Content

Clinical Study of Prolistem® Supplement in Men with Non-Obstructive azoospermia (Primary testicular Failure).

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Abstract

Introduction: Non-obstructive azoospermia is a cause of male infertility and despite the advancement in gynecology it is still one of the most challenging conditions to treat. Prolistem is a novel treatment for this condition with unique mechanism called "Spermatogenesis Restarting Process"

Objectives: To evaluate the effectiveness of Prolistem supplement in the therapy of infertile men with nonobstructive Azoospermia.

Methods: Eighty-nine patients received Prolistem supplement for six months. Hormones parameters such as FSH, LH and Testosterone levels of the patients were measured before and after the procedure. Semen test was performed after the treatment. In case of no sperm found semen, the patients recommended to perform sperm retrieval such as TESE or micro-TESE. All the required data for the study was collected retrospectively from the patient or the hospital records.

Results: 23% of NOA cases success to find sperm in semen after the six months supplement. 25% of NOA cases success to extract sperm from the testis with the help of sperm retrieval techniques.

Conclusion: Numerous studies and our previous studies using animal models have proven that that testosterone had an inhibitory effect on spermatogonial differentiation in azoospermia cases. Prolistem supplement has been successful for the treatment of cases of non-obstructive azoospermia type primary testicular failure

Introduction

Infertility in the male partner contributes to approximately half of all cases. To date, various techniques, such as in vitro fertilization (particularly, intracytoplasmic sperm injection or ICSI) and so-called TESE-ICSI involving the harvesting of sperm from the testes, have been developed for male infertility. Although these methods are steadily producing results, no technique has proven effective for patients with non-obstructive azoospermia, in which there is an absence of mature sperm in the testes. Evidence suggests that many patients with azoospermia have a genetic predisposition to the condition, although the cause has not been elucidated in the vast majority of cases [1]. Conversely, studies using knockout mouse models have recently linked many genes to spermatogenesis, the mechanisms of which are currently being clarified. These animal findings have yet to be shown applicable to most human cases. This is because identifying the affected genes in humans requires a retrograde genetic approach and because the knockout mouse phenotype is not always faithfully reproduced in humans.

Human Male Infertility and Reasons

Many researchers and clinicians have asserted that societal progress in advanced countries and worsening of the natural environment have likely resulted in decreased male fertility. Long-reported risk factors include working in high temperatures [2], noise associated with manufacturing [3], exposure to radiation [4], electromagnetic waves [5], and a variety of chemical substances [6]. Numerous studies have compared patients with male infertility (oligospermia or azoospermia) to healthy subjects (normal sperm count). To date, proposed risk factors include air temperature [7], automobile driving time per day [8], air pollution [9], regional differences in residential population density [10], mumps [11], stress [12], and alcoholism [13]. On the contrary, many reports indicate the absence of a correlation between environmental factors and male infertility [14, 15]. Thus, there is presently no consistent view on the role of environmental factors and male infertility.

In 1976, Tiepolo and Zuffardi first proposed an explanation for the role of the human Y chromosome in spermatogenesis [16]. They microscopically identied the presence of micro deletions on the long arm of the Y chromosome in six patients with azoospermia and proposed an important spermatogenesis gene in this region. They named this the azoospermia factor (AZF) region. Various subsequent studies have been conducted, particularly by Vogt et al. [17], and in 1995, Reijo et al. examined 89 patients with non-obstructive azoospermia and found that 12 (13%) had a deletion in the AZF region.
These results brought recognition to the close relationship between human azoospermia and this region [18]. Vogt et al. further showed the micro deletions to be concentrated in three regions according to the testicular tissue type and divided the AZF region into subregions, AZFa, AZFb, and AZFb [19].

Non-Obstructive Azoospermia

In non-obstructive azoospermia the testes are abnormal, atrophic, or absent, and sperm production severely disturbed to absent. FSH levels tend to be elevated (hypergonadotropic) as the feedback loop is interrupted. The condition is seen in 49-93% of men with azoospermia [20]. Testicular failure includes absence of failure production as well as low production and maturation arrest during the process of spermatogenesis.

Causes for testicular failure include congenital issues such as in certain genetic conditions (e.g. Klinefelter syndrome), some cases of cryptorchidism or Sertoli-cell-only-syndrome as well as acquired conditions by infection (orchitis), surgery (trauma, cancer), radiation [21] or other causes that we don't know yet. Mast cells releasing inflammatory mediators appear to directly suppress sperm motility in a potentially reversible manner, and may be a common pathophysiological mechanism for many causes leading to inflammation [22] generally, men with unexplained hypergonadotropic azoospermia need to undergo a chromosomal evaluation.

Until recently, it was assumed that men with non-obstructive azoospermia were untreatable. The only options offered to these couples to have children were the use of donor spermatozoa or adoption. Several clinically relevant findings have changed our approach to this condition. Direct evaluation of testis biopsy specimens often demonstrates sperm in men with non-obstructive azoospermia, despite severe defects in spermatogenesis.

Current Azoospermia Treatment

The method of sperm retrieval may be critical in the management of NOA. Because testicular sperm production, when present, is randomly and heterogeneously distributed throughout one or both testes, surgical methods for sperm retrieval have been developed to achieve wide sampling of the testicular parenchyma. Percutaneous, incisional, and microsurgically assisted techniques have been described. Percutaneous methods such as testicular sperm aspiration (TESA) involve aspiration of testicular tissue using small- or large-bore needles. The needle is typically attached to a syringe that is used to create suction while the needle tip is moved around within each testis to achieve wide sampling of the seminiferous tubular tissue. Incisional methods are generally referred to as conventional testicular sperm extraction (cTESE) or microdissection testicular sperm extraction (mTESE). In cTESE, seminiferous tubular tissue is extracted through one or more testicular incisions. Microdissection TESE is performed by making a large testicular incision and then selectively sampling the largest-diameter seminiferous tubules using optical magnification provided by an operating microscope.

The most important outcome when assessing sperm extraction is sperm-retrieval rate. No randomized controlled trials have been performed to compare techniques of sperm extraction. Two recent systematic reviews have been performed examining surgical sperm-extraction techniques in men with NOA; both identified the same seven studies comparing mTESE to cTESE. The authors report successful sperm retrieval in 35% of cTESE cases (range: 17%–45%) and 52% of mTESE cases (range: 45%–63%), estimating that the performance of a mTESE was 1.5 times more likely to retrieve sperm (95% confidence interval) [23, 24]. Using a combination of prospective and retrospective data, the authors of both reviews concluded that mTESE was superior to cTESE for surgical sperm extraction in men with NOA. It was noted that the greatest advantage seemed to be in men with limited sperm production such as Sertoli cell−only pattern. In addition, seven studies were also pooled to provide a comparison in sperm-retrieval rates between TESA (28%, range: 7%–42%) and cTESE (56%, range: 43%–64%), concluding the superiority of cTESE vs. TESA (relative risk [RR] 2.0, 95% CI 1.8–2.2). Although sperm-retrieval rates were different for cTESE in each of the comparison groups, the conclusions suggest the superiority of mTESE over cTESE and of cTESE over TESA. When a repeat procedure is necessary, data suggest that allowing at least 6 months to pass increases the retrieval rate (80% vs. 25%, P=.02 [calculated]) [25].

A diagnostic biopsy (either open or percutaneous) has also been advocated. Although it may allow men to avoid a more extensive procedure to identify sperm, a diagnostic biopsy obligates men to undergo a second procedure to obtain sperm for reproduction. Data suggest that a diagnostic biopsy may provide information about the likelihood of sperm retrieval at the time of sperm extraction. Men in whom biopsy results demonstrate hypospermatogenesis (79%–98%), maturation arrest (47%–94%), and Sertoli cell−only (5%–24%) have different sperm-retrieval rates [26, 27, 28].

In addition to the sperm-retrieval rate, safety and complication rates are also important considerations. Overall, complications from all sperm-retrieval techniques are uncommon and minor [29]. Percutaneous approaches are thought to have the lowest rate, with many studies reporting no complications [30-31]. However, a study of 267 procedures reported a 3% complication rate including hematoma and syncope during the procedure [32]. Complications of TESE have been reported as hematoma, hypogonadism, and wound infection. Few studies have been reported that compare complications rates between TESE groups. However, higher

postoperative intratesticular hematoma formation with cTESE compared to mTESE as assessed by scrotal ultrasonography has been suggested by several studies [33-35]. The use of the microsurgical technique may allow decreased testicular parenchyma harvest and reduced sequelae including hypogonadism. Serum testosterone levels do fall acutely after TESE but return to 95% of baseline after healing is complete [36, 37].

Timing of sperm retrieval

Another important consideration in the management of NOA is the timing of sperm retrieval. Surgical sperm retrieval can be performed during an IVF cycle to coincide with oocyte retrieval with the intent of using fresh sperm, if identified, for ICSI. Alternatively, sperm retrieval can be performed before ovarian stimulation with the plan for cryopreservation if sperm are identified for use in future IVF cycles. There are theoretical advantages of each strategy. The use of freshly extracted sperm allows sperm to avoid the stress of cryopreservation. Freezing the extracted sperm for later use separates timing of the IVF from sperm extraction so that if sperm is not found, the female partner can potentially avoid an unnecessary ovarian stimulation. In addition, both members of the couple will be undergoing gamete retrieval on separate days, allowing each to help the other rather than involving a third party for transportation/assistance. Moreover, due to the inherent work flows of coordinating an operating room, scheduling a sperm extraction for a precise day or time can be challenging when the exact timing is known only a few days prior. Establishing the efficacy of frozen sperm can also allow men to undergo a single sperm extraction rather than a separate procedure for each cycle.

Outcomes for the use of fresh vs. frozen sperm for ART in men with NOA have been compared. A meta-analysis compiled data from 11 studies reporting on 574 ICSI cycles (275 fresh and 299 frozen) that involved injection of 4,177 oocytes [38]. No difference between fresh and frozen sperm was identified in clinical pregnancy rate (RR 1.00, 95% CI 0.75–1.33) or fertilization rate (RR 0.97, 95% CI 0.92–1.02). Three additional studies involving 401 cycles also failed to identify a difference in outcomes using fresh vs. frozen sperm in men with NOA [39-41]. Identification of sperm after cryopreservation was not reported by all studies, but five groups report identification ranging from 79% to 100% [42-45]. Three studies reported post-thaw identification rates of 100% with an overall weighted average of 87% for all studies. Laboratory comfort and experience with cryopreservation of testicular tissue in men with spermatogenic failure are crucial to success.

Many cases of prolonged azoospermia appear to be a result of killing all the spermatogonial stem cells inside the testis by infection or cytotoxic agents [46] or others reasons that we don't know. In other instances, however, the stem spermatogonia survive but fail to differentiate into sperm, as evidenced by the spontaneous re-initiation of spermatogenesis in some patients after many years of azoospermia [47]. There is evidence of arrest at the spermatogonial [48] or the spermatocyte [49] stages during the azoospermic period caused by cytotoxic agents or other reasons. The trigger for the spontaneous recovery is not known, but its existence supports the proposal that more rapid or additional recovery from surviving but arrested germ cells can be induced.

Examination of the hormonal status of Azoospermic cases revealed that the failure of differentiation of spermatogonia could not be a result of insufficient stimulation by

gonadotropins or testosterone. Follicle-stimulating hormone (FSH) levels were 1.5-fold normal, luteinizing hormone (LH) and testosterone levels remained unchanged fold normal [50,51]

Researches hypothesized that, in azoospermia cases, testosterone might actually be inhibiting spermatogonial differentiation. Based on this hypothesis, many studies have been done and researches suppressed testosterone by treating Azoospermic rats with gonadotropin releasing hormone (GnRH) antagonists that [52] prevented the block in spermatogonial differentiation. Although the spermatogonia differentiated, they could not progress past the round spermatid stage as long as testosterone was suppressed.

Studies have shown that testosterone is critical for the late stages of spermatogenesis, Spermatogonia Stem Cells in the seminiferous tubules do not need testosterone to divide [53]. Researchers have also found that testosterone is involved in the blockage of Spermatogonia Stem Cells in abnormal conditions such as azoospermia [54].
Suppression of testosterone restores the spermatogenesis process, and in some cases, spermatogenesis was maintained after the cessation of hormonal treatment and fertility restoration [54].
Hormones are responsible for the maintenance of sperm production in normal conditions; however, in abnormal conditions the testosterone inhibits the spermatogonial differentiation [55-58].

Prolistem® Idea

It was surprising that the testosterone, which is required to support normal spermatogenesis, appeared to inhibit this process in azoospermia conditions. Therefore, more studies were performed to confirm that testosterone was indeed inhibitory in azoospermia cases [59, 60]. It should be pointed out that the action of testosterone in normal spermatogenesis is to support spermatocyte and spermatid development; it has only small quantitative effects on spermatogonia [61,62]. So, the newly discovered inhibitory phenomenon is an additional action of testosterone and does not replace his usual action. Furthermore, it should be noted that the germ cells are generally believed to lack androgen and FSH receptors, so that the hormones act on the somatic cells, likely the Sertoli cell, which then affects the spermatogonia by paracrine or juxtacrine interactions.

Prolistem® is a natural supplement that designed to support non-obstructive azoospermia (primary testicular failure) by a unique mechanism called “Spermatogenesis Restarting Process”. Prolistem® works by temporarily reducing testosterone levels to allow for the crucial early stages of spermatogenesis to take place.
Prolistem® Stage one and two works on the reduction of testosterone that will push the body to restart sperm production while Prolistem® Stage three supplies the body with natural components and vitamins to increase the production of healthy sperm. The testosterone-induced block to Spermatogonial Stem Cells mechanisms is still unknown

Prolistem® Rats Experiment

Animals and Non-Obstructive Azoospermia

LBNF rats were anesthetized and affixed to an acrylic board with surgical tape; then the lower part of the body was irradiated by a 60Co gamma ray unit. The field extended distally from a line about 6 cm above the base of the scrotum. Dose 6 Gy was given at a dose rate of approximately 1 Gy/min, the radiation caused permanent azoospermia to the LBNF1 rats (Non-Obstructive Azoospermia).

Prolistem® treatment

Prolistem® treatment was performed after 10 weeks of radiation; Prolistem® (stage one) was dissolved in water and administered as daily by gavag for one and two months (by the mouth). Control group received only water.

Tissue processing

Rats were killed by an overdose of a ketamine-acepromazine mixture after 1 month and 2 months. Each testis was surgically excised and weighed with the tunica albuginea intact. The right testis was fixed overnight in Bouin's fluid. The testis was suspended by silk sutures and centrifuged for 30 min at 60 × g at 4°C, and the weight of the fluid collected was determined. The remaining weight of the testis parenchymal tissue was measured after removing the tunica albuginea. The tissue was then homogenized in water for sperm head counts.

Evaluation of Spermatogenesis

For histological analysis, the fixed right testis was embedded in glycol methacrylate plastic and 4-µm sections were cut and stained with periodicacid Schiff's (PAS) and hematoxylin. To evaluate the recovery of spermatogenesis from irradiation, we scored a minimum of 200 seminiferous tubules in one section from each animal for the most advanced germ-cell stage present in each tubule. we computed the tubule differentiation index, which is the percentage of tubules containing 3 or more cells that had reached type B spermatogonial stage or later.

Rats Experiment Results

We examined the effects of Prolistem® mix on spermatogenic recovery in LBNF1 rats; Prolistem® treatment starting after 10 weeks after irradiation with 6 Gy restored the production of differentiated cells in 9% after one month and 18% after two months (fig.1). Control rats didn’t show any recovery (fig. 2).

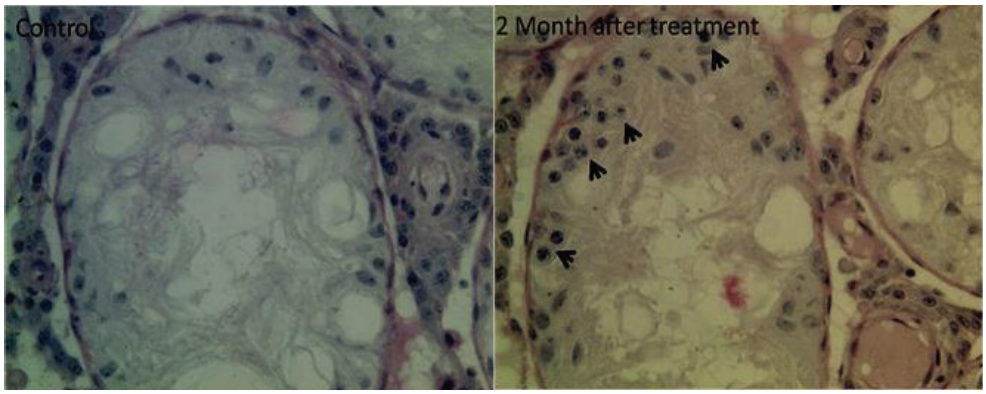

FIG. 1. Recovery of spermatogenesis at 10 weeks after Prolistem treatment. Tubule differentiation index (TDI), defined as percentage of tubules differentiating to the B spermatogonial stage or beyond.

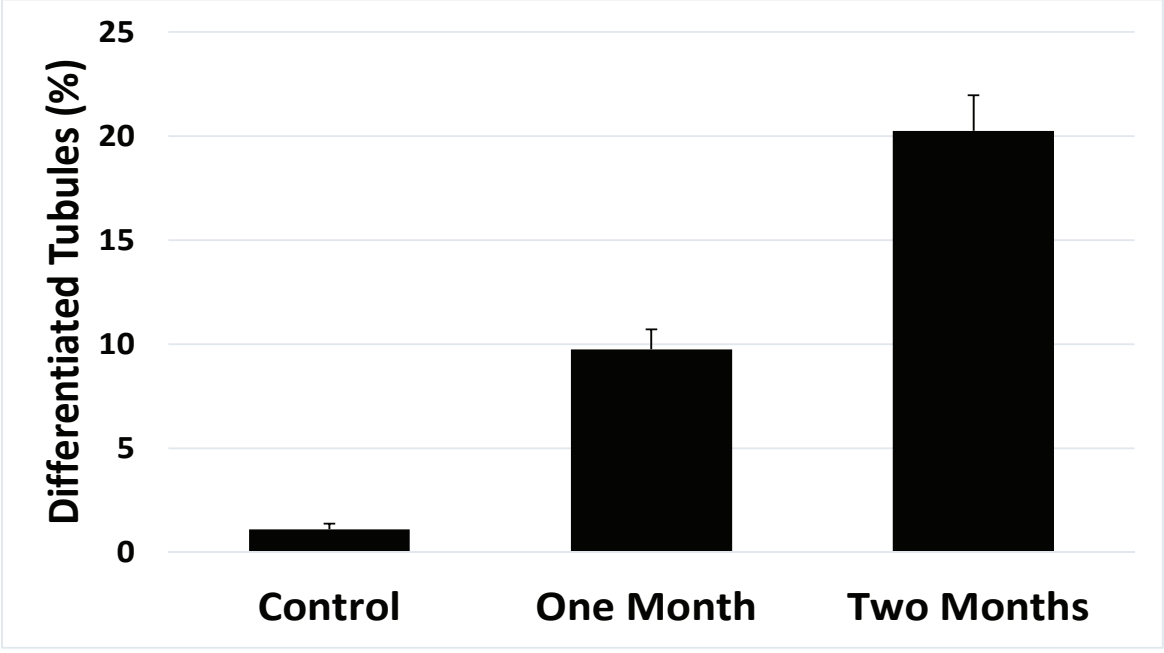

FIG. 2: Histology of LBNF1 rat testes 2 months without (Control) or with (treated) Prolistem treatment. Control rats showed atrophic tubules and interstitial edema, most tubules contained only Sertoli cells (SC) but some contained a few type A spermatogonia. Prolistem treatment for two months induced recovery of spermatogenesis.

Control and treated rats did not show any sperm count after one month of treatment. However, treated rats with Prolistem® showed increase in sperm count from zero to 100,000 sperm cells / testis (Figure 3).

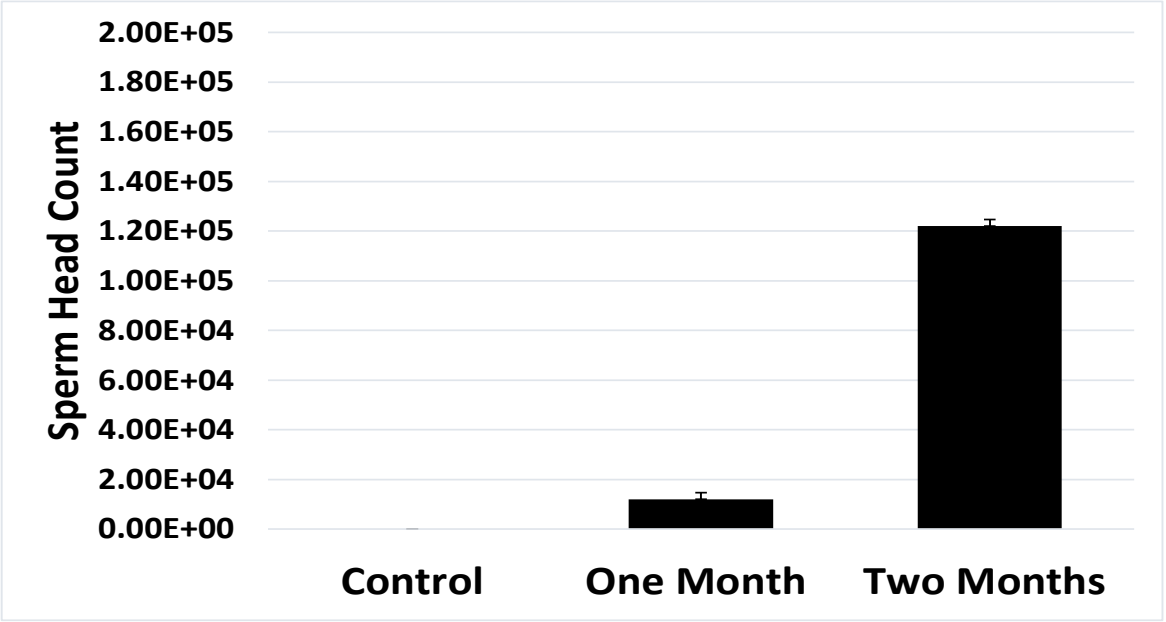

FIG. 3: Testicular sperm production: numbers of sonicationresistant late spermatids per testis.

Prolistem® Clinical Study

Purpose:
The purpose of this trail was to determine whether 6 months treatment with Prolistem® improve sperm production in semen or increase the sperm extraction from patients with non-obstructive azoospermia (primary testicular failure).

Participants
Adults with non-obstructive azoospermia (primary testicular failure), patients with known genetic issues didn't included in the study.

Treatment with Prolistem®
The treatment of six months of Prolistem® shipped to 89 patients around the world (such as: USA, Nigeria, Jordan, Israel, India…), the patients of azoospermia contact us directly or through their clinic.

Data Analysis:
The following parameters were collected from the patients basically through email or their doctor that collaborate with us:

- Levels of FSH, LH and Total Testosterone before starting the treatment and after

the treatment (because the variability in reporting methods used by the various laboratories we used the terms “Norma” and “High” as an indication of hormones levels.

- Semen test (all reports were zero sperm in semen)
- Biopsy report if available

Results of Prolistem® Clinical Study

After six months the patients performed semen analysis, if no sperm found then they performed TESE or micro-TESE directly after the treatment based on our recommendations.
23% of the patients found sperm in their semen (from few sperm to few millions) and 25% of the patients performed success TESE or micro-TESE (sperm were extracted by surgery) There is no effect in 52% of the patients that used our treatment for six months, this could be related to unknown genetic issues. Example of success reports described in table 1 to 10.

Table 1:

Case # Az88932	**Age:** 43		**Country:** Saudi Arabia
Patient History	*FSH*	12.2	Range (1.27 – 19.26) MIU/ML
	LH	7.4	Range (1.24 – 8.62) MLIU/ML
	Testosterone	0.8	Range (1.75– 7.81) ug/L
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No family members with infertility	
Treatments History	Clomid tab, Proxeed, Evit 400mg, Merional 75 and choriomon 5000 IU		
Biopsy History	Not done		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	11.56	Range (1.27 – 19.26) MIU/ML
	LH	10.16	Range (1.24 – 8.62) MLIU/ML
	Testosterone	1.37	Range (1.75– 7.81) ug/L
Sperm in Semen	**200 sperm**		
Sperm Retrieval	Not done		

Table 2:

Case # Az88965	**Age:** 40		**Country:** India
Patient History	*FSH*	6.60	Range (1.4 – 18) mIU/mL
	LH	4.85	Range (1.5 – 9.3) mIU/mL
	Testosterone	338	Range (241– 827) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No family members with infertility	
Treatments History	Sperm Retrieval No Success		
Biopsy History	Maturation Arrest		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	Normal	Range (1.27 – 19.26) MIU/ML
	LH	Normal	Range (1.24 – 8.62) MLIU/ML
	Testosterone	Normal	Range (1.75– 7.81) ug/L
Sperm in Semen	**15 million in semen**		
Sperm Retrieval	Not done		

Table 3:

Case # Az88976	**Age:** 30		**Country:** USA
Patient History	*FSH*	6.88	Range (1.55 – 9.47) muI/mL
	LH	2.65	Range (0.8 – 7.6) muI/mL
	Testosterone	202	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	**AZFc deletion**	
	Family	-	
Treatments History	Sperm Retrieval No Success		
Biopsy History	Sertoli cells only		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	-	-
	LH	-	-
	Testosterone	-	-
Sperm in Semen	-		
Sperm Retrieval	Sperm found by Micro-TESE		

Table 4:

Case # Az88522	**Age:** 35		**Country:** USA
Patient History	*FSH*	23	Range (1.55 – 9.47) muI/mL
	LH	5.8	Range (0.8 – 7.6) muI/mL
	Testosterone	581	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	-	
Treatments History	Sperm Retrieval No Success		
Biopsy History	Biopsy Tubular findings; Azoospermia in 100 % of tubules and Sertoli cells only. Conclusion: Germ cell aplasia /Azoospermia		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	37	Range (1.55 – 9.47) muI/mL
	LH	6	Range (0.8 – 7.6) muI/mL
	Testosterone	620	Range (262– 1593) ng/dl
Sperm in Semen	**10 sperm in semen**		
Sperm Retrieval	-		

Table 5:

Case # Az88721	**Age:** 36		**Country:** Israel
Patient History	*FSH*	24.7	Range (1.55 – 9.47) muI/mL
	LH	8.4	Range (0.8 – 7.6) muI/mL
	Testosterone	142	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No Family History	
Treatments History	-		
Biopsy History	-		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	-	Range (1.55 – 9.47) muI/mL
	LH	-	Range (0.8 – 7.6) muI/mL
	Testosterone	-	Range (262– 1593) ng/dl
Sperm in Semen	**-**		
Sperm Retrieval	**11 Sperm Found by Micro-TESE**		

Table 6:

Case # Az88211	**Age:** 34		**Country:** New Zealand
Patient History	*FSH*	Normal	Range (1.55 – 9.47) muI/mL
	LH	Normal	Range (0.8 – 7.6) muI/mL
	Testosterone	Normal	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No Family History	
Treatments History	TESA in march 2012/ Micro-TESE in July 2012		
Biopsy History	Maturation Arrest		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	Normal	Range (1.55 – 9.47) muI/mL
	LH	Normal	Range (0.8 – 7.6) muI/mL
	Testosterone	Normal	Range (262– 1593) ng/dl
Sperm in Semen	**-**		
Sperm Retrieval	**10 Sperm Found by Micro-TESE on April 2013**		

Table 7:

Case # Az88981	**Age:** 33		**Country:** Algeria
Patient History	*FSH*	21.83	Range (1.55 – 9.47) muI/mL
	LH	10.05	Range (0.8 – 7.6) muI/mL
	Testosterone	760	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No Family History	
Treatments History	-		
Biopsy History	-		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	14.86	Range (1.55 – 9.47) muI/mL
	LH	Not tested	Range (0.8 – 7.6) muI/mL
	Testosterone	684	Range (262– 1593) ng/dl
Sperm in Semen	**-**		
Sperm Retrieval	**Few Sperm Found by Micro-TESE**		

Table 8:

Case # Az88233	**Age:** 40		**Country:** Dominican Republic
Patient History	*FSH*	37.9	Range (1.55 – 9.47) muI/mL
	LH	10.29	Range (0.8 – 7.6) muI/mL
	Testosterone	Not tested	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No Family History	
Treatments History	-		
Biopsy History	-		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	-	Range (1.55 – 9.47) muI/mL
	LH	-	Range (0.8 – 7.6) muI/mL
	Testosterone	-	Range (262– 1593) ng/dl
Sperm in Semen	**-**		
Sperm Retrieval	**Successful TESE and IVF (baby girl)**		

Table 9:

Case # Az88777	**Age:33**	**Country:** Iraq	
Patient History	*FSH*	21	Range (1.55 – 9.47) muI/mL
	LH	Normal	Range (0.8 – 7.6) muI/mL
	Testosterone	Normal	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No Family History	
Treatments History	Different supplements were prescribed (Proxeed Plus , fertiman and others)		
Biopsy History	-		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	21.9	Range (1.55 – 9.47) muI/mL
	LH	Not tested	Range (0.8 – 7.6) muI/mL
	Testosterone	246	Range (262– 1593) ng/dl
Sperm in Semen	**20,000 Sperm in semen**		
Sperm Retrieval	**-**		

Table 10:

Case # Az88254	**Age: 35**	**Country: Palestine**	
Patient History	*FSH*	Normal	Range (1.55 – 9.47) muI/mL
	LH	Normal	Range (0.8 – 7.6) muI/mL
	Testosterone	Normal	Range (262– 1593) ng/dl
	Ultrasound	Normal	
Genetic History	*karyotype test*	Normal	
	AZF test	Normal	
	Family	No Family History	
Treatments History	Micro-TESE no sperm found		
Biopsy History	Sertoli cell only		
After taking Prolistem® for six months			
Hormones After Prolistem®	*FSH*	Normal	Range (1.55 – 9.47) muI/mL
	LH	Normal	Range (0.8 – 7.6) muI/mL
	Testosterone	Normal	Range (262– 1593) ng/dl
Sperm in Semen	**-**		
Sperm Retrieval	**Micro-TESE: Few sperm for IVF**		

Conclusion

Our animals model using azoospermic rats showed 100% response to the Prolistem treatment. The same effect also reported in our previous studies [63,64] while our clinic trail showed about 48% success and this may be due to unknown genetic issues.

Various studies have indicated that testosterone had an inhibitory effect on spermatogonial differentiation in azoospermia cases. The stimulation of spermatogonial differentiation by suppression of testosterone with GnRH antagonist was reversed by exogenous testosterone.

In azoospermia conditions, it appears that testosterone act additively to inhibit the differentiation of spermatogonia, whereas in normal spermatogenesis testosterone act to support survival and differentiation of spermatocytes and spermatids.

Chemicals drugs that lower testosterone would be ideal for use to treat azoospermia in humans but we don't know what are the major side-effects. For example, in low testosterone cases, physicians trying to avoid testosterone supplementation or at least delayed and given in as low a dose as possible, because testosterone supplementation has many side-effects that we trying to avoid, researches and physicians recommend to increase the testosterone levels naturally if it possible. For the same reason we found formula from natural sources that reduce the testosterone levels in human body to acceptable levels with no side-effects to treat non-obstructive azoospermia.

Until recently, it was assumed that non-obstructive azoospermia was untreatable, here we showed that Prolistem® has the ability to restore fertility by reducing the testosterone levels naturally.

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Rat Models of Post-Irradiation Recovery of Spermatogenesis: Interstrain Differences

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Abstract

Recently we reported large differences between rat strains in spermatogenesis recovery at 10 weeks after 5-Gy irradiation suggesting that there are interstrain as well as interspecies differences in testicular radiation response. To determine whether these interstrain differences in sensitivity might be a result of the particular dose and time-point chosen, we performed dose-response and time-course studies on sensitive Brown-Norway (BN) and more resistant spontaneously hypertensive (SHR) and Sprague-Dawley (SD) rats. Type A spermatogonia were observed in atrophic tubules at 10 weeks after irradiation in all strains indicating that tubular atrophy was caused by a block in their differentiation, but the doses to produce the block ranged from 4.0 Gy in BN to 10 Gy in SD rats. Although the numbers of type A spermatogonial were unaffected at doses below 6 Gy, higher doses reduced their number, indicating that stem cell killing also contributed to the failure of recovery. After 10 weeks, there was no further recovery and even a decline in spermatogonial differentiation in BN rats, but in SHR rats, sperm production returned to control levels by 20 weeks after 5.0 Gy and, after 7.5 Gy, differentiation resumed in 60% of tubules by 30 weeks. Suppression of testosterone and gonadotropins after irradiation restored production of differentiated cells in nearly all tubules in BN rats and in all tubules in SHR rats. Thus the differences in recovery of spermatogenesis between strains were a result of both quantitative differences in their sensitivities to a radiation-induced, hormone-dependent block of spermatogonial differentiation and qualitative interstrain differences in the progression of post-irradiation recovery. The progression of recovery in SHR rats was similar to the prolonged delays in recovery of human spermatogenesis after cytotoxic agent exposure and thus may be a system for investigating a phenomenon also observed in men.

Keywords

ionizing radiation; spermatogenesis; rat strains; spermatogonia

INTRODUCTION

The mammalian testis is sensitive to ionizing radiation: low doses can temporarily reduce sperm production, moderate doses can cause prolonged reductions in sperm count, and high

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doses can result in permanent azoospermia. In humans, the testis appears more sensitive and exhibits longer delays before spermatogenesis recovers than in most rodent models. Single doses as low as 0.15 Gy cause temporary reductions in spermatogonial numbers and sperm count that can last as long as 6 months (Clifton & Bremner, 1983; Paulsen, 1973; Rowley *et al.*, 1974). Higher doses can produce azoospermia that lasts from about 8 months after 0.5 Gy to about 2 years after 6 Gy, and it then takes several years for sperm production to return to normal. The delays indicate that there are surviving spermatogonial stem cells that are blocked at some point in their differentiation, but the mechanisms of the block and subsequent recovery of spermatogenesis in human are not known. The fractionated radiation therapy used in cancer treatment is more toxic to the testis than the single doses (Meistrich & van Beek, 1990) and can result in no recovery of spermatogenesis occurs even 5 years after treatment (Hahn *et al.*, 1982; Sandeman, 1966; Speiser *et al.*, 1973), suggesting that all stem cells may have been killed.

An animal model that simulates the response of the human testis to radiation is needed to improve our understanding of this process. Non-human primates (macaques) show the most similarities to human including the histological types of spermatogonia (Ehmcke & Schlatt, 2006) and drastic declines in spermatogonial numbers and sperm count after 2 or 4 Gy lasting 6 months before recovery begins, and incomplete recovery even after 18 months (Foppiani *et al.*, 1999; Kamischke *et al.*, 2003; van Alphen *et al.*, 1988). However, studies on primates are limited by theirs cost and lack of genetic tools. Rodent models are inexpensive, have more detailed literature, inbred lines, and genetic tools, and are most amenable to laboratory studies but they have not so far shown the delayed recovery phenomenon.

The recovery from toxic effects of radiation in mice is much more rapid and robust than in humans. The stem spermatogonia surviving irradiation begin to differentiate almost immediately after doses even as high as 6 Gy and restore spermatogonial numbers to control levels after only 2 weeks (Erickson & Hall, 1983). Sperm production begins to increase within 7 weeks after 2 Gy and within 11 weeks after 6 to 12 Gy and reaches 60% of control values within 23 weeks after 6 Gy (Meistrich *et al.*, 1978; Meistrich & Samuels, 1985; Searle & Beechey, 1974). Killing of stem spermatogonia first becomes significant at a dose of 4 Gy (de Ruiter-Bootsma *et al.*, 1976; Erickson, 1981), and their numbers are further reduced with higher doses, with 9 Gy resulting in only 25% of tubules recovering production of differentiated cells within 5 weeks (Lu *et al.*, 1980; Withers *et al.*, 1974). Since a negligible number of the atrophic tubules contain spermatogonia (Kangasniemi *et al.*, 1996a), these atrophic tubules are primarily due to stem cell killing and not a block in spermatogonial differentiation, although there is some reduction in the yield of later differentiated cells after high doses (van den Aardweg *et al.*, 1983).

The rat testis appears somewhat more sensitive to damage produced by irradiation than the mouse and shows less recovery. However in Sprague-Dawley (SD) rats, the most widely-studied and most resistant strain, spermatogonial numbers recovered after 3 Gy to control levels within 5 weeks (Dym & Clermont, 1970), and epididymal sperm counts to 40% of control after 19 weeks (Jégou *et al.*, 1991). But after 6 Gy, recovery was far from complete at 16 weeks, as testis weights were only 52% of control and 44% of the tubules had incomplete spermatogenesis (Erickson & Hall, 1983). Following 9 Gy of radiation, less than 10% of tubules showed differentiating cells at 8 weeks (Delic *et al.*, 1986), and not until 26 weeks did sperm production reach 10% of control (Pinon-Lataillade *et al.*, 1991). Other strains of rats, such as LBNF1 (F1 hybrids of Lewis and Brown-Norway), were much more sensitive and, despite the survival and maintenance of stem spermatogonia, the testis showed progressive failure of recovery (Kangasniemi *et al.*, 1996b; Shetty *et al.*, 2000). Although some recovery of differentiated cells was transiently observed at 6 weeks after irradiation, this declined progressively to zero at 60 weeks after 3.5 Gy and by 10 weeks after 5 Gy, thus

indicating a permanent failure of spermatogenic recovery. The only other rats previously studied, various Wistar substrains, did show some recovery after 5 Gy, but to levels below that of Sprague-Dawley (Delic *et al.*, 1986; Delic *et al.*, 1987).

To systematically characterize these strain differences, we directly compared the recovery of spermatogenesis at 10 weeks after 5-Gy irradiation in seven rat strains and observed dramatic differences (Abuelhija *et al.*, 2012). There was no recovery of differentiating germ cells in the Lewis and Brown Norway (BN) stains despite the presence of type A spermatogonia in many tubules. Thus they showed the complete block in spermatogonial differentiation as had been previously observed in the LBNF1 hybrids (Kangasniemi *et al.*, 1996b). In contrast, in two Wistar-derived inbred strains, Wistar-Kyoto and spontaneously hypertensive rats (SHR), recovery of spermatogenesis was observed in 55% and 94% of the tubules, respectively. Sperm production was still markedly reduced, as it was only 3% of control levels in both Wistar strains. SD rats showed the best recovery of spermatogenesis, as 98% of tubules showed recovery and sperm production was 6% of controls. Nevertheless, the atrophic tubules in all strains contained type A spermatogonia, indicating that the tubular atrophy observed after 5 Gy was due primarily to a block in spermatogonial differentiation and not stem cell killing.

However, questions remain regarding the differences in sensitivity between strains. For example, is not known whether the different strains would show the same qualitative patterns of recovery, but differ in quantitative doses to produce blocks in recovery, and could we find a strain and dose that results in recovery after a delay. Furthermore, the role of testosterone in blocking spermatogenic recovery in different strains needs to be investigated to determine whether the recovery in the resistant strains is a result of their insensitivity to the action of testosterone, which we have previously shown is responsible for the block in spermatogonial differentiation in LBNF1 rats (Shetty *et al.*, 2000). In addition, we must determine whether the presence of tubules at different stages of differentiation at 10 weeks after irradiation represented a block at a later stage of differentiation or just a delay in initiation of differentiation. Finally we wanted to identify a strain showing some characteristics of the transient block in spermatogenic cell differentiation and the delayed recovery process observed in human testes.

To address these questions, we performed a dose-response, time-course, and hormone-effect study of the recovery of spermatogenesis after irradiation in three strains of rats. We performed all studies comparing BN and SHR since these strains are most amenable to future genetic studies as recombinant inbred rats between these two strains are already available to identify quantitative trait loci responsible for the differences in radiation sensitivity (Tabakoff *et al.*, 2009) and their genomes have been sequenced (Atanur *et al.*, 2010; Gibbs *et al.*, 2004). In addition, dose-response studies were performed on SD rats as this strain is the most resistant and is most widely used in toxicological studies.

MATERIALS AND METHODS

Animals and Irradiation Exposure

Brown Norway (BN/SsNHsd) and Sprague-Dawley (Hsd:Sprague Dawley SD) rats were obtained from Harlan Laboratories; SHR (SHR/NCrl) rats were obtained from Charles River Laboratories. We obtained the rats at 7 wk of age and allowed them to acclimatize in our facility for 1 wk prior to use. Rats were housed under standard lighting (12 h light, 12 h dark) and were given food and water *ad libitum*. All procedures were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Rats were irradiated as described previously (Shetty *et al.*, 2000). Briefly, they were anesthetized and affixed to an acrylic board with surgical tape; then the lower part of the body was irradiated by a ^{60}Co gamma ray unit (Eldorado 8; Atomic Energy Canada Ltd., Ottawa, ON, Canada). The field extended distally from a line about 6 cm above the base of the scrotum. Different doses (2.7 Gy to 12.5 Gy) were given at a dose rate of approximately 1 Gy/min; dose ranges were chosen for each strain based on the sensitivity observed previously (Abuelhija *et al.*, 2012). Testis tissue was harvested at various times between 10 and 40 weeks after irradiation (Table 1). Each dose and time point represents the mean and standard error of between 3 and 10 rats.

Hormone treatment

Hormone suppressive treatment was performed with the GnRH antagonist (GnRH-ant) acyline (National Institute of Child Health and Human Development) and the androgen receptor-antagonist flutamide starting immediately after radiation and continuing until tissue harvest, Acyline was dissolved in water and administered as weekly subcutaneous injections of 1.5 mg/kg (Porter *et al.*, 2006). Flutamide was administered by subcutaneous implantation of four 5-cm-long Silastic capsules calculated to deliver 20 mg/kg/day (Porter *et al.*, 2009). Each treatment group (time and dose point) consisted of a minimum of 4 rats.

Intratesticular interstitial fluid and tissue processing

Rats were killed by an overdose of a ketamine-acepromazine mixture. Each testis was surgically excised and weighed with the tunica albuginea intact. The right testis was fixed overnight in Bouin's fluid.

Interstitial tubule fluid was collected from the left testis as we had done previously (Abuelhija *et al.*, 2012) using a modification of methods described earlier (Porter *et al.*, 2006; Rhenberg, 1993). Briefly, the testis was suspended by silk sutures and centrifuged for 30 min at 60 × g at 4°C, and the weight of the fluid collected was determined. The remaining weight of the testis parenchymal tissue was measured after removing the tunica albuginea. The tissue was then homogenized in water for sperm head counts.

Evaluation of Spermatogenesis

For histological analysis, the fixed right testis was embedded in glycol methacrylate plastic (JB4, Polysciences Inc., Warrington, PA), and 4-µm sections were cut and stained with periodic-acid Schiff's (PAS) and hematoxylin. To evaluate the recovery of spermatogenesis from irradiation, we scored a minimum of 200 seminiferous tubules in one section from each animal for the most advanced germ-cell stage present in each tubule. Unless otherwise stated, we computed the tubule differentiation index (TDI), which is the percentage of tubules containing 3 or more cells that had reached type B spermatogonial stage or later (Meistrich & van Beek, 1993). To obtain a more complete description of the stages of differentiation present in the testis, we also determined the percentages of tubules with 3 or more cells reaching the leptotene spermatocyte stage or later (TDI-spermatocyte) or the round spermatid stage or later (TDI-spermatids), or with 10 or more cells reaching the elongating or elongated spermatid stage (TDI-late spermatids).

We counted all type A spermatogonia, which includes the stem, chains of undifferentiated, and differentiating spermatogonia to type A_4 (Chiarini-Garcia *et al.*, 2003) and Sertoli cells, in atrophic seminiferous tubule cross-sections of irradiated rat testes at 1000× magnification (n=3-7/group). For samples with almost complete seminiferous tubule atrophy, cells were counted using systematic random sampling (Stereo Investigator version 8.0 software, MicroBrightField, Inc., Williston, VT), by counting A spermatogonia and Sertoli cells in 300 randomly selected 100 µm × 80 µm fields. In samples with few atrophic seminiferous

tubules, these tubules were identified visually using light microscopy, and all cells in the tubules were counted. A minimum of 500 Sertoli cells were counted per testis. Results were presented as A spermatogonia per 100 Sertoli cells.

Testicular sperm production was evaluated by counting sonication-resistant sperm heads, which represent nuclei of step 12–19 spermatids, in testicular homogenates. An aliquot of the homogenate of the left testis was sonicated, and the sperm heads were counted in a hemacytometer using phase contrast optics (Meistrich & van Beek, 1993).

Hormone Assays

Serum testosterone and interstitial fluid testosterone (IFT) concentrations were measured using a coated-tube radioimmunoassay kit (Coat-A-Count Total Testosterone, Cat No. TKTT1, Siemens, Los Angeles, CA) similar to procedures described previously (Porter *et al.*, 2006; Shetty *et al.*, 2000). Rat serum follicle-stimulating hormone (FSH) was measured by radioimmunoassay, and luteinizing hormone (LH) was measured by a sensitive two-site sandwich immunoassay. Both FSH and LH were measured by the University of Virginia, Center for Research in Reproduction, Ligand Assay and Analysis Core, using previously described methods (Gay *et al.*, 1970).

Statistical analysis

Results were presented as either mean ± SEM calculated from untransformed data or, in the case of sperm head counts, testosterone, and LH as the mean ± SEM calculated from log-transformed data obtained from individual rats. The statistical significance of differences between two groups was determined using the *t*-test with $P < 0.05$ being considered significant.

RESULTS

Spermatogenesis at 10 Weeks after Irradiation (Dose-Response)

To identify the doses that induce the declines in spermatogenic recovery and the accumulation of testicular interstitial fluid, which had been correlated with the block to recovery (Porter *et al.*, 2006), BN, SHR, and SD rats were given different ranges of doses of radiation depending on the sensitivity of the strain, and tissue was harvested 10 weeks later. Radiation reduced the testicular parenchymal weights in all the strains in a dose-responsive manner, with a steep initial decline, corresponding to the major phase of germ cell loss, followed by a shallower slope reaching 15–20% of control at high doses (Fig. 1A). The steep decline occurred in BN rats at doses below 3 Gy, but 5 to 6 Gy were required in SHR and SD rats to complete the steep decline.

As shown previously (Abuelhija *et al.*, 2012), there were large increases (~0.2 g) in testicular interstitial fluid in BN rats at 5 Gy of irradiation but small or negligible increases in SHR and SD rats. In all strains, the increases in interstitial fluid were dose-responsive (Fig. 1B). The increase reached a maximum at 3.3 Gy in BN, but 5.7 Gy and 10 to 11 Gy were required in SHR and SD, respectively, to reach maximal levels, which were less than that observed in BN.

To assess whether the radiation-induced decline in spermatogenesis was quantitatively different in the different strains, the dose-response of the recovery of spermatogenesis from surviving stem cells was assessed by the percentage of tubules showing differentiated cells (tubule differentiation index, TDI) in histological sections (Fig. 2A) and the numbers of late spermatids produced (Fig. 2B). Control rats showed differentiation in 100% of the tubules and 2×10^8 late spermatids per testis. Both parameters showed dose-responsive declines, with

the BN rats being most sensitive to irradiation, SHR showing intermediate sensitivity, and SD displaying the most resistance. It was noted that doses that reduced the TDI to between 30% and 60% of control reduced late spermatid counts to between 0.05% and 1% of control.

The atrophic tubules showing no differentiated cells at 10 weeks after irradiation were then examined to determine whether the absence of differentiated cells was a consequence of killing of all stem spermatogonia or a block in spermatogonial differentiation. In the sensitive BN strain, atrophic tubules were observed at all doses tested, but in the resistant strains they could only be observed after 5.0 Gy in SHR and after 6.5 Gy in SD rats. At the lower doses of radiation, atrophic tubules in all the strains contained between 2.5 and 2.8 type A spermatogonia per 100 Sertoli cells (Fig. 3A). Increasing the radiation exposure produced a dose-responsive reduction in the numbers of type A spermatogonia in the three strains resulting in about 0.5 type A spermatogonia per 100 Sertoli cells after 10 Gy of irradiation. This reduction suggests that stem cells were killed at these higher doses of radiation. However, the presence of some type A spermatogonia in atrophic tubules demonstrated that a block in spermatogonial differentiation (Meistrich & Shetty, 2003) also contributed to the failure of spermatogenesis to recover.

Recovery of Spermatogenesis after Irradiation (Time-Course)

To identify strains with permanent or reversible blocks in spermatogonial differentiation, we examined recovery at times longer than 10 weeks. In BN rats there was no significant histological recovery of spermatogonial differentiation between 10 and 20 weeks after irradiation with the doses ($\geq$3.3 Gy) that were tested (Fig. 4C). The numbers of late spermatids remained low ($\leq 10^5$) (Fig. 4E); values in the 10^4–10^5 range were occasionally observed despite the lack of histological evidence of differentiation in the testis and may have represented sperm heads retained in the testis. The lack of recovery can be attributed to a continued block in spermatogonial differentiation and not a loss of stem cells, as the numbers of type A spermatogonia did not show any decrease between weeks 10 and 20 (Fig. 3B)

In contrast, SHR rats showed progressive recovery at all doses up to 7.5 Gy. With doses up to 5 Gy, sperm production approached control levels by 15 weeks after irradiation (Fig. 4F). After higher doses, the percentage of tubules with differentiated cells was less than 7% at 10 weeks after irradiation, but steadily increased reaching 60% by 20 weeks after 6.5 Gy and by 30 weeks after 7.5 Gy (Fig. 4D). Although most tubules showed differentiation, it was generally only to the B spermatogonial or spermatocyte stages (Fig. 5). After 6.5 Gy appreciable differentiation to the round or late spermatid stages was observed in only one rat at 15 weeks (out of 4 examined) and one at 20 weeks (out of 5). After 7.5 Gy, few tubules progressed to the round spermatid stage and almost none to the late spermatid stage, indicating a block at a later stage of differentiation. Sperm production measured in the contralateral testis, surprisingly, appeared to slightly increase at 15 weeks after 7.5 Gy but then remained low at later times (Fig. 4F). Furthermore, after 7.5 Gy (Fig. 5B), there was no further histological recovery of spermatogenesis between 30 and 40 weeks.

Hormone analyses

To determine whether differences in testosterone or FSH levels might be related to the differential induction of the block in spermatogonial differentiation in the strains, hormone analyses were performed on the three strains of rats before and after irradiation. Serum testosterone showed a modest trend toward reduction at 10 weeks after irradiation in all strains (Fig. 6A), but this was only significant in SHR and SD rats. Interstitial fluid testosterone (IFT) levels were unaffected by the radiation in all 3 strains (Fig. 6B). Serum FSH levels significantly increased by about 2-fold 10 weeks after radiation in all strains

(Fig. 6C) as expected owing to the germ cell loss that occurs. LH levels (data not shown) also appeared to be elevated by irradiation.

The levels of serum testosterone, interstitial fluid testosterone, and serum FSH levels in SHR rats were significantly higher than the corresponding values in BN rats both in unirradiated rats and after nearly all dose (Fig.6) and time points (Fig. 7). The values in SD rats were generally intermediate between those of the other two strains (Fig. 6A–C). Although testosterone and FSH were previously shown to contribute to the spermatogonial differentiation block in LBNF1 rats (Shetty *et al.*, 2006), the greater sensitivity of BN rats than of SHR or SD to induction of a spermatogonial block by radiation cannot be attributed to higher levels of testosterone or FSH.

Suppression of hormone levels and spermatogenesis recovery

To confirm that the action of testosterone and/or FSH was involved in the radiation-induced block of spermatogonial differentiation in these strains, we examined the effects of hormone suppression on spermatogenic recovery in BN and SHR rats at different times after 7.5 Gy (Fig. 7) and at 10 weeks after 5 and 10 Gy (data not shown). Hormone suppression decreased serum testosterone to below the limits of detection in both strains (Fig. 7A). IFT levels were reduced in BN rats to ~1 ng/ml and were reduced even more in SHR rats (Fig. 7B). However, these residual levels of intratesticular testosterone would not be expected to have significant effects on spermatogenesis because the rats were also treated with flutamide. The suppressive treatment also markedly reduced serum FSH levels to about 1 ng/ml in all groups of rats (Fig. 7C) and reduced LH to undetectable levels (not shown).

Although hormonal suppression in control and treated rats markedly decreased testicular parenchymal weights to about 7% of control in both strains at the various dose and time points (Fig 8A,D), which was also evident by the decrease in tubule diameter (compare Fig. 9A and C), it induced differentiation in a high percentage of tubules in irradiated rats of both strains (Figs. 8B and 9C). In BN rats, irradiation with 5 Gy and above almost completely eliminated the differentiating spermatogenic cells (TDI < 2%); nevertheless, hormone suppression starting immediately after irradiation with 5 Gy restored the production of differentiated cells in 100% of tubules; however, with the low testosterone and FSH levels, differentiation could only proceed to the spermatocyte stage (Fig. 9D). There was incomplete recovery of spermatogonial differentiation at 10 weeks after 7.5 Gy, as only 88% of tubules showed differentiating cells, but recovery progressed with time so that by week 20 100% of tubules were differentiated (Fig. 8E). The higher dose of 10 Gy reduced the percentage of tubules showing differentiation at 10 weeks to 48%. In SHR rats, after the 7.5- and 10-Gy doses, which blocked all spontaneous recovery at 10 weeks after irradiation, hormonal suppression stimulated the production of differentiated cells in 100% and 90% of tubules, respectively.

Hormone suppression completely reversed the large increase in interstitial fluid accumulation observed in BN rats (Fig 8C,F). The modest increases in interstitial fluid observed in SHR rats after irradiation were also reversed by the hormone suppression.

DISCUSSION

The human testis is characterized by high sensitivity to and delayed recovery of spermatogenesis after moderate doses of radiation. Here we compared 3 rat strains and found that the BN rats were also very sensitive to the gonadotoxic effects of radiation, but they showed no recovery of spermatogenesis. SD rats displayed the most resistance to radiation as high doses were required to produce severe gonadotoxic effects. However, SHR rats showed marked and prolonged gonadotoxic effects to doses of about 6 Gy, and may

indicate that Wistar-derived rats might have some of the sensitivity characteristics similar to human testes.

The present study clarifies the question of whether the data of our previous study (Abuelhija *et al.*, 2012), showing that at 10 weeks after 5-Gy irradiation there was no recovery of spermatogenesis in BN rats, whereas in SHR and SD rats nearly all of the tubules contained differentiating germ cells, are a result of qualitative or quantitative differences between the strains. The dose-response studies showed that at 10 weeks after irradiation, BN rats failed to show recovery even after low doses (4 Gy) (Fig. 2), similar to the sensitivity described previously in LBNF1 rats (Kangasniemi *et al.*, 1996b), which are F1 hybrids of Brown-Norway and Lewis, another very sensitive strain (Abuelhija *et al.*, 2012). At these low doses, the atrophic tubules are almost exclusively due to a block in spermatogonial differentiation, as type A spermatogonia were present and their numbers were maintained in the atrophic tubules. In the resistant SHR and SD strains, low to intermediate doses ($\leq$5 Gy) did not produce a significant block in spermatogonial differentiation. However higher doses of irradiation induced radiation-induced blocks in spermatogonial differentiation, similar to that observed in BN rats at the lower doses, in SHR and SD rats after 6.5 Gy and 8 Gy, respectively. Thus the major contribution to the differences in recovery of spermatogenesis between strains is the quantitative difference in their sensitivities to a radiation-induced block of spermatogonial differentiation.

The time-course studies addressed whether this block was reversible at later times in the different strains. The block was not reversible at all between 10 and 20 weeks in BN rats even at doses as low as 3.3 or 4 Gy (Fig. 4A). Based on results with LBNF1 rats, which were followed for 60 weeks to demonstrate the permanence of the block, we suggest that no recovery will occur with BN rats even after longer periods of time (Kangasniemi *et al.*, 1996b). Furthermore, in LBNF1 rats, the incomplete block produced at 3.5 Gy became even more severe between 10 and 60 weeks, with spermatogonial differentiation steadily declining to a complete block; a similar decline occurred in BN rats after 3.3 Gy (Fig. 4C).

In contrast in the more resistant SHR rats, doses above 5 Gy were required to produce a block in spermatogonial differentiation at 10 weeks after irradiation. This block that was observed after doses of 6.5 and 7.5 Gy was reversible, as demonstrated by the progressive increase in the number of spermatogonia in the atrophic tubules at 15 and 20 weeks (Fig. 3B), and in differentiating tubules at 20 and 30 weeks after irradiation (Fig. 4D). Thus there is a qualitative difference between strains, as the more resistant strains, like SHR, showed a delayed but progressive recovery of spermatogonial differentiation, whereas the block in spermatogonial differentiation in the sensitive strains like BN and LBNF1 was permanent.

Although the block in spermatogonial differentiation in SHR rats was reversed at later post-irradiation times, there still was a prolonged decrease in spermatogenesis as exemplified by the reduction in testis weights, later differentiated cells, and sperm production at doses >5 Gy (Figs. 4B,F, 5). The reversible, but incomplete recovery in the SHR strain appears to be similar to that previously reported in SD rats (see Introduction). In SHR rats, the presence of appreciable numbers of tubules containing B spermatogonia and spermatocytes at 20 weeks after 7.5-Gy irradiation, but almost no spermatids at weeks 30 and 40, demonstrates that the absence of late stage germinal cells is a result of a decreased efficiency or even a block in development to later differentiation steps, and not just a result of the delay in the initiation of spermatogonial differentiation. It is highly unlikely that these differentiated germ cells were arrested in development since the spermatogonia were mitotically active and, when present, the later cells were arranged according to the stages of the cycle of the seminiferous epithelium. We believe that a damaged somatic environment, as previously observed to produce the block in spermatogonial differentiation in LBNF1 rats (Zhang *et al.*, 2007), is

unable to properly support spermatogenic cell differentiation. Hence the recovery observed between 15 and 30 weeks may be due to restoration of a favorable somatic environment, like that which occurs when hormones are suppressed, but a mechanism for this spontaneous recovery is not known.

Since doses of ≥5.7 or 6.5 Gy were necessary to produce a block in spermatogonial differentiation in resistant strains like SHR and SD, respectively (Fig. 2A), the possible role of stem cell killing could also be considered as a cause of the atrophic tubules at higher doses. But as the numbers of type A spermatogonia were still maintained in the atrophic tubules at doses up to 5.7 Gy or 6.5 Gy for SHR and SD respectively (Fig. 3A), the block in spermatogonial differentiation must be the principal cause of tubular atrophy at these doses. However at higher doses, there was a decline in the numbers of A spermatogonia (Fig. 3A), suggesting that stem cell killing is also a cause of tubular atrophy, but cannot be the only cause since A spermatogonia were still observed. These results are consistent with direct counts of isolated type A spermatogonia, the putative stem cells, in SD rats, which indicated that although there was a transient loss of these cells after doses as low as 2 Gy, 6 Gy was required to cause a more prolonged loss of the these stem cells for 26 days (Erickson, 1976).

The block in spermatogonial differentiation in irradiated LBNF1 rats was previously shown to be mediated by the action of testosterone and also to some extent by FSH (Shetty *et al.*, 2006). This inhibitory action of the hormones is in contrast to the situation in normal rats, in which spermatogonial differentiation is qualitatively independent of both testosterone and FSH (Huang & Nieschlag, 1986). Here we show that hormones were also responsible for the spermatogonial block in BN rats, as the production of differentiated cells in all tubules could be restored by hormone suppression for 10 weeks after 5-Gy irradiation (Fig. 8B). The lack of sensitivity of SHR or SD rats to the radiation-induced block in spermatogonial differentiation at 5 Gy cannot be a result of lower levels of testosterone and FSH since irradiated rats of these strains actually had higher levels of these hormones than did BN (Fig. 6), or of the absence of the hormone-dependence of the block in spermatogonial differentiation, which was demonstrated in 7.5-Gy irradiated SHR rats (Fig. 8B). Thus the differences between the sensitive and resistant strains appear to be a result of differences in the dose required to render the testis sensitive to this block of spermatogonial differentiation.

Another factor that may be involved in this block in spermatogonial differentiation appears to be the accumulation of testicular interstitial fluid as irradiation of LBNF1 rats dramatically increased testicular interstitial fluid at the time the block in spermatogonial differentiation occurred and hormonal treatments to restore spermatogonial differentiation reduced interstitial fluid (Porter *et al.*, 2006) However, other sensitive (Lewis) or intermediate (Wistar-Kyoto) strains also had low interstitial fluid accumulation, indicating that fluid accumulation could not be the cause of the block in those strains (Abuelhija *et al.*, 2012). In the present study dose-responsive increases in interstitial fluid levels (Fig. 1B), although lower in magnitude in SHR and SD than in BN, occurred in all 3 strains at doses corresponding to the decline in spermatogonial differentiation (Fig. 2A). This result and the reversal of the radiation-induced fluid increase in BN and SHR rats with hormone suppression (Fig. 8C,F) further support a correlation between increases in interstitial fluid and the block in spermatogonial differentiation in these strains.

Finally, the demonstration of a delay in the recovery of spermatogonial differentiation (15–20 weeks) in SHR rats irradiated with 7.5 Gy (Fig. 4D) and an even longer delay in the production of late spermatids in the testis (30–40 weeks) appears to provide a rat model for the prolonged delays in recovery of human spermatogenesis after radiation and other cytotoxic exposures. The relative roles of stem cell renewal/spermatogonial differentiation, which show differences between rodents and primates (Ehmcke & Schlatt, 2006), and the

changing ability of the somatic environment to support spermatogenic cell differentiation in the delayed recovery phenomenon is not known. Further studies at longer times are needed in SHR, other Wistar-derived strains, or SD rats (Pinon-Lataillade *et al.*, 1991) to determine if the recovery continues to progress and will lead to increases in epididymal sperm counts and to investigate the mechanisms underlying the delay or block.

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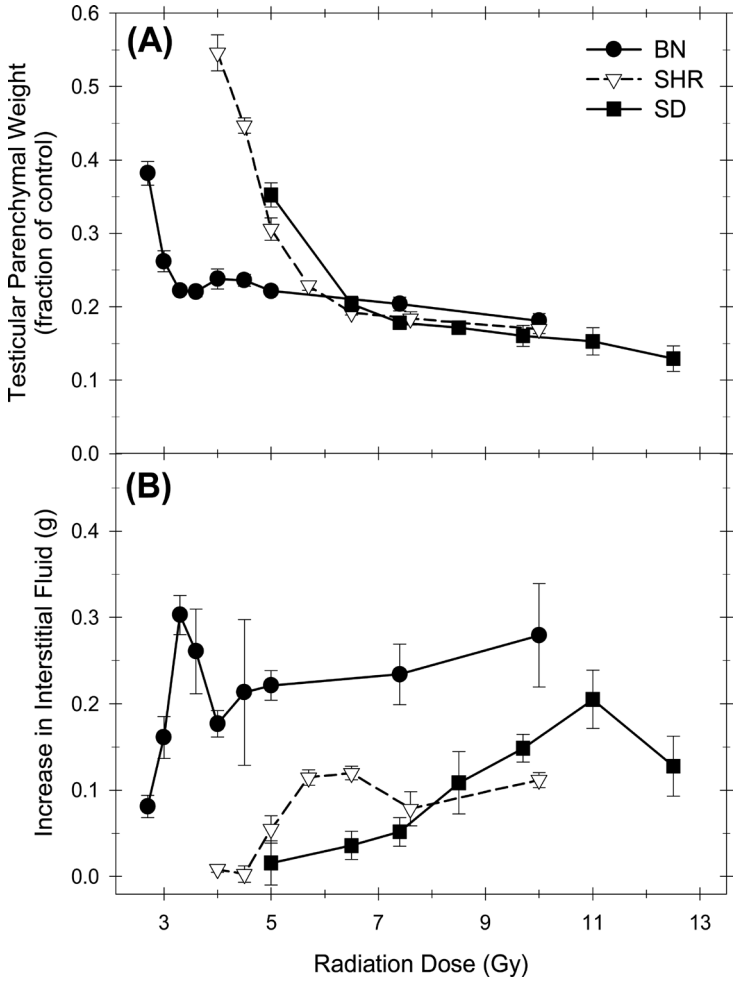

FIG 1.
Weights of testis parenchymal tissue and interstitial fluid of BN, SHR and SD rats 10 weeks after irradiation. (A) Testis weights relative to those of unirradiated controls of same strain. Control values were 1.50 g, 1.27 g, and 1.66 g for BN, SHR, and SD, respectively. (B) Increase in interstitial fluid weights from unirradiated control levels.

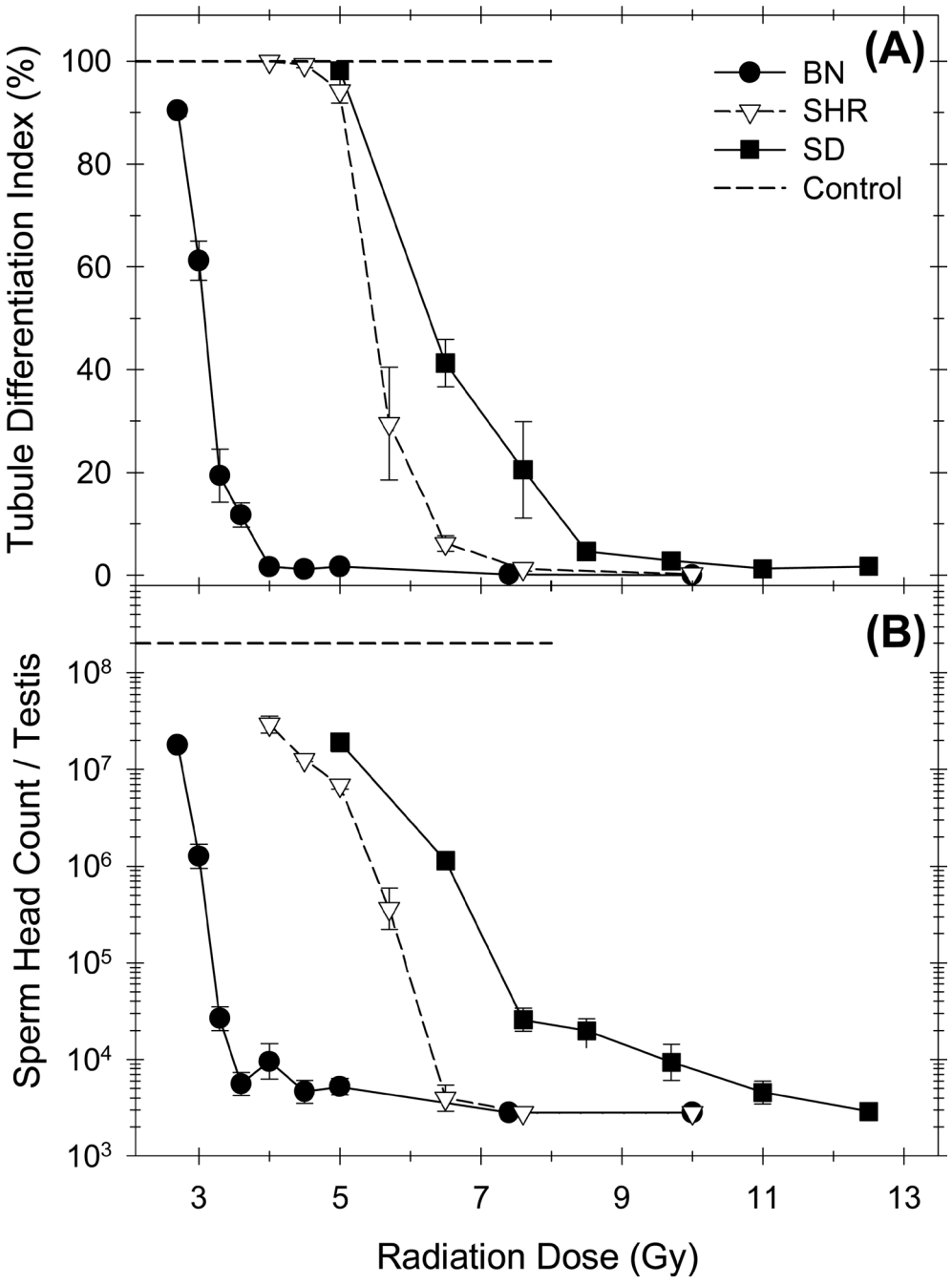

FIG 2.
Recovery of spermatogenesis at 10 weeks after various doses of radiation. (A) Tubule differentiation index (TDI), defined as percentage of tubules differentiating to the B spermatogonial stage or beyond. (B) Testicular sperm production: numbers of sonication-resistant late spermatids per testis. The dashed lines indicate the control values.

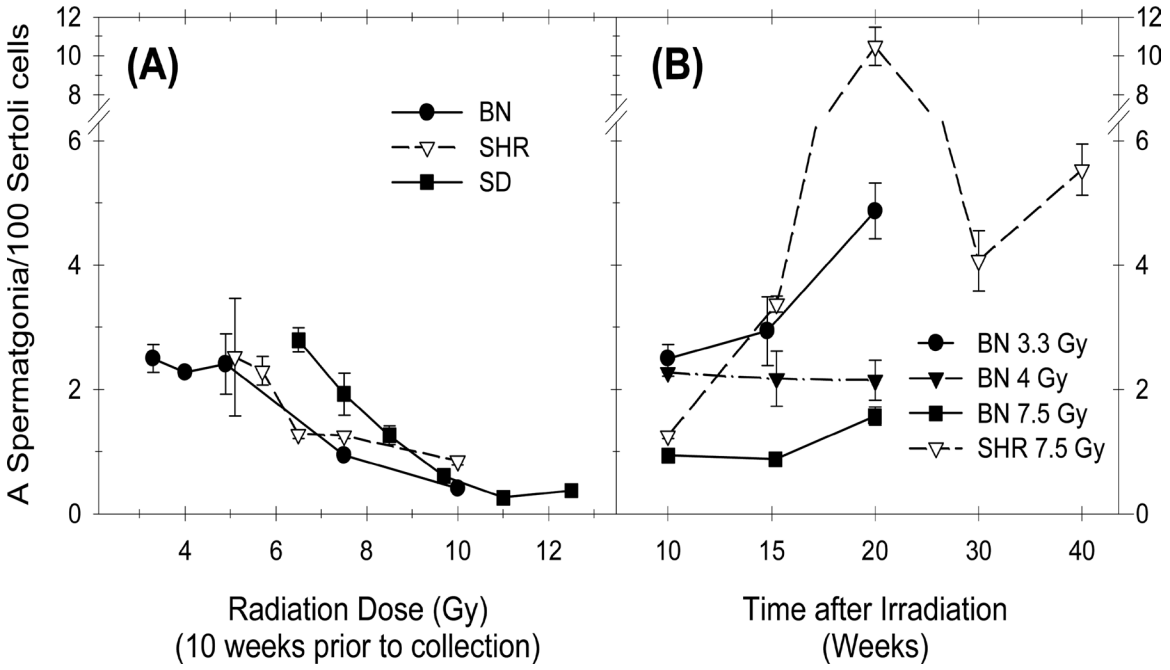

FIG 3.
Numbers of type A spermatogonia per 100 Sertoli cells in nonrepopulating tubules of (A) BN, SHR, and SD rats 10 weeks after irradiation (dose-response), and (B) BN and SHR rats at longer periods of time after different doses of irradiation (time course).

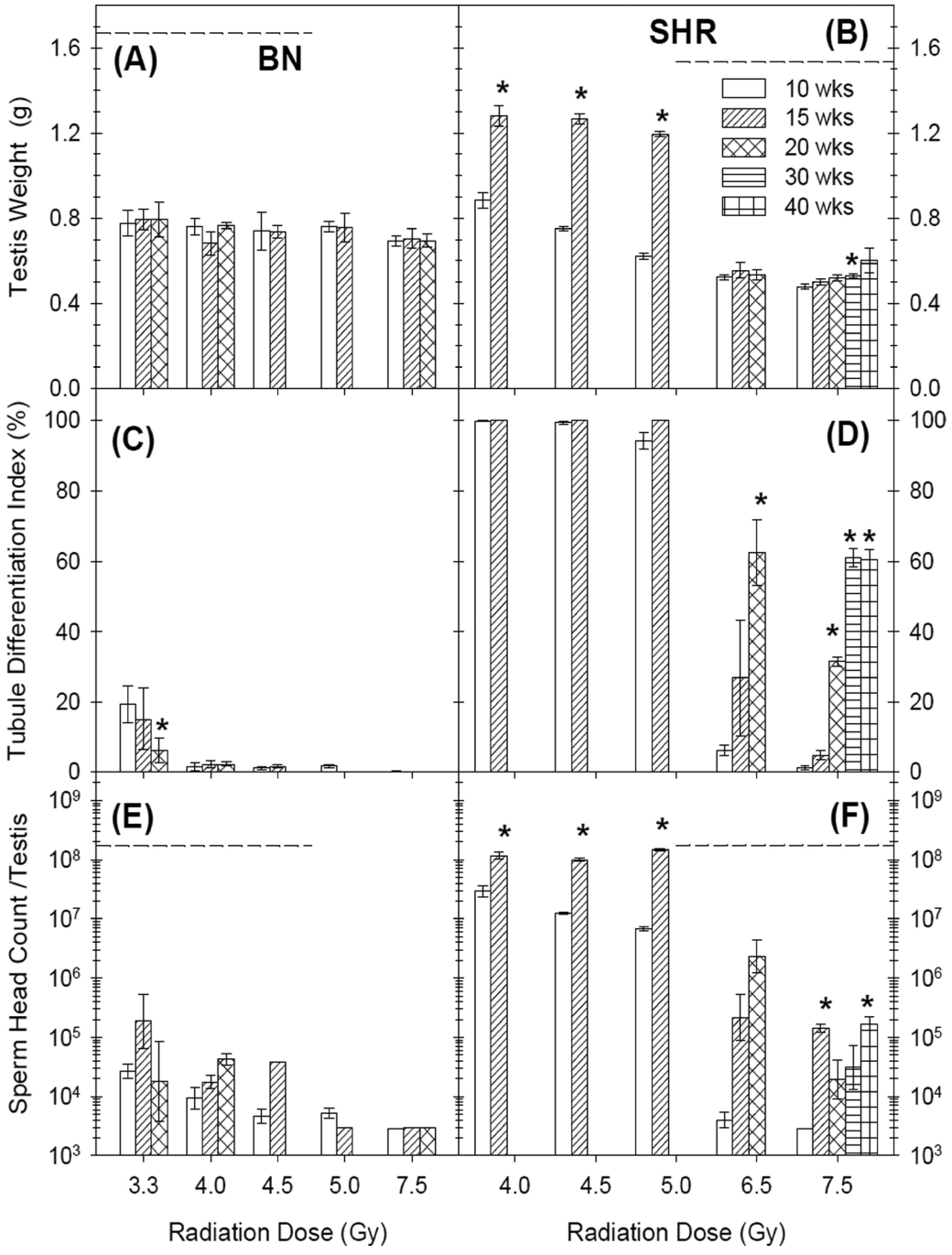

FIG 4.
Time courses of changes in (A,B) absolute testis weights, (C,D) tubule differentiation indices, and (E,F) sperm head counts of BN (A,C,E) and SHR (B,D,F) rats after different doses of radiation. The dashed lines indicate the control values. (*) indicates significantly different from value at 10 weeks ($P<0.05$, *t*-test).

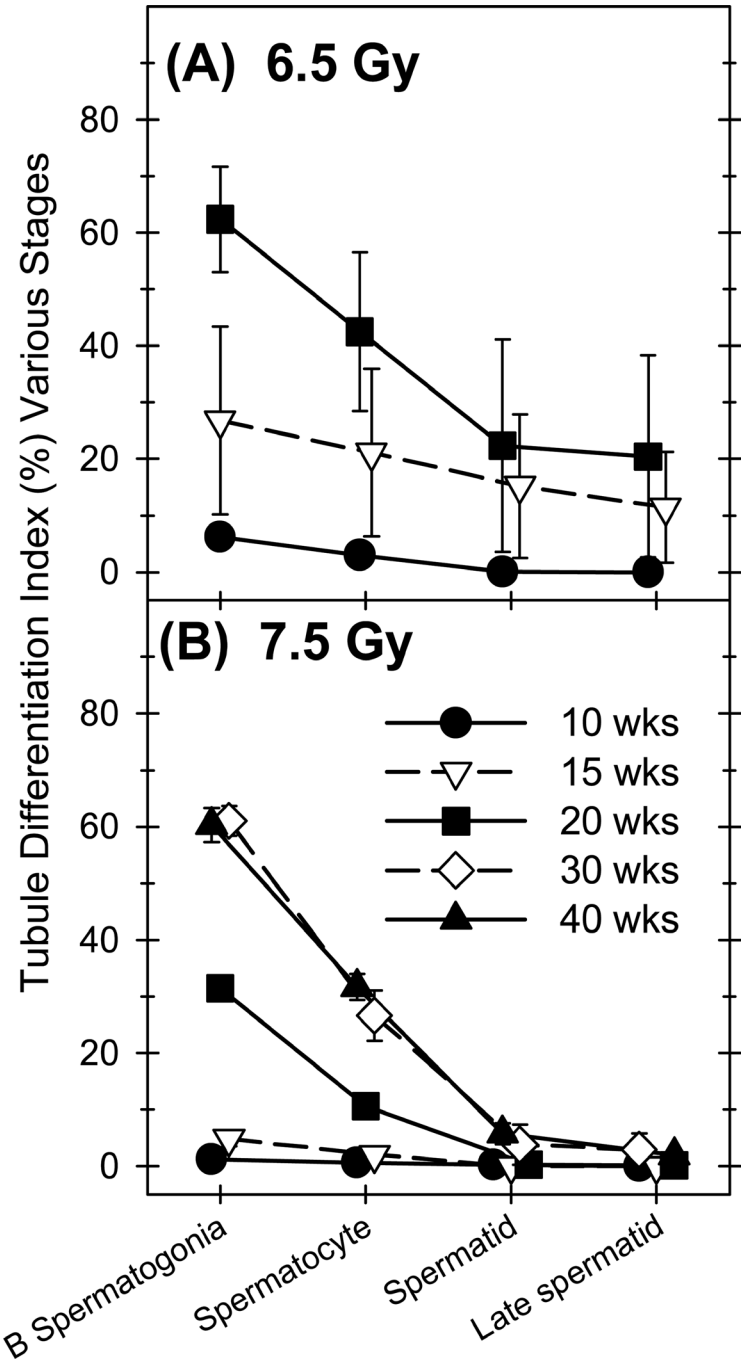

FIG 5.
Recovery of progression of spermatogenesis as measured by the percentage of tubules with morphologically differentiated cells reaching indicated stage of differentiation or beyond for SHR rats at various times after (A) 6.5 Gy or (B) 7.5 Gy.

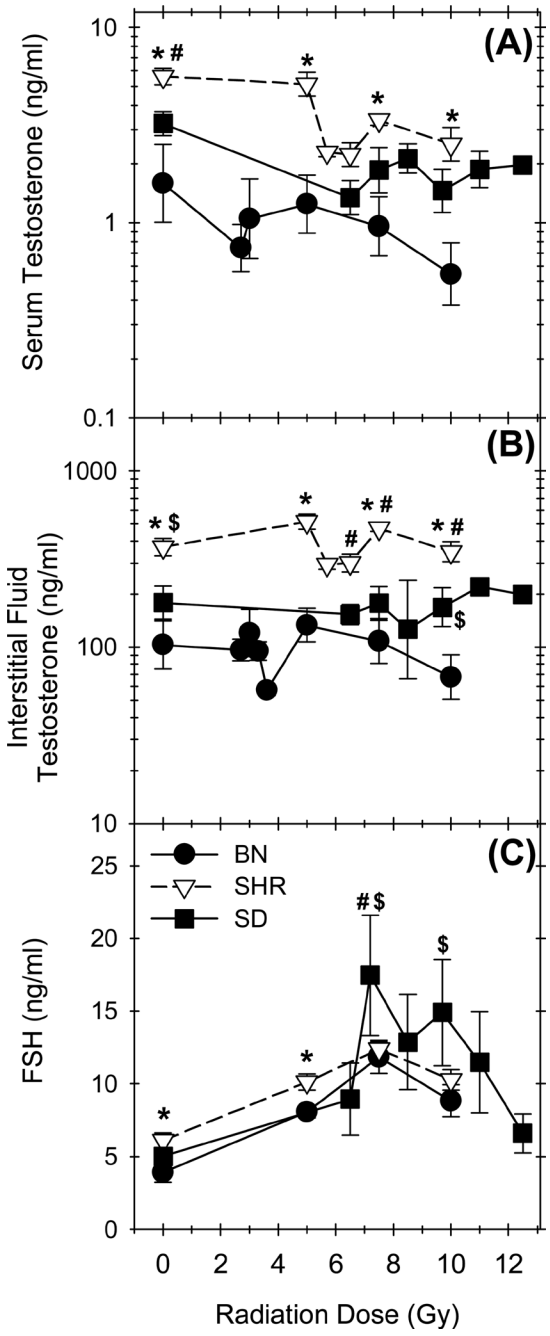

FIG 6.
Hormones levels in BN, SHR, and SD rats measured 10 weeks after different doses of radiation. (A) Serum testosterone. (B) Intratesticular fluid testosterone. (C) Serum FSH. (*) indicates values in SHR are significantly different from those in BN. (#) indicates values in SHR are significantly different from those in SD. ($) indicates values in SD are significantly different from those in BN ($P<0.05$, t-test).

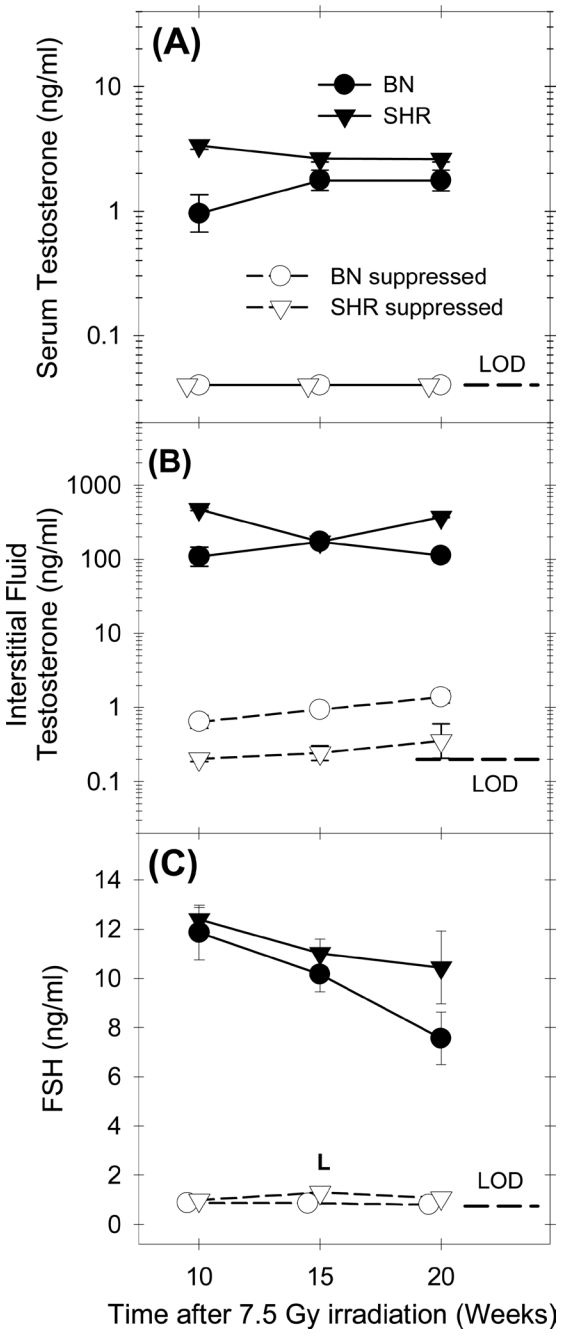

FIG 7.
Hormone levels in BN and SHR rats without hormone suppression (filled symbols) and after hormone suppression (open symbols) at different times after 7.5 Gy irradiation. (A) Serum testosterone. (B) Intratesticular fluid testosterone. (C) Serum FSH. LOD indicates limit of detection of the assay, (L) Indicates undetectable values of some but not all samples in the group.

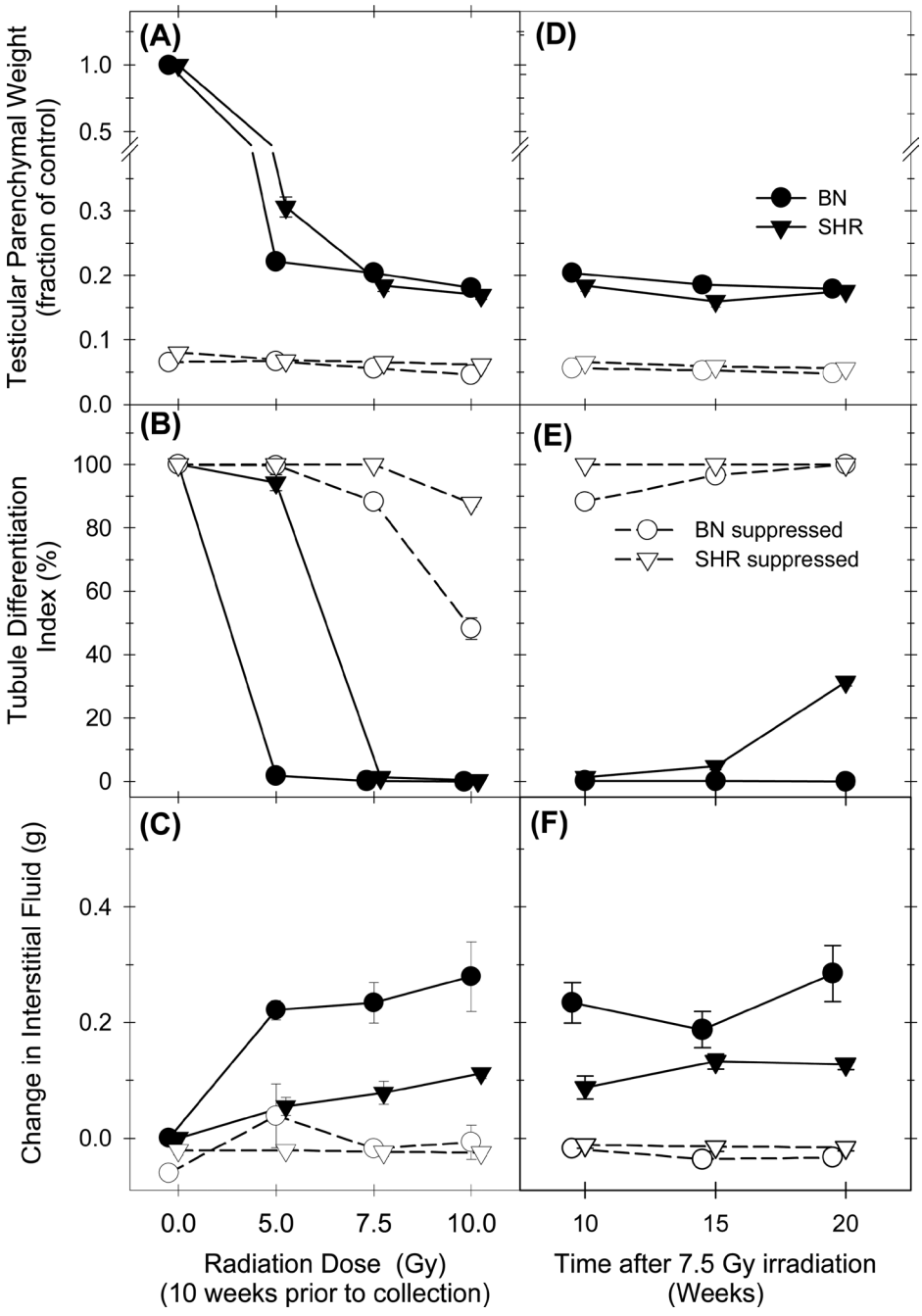

FIG 8.
Dose-response and time-course of changes in testis weights, differentiation in tubules, and interstitial fluid in BN and SHR rats without hormone suppression (filled symbols) and after hormone suppression (open symbols). (A,D) Testis weights relative to unirradiated controls of same strain. (B,E) Percentage of tubules with differentiated cells. (C,F) Change in interstitial fluid weights from unirradiated control levels.

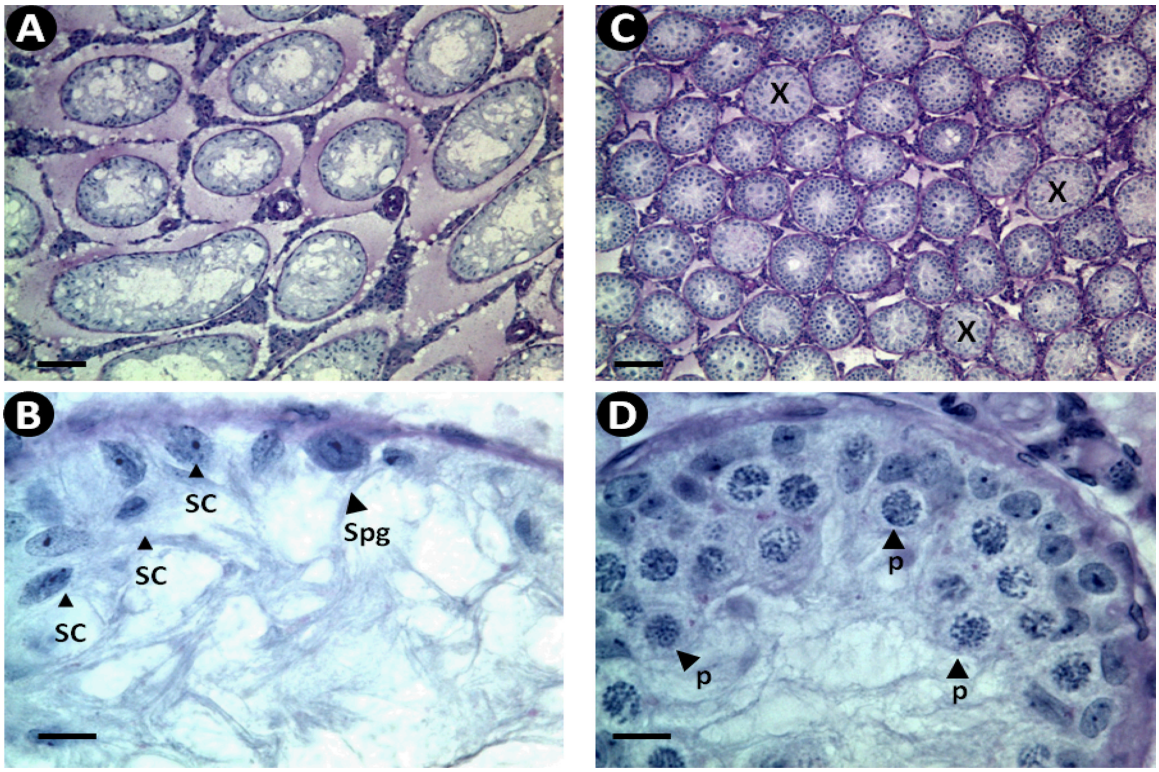

FIG 9.
Histology of BN rat testes 10 weeks after irradiation with 7.5 Gy without (A,B) or with (C,D) hormone suppression. (A) Irradiation produced atrophic tubules and interstitial edema. (B) Most tubules contained only Sertoli cells (SC) but some contained a few type A spermatogonia (Spg). (C) Hormone suppression after irradiation induced recovery of spermatogenesis in nearly all tubules, except those marked with. (X). (D) The recovering tubules showed development to only the pachytene spermatocyte stage (p). (A,C) bar: 100 µm, (B,D) bar: 10 µm.

Table 1

Radiation Doses, Analysis Times, and Strains Analyzed

Rat Strain	Doses (in Gy) used and time of tissue harvest after irradiation				
	10 weeks	15 weeks	20 weeks	30 weeks	40 weeks
BN	2.7, 3.0, 3.3, 3.6, 4.0, 4.5, 5.0, 7.5, 10.0	3.3, 4.0, 4.5, 5.0,7.5,	3.3, 4.0, 7.5		
SHR	4.0, 4.5, 5.0, 5.7, 6.5, 7.5, 10.0	4.0, 4.5, 5.0, 6.5, 7.5	6.5, 7.5	7.5	7.5
SD	5.0, 6.5, 7.5 8.5, 9.7, 11.0, 12.5				

Inhibition of Spermatogonial Differentiation by Testosterone

Review

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In this review we describe a variety of pathological conditions in rodents that result in seminiferous tubule atrophy, and which are characterized by the absence of all germ cells except for type A spermatogonia. In many such cases, suppression of gonadotropins and testosterone with gonadotropin-releasing hormone (GnRH) analogues restores spermatogonial differentiation and spermatogenic progression. In some cases, spermatogenesis is maintained after the cessation of hormonal treatment and fertility is restored. We and others have shown that the hormones normally responsible for the maintenance of spermatogenesis—testosterone, and in some cases, follicle-stimulating hormone (FSH)—actually inhibit spermatogonial differentiation in these conditions. This inhibitory function is a completely new role for androgens in the testis. It has long been known that systemic administration of low levels of testosterone can inhibit the completion of spermatogenesis (Steinberger, 1971) as a result of decreasing gonadotropin levels, thereby reducing testosterone production by the Leydig cells and actually reducing intratesticular testosterone (ITT) concentrations. In the pathological conditions we and others have described, however, the ITT concentration is not reduced and it is responsible for the inhibition of spermatogonial differentiation.

As will be described later, it is not clear whether the direct action of testosterone is to block an actual step of differentiation of the spermatogonia or to cause the apoptosis of the spermatogonia prior the step at which they would differentiate. However, throughout this review we will use the concept of ''inhibition of spermatogonial differentiation'' to encompass both possibilities.

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Blocks of Spermatogonial Differentiation

The stem spermatogonia, designated A_s, maintain their numbers by self-renewal, and some differentiate to form by sequential divisions A_{pr} (A-paired), and A_{al}-4 and A_{al}-8 (A-aligned) spermatogonia, which go on to produce A_1 spermatogonia.

This differentiation may be blocked in 3 ways. In one way, which is the focus of this review, undifferentiated spermatogonia proliferate but their numbers remain relatively constant because of apoptosis (Figure 1) (Allard and Boekelheide, 1996; Shuttlesworth et al, 2000). We will call this the proliferation-apoptosis (PAp) block to distinguish it from the other 2 ways. The second type of block in spermatogonial differentiation, which is caused by vitamin A deprivation, is characterized by spermatogonial differentiation to the A_{al} stage, but then proliferation ceases and spermatogonia can remain at this stage for a period of only several weeks (van Pelt and de Rooij, 1990) and is designated Ar (arrest). In the third kind of block, which is observed in several types of transgenic mice, including *bax*-deficient, or *bcl-2*-overexpressing, or glial cell line-derived neurotropic factor (GDNF)-overexpressing mice (Knudson et al, 1995; Furuchi et al, 1996; Meng et al, 2000), type A spermatogonia proliferate and accumulate but produce few differentiated cells, and is designated proliferation-accumulation (PAc).

The precise relationship between these 3 blocks in rodents and the clinical phenotype of spermatogonial arrest in humans, which is often the result of hypogonadotropism (Johnsen, 1970), is not known. However, the types of spermatogonia present in humans and their proliferative status have not been studied.

Conditions Causing the Proliferation-Apoptosis Block in Spermatogonial Differentiation

A variety of testicular toxicants produce similar testicular histology in rats consistent with the PAp type of block. These agents include hexanedione (Boekelheide and Hall, 1991), boric acid (Ku et al, 1993), radiation (Kangasniemi et al, 1996), procarbazine (Meistrich, 1999), dibromochloropropane (DBCP; Meistrich, unpublished results), and indenopyridines (Hild et al, 2001). The type A spermatogonia proliferate in atrophic tubules but they do not accumulate because they continue to be lost by apoptosis many months after the original acute or subchronic exposure. Atrophic tubules with actively dividing stem type A spermatogonia were also observed in testis cross-sec-

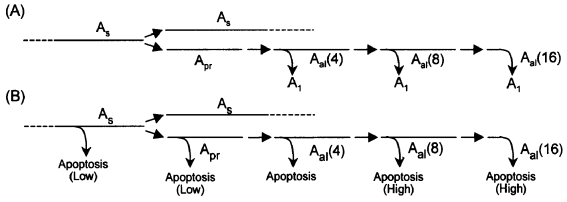

Figure 1. Outline of stem cell kinetics in **(A)** normal rodents and **(B)** rodents with a proliferation-apoptosis block in spermatogonial differentiation as described for toxicant-treated rats, cryptorchid mice, and some mutant mice. In normal rodents, no apoptosis is observed at these stages, and the A_{al} and some A_{pr} spermatogonia are induced to undergo differentiation into A_1 spermatogonia at stage VII–VIII of the cycle of the seminiferous epithelium. In the rodents with the PAp block, spermatogonia of all clonal sizes undergo apoptosis, with the probability of undergoing an apoptotic event, as opposed to a mitotic division, increasing with chain length.

tions from 27-month-old Brown-Norway rats (Schoenfeld et al, 2001). The failure of these cells to differentiate is in part responsible for the decline in spermatogenesis with age in these rats.

In contrast to that of rats, brief exposures to such toxicants do not induce such a block in spermatogonial differentiation in mice. Whereas 3.5 Gy of irradiation was sufficient to induce this block in $LBNF_1$ rats, mouse spermatogonia maintain their ability to differentiate even after doses of 12 Gy (Meistrich et al, 1978). But a block in spermatogonial differentiation can be induced by continuous elevation of temperature. In cryptorchid C57BL/6 mouse testes, spermatogenesis fails to progress past the A_{al} spermatogonial stage (Haneji et al, 1983); these cells actively proliferate but die by apoptosis (de Rooij et al, 1999). Similar blocks in the differentiation of A spermatogonia were also observed in *jsd* (juvenile spermatogonial depletion) mice (Beamer et al, 1988) and in Sl^{17H} mice, which have an altered form of stem cell factor in Sertoli cells (Brannan et al, 1992). In these mice, an initial wave of spermatogenesis is not maintained, so that the adult testis tubules contain only Sertoli cells and type A spermatogonia; the latter proliferate but die by apoptosis (de Rooij et al, 1999). In addition, certain other mouse mutants, including XO-Sxr^b (Sutcliffe and Burgoyne, 1989) and *Dazl* (Schrans-Stassen et al, 1999), have PAp blocks in spermatogonial differentiation but they differ from the above 2 models in that this condition is apparent by postnatal day 10 and there is no initial wave of spermatogenesis.

It was indeed surprising that such a wide variety of toxicant exposures, conditions, and genetic mutations produced such a similar phenotype. For example, some of these toxicants, such as irradiation, are believed to act directly on germ cells (Lee et al, 1999), whereas other toxicants, such as hexanedione, are believed to act on Sertoli cells. Furthermore, at least in some instances, the block to spermatogonial differentiation does not begin to develop for almost 6 weeks after the insult. Both these observations imply that the block to spermatogonial differentiation is not a direct consequence of the initial event, but that different initiating events produce a common outcome, which in turn, leads to the block.

Characterization of the Proliferation-Apoptosis Block of Spermatogonial Differentiation

Although the PAp blocks in spermatogonial differentiation caused by different agents have much in common (Figure 1), they show some quantitative differences in terms of the stage to which spermatogonia differentiate before undergoing apoptosis.

The type A spermatogonia in atrophic testes were first identified following exposure of either Sprague-Dawley or Fischer F344 rats to hexanedione (Boekelheide and Hall, 1991). Stem cells (isolated type A spermatogonia), although reduced in number from controls, still constituted a substantial proportion of these remaining A spermatogonia (Allard et al, 1995). The A spermatogonia were in active proliferation, but their numbers remained constant because they underwent apoptosis (Allard and Boekelheide, 1996). Calculations based on numbers of stem cells and total spermatogonia indicated that the cells were progressing to the A_2 or A_3 spermatogonial stage (Allard and Boekelheide, 1996).

In contrast, direct, whole-mounted tubule analysis of mitotic clones of A spermatogonia in irradiated $LBNF_1$ rats revealed that most of the clones were isolated or paired A spermatogonia and few had a clone size greater than 4, indicating that they were early progeny from the stem cells (Shuttlesworth et al, 2000). Very few clones progressed to become A_{al}-8 and A_{al}-16, which are the clone sizes that most often undergo differentiation in normal rats (Figure 1A) because the probability of apoptosis increased as clone size increased (Figure 1B). Thus, failure of spermatogonia to differentiate appeared to be a consequence of their undergoing apoptosis first.

In *jsd,* Sl^{17H}, and cryptorchid mice, the clones of A spermatogonia in whole-mounted tubules were arranged as 1 to 16 cells (de Rooij et al, 1999). There were appreciable and similar numbers of clones of A_{al}-8 and A_{al}-16 in all 3 models. These undifferentiated A spermatogonia were proliferating, but they did not accumulate, and the larger clones in particular underwent apoptosis. Because the clone sizes indicate that spermatogonia develop to the point at which the A_{al} cells should differentiate into A_1 spermatogonia, the failure to do so indicates the lack of a signaling system rather than prior apoptosis.

The difference in numbers and stage of development of spermatogonia between the irradiated rat and the mouse models appears to be real because the same methodology was employed. It is not known whether these differences are due to how mice and rats respond to

blocks at the spermatogonial level or whether differences in the cause of the blocks. The difference in stage at which the block was reported to occur in irradiated vs hexanedione-treated rats could be a result of the different analytical methods employed, in addition to the possible contributions of rat strain or the nature of the original toxic insult.

Hormone Levels During the Proliferation-Apoptosis Block to Spermatogonial Differentiation

As is typical in cases of testicular tubular atrophy, FSH and luteinizing hormone (LH) levels rise in most cases in which only type A spermatogonia remain in the tubules. FSH levels were elevated 1.5-fold to 2-fold and LH levels were elevated 2-fold to 4-fold after treatment of rats with hexanedione (Boekelheide and Hall, 1991), gamma radiation (Kangasniemi et al, 1996), procarbazine (Meistrich et al, 1999), indenopyridine (Hodel and Suter, 1978), boric acid (Ku et al, 1993), and DBCP (Meistrich, unpublished results) and in *jsd* mice (Shetty et al, 2001).

In all the cases studied, serum testosterone remained unchanged. It has been shown that when the germ cells in the testes are lost, testicular mass and, consequently, blood flow decline (Wang et al, 1983). The maintenance of serum testosterone levels is a result of the hypothalamic-pituitary axis acting to keep serum testosterone constant when there is a decline in testicular blood flow by adjusting LH levels accordingly. This results in a 2.5-fold to 3-fold increase in ITT concentrations, which was confirmed in irradiated, procarbazine-treated, and DBCP-treated rats and *jsd* mice. The greater proportion of Leydig cells (their numbers are not decreased) in the testis, the decreased clearance rate of newly produced testosterone from the testis, and the elevated LH levels are all responsible for the increase in ITT concentrations.

There were 2 exceptions to this pattern of hormone changes. First, in cryptorchid mice, FSH was elevated 1.5-fold, but LH was unchanged (Mendis-Handagama et al, 1990). Second, in aged rats, both serum and testicular interstitial fluid testosterone levels were depressed (Schoenfeld et al, 2001). This depression in testosterone levels may be a combined result of the general depression with aging in LH and Leydig cell function, which can no longer respond by increasing testicular testosterone production. Nevertheless, these results show that above normal levels of ITT are not necessarily required for inhibition of spermatogonial differentiation, which will be discussed later.

GnRH Analogue Treatment Reverses Proliferation-Apoptosis Blocks in Spermatogonial Differentiation

We first demonstrated the stimulation of recovery of spermatogenesis in rats using hormone treatment given after irradiation (Meistrich and Kangasniemi, 1997). All previous studies had focused on the possible protective effect of giving the suppressive hormones before the toxicant exposure (Ward et al, 1990). However, we ruled out many possible mechanisms (Meistrich et al, 1997) by which the hormone treatment could have protected the survival of the spermatogonia and concluded that the only explanation that fit the data was that the hormonal treatment given before the toxic insult helped somatic cells to support sustained recovery of spermatogenesis from surviving stem cells after the toxicant exposure (Meistrich et al, 2000). In all subsequent work we have focused on giving the GnRH analogue treatment after toxicant exposure, although others have given the hormones before and after the toxicant.

In our initial study (Meistrich and Kangasniemi, 1997), the tubule differentiation index (TDI; the percentage of tubule cross-sections containing differentiated cells) was only 37% at 10 weeks after 3.5 Gy irradiation in the absence of hormone treatment. When GnRH agonist treatment was started immediately after irradiation, the TDI at 10 weeks was dramatically increased to 91%. However, because GnRH analogue treatments suppress testosterone, which is required for spermatid differentiation, there is histological recovery to the round spermatid stage, but no sperm are produced. The production of sperm after cessation of a transient GnRH analogue block will be discussed below. We also showed that systemic exogenous administration of testosterone, which suppresses ITT concentrations, also maintains spermatogonial differentiation after irradiation.

In other cases involving a toxicant-induced PAp block to spermatogonial differentiation (Table 1) maintenance or recovery of spermatogenesis was enhanced by giving GnRH analogues after the toxicant treatment (Table 2). These include hexanedione, procarbazine, or DBCP.

In some other cases, GnRH analogue treatment has also proved beneficial to the maintenance or recovery of spermatogenesis after exposure to a toxicant for which the blocks in spermatogonial differentiation were not well characterized. Treatment with GnRH agonist for about 12 weeks after exposure to the anticancer agent busulfan significantly increased the TDI at week 18 (Udagawa et al, 2001). However, a 4-week hormone treatment prior to busulfan injection was ineffective. The irreversible loss of spermatogenic function that occurred after a single dose of heat to rat testes was likewise counteracted by GnRH analogue posttreatment (Setchell et al, 2001), and treatment with GnRH agonist before heating was also effective (Setchell et al, 2002). Finally, prevention of the indenopyridine-induced block to spermatogonial differentiation was achieved when GnRH analogues were given both before and after drug treatment (Hild et al, 2001). However, in a subsequent study using a GnRH antagonist, only prior, but not subsequent, treatment with the GnRH

Table 1. *Examples of pathological conditions causing a proliferation-apoptosis block in spermatogonial differentiation in rodents*

Species	Treatment* or Gene	Duration of Exposure	Reference
Rat	Hexanedione	Subchronic (5 wk)	(Boekelheide and Hall, 1991)
Rat	Radiation (gamma)	Acute	(Kangasniemi et al, 1996)
Rat	Radiation (neutron)	Acute	(Wilson et al, 1999)
Rat	Procarbazine	Acute	(Meistrich 1999)
Rat	Indenopyridine (CDB-4022)	Acute	(Hild et al, 2001)
Rat	Boric acid	Subchronic (9 wk)	(Ku et al, 1993)
Rat	DBCP	Acute	Meistrich, unpublished
Rat	Aging	Chronic	(Schoenfeld et al, 2001)
Mouse	*jsd* mutation	Permanent (genetic)	(Beamer et al, 1988; de Rooij et al, 1999)
Mouse	Heating (cryptorchid)	Chronic	(Nishimune et al, 1978; de Rooij et al, 1999)
Mouse	*Sl*^{17H} mutation	Permanent (genetic)	(Brannan et al, 1992; de Rooij et al, 1999)
Mouse	X*Sxr*^{b}O (*Eif2s2y* mutation)	Permanent (genetic)	(Sutcliffe and Burgoyne, 1989)
Mouse	*Dazl* −/− mutation	Permanent (genetic)	(Schrans-Stassen et al, 2001)

* In all cases, except for indenopyridine treatment, spermatogonia proliferated but were lost by apoptosis.

analogue was effective at restoring recovery of spermatogenesis following indenopyridine treatment (S.A. Hild, personal communication).

GnRH analogue treatment also enhanced the stimulation of recovery of spermatogenesis from stem cells following spermatogonial transplantation. When mouse testicular cells were transplanted into busulfan-treated mouse recipients, the efficiency of differentiated germ cell production from transplanted stem cells in the recipient tubules was enhanced with GnRH analogue treatment (Ogawa et al, 1998; Dobrinski et al, 2001). However, a significant benefit was derived only from pretreatment with GnRH analogue, indicating that the hormone treatment may be important for the stem cells to attach in their proper niche in the seminiferous tubules, but not for the initiation of differentiation. The importance of suppressing ITT levels with either GnRH agonist or exogenous testosterone treatment was also demonstrated in studies in which rat or mouse spermatogonia were transplanted into busulfan-treated rat hosts (Ogawa et al, 1999).

When GnRH treatment is given relative to the toxic exposure is important. Data from irradiated and hexanedione-treated rats showed that treating immediately after exposure to a toxicant was more effective than delayed treatments in the restoration of spermatogonial differentiation (Meistrich et al, 1999). However, there has not been a strict comparison between the effects of pretreatments and posttreatments in any of the models in which both treatments are effective.

Fertility can be restored in these pathological situations by GnRH analogue treatment. When a 10-week GnRH agonist or GnRH antagonist treatment was started immediately after 3.7-Gy irradiation, fertility was maintained at week 20 in the GnRH agonist and GnRH antagonist treated rats at normal and nearly normal levels, respectively, whereas none of the irradiated-only rats were fertile (Meistrich et al, 2001b). When treatment was initiated 10 weeks after 5 Gy irradiation, at which point spermatogenesis had completely declined, fertility was restored at week 30 to subnormal levels in 83% of GnRH agonist and 50% of GnRH antagonist treated rats. Thus we conclude that normal fertility can be restored by GnRH treatment after irradiation, although that may depend on initiation of the GnRH analogue treatment soon after a toxicant exposure that is not too severe. We have also demonstrated that GnRH analogue posttreatment significantly increases recovery of fertility in rats after procarbazine treatment (Meistrich et al, 1999). In contrast in the *jsd* mice, a transient increase in spermatogonial and spermatocyte differentiation was produced by the GnRH antagonist treatment; testicular sperm extraction and intracytoplasmic sperm injection (ICSI) were both required to produce offspring (Tohda et al, 2002).

Maintenance of Spermatogenesis After Reversal

Although the TDI in rats receiving 3.5 Gy of radiation and GnRH agonist for 10 weeks was 91%, testicular sperm head counts were only 0.1% of controls because the hormone treatment suppressed spermiogenesis. However, when additional time without further GnRH treatment was allowed before the rats were killed, the TDI recovered to 100%, and sperm counts reached about 50% of normal control levels at 6.5 weeks after stopping treatment and were maintained at this level for at least another 3.5 weeks.

The maintenance of spermatogenesis in irradiated rats after GnRH analogue treatment is stopped depends on the toxicant dose and time of initiation and duration of the hormone treatment. For example, when a 7-week GnRH analogue treatment was initiated at week 15 after 6 Gy of irradiation, the TDI was elevated from 0% in irradiated-only rats to 95% at week 24 (2 weeks after stopping the GnRH treatment), but then declined to 50% at week 36 (14 weeks after stopping GnRH; G.A. Shuttlesworth and M.L. Meistrich, unpublished data). Thus permanent progression and maintenance of spermatogenesis is not

Table 2. *Treatments or mutations in which the block of spermatogonial differentiation can be reversed with hormone treatment*

Species	Treatment or Mutation*	Hormone treatment and timing relative to inducing event			References
		Before	Immediately After	After a Delay	
Rat	Hexanedione*		GnRH-agonist	GnRH-agonist	(Blanchard et al, 1998)
Rat	Radiation (gamma)*	T+E2	GnRH-agonist, GnRH-antagonist, testosterone	GnRH-agonist, GnRH-antagonist, testosterone, androgens	(Meistrich and Kangasniemi, 1997; Meistrich et al, 2000, 2001b; Shetty et al, 2000; Shetty et al, 2002)
Rat	Procarbazine*	GnRH-agonist + flutamide, GnRH-antagonist + flutamide, T, T+E2	GnRH-agonist		(Parchuri et al, 1993; Kangasniemi et al, 1995a,b; Meistrich, 1999)
Rat	Indenopyridine (CDB-4022)*	GnRH-agonist†	GnRH-agonist†		(Hild et al, 2001)
		GnRH-antagonist	{GnRH-antagonist}‡		S.A. Hild, personal communication
Rat	DBCP*		GnRH-agonist	GnRH-agonist	M.L. Meistrich, unpublished
Rat	Acute heating	GnRH-agonist + flutamide	GnRH-agonist + flutamide		(Setchell et al, 2001)
Rat	Aging*			GnRH-agonist (at 27 mo)	(Schoenfeld et al, 2001)
Rat	Busulfan	{GnRH-agonist}‡	GnRH-agonist		(Udagawa et al, 2001)
Rat	Busulfan, spermatogonial transplant	GnRH-agonist†	GnRH-agonist†		(Ogawa et al, 1999)
Mouse	*jsd* mutation*		GnRH-antagonist (at age 5 weeks)	GnRH-antagonist (at age 10 weeks)	(Matsumiya et al, 1999; Shetty et al, 2001)
Mouse	Busulfan, spermatogonial transplant	GnRH-agonist	{GnRH-agonist}‡		(Ogawa et al, 1998; Dobrinski et al, 2001)

* Instances in which it has been shown that type A spermatogonia are present in the atrophic tubules but do not undergo differentiation.
† Hormone treatment was given both before and after the toxicant treatment.
‡ The hormone in brackets {hormone} indicates the treatment was tried but was unsuccessful.

assured by this technique. Although no time course studies were done, extensive recovery of spermatogenesis in tubules after hexanedione treatment was observed 9 weeks after the end of a 10-week GnRH agonist treatment, and the degree of recovery was inversely correlated with the dose of hexanedione (Blanchard et al, 1998).

In contrast to the toxicant-treated rat models, spermatogenesis degenerated rapidly in *jsd* mice after withdrawal of the GnRH antagonist. Whereas a 6-week GnRH antagonist treatment increased the TDI from 11% in non-hormone treated mice to 95%, 5 weeks after cessation of the treatment the TDI progressively declined to 78% and to 8% after 13 weeks (Shetty et al, 2001). Although one wave of late spermatids was produced from the differentiating spermatogonia and spermatocytes that developed during the GnRH antagonist treatment, the maximum percentage of tubules that contained elongated spermatids was only 20% at week 4 after the hormone treatment was stopped (Tohda et al, 2002). However, these elongated spermatids were used in ICSI to effect a pregnancy.

The difference between the maintenance of spermatogenesis in the irradiated rat model and *jsd* mice is that the former likely involves an epigenetic change, whereas the latter is a genetic alteration. The epigenetic change caused by irradiation to render spermatogonial differentiation sensitive to inhibition by testosterone can be largely reversed by hormonal treatment. But the underlying defect in a genetic disorder manifests itself again as soon as the hormone treatment is stopped.

Role of Testosterone in Block of Spermatogonial Differentiation

Because the GnRH analogues that were used to stimulate or maintain spermatogonial differentiation in the various cases described above generally suppress LH, FSH, and testosterone, these hormones were implicated in the inhibition of spermatogonial differentiation. Using irradiated rat and *jsd* mouse models, we and others investigated the roles of these hormones in the regulation of spermatogonial differentiation.

One study involved the administration of exogenous LH to GnRH antagonist–treated *jsd* mice (Tohda et al, 2001). Whereas the GnRH antagonist restored spermatogonial differentiation, the addition of exogenous LH inhibited it. However, other experiments with *jsd* mice (Shetty et al, 2001; Tohda et al, 2001) and with irradiated rats indicated that it was the testosterone production stimulated by the LH, and not the LH itself, that inhibited spermatogonial differentiation. For example, GnRH agonist treatment of $LBNF_1$ rats did not suppress LH levels, but it did suppress ITT, serum testosterone, and FSH levels and stimulated spermatogonial differentiation (Meistrich and Kangasniemi, 1997; Meistrich et al, 1999). In another study, when irradiated rats treated with GnRH agonist were given exogenous testosterone, spermatogonial differentiation was inhibited despite a suppression of LH levels (Shetty et al, 2001). This led us to further investigate the precise roles of testosterone and FSH in the inhibition of spermatogonial differentiation after irradiation.

Various studies have indicated that testosterone had an inhibitory effect. Because there is a major increase in the ITT concentration in mice between 30 and 40 days of age (Jean-Faucher et al, 1978), the large decline in the numbers of B spermatogonia in *jsd* testes, which occurs between 6 and 7 weeks of age (Kojima et al, 1997), could very well be a consequence of the increase in ITT. In addition in these mice, the stimulation of spermatogonial differentiation by suppression of testosterone with GnRH antagonist was reversed by exogenous testosterone (Shetty et al, 2001). Furthermore, that inhibition by testosterone was reversed by treatment with the androgen-receptor antagonist flutamide.

In irradiated rats, we have shown that testosterone dose-dependently reduced the GnRH antagonist-stimulated spermatogonial differentiation. (Shetty et al, 2000, 2002). Further, the stimulatory action of low-dose testosterone alone, which reduces ITT concentrations, was also reduced with increasing doses of testosterone that increased both ITT and serum testosterone concentrations. The TDIs and the serum and ITT levels were similar for each given dose of testosterone, with or without the GnRH antagonist, showing that the testosterone levels in the testis or the serum, or both, limit the ability of spermatogonia to differentiate. The inhibition of spermatogonial differentiation by testosterone was further confirmed by showing that flutamide reversed the inhibition induced by exogenous testosterone in GnRH antagonist–treated, irradiated rats (Shetty et al, 2000). Further support for our hypothesis that it is indeed testosterone acting through the androgen receptor and not a nonandrogenic metabolite of testosterone that inhibits spermatogonial differentiation was obtained by showing that various androgens, including 5α-dihydrotestosterone (a 5α-reduced androgen), 7α-methyl-19-nortestosterone (a non-5α-reducible androgen but one that can be aromatized), and methyltrienolone (a nonmetabolizable androgen) also suppressed spermatogonial differentiation in GnRH antagonist–treated irradiated rats (Shetty et al, 2002). In the same study, we showed that estradiol (E_2) was not inhibitory.

When testicular testosterone levels in irradiated rats treated with various GnRH analogues and testosterone combinations were compared with the TDI, an excellent negative correlation was observed (Figure 2) with only 1 point deviating significantly from each of the fitted curves (Figure 2, B and D). Although a general negative trend

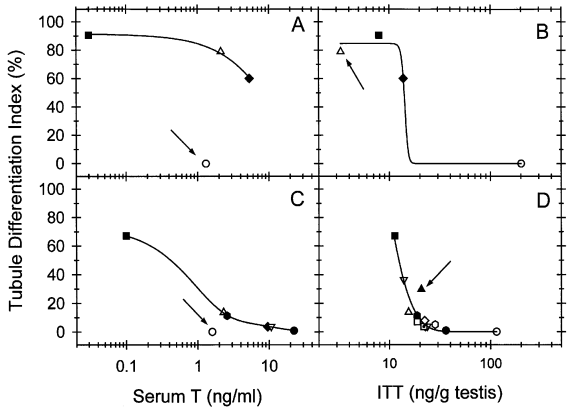

Figure 2. Correlation between serum testosterone **(A, C)** and ITT **(B, D)** during hormone treatment and the levels of recovery of spermatogenesis at the end of experiment. **(A, B)** Data from combinations of testosterone with GnRH agonist, given during weeks 0–10 after 6 Gy irradiation. TDI analysis was performed on testicular histological sections prepared on week 20. **(C, D)** Data from combinations of testosterone with GnRH antagonist, given during weeks 3–7 after 5 Gy irradiation. TDI analysis was performed on testicular histological sections prepared on week 13. Equivalent symbols in **(A** through **D)** are from the same treatments. Regression curves were fitted to the data with the exception of the deviant points (open circles) in **(A** and **C).** Arrows indicate discrepancies from complete correlations. Data from 2 reports (Shetty et al, 2000, 2002) were combined.

was also noted for serum testosterone vs TDI, there was a very significant deviation (Figure 2, A and C, arrow and open circles) in which the irradiated rats with a moderate amount of serum testosterone showed no differentiation. However, irradiated rats treated with GnRH analogues and testosterone (Figure 2, A and C, open upward triangles and filled diamonds) showed higher serum testosterone, but a significantly higher percentage of the tubules contained differentiating cells. This led us to conclude that ITT is the major factor, as the irradiated-only rats (open circles) had much higher ITT concentrations than those also treated with GnRH analogues and testosterone (Figure 2, B and D, open upward triangle and filled diamond). However, there were some small but significant discrepancies in the correlation between ITT and TDI. For example, GnRH agonist–treated, irradiated rats (Figure 2B, filled square) showed a higher TDI but also higher ITT than a similar group of rats that also received testosterone implants (Fib. 2B, open triangle). Because the former group had much lower serum testosterone levels (Figure 2A), we suggested that although the ITT was the major factor inhibiting spermatogenic recovery, serum testosterone seemed to have a minor additive inhibitory role. The point that deviated from the curve in Figure 2D (filled triangle) was a result of treatment of irradiated rats with GnRH antagonist and daily injections of testosterone proportionate, which may result in varying levels of testosterone throughout the course of treatment.

In all these situations, ITT concentrations in the normal range (about 50 ng/g testis) seem to inhibit the differentiation of spermatogonia. Figure 2D shows that even ITT concentrations of 15–30 ng/g of testis inhibited spermatogonial differentiation. Further, the observed block in the spermatogonial differentiation in aged rats that had ITT concentrations below normal and spermatogonial differentiation was stimulated by further suppression of ITT with a GnRH agonist show that above normal levels of ITT are not necessarily required for the inhibition of spermatogonial differentiation. Rather, in these circumstances, spermatogonial differentiation becomes sensitive to physiological levels of testosterone.

Based on the concept that testosterone inhibited spermatogenesis in toxicant-treated rats, hexanedione-exposed rats were treated with ethane dimethane sulfonate (EDS), which specifically eliminates Leydig cells, followed by GnRH agonist, which prevented Leydig cell regeneration (Richburg et al, 2002). Even though EDS reduced testosterone levels to undetectable levels, the EDS treatment inhibited the recovery of spermatogonial differentiation that the GnRH agonist would normally induce. Although the results of this study seemed to contradict the hypothesis that testosterone inhibits spermatogonial differentiation, that hypothesis could still be valid if a Leydig cell factor is required for the stimulation of spermatogenic recovery in the atrophic testis and this factor were eliminated by EDS, but not by GnRH analogue treatment.

Role of FSH in Block of Spermatogonial Differentiation

The elevated FSH levels in these pathological models of testicular atrophy could contribute to the inhibition of spermatogonial differentiation. Although as shown above, testosterone appears to be an inhibitory factor, it is necessary to determine whether FSH also has a role.

The possible contribution of serum testosterone to inhibiting spermatogonial differentiation suggests that testosterone may act at an extratesticular site. One such likely site is the pituitary, where it could act by altering gonadotropin levels. We have already ruled out LH as having a significant contribution to the inhibition of spermatogonial differentiation, so we focused on a possible role for FSH. However, testosterone has a complex action on pituitary production of FSH. When testosterone is given to rats or mice that have normal GnRH production and action, it suppresses FSH levels by having a combined action on the hypothalamus and pituitary. However, when testosterone is given to GnRH antagonist–treated rats, but not mice (Shetty et al, 2001), it reverses the GnRH antagonist-induced reduction of FSH levels in these rats by direct up-regulation of FSHβ gene transcription in the pituitary (Perheentupa et al, 1993). The levels of FSH in the presence of exogenous testosterone appear

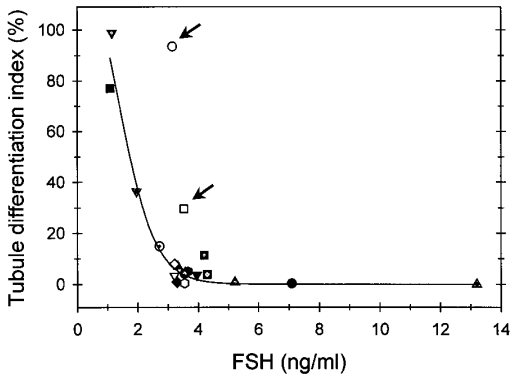

Figure 3. Correlation between serum FSH levels during hormone treatment and the subsequent levels of recovery of spermatogenesis in irradiated rats treated with various combinations of GnRH antagonist, 1 of the androgens, and the antiandrogen flutamide. The points indicated by arrows deviated from the curve fitted to the other points. The deviant points are from rats treated with GnRH antagonist, testosterone, and flutamide (open circle), or GnRH antagonist and daily injections of testosterone propionate (open square). Data from 2 reports (Shetty et al, 2000, 2002) were combined.

to be independent of whether or not a GnRH antagonist is also given (Shetty et al, 2000).

There was good inverse correlation between TDI and FSH levels (Figure 3), which could be due in part to the concomitant rise in FSH when testosterone was given. Several points did deviate from this correlation curve (Figure 3, arrow). Although TDI and ITT were even more closely correlated, (Figure 2), a role for FSH in inhibition of spermatogonial differentiation could not be ruled out. We directly tested the role of FSH by giving exogenous FSH to irradiated rats while suppressing levels and actions of androgens with GnRH antagonist and flutamide. Exogenous FSH significantly inhibited the tubule differentiation stimulated by GnRH antagonist-flutamide treatment, although not as drastically as did androgens (G. Shetty, unpublished data). From these data and the overall relationship between hormone levels and TDI (Figure 4), we conclude that primarily ITT, but also FSH, which is regulated by serum testosterone, inhibits spermatogonial differentiation in irradiated rats.

In contrast to the results with rats, administration of exogenous FSH in *jsd* mice during suppression of gonadotropins did not inhibit spermatogonial differentiation (Tohda et al, 2001). Further confirmation of the inability of FSH to inhibit spermatogonial differentiation in *jsd* mice was shown by the lack of correlation between FSH levels and the TDI (Shetty et al, 2001) and the lack of correlation in timing of the rise in FSH levels, which reaches near adult levels during the first 2 weeks after

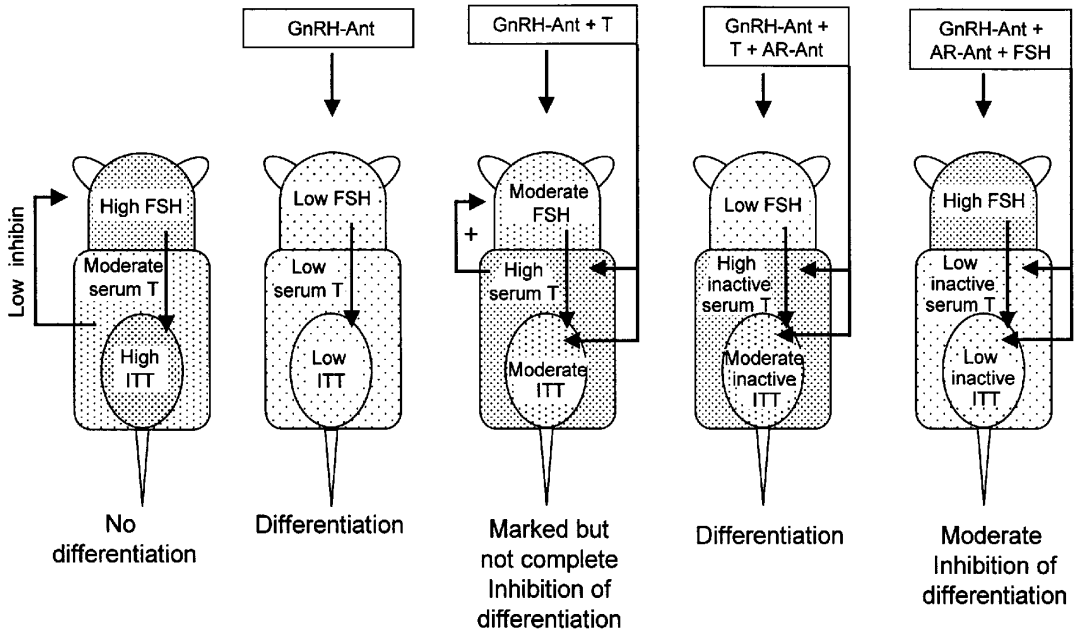

Figure 4. Schematic of observations in irradiated rats showing the different levels of FSH and testosterone in the serum and testis during various hormone treatments and the resulting changes in the differentiation of spermatogonia. Whereas no spermatogonial differentiation is observed in irradiated rats not treated with hormones **(left panel),** likely due to the high levels of ITT and FSH, differentiation is induced by suppression of testosterone and FSH **(second panel).** The fact that there is some differentiation in the third panel indicates that either ITT or FSH are inhibitory. The fourth panel indicates that testosterone is acting through the androgen receptor, although it could be acting at the pituitary or testis. The fifth panel (compare with the second panel) shows that FSH has an inhibitory role.

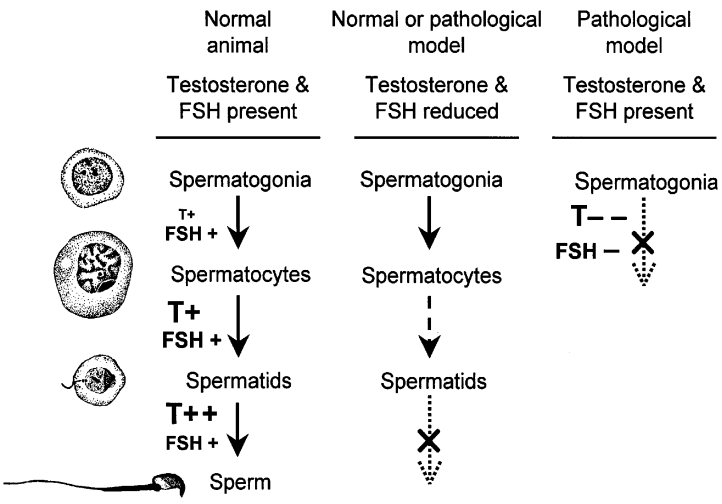

Figure 5. Roles of testosterone and FSH in normal spermatogenesis and in pathological models involving a proliferation-apoptosis block in spermatogonial differentiation. Stimulatory and inhibitory roles are indicated by pluses and minuses, respectively, with the strength of the stimulatory or inhibitory action indicated by the numbers of pluses or minuses and the font size.

birth (Slegtenhorst-Eegdeman et al, 1998), and the major block in spermatogonial differentiation, which does not occur until between weeks 6 and 7 after birth (Kojima et al, 1997).

Relationship to Roles of Hormones in Normal Spermatogenesis

In the various pathological conditions we have described, it appears that testosterone and FSH may act additively to inhibit the differentiation of spermatogonia, whereas in normal spermatogenesis they act additively to support survival and differentiation of spermatocytes and spermatids. Thus the differences between the action of the hormones in the 2 situations involves not only the direction of action but also their targets during spermatogenesis (Figure 5). In spermatocyte and spermatid differentiation, the normal requirement for primarily testosterone, but also with some additive effects of FSH (O'Donnell et al, 1994; El Shennawy et al, 1998), appears not to be altered in the pathological situation, in which differentiation does not proceed past the spermatocyte or early spermatid stage during suppression of testosterone and FSH. However, in these pathological models the hormones act at an additional checkpoint. Spermatogonial survival and differentiation, which in normal rats can proceed in the absence of testosterone and FSH but is augmented by these hormones (Huang and Nieschlag, 1986; Meachem et al, 1999), becomes, in these pathological models, sensitive to inhibition by testosterone and in some cases, to inhibition by FSH as well. Possible mechanisms for the development of this checkpoint will be described in the next section.

Possible Mechanisms for Block in Spermatogonial Differentiation

Although testosterone and FSH have effects on spermatogonial differentiation in these pathological models, spermatogonia are not known to have receptors for these hormones. According to currently accepted dogma, in normal animals, FSH receptors (FSHRs) are localized exclusively in the Sertoli cell (Kliesch et al, 1992) and androgen receptors (ARs) are localized in a variety of somatic cell types, including Sertoli, Leydig, peritubular myoid, and vascular smooth muscle cells (Bremner et al, 1994). Furthermore, normal development of germ cells that lack an AR is possible (Johnston et al, 2001). Because germ cells lack AR and FSHR, these hormones must act via paracrine or juxtacrine routes between the cells that contain the receptors for these hormones and the spermatogonia.

The model chosen to explain the apparent contradiction, that testosterone inhibits spermatogonial differentiation after some pathological insults or genetic defects but not in normal spermatogenesis, depends on whether the pathology directly alters the spermatogonia or the androgen-responsive somatic cell. In most cases the target is not known. Although certain toxicants are believed to act primarily on Sertoli cells (eg, hexanedione; Boekelheide, 1988) or germ cells (eg, radiation; Lee et al, 1999), it is not possible to prove that the long-term effects are due to action on these cells. Hence, Figure 6, which lays out our model, is divided into two parts, A and B, which assume the defect lies in the spermatogonia, whereas Figure 6C and D, assume it lies in the Sertoli cells. The Sertoli cell was used as the example of the androgen-

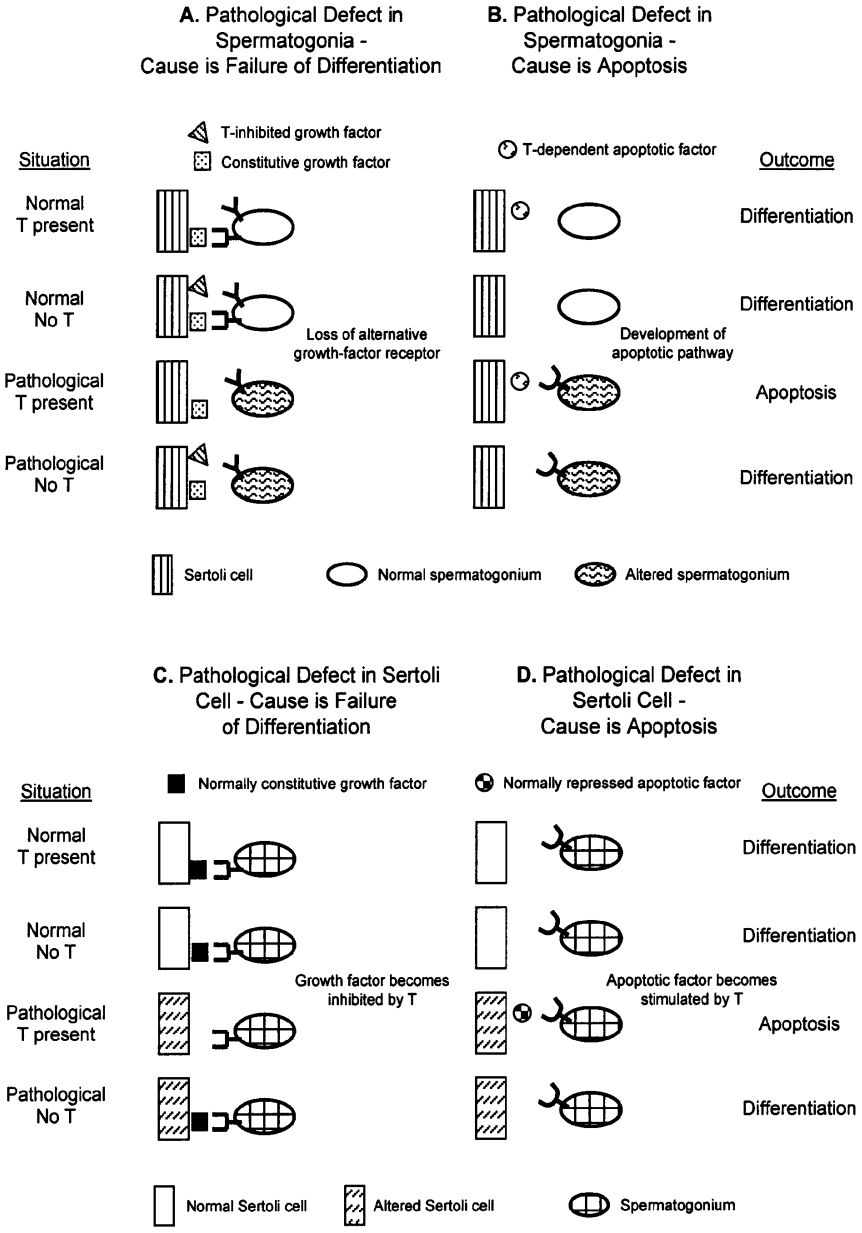

Figure 6. Models to explain testosterone-dependent inhibition of spermatogonial differentiation in pathological situations in mice and rats but not normal rodents. **(A)** It is assumed that the defect is in spermatogonia and they do not differentiate because a growth, survival, or differentiation factor is missing. Normal spermatogonia could have 2 pathways that support this step, but altered spermatogonia lack one receptor (square symbol) (or intracellular component of signal-transduction pathway) and therefore require the second receptor, the ligand (triangle), which is suppressed by the presence of testosterone. **(B)** It is assumed that the defect is in spermatogonia and they do not differentiate because they are killed by apoptosis. Sertoli cells could secrete an apoptotic effector (circle) in the presence of testosterone but normal spermatogonia lack the receptor or pathway for this ligand. The altered spermatogonia possess this receptor and therefore become sensitive to apoptosis in the presence of testosterone. **(C)** It is assumed that the defect is in somatic (Sertoli) cells and the reason spermatogonia do not differentiate is that a growth or differentiation factor is absent. Normal Sertoli cells make this growth factor constitutively, but in the altered Sertoli cells, it could be inhibited by testosterone. **(D)** It is assumed that the defect is in somatic (Sertoli) cells and the reason spermatogonia do not differentiate is that they are killed by apoptosis. Whereas normal Sertoli cells do not make an effector for this apoptotic process, the altered Sertoli cells could make this effector, but only in the presence of testosterone.

responsive somatic cell because it is most likely, but we cannot rule out the possibility that the peritubular, Leydig, or vascular smooth muscle cells are instead involved in some cases. In Sl^{17H} mice the defective gene, stem cell factor, is indeed specifically produced by Sertoli cells. However, for the *jsd* mutation, transplantation experiments have shown conclusively that the defect is expressed in the spermatogonia, not in the somatic cells (Boettger-Tong et al, 2000; Ohta et al, 2001).

The appropriate choice of model also depends on whether the cause of the block is spermatogonial apoptosis (Figure 6, B and D), or the lack of a functional signal for the spermatogonia to differentiate (Figure 6, A and C). In the first case, the failure to differentiate is a secondary consequence of the failure of the cells to survive to an appropriate stage. In the second instance, the observed apoptosis would be a secondary consequence of cells remaining undifferentiated for too long.

Alterations in spermatogonia could make these cells either more sensitive to testosterone-induced proapoptotic factors from the Sertoli cell (Figure 6B) or more dependent on testosterone-suppressible growth and differentiation factors from their surroundings (Figure 6A). Alternatively, testosterone could act on somatic cells to induce proapoptotic factors (Figure 6D) or to inhibit normally secreted growth factors (Figure 6C). In any case, all of these models predict that there should be at least one gene or gene product specifically regulated by testosterone in the target somatic cell.

Possibilities for Clinical Application

The above animal models may be applicable to 4 areas of human infertility or fertility control: idiopathic male infertility involving spermatogenic arrest, infertility due to treatment of cancer and autoimmune diseases with chemotherapy or radiotherapy, infertility due to environmental or occupational exposures, and development of a male reversible contraceptive.

Many cases of male infertility involve testicular disorders with arrest at various stages of spermatogenesis, including arrest at the spermatogonial stage in 10% of such cases (Skakkebaek et al, 1973). If testosterone inhibits spermatogenesis as was the case with *jsd* mice (Tohda et al, 2002), the late spermatids might be produced with intermittent testosterone suppression and be used for ICSI.

Chemotherapy or radiotherapy induces prolonged or permanent azoospermia in 3000 men of reproductive age in the United States each year (Meistrich et al, 2001a). Azoospermia also results from cyclophosphamide treatment for autoimmune diseases (Watson et al, 1985). Although some of these treatments may kill all of the stem cells, sometimes stem spermatogonia do survive but they fail to differentiate, as was observed in the rodent models. This is evidenced by the spontaneous reinitiation of spermatogenesis in some patients after many years of azoospermia (Meistrich et al, 1992). There are also histological examples of failure to differentiate past the spermatogonial (Kreuser et al, 1989) or the spermatocyte (Meistrich and van Beek, 1990) stages during the azoospermic period. The trigger for the spontaneous recovery is not known. Although several earlier attempts to enhance recovery of spermatogenesis by treatment with GnRH analogues before and during chemotherapy or radiotherapy were unsuccessful (Morris and Shalet, 1990), low-dose systemic testosterone to suppress intratesticular testosterone levels did induce recovery of spermatogenesis in all men treated with cyclophosphamide (Masala et al, 1997). However, there has been only one trial of the use of hormonal suppression after the completion of chemotherapy, and in that trial, no recovery was observed (Thomson et al, 2002). It should be noted that all the patients had been treated before puberty with high doses of procarbazine or radiation, which likely led to a complete loss of stem cells. A study using GnRH analogues for adult patients whose azoospermia resulted from lower doses of cytotoxic agents should be conducted next.

Environmental and occupational exposures to toxicants that block spermatogonial differentiation in rats may also produce similar effects in men. Boric acid is in widespread commercial and consumer use. Hexanedione is the active metabolite of the widely used solvent n-hexane. As yet there are no reports of effects of these chemicals on human spermatogenesis. However, DBCP, which is now banned, produced azoospermia in all highly exposed workers involved in its production (Whorton et al, 1979), and many thousands of agricultural workers who were exposed to DBCP appear to have an increased incidence of azoospermia (Slutsky et al, 1999). That azoospermia in men following exposure to moderate doses of DBCP may be spontaneously reversible years later (Potashnik and Porath, 1995) indicates that the stem cells may have survived and that DBCP may cause azoospermia by producing a prolonged block in spermatogenic differentiation.

The ability to reversibly block the differentiation of spermatogonia has potential for use as a male contraceptive. Compounds such as indenopyridines, in a single dose produce apparently irreversible sterility in rats, mice, and dogs without other toxicity (Cook et al, 1995), but their development as contraceptives is limited by the irreversibility of the spermatogenic block. However, the presence of type A spermatogonia in the tubules of indenopyridine-treated rats suggests that it could be reversed. Although spermatogonial differentiation and recovery of spermatogenesis was enhanced by treating rats with GnRH analogues before and after indenopyridine treatment (Hild et al, 2001), further studies showed that

only the treatment before indenopyridine was effective (S.A. Hild, personal communication). It is now important to determine whether spermatogonial differentiation can be reinitiated by hormonal or other forms of treatment given after the induction of a block to differentiation by the indenopyridine.

Although GnRH analogues and gonadal steroids have similar actions in humans and rodents, we do not know whether they will stimulate recovery of spermatogenesis in men with genetic or toxicant-induced blocks in spermatogonial differentiation because we do not know whether the mechanisms of the block are the same in the different species. Preliminary analyses of studies in irradiated monkeys show that GnRH antagonist treatment failed to prevent or reverse the reductions in spermatogenesis produced by radiation (A. Kamischke, personal communication; Richburg et al, 2002). Therefore, it is important to elucidate the mechanism by which testosterone inhibits spermatogonial differentiation in rodents to evaluate its application to men. Mechanistic knowledge can be used to find targets downstream from the initial action of androgen to develop restorative treatments that allow maintenance of androgen levels.

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Differences in Radiation Sensitivity of Recovery of Spermatogenesis Between Rat Strains

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Previous studies with Lewis/Brown-Norway (BN) F1 hybrid rats indicated that spermatogenesis was much more sensitive to ionizing radiation than in the widely studied outbred Sprague Dawley stock, suggesting that there were genetically based differences; however, the relative sensitivities of various inbred strains had not been established. As a first step to defining the genes responsible for these differences, we compared the sensitivities of seven rat strains to radiation damage of spermatogenesis. Recovery of spermatogenesis was examined 10 weeks after 5-Gy irradiation of seven strains (BN, Lewis, Long-Evans, Wistar Kyoto, spontaneously hypertensive [SHR], Fischer 344, and Sprague Dawley). The percentages of tubules containing differentiated cells and testicular sperm counts showed that BN and Lewis were most sensitive to radiation (< 2% of tubules recovered, $< 2 \times 10^5$ late spermatids per testis), Long-Evans, Wistar Kyoto, Fischer, and SHR were more resistant, and Sprague Dawley was the most resistant (98% of tubules recovered, 2×10^7 late spermatids per testis). Although increases in intratesticular testosterone levels and interstitial fluid volume after irradiation had been suggested as factors inhibiting recovery of spermatogenesis, neither appeared to correlate with the radiation sensitivity of spermatogenesis in these strains. In all strains, the atrophic tubules without differentiated germ cells nevertheless showed the presence of type A spermatogonia, indicating that their differentiation was blocked. Thus, we conclude that the differences in radiation sensitivity of recovery of spermatogenesis between rat strains of different genetic backgrounds can be accounted for by differences in the extent of the radiation-induced block of spermatogonial differentiation.

Key Words: **ionizing radiation; spermatogenesis; rat strains; spermatogonia.**

Identification and quantification of risks that particular toxicants will damage the human male reproductive system are based on results from animal model systems. Rodents have been the primary model system used in reproductive toxicology because they are small, inexpensive, and genetically well characterized. However, it is important for qualitative extrapolation to human that the mechanisms of the toxicity in the test species have the same characteristics as in the human. For quantitative extrapolation, it also is necessary to consider the doses to produce equivalent effects (Meistrich, 1992). Within a test species, the strain chosen is important because there may be quantitative differences in the response with different strains. Furthermore, there might be qualitative differences in mechanisms with different strains.

For many decades, the rat had been the primary rodent model used for reproductive toxicology. However, the mouse has been used increasingly in recent years because more genetic tools are available in this species to elucidate mechanisms, and there have been numerous studies of strain differences in effects of toxicants on spermatogenesis in mice (Bianchi *et al*., 1985; Meistrich *et al*., 1984; Spearow *et al*., 1999). In contrast, there have been very few reports characterizing strain differences in sensitivities of various of rat strains (Delic *et al*., 1987; Parchuri *et al*., 1993; Sotomayor *et al*., 1996), and some of these studies often included outbred rat stocks rather than inbred strains. However, the genetic knowledge and techniques in the rat are now progressing with the sequencing of the genome (Gibbs *et al*., 2004), the existence of sets of recombinant inbred lines (Tabakoff *et al*., 2009; Voigt *et al*., 2008), and the ability to produce gene knockouts (Jacob *et al*., 2010), so that studies of strain differences in rats have the potential to lead to discovery of gene function.

Previously, we reported that there were dramatic interstrain differences in the recovery of spermatogenesis in rat testes from the chemotherapy drug procarbazine (Parchuri *et al*., 1993). Whereas spermatogenesis in most outbred Sprague Dawley rats was nearly completely resistant to prolonged effects of multiple injections of procarbazine on the testis, about 25% of the rats were quite sensitive to that treatment. In contrast, both Lewis and LBNF1 (F1 hybrids of Lewis and Brown-Norway [BN] inbred strains) were extremely sensitive to the same doses of procarbazine. With chemical treatment, it is not known whether the differences in sensitivity were due to

target organ sensitivities as opposed to differences in pharmacokinetics or systemic effects.

To more specifically examine differences in target organ sensitivities, we compared the data on radiation sensitivities of different strains. Radiation is highly toxic to the human testis and 4–6 Gy can produce total loss of sperm production for about 2 years (Clifton and Bremner, 1983).

The most sensitive targets for radiation damage to the testis are the proliferating differentiating spermatogonia (A_1–A_4, intermediate, and B spermatogonia) (Erickson, 1976). The loss of these cells results in a progressive depletion of differentiating germ cells (Dym and Clermont, 1970). The spermatogonial stem cells (undifferentiated type A) are more resistant and can survive moderate radiation doses, and, if the dose is not too high, they can eventually produce complete recovery of spermatogenesis in resistant strains (Dym and Clermont, 1970). However, at high doses or in sensitive strains, the recovery may be incomplete or permanent testicular atrophy may occur (Kangasniemi *et al*., 1996). For example, LBNF1 rats were much more sensitive to prolonged spermatogenic damage from irradiation (Kangasniemi *et al*., 1996) than were Sprague Dawley rats, based on comparison of similar doses and endpoints gathered from the literature (Delic *et al*., 1987; Erickson and Hall, 1983; Huckins, 1978). Whereas LBNF1 rats showed atrophic seminiferous tubules with only A spermatogonia, indicating a block in their differentiation, Sprague Dawley rats showed progressive recovery of spermatogenesis at similar doses. A block in spermatogonial differentiation after exposure to a variety of therapeutic and environmental toxicants, including hexanedione and dibromochloropropane, has been observed in Sprague Dawley and Fischer 344, in addition to LBNF1, rats (Meistrich and Shetty, 2003).

The radiation-induced block in spermatogonial differentiation in LBNF1 rats was not due to damage to the stem cells as they differentiated into spermatozoa after transplantation into the depleted testes of nude mice (Zhang *et al*., 2006). It was also not due to failure of the stem cells to proliferate, as they were actively cycling in atrophic tubules of several models with spermatogonial blocks induced by irradiation, hexanedione, or age, but was rather due to apoptosis of these cells when they began to differentiate (Allard *et al*., 1995; Schoenfeld *et al*., 2001; Shuttlesworth *et al*., 2000). This block was due to damage to the somatic environment as transplanted spermatogonia from normal immature rats failed to differentiate in the irradiated testis tubules (Zhang *et al*., 2007). The cause of the block is not known but several candidate genes whose expression changes in somatic cells of LBNF1 rats after radiation have been identified (Zhou *et al*., 2010, 2011).

To extend these anecdotal observations, we directly compared the sensitivities of seven different strains or stocks of rats treated with the same doses of radiation. Strains were chosen on the basis of their usefulness in toxicological or endocrine studies, previous indications of strain differences, and the existence of recombinant inbred lines to facilitate identification of loci contributing to the phenotype. We identified very marked differences in the sensitivity of the strains to radiation.

MATERIALS AND METHODS

Animals and irradiation exposure. We examined seven strains of rats to measure the recovery of spermatogenesis after irradiation. These included five inbred strains: BN (BN/SsNHsd) and Lewis (LEW/SsNHsd) obtained from Harlan Laboratories; Fischer 344 (F344/NCrl), Wistar Kyoto (WKY/NCrl), and SHR (SHR/NCrl) obtained from Charles River Laboratories; and two outbred stocks: Long-Evans (Crl:LE) from Charles River and Sprague Dawley (Hsd:Sprague Dawley SD) from Harlan. We obtained the rats at 7 weeks of age and they were allowed to acclimatize in our facility for 1 week prior to use. Rats were housed under standard lighting (12-h light, 12-h dark) and were given food and water *ad libitum*. All procedures were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee, and the housing facilities were approved by the American Association of Laboratory Animal Care.

Rats were anesthetized with a mixture of ketamine (0.72 mg/kg) and acepromazine (0.022 mg/kg) im and affixed to an acrylic board with surgical tape; then, the lower part of the body was irradiated by a ^{60}Co gamma ray unit (Eldorado 8; Atomic Energy Canada Ltd., Ottawa, ON, Canada). The field extended distally from a line about 6 cm above the base of the scrotum. A single dose of 5 Gy was given at a dose rate of approximately 1 Gy/min (Shetty *et al*., 1989). Rats were euthanized 10 weeks after irradiation; serum was collected for hormone measurements, and the testis tissue was harvested for analysis as indicated below because all tubules in Sprague Dawley rats showed recovery of spermatogenesis at this dose, another group of Sprague Dawley rats were given 6.5 Gy of irradiation, and testis tissue was harvested 10 weeks later.

For each strain/stock, (n = 5–10) irradiated rats and (n = 3) age-matched unirradiated controls were analyzed.

Intratesticular interstitial fluid and tissue processing. Rats were killed by an overdose of the ketamine-acepromazine mixture. Each testis was surgically excised and weighed with the tunica albuginea intact. The right testis was fixed overnight in Bouin's fluid.

Interstitial tubule fluid was collected from the left testis using a modification of previously described methods (Porter *et al*., 2006; Rhenberg, 1993). Briefly, a silk suture was attached to the caput end of the testis with a wound clip. Four 1-mm incisions that did not intersect were cut into the caudal end of the testis. The testis was suspended inside a 10-ml syringe barrel by the attached suture, which was taped to the outside of the syringe. A preweighed silicone-coated microcentrifuge tube was attached to the luer lock tip of the syringe. The syringe assembly was centrifuged inside a 50-ml tube for 30 min at 60 × g at 4°C, and the weight of the microfuge tube containing the fluid was determined. The remaining weight of the testis parenchymal tissue was measured after removing the tunica albuginea. The tissue was then homogenized in water for sperm head counts.

Evaluation of spermatogenesis. For histological analysis, the fixed right testis was cut in half, testis section was taken from the middle from one of the two pieces and then embedded in glycol methacrylate plastic (JB4; Polysciences Inc., Warrington, PA), and 4-μm sections were cut and stained with periodic acid Schiff and hematoxylin. To evaluate the recovery of spermatogenesis from irradiation, we scored a minimum of 200 seminiferous tubules from the whole testis cross section from each animal for the most advanced germ cell stage present in each tubule. Unless otherwise stated, we computed the tubule differentiation index (TDI), which is the percentage of tubules containing three or more cells that had reached type B spermatogonial stage or later (Meistrich and van Beek, 1993). To obtain a more complete

description of the stages of differentiation present in the testis, we also determined the percentages of tubules with three or more cells reaching the leptotene spermatocyte stage or later (TDI-spermatocyte) or the round spermatid stage or later (TDI-spermatids) or with 10 or more cells reaching the elongating or elongated spermatids stage (TDI-late spermatids).

Although there are multiple subtypes of A spermatogonia in the rat testis (Chiarini-Garcia *et al*., 2003; van Bragt *et al*., 2008), they cannot be reliably distinguished in Bouin's-fixed methacrylate-embedded sections. Therefore, we counted all type A spermatogonia and Sertoli cells in atrophic seminiferous tubule cross-sections of irradiated rat testes at ×1000 magnification ($n = 3$–7 per group). For samples with almost complete seminiferous tubule atrophy, cells were counted using systematic random sampling (Stereo Investigator version 8.0 software; MicroBrightField, Inc., Williston, VT), by counting A spermatogonia and Sertoli cells in 300 randomly selected 100 × 80 μm fields. Results were presented as A spermatogonia per 100 Sertoli cells. In samples with few atrophic seminiferous tubules, these tubules were identified visually using light microscopy, and all cells in the tubules were counted. A minimum of 500 Sertoli cells was counted per testis.

Testicular sperm production was evaluated by counting sonication-resistant sperm heads, which represent nuclei of step 12–19 spermatids, in testicular homogenates. An aliquot of the homogenate of the left testis was sonicated and the sperm heads were counted in a hemocytometer using phase contrast optics (Meistrich and van Beek, 1993).

Hormone assays. Serum testosterone and intratesticular fluid testosterone concentrations were measured using a coated-tube radioimmunoassay kit (Coat-A-Count Total Testosterone; Cat No. TKTT1; Siemens, Los Angeles, CA) similar to procedures described previously (Porter *et al*., 2006; Shetty *et al*., 2000). Rat serum follicle–stimulating hormone (FSH) was measured by radioimmunoassay, and luteinizing hormone (LH) was measured by a sensitive two-site sandwich immunoassay. Both FSH and LH were measured by the University of Virginia, Center for Research in Reproduction, Ligand Assay and Analysis Core, using previously described methods (Gay *et al*., 1970).

Statistical analysis. Results were presented as either mean ± SEM calculated from untransformed data or, in the case of sperm head counts, testosterone, and LH, as the mean ± SEM calculated from log-transformed data obtained from individual rats. The statistical significance of differences between two groups was determined using SPSS version 19 software (Lead Technologies, Chicago, IL) using one-way ANOVA and the Student-Newman-Keuls *post hoc* test with $p < 0.05$ being considered significant.

RESULTS

Recovery of Spermatogenesis After Irradiation

Ten weeks after 5-Gy irradiation, rats were killed and testis tissue was harvested and serum removed for hormone analysis. Whereas the control testis parenchymal weights ranged from 1.04 g (Lewis and Fischer 344) to 1.74 g (Long-Evans), radiation markedly reduced testicular weights in all strains to between 0.29 g (Lewis) and 0.59 g (Sprague Dawley) (Fig. 1A). Expressing the parenchymal weight as a fraction of the control for each strain showed small but significant differences between strains. BN, Lewis, and Long-Evans appeared most sensitive as testicular weights decreased to between 24 and 28% of control. Fischer, Wistar Kyoto, and SHR had testicular weights of about 30% of control. Sprague Dawley was most resistant, with a testicular weight of 36% of control.

Interstitial fluid weights of control rats ranged from 0.06 g in Lewis to 0.11 g in Sprague Dawley, but no significant differences between strains were observed. Interstitial fluid weights were measured after irradiation (Fig. 1C) and showed negligible increases of only 0.01 g from the control in the Wistar Kyoto and SHR strains, marginal increases of 0.04–0.06 g in the Lewis, Fischer, and Sprague Dawley strains, but large significant increases of 0.15 g in Long-Evans and 0.22 g in the BN rats (Fig. 1D).

Despite only small differences in testis weights, the histological appearances of the testes were markedly different. Some strains, such as BN and Lewis, showed complete tubular atrophy with no differentiated germ cells present in any of the seminiferous tubules (Figs. 2A and B). However, the two strains differed in that there were large cellular interstitial spaces in BN, indicative of interstitial edema corresponding to the fluid accumulation in this strain (Fig. 1C), but not in the Lewis strain (not shown). Other strains such as SHR and Sprague Dawley showed recovery of spermatogenesis in essentially all tubules (Fig. 2C). Although late spermatids were observed in some tubules, other tubules showed incomplete recovery only to the spermatocyte or round spermatid stage (Fig. 2D).

The recovery of spermatogenesis was quantified by calculation of the TDI in histological sections (Fig. 3A). BN and Lewis were the most sensitive with less than 2% of tubules having evidence of differentiated germ cells, Long-Evans, Wistar Kyoto, and Fischer had between 50 and 75% of tubules with differentiated cells, whereas SHR and Sprague Dawley were more resistant, with evidence of differentiation in nearly all tubules. Long-Evans rats showed high variation in tubule differentiation (standard deviation: 31%), whereas the inbred strains and the outbred Sprague Dawley rats had standard deviations of < 10%.

The atrophic tubules were examined to determine whether they were a result of killing of stem spermatogonia or a block in their differentiation as previously observed with LBNF1 rats (Kangasniemi *et al*., 1996). The atrophic tubules observed in 5-Gy irradiated BN, Lewis, Long-Evans, Wistar Kyoto, Fischer 344, and SHR rats contained between 2.2 and 3.9 type A spermatogonia per 100 Sertoli cells (Table 1, Fig. 2C), indicating that the stem cells were not killed but their differentiation was blocked. Although residual A spermatogonia in Sprague Dawley rats exposed to 5 Gy could not be counted because less than 2% of tubules were atrophic, at 6.5 Gy, there were atrophic tubules and they did contain 2.8 type A spermatogonia per 100 Sertoli cells.

Among the recovering tubules, there was heterogeneity in the stages to which differentiation was observed. For example, of the 54% of the tubules showing differentiation in irradiated Long-Evans rats, 1% recovered only to the B spermatogonial stage, 17% reached the spermatocyte stage, 32% recovered to the round spermatid stages, and only 5% of the tubules reached the late spermatid stage. We used these data to plot the percentages of tubules reaching each stage of differentiation or beyond (Fig. 3B). These plots revealed differences between strains in the ability of differentiating tubules to progress. For

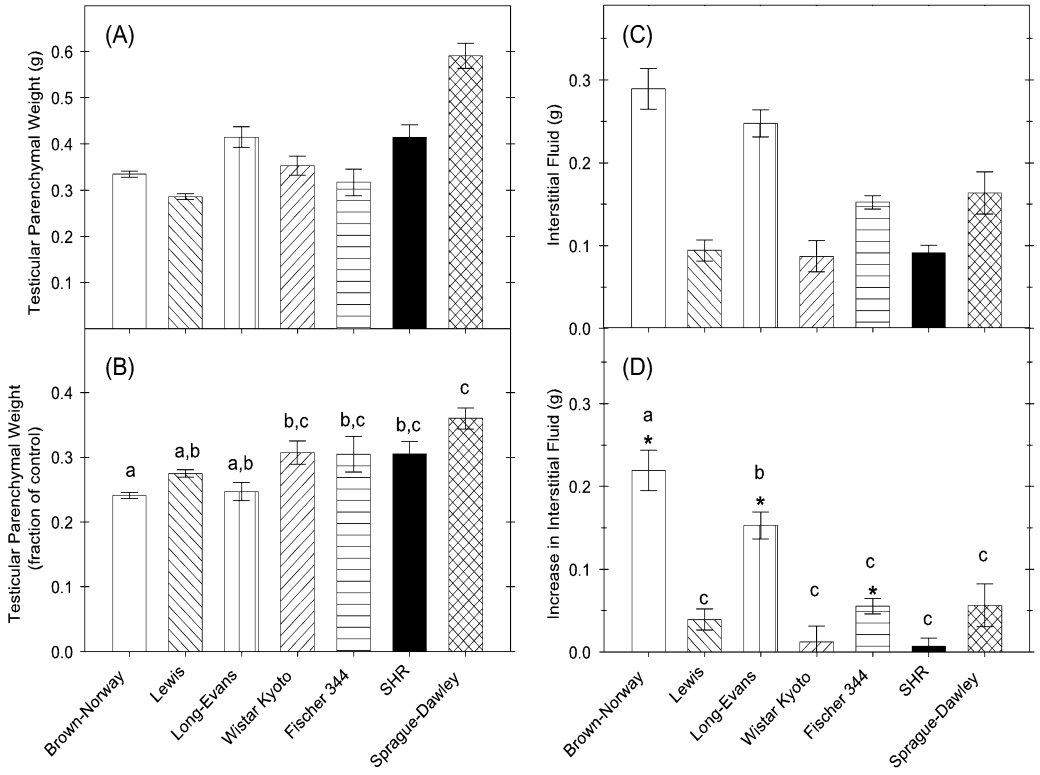

FIG. 1. Weights of testis parenchymal tissue and interstitial fluid for rats of seven different strains 10 weeks after irradiation with 5 Gy. (A) Absolute testis weights. (B) Testis weights relative to unirradiated controls of same strain. (C) Absolute testis interstitial fluid weights. (D) Increase in interstitial fluid weights from unirradiated control levels. In (B) and (D), the values for groups of irradiated rats with different letters (a, b, and c) are significantly different from each other ($p < 0.05$) and groups with the same letter are not. In (D), * is used to indicate strains that showed significant increases ($p < 0.05$) in testicular interstitial fluid resulting from irradiation.

example, whereas in both SHR and Sprague Dawley rats germ cell differentiation reached the spermatocyte stage or beyond in over 90% of tubules, in SHR only 20% of tubules recovered to the late spermatid stage but in Sprague Dawley 42% showed late spermatids.

Although the histological data provide an indication of sperm production, a more quantitative measure is the number of sonication-resistant sperm heads per testis. Irradiated rats showed huge differences between strains, with over 1000-fold differences in testicular sperm production, varying from about 10^4 in BN and Lewis to almost 2×10^7 in Sprague Dawley (Fig. 4A). As there were some differences in control values, ranging from 1.4×10^8 in Lewis to 2.4×10^8 in Long-Evans, the counts were normalized to the control values (Fig. 4B). BN and Lewis were the most sensitive rat strains with more than a 10,000-fold reduction in sperm production, and Sprague Dawley was most resistant with sperm count remaining at 9% of control. Long-Evans rats were more resistant than Lewis and BN but more sensitive than Wistar Kyoto, Fischer, and SHR; they also showed the largest standard deviation in the counts. These strain differences were consistent with the percentages of tubules with late spermatids in the histological sections (Fig. 3B).

Hormone analyses were performed on one sensitive strain, BN, and one resistant strain, SHR. Serum testosterone, interstitial fluid testosterone, and serum FSH levels in control and irradiated SHR rats were significantly higher than the corresponding values in BN rats, and serum LH levels were significantly higher in control SHR rats than BN rats (Fig. 5). Although there were no significant changes in serum testosterone and LH levels in either BN or SHR rats as a result of irradiation, interstitial fluid testosterone levels were significantly increased after radiation in SHR rats by1.4-fold, and serum FSH levels significantly increased after radiation in both SHR (1.7-fold) and BN rats (2-fold).

DISCUSSION

In this study, we directly compared the recovery of spermatogenesis at 10 weeks after 5 Gy of irradiation in seven different strains or stocks of rats. The results showed that the recovery of spermatogenesis was incomplete in all strains of rats analyzed. Even in the most resistant strain, Sprague Dawley, sperm counts had not even recovered to 10% of control levels.

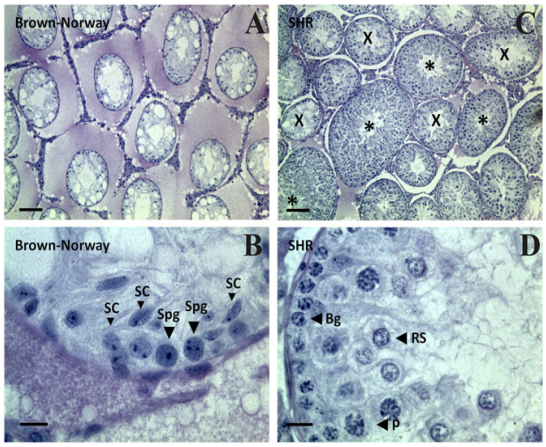

FIG. 2. Histology of rat testes 10 weeks after irradiation with 5 Gy. (A) BN testis showing atrophic tubules and interstitial edema. (B) The tubules in BN contained mostly Sertoli cells (SC) but some contained a few type A spermatogonia (Spg). (C) SHR testis showing recovery of spermatogenesis in nearly all tubules. Some tubules in SHR testes (*) showed complete spermatogenesis; other tubules (X) only showed development to the early spermatid stage. (D) Higher magnification image of tubule from irradiated SHR rat showing development to only the early spermatid stage. (Bg) Type B spermatogonia, (P) pachytene spermatocyte, and (RS) round spermatid. Scale (A, C) bar: 100 µm, Scale (B, D) bar: 10 µm.

TABLE 1
Number of Type A Spermatogonia per 100 Sertoli Cells in Nonrepopulating Tubules at 10 Weeks After 5-Gy Irradiation[a] in Different Rat Strains (n = 3–7 per Group)

Strain[a]	Spermatogonia per 100 Sertoli cells[b]
BN	2.4 ± 0.5
Lewis	2.2 ± 0.3
Long-Evans	3.4 ± 0.3
Wistar Kyoto	3.9 ± 0.3
Fischer 344	2.5 ± 0.4
SHR	2.5 ± 0.9
Sprague Dawley[a]	2.8 ± 0.2

[a]There were insufficient (< 2%) nonrepopulating tubules in 5-Gy irradiated Sprague Dawley rats to perform these counts, so the group irradiated with 6.5 Gy was used for these counts.
[b]No significant differences were observed between different rat strains.

The contribution of the block in spermatogonial differentiation, previously described in LBNF1 rats, to the failure of recovery was assessed. The atrophic tubules observed in all strains of rats contained similar numbers of type A spermatogonia (Table 1). These results indicate that the failure of recovery was not due to loss of stem cells but rather to treatment-induced block in the ability of the spermatogonia in these tubules to differentiate and that the major component of the difference in sensitivity between the strains was in the percentage of tubules with evidence of a block in spermatogonial differentiation at a given dose (Fig. 3). Thus, the radiation-induced block in spermatogonial differentiation is a characteristic of all strains but had not been observed before in Sprague Dawley rats either because the radiation doses were low (Dym and Clermont, 1970; Erickson and Hall, 1983; Huckins, 1978) or the spermatogonia in the atrophic tubules were not noticed in paraffin-embedded tissues (Delic *et al.*, 1987).

Even in the tubules showing differentiated germ cells, there was heterogeneity in the ability to differentiate into the various

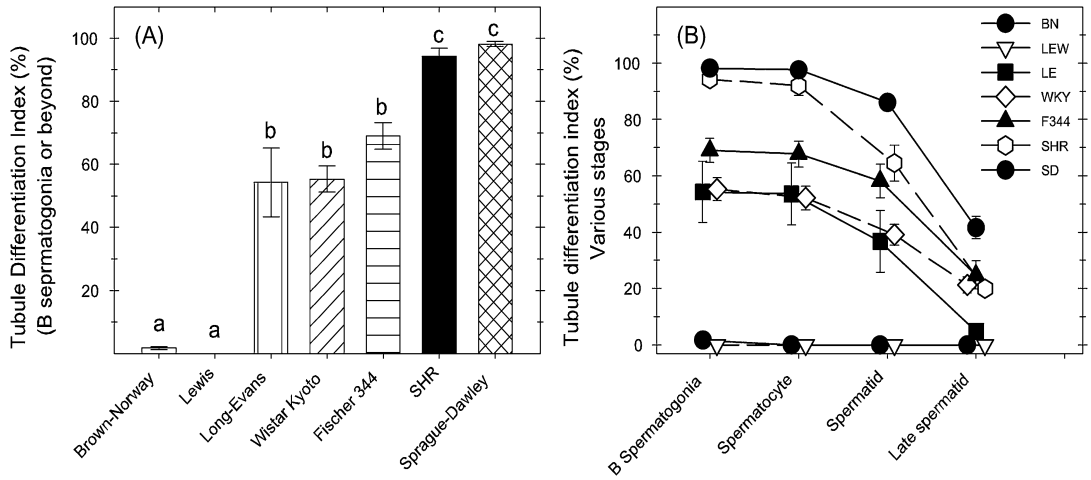

FIG. 3. Recovery of spermatogenesis as measured by the percentage of tubules with morphologically differentiated cells at a specific stage of differentiation or beyond for different strains of rats. (A) TDI defined as differentiation to the B spermatogonial stage or beyond, unless otherwise noted. (B) Percentage of tubules reaching differentiation to specific stages or beyond. The values for groups of irradiated rats with different letters (a, b, and c) are significantly different from each other ($p < 0.05$) and groups with the same letter are not.

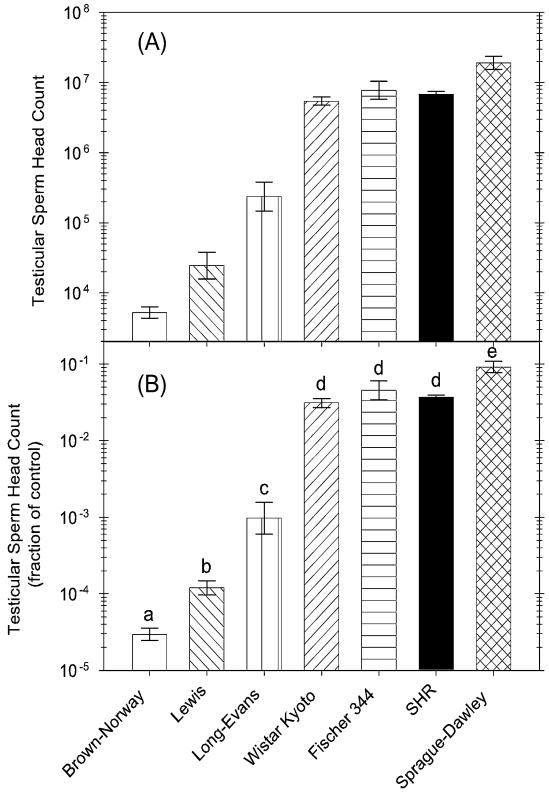

FIG. 4. Testicular sperm production. (A) Numbers of sonication-resistant late spermatids per testis in rats at 10 weeks after irradiation with 5 Gy. (B) Numbers of sonication-resistant late spermatids relative to unirradiated controls of same strain. The values for groups of irradiated rats with different letters (a, b, c, d, and e) are significantly different from each other ($p < 0.05$) and groups with the same letter are not.

stages (Figs. 2C and 3B). We attribute this to damage to the somatic environment, with some tubules being able to support differentiation to only the spermatocyte or early spermatid stage. Although the present data do not rule out the possibility that this heterogeneity reflects variable delays in initiation of differentiation in various tubules, other data (Kangasniemi *et al*., 1996) (our unpublished results) show that the differentiation in some tubules does not progress beyond a certain stage even at later postirradiation time points.

Because radiation treatment with 5 Gy produced consistent results on the recovery of spermatogenesis within inbred strains of rats but produced differing results between strains, the differences in radiation sensitivity must be attributable to genetic variations between the strains. Consistent with this idea, we found that the standard deviations of the sperm count and tubule differentiation data after irradiation were greater in the outbred Long-Evans rats than in any of the inbred strains, although such a difference was not observed in the outbred Sprague Dawley rats. However, whereas the outbred Sprague Dawley rats were most resistant to radiation effects on spermatogenic recovery, the outbred Long-Evans rats were moderately sensitive, so we cannot conclude that outbred rats are more resistant than inbred ones.

The rat strains were classified according to their differing sensitivities to radiation-induced inhibition of spermatogenic recovery: BN and Lewis were the most sensitive; Long-Evans was intermediate; Wistar Kyoto, Fischer, and SHR were moderately resistant; and Sprague Dawley was most resistant. To investigate a basis for this grouping, we compared the phylogenetic relationships among strains (Saar *et al*., 2008; Thomas *et al*., 2003) to resistance levels. The SHR and Wistar Kyoto rats are most closely related and their similar resistance to radiation likely is due to a common set of genes. In contrast, Lewis and Fischer rats, which also are derived from a common ancestor and are relatively closely related, showed a dramatic difference in radiation sensitivity. Lewis are also much more closely related to the more resistant Sprague Dawley rats than they are to the highly sensitive BN strain, which is most genetically distinct of all the rat strains and diverged first in the evolution of strains. Thus, the cause of radiation sensitivity in BN may be different from that in the Lewis rats as it is more likely that two different mutations related to sensitivity would have arisen in the BN and Lewis strains than that mutations to produce resistance arose in all of the five other strains after divergence from the common ancestor with BN. In contrast to the lack of a close relationship between radiation sensitivity and phylogenetic relationship, interstitial fluid accumulation was more closely associated in related strains. The low levels of increase in fluid after irradiation in Lewis, Fischer, Sprague Dawley, and particularly SHR and Wistar Kyoto (Fig. 1D) are consistent with their close phylogenetic relationship; significantly greater increases were observed in Long-Evans and BN, which are more distantly related to the first five strains.

Our results on differential sensitivities of various strains of rats are in general agreement with previous studies using radiation and different toxicant models. The recovery of spermatogenesis after irradiation in Sprague Dawley rats has been shown to be greater than in Wistar rats (Delic *et al*., 1987). We previously reported that the recovery of spermatogenesis after treatment with the procarbazine was much greater in Sprague Dawley than in Lewis or LBNF1 rats (Parchuri *et al*., 1993). In addition, Sprague Dawley rats showed greater recovery of spermatogenesis than did Fischer rats after treatment with 2,5-hexanedione, a Sertoli cell toxicant (Blanchard *et al*., 1996; Boekelheide, 1988; Boekelheide and Hall, 1991). Thus, the interstrain differences appear to be related to the sensitivity to induction of a spermatogonial block after different toxic stresses rather than the sensitivity of the testis to a particular toxicant.

The role of hormones in the strain differences in radiation sensitivity was investigated next. In normal rats, spermatogonial differentiation is qualitatively independent of both testosterone and FSH and occurs even when these hormones are suppressed (Huang and Nieschlag, 1986). However, in

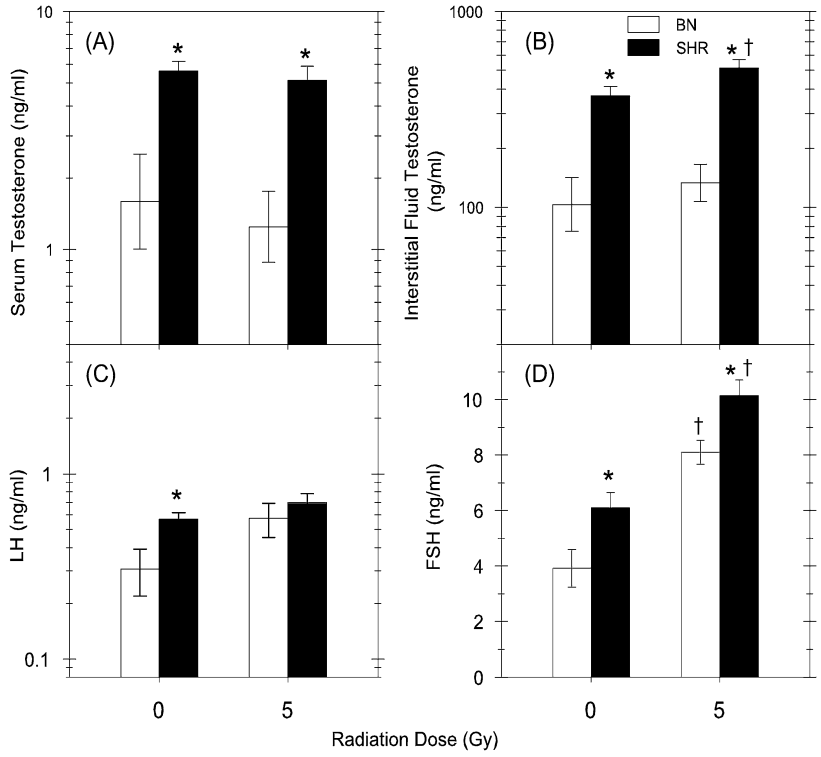

FIG. 5. Testosterone, LH, and FSH levels in BN and SHR rats at 10 week after irradiation with 5 Gy. (A) Serum testosterone. (B) Intratesticular fluid testosterone. (C) Serum LH. (D) Serum FSH. * Indicates values in SHR are significantly different from those in BN, and † indicates value in irradiated is significantly different from that in unirradiated testes ($p < 0.05$, *t*-test).

irradiated rats, we demonstrated that the differentiation of type A spermatogonia can be completely inhibited by moderate levels of testosterone alone, independently of the pituitary hormones, or partially inhibited by high levels of FSH (Shetty *et al*., 2006). In fact, suppression of testosterone for 10 weeks after irradiation of LBNF1 rats (Meistrich *et al*., 2001) or BN rats (data not shown) can restore the production of differentiated cells in nearly all tubules, as we observed with the more resistant strains without the need for hormonal suppression (Fig. 3A). We therefore tested whether the block in BN rats but not SHR rats could be due to higher levels of testosterone or FSH. To the contrary, there were lower levels of serum and intratesticular testosterone and FSH in BN rats than in SHR rats both before and after irradiation. An alternative hypothesis, that the high levels of testosterone in SHR are responsible for the greater recovery of spermatogenesis, is not consistent with all of our data, as Sprague Dawley rats, the most resistant strain, had levels of testosterone intermediate between the levels in SHR and BN rats (data not shown).

The possible role of the increase in interstitial fluid levels in the inhibition of spermatogonial differentiation was also evaluated because we previously identified a correlation between the two parameters in irradiated LBNF1 rats (Porter *et al*., 2006). Radiation induced significant increases in interstitial fluid levels in three of the rat strains, most dramatically in BN, a sensitive strain, and Long-Evans, a strain with intermediate sensitivity. In contrast to BN, the other radiation-sensitive strain, Lewis, showed only a small nonsignificant increase in fluid levels. Examination of the relationship between the increase in interstitial fluid and TDI in the various strains (Fig. 6) failed to indicate any significant correlation between the increase in fluid after irradiation and sensitivity of the different strains to the radiation-induced block in spermatogonial differentiation.

The genetic alterations that are responsible for the differences in the recovery of spermatogenesis after radiation in the various strains are not known. The sensitive and resistant strains identified in this study could be used to determine which specific changes in gene expression that occurred after radiation in LBNF1 rats (Zhou *et al*., 2010) also occur in a sensitive inbred strain identified in this study but not in a resistant strain. In addition, the regions of the genome (quantitative trait loci, QTL) that contain the candidate genes for the interstrain differences in radiation sensitivity can be determined from genetic crosses between strains. Fortunately the BN and SHR rats, a pair of strains for which recombinant inbred rats already available (Tabakoff *et al*., 2009) showed

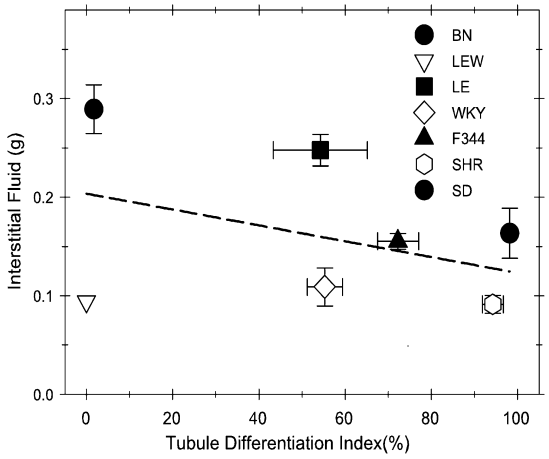

FIG. 6. Radiation-induced increase in testicular interstitial fluid plotted against the recovery of spermatogenesis at 10 weeks after 5 Gy of irradiation. A linear regression was performed on the data points and the correlation was weak ($r^2 = 0.25$) and not statistically significant ($p = 0.25$).

large differences in radiation sensitivity, and we are using these strains to identify QTL related to the radiation sensitivity.

The difference between strains in radiation response highlights the importance of knowledge of this information in choosing an animal species and strain within that species for evaluation of risks to human. Blocks in spermatogonial and later germ cell differentiation were observed in all strains and may correspond to the human situation in which no sperm is produced for a prolonged periods after single doses of 1–6 Gy of irradiation to the testis, despite the presence of surviving stem cells from which there is eventual recovery of spermatogenesis (Clifton and Bremner, 1983). In this study, which used only one dose at one time point (5 Gy, 10 weeks), we found very large differences in recovery of differentiation (0–100% of tubules) and sperm production (100-fold differences). It is not known whether there are qualitative differences between strains or only quantitative differences in the dose at which the complete block occurs or differences in the time course of possible subsequent recovery. These questions are being addressed in further experiments.

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ORIGINAL ARTICLE

Differentiation of murine male germ cells to spermatozoa in a soft agar culture system

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Establishment of an *in vitro* system that allows the development of testicular germ cells to sperm will be valuable for studies of spermatogenesis and future treatments for male infertility. In the present study, we developed *in vitro* culture conditions using three-dimensional agar culture system (SACS), which has the capacity to induce testicular germ cells to reach the final stages of spermatogenesis, including spermatozoa generation. Seminiferous tubules from testes of 7-day-old mice were enzymatically dissociated, and intratubular cells were cultured in the upper layer of the SACS in RPMI medium supplemented with fetal calf serum (FCS). The lower layer of the SACS contained only RPMI medium supplemented with FCS. Colonies in the upper layer were isolated after 14 and 28 days of culture and were classified according to their size. Immunofluorescence and real-time PCR were used to analyse specific markers expressed in undifferentiated and differentiated spermatogonia (*Vasa*, *Dazl*, *OCT-4*, *C-Kit*, *GFR-α-1*, *CD9* and *α-6-integrin*), meiotic cells (*LDH*, *Crem-1* and *Boule*) and post-meiotic cells (*Protamine-1*, *Acrosin* and *SP-10*). Our results reveal that it is possible to induce mouse testicular pre-meiotic germ cell expansion and induce their differentiation to spermatozoa in SACS. The spermatozoa showed normal morphology and contained acrosomes. Thus, our results demonstrate that SACS could be used as a novel *in vitro* system for the maturation of pre-meiotic mouse germ cells to post-meiotic stages and morphologically-normal spermatozoa.

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Keywords: acrosome; agar; *in vitro* culture; meiosis; spermatogenesis; spermatogonia; spermatozoa; testis

INTRODUCTION

In mammalian species, spermatogenesis occurs in the seminiferous tubules of the testis and relies on the appropriate expansion of undifferentiated and differentiated spermatogonia prior to the entry of germ cells into meiosis and subsequent spermiogenesis.[1,2]

Several attempts have previously been made to establish and optimize germ cell cultures using specific culture media, growth factors, sera, conditioned media of testicular or non-testicular origin and feeder layers.[1,3–12] However, none of these conditions have successfully generated spermatozoa.

Most attempts to culture male germ cells have been performed using two-dimensional cell culture systems. We recently described a novel three-dimensional cell culture system using soft agar (SACS)[11] (**Figure 1**). This culture system is more representative of the *in vivo* conditions as it mimics some aspects of the natural three-dimensional environment a cell is exposed to in an organ.[13,14] In the past, the three-dimensional SACS has been used to investigate proliferation and differentiation of bone marrow and haematopoietic cells *in vitro*.[15,16] We hypothesize that this approach is adaptable to male germ cells and will optimize the microenvironment for clonal expansion and differentiation of germ cells. In a recent review, we provided preliminary evidence that pre-meiotic mouse germ cells differentiated into morphologically normal sperm using SACS.[17] Here, we provide all experimental details, and we present additional evidence by detailed analysis of germ cell clusters, using real-time PCR and immunofluorescence. We provide evidence that a progressive development of germ cells occurs *in vitro* and that SACS is an appropriate strategy for the expansion and differentiation of immature mouse testicular germ cells. Starting with pre-meiotic germ cells, SACS supports the development of mature spermatozoa with intact acrosomes.

MATERIALS AND METHODS

Animals

This investigation was conducted in accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by the Society for the Study of Reproduction. Sexually mature (4- to 8-week-old) or immature (7- and 14-day-old) BALB/c mice (Harlan Laboratories, Jerusalem, Israel) were used.

Chemicals and reagents

Collagenase V and DNAase (2000 KU) were obtained from Sigma (St Louis, MO, USA). RPMI, penicillin, streptomycin and fetal calf serum (FCS) were purchased from Beit Haemek Biological Industries (Beit Haemek, Israel). Agar was purchased from Bacto-Agar (Difco Laboratories, Detroit, MI, USA).

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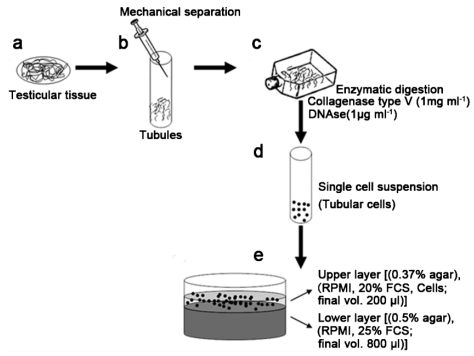

Figure 1 Scheme of the SACS. The SACS was composed of two layers: the solid lower layer (0.5% (w/v) agar) and the soft upper layer (0.37% (w/v) agar), which were cultured in 24-well plates. Testicular tissue from immature mice (**a**) was mechanically separated to obtain interstitial tissue and tubules (**b**). The tubules were enzymatically digested (**c**), and the isolated tubular cells (**d**) were used for culture in the upper phase of the SACS (**e**). Tubular cells (10^6 cells per well per 200 µl) were cultured in the upper layer of the soft agar medium. Cultures were incubated in 5% CO_2 incubator at 37 °C. FCS, fetal calf serum; SACS, Soft Agar Culture System.

Isolation of mouse spermatogonial cells

Tubular cells were isolated from the testes of 7-day-old male BALB/c mice. At this age, the testis does not contain any meiotic germ cells and the seminiferous epithelium comprises proliferating Sertoli cells and a mixture of undifferentiated and differentiating type A spermatogonia. Testicular cell suspensions were obtained as described by Zeyse *et al.*[18] Briefly, the testes were decapsulated and mechanically digested by multiple aspirations through pipette tips (eight aspirations through 2-mm diameter tips followed by 10 aspirations through 1-mm diameter tips) into a 50-ml syringe following the addition of 20 ml phosphate-buffered saline (PBS). Mechanical digestion was continued until the tubules were completely dissociated. Thereafter, the tubules were allowed to settle by gravity and washed three times with PBS. Supernatants containing the interstitial cells were discarded. The tubules were transferred to a 50-ml culture flask and subjected to digestion in saline containing collagenase type V (1 mg ml^{-1}) and DNAse (1 µg ml^{-1}) for 25 min in a shaking water bath (120 cycles min^{-1} at 37 °C). The suspension was then aspirated three times with a pipette and incubated for an additional 5 min in a 37 °C shaking water bath. This isolation procedure resulted in a single cell suspension consisting of Sertoli cells and spermatogonia (as these are the only germ cells present at postnatal day 7) as well as a small proportion of peritubular cells.

The intratubular cell suspension was filtered through sterile surgical gauze and washed by centrifugation for 5 min at 200*g* at room temperature. The cells were suspended in RPMI and counted.

The same method, using testes from 14-day-old and mature mice, was used to prepare a suspension of tubular cells to be used as a positive control for immunostaining and real-time PCR analysis. The suspension from adult mice contains germ cells of all spermatogenic stages (undifferentiated spermatogonia to spermatozoa).

SACS

The conditions for the clonogenic culture of testicular cells in SACS were selected in accordance with previous experiments performed on haematopoietic stem cells.[16] Briefly, the SACS was composed of two layers (**Figure 1**): the solid lower layer (0.5% (w/v) agar) and the soft upper layer (0.37% (w/v) agar) and cultured in 24-well plates. To establish definite concentrations of agar and cells, 0.7% (w/v) agar and 1% (w/v) agar were mixed with distilled water during the preparation of the upper and lower phases, respectively. Tubular cells (10^6 cells per well per 200 µl) were cultured in the upper layer of the soft agar medium (0.37% agar+RPMI+20% (v/v) FCS, tubular cells; final volume of 200 µl). Cell suspensions were added to the RPMI prior to mixing with the agar. The agar and the RPMI/cells were mixed at 37 °C to avoid heat-induced cellular stress and premature agar coagulation. This layer was added on top of the lower layer after it had solidified. The solid agar base (lower layer; final volume of 800 µl) contained both RPMI and 25% (v/v) FCS and 0.5% (w/v) agar only. All culture experiments were maintained in standard cell culture incubators at 37 °C and 5% CO_2 for up to 4 weeks.

Cells in the culture dish were defined as colonies when more than 50 cells were present and were further classified into colonies containing more than 50 cells but less than 150 cells (small; S), more than 150 but less than 300 cells (medium; M) and more than 300 cells (large; L). The analysis of colonies was performed microscopically after incubation for two or four weeks. Colonies (S, M and L) were counted separately in each well of the same treatment group (4–6 wells in each treatment group).

Colonies (S, M and L) were manually picked under microscopic observation using a microtip, and the cells in this sample were processed for RNA detection and immunofluorescence analysis. Colonies from all wells of the same treatment were determined with respect to their size and were collected into the same tube for RNA extraction. Cells from different experiments were stored in different tubes and analysed separately. In addition, extracted RNA from the colonies was examined for the expression of androgen binding protein (*ABP*) (a specific marker for Sertoli cells), alpha-smooth muscle (*α-Sm*) (a specific marker for peritubular cells; P) and immune cell markers, such as those for macrophages (*CD11-b*).

Analysis to evaluate spermatogonial cell differentiation stages

Extraction of total RNA for reverse transcription (RT-) PCR and real time PCR analysis. First-strand complementary DNAs (cDNAs) were synthesized from 2.5 µg total RNA (from testicular homogenates) or using the entire extracted RNA when colonies were picked) with 0.5 µg random oligonucleotide primers (Roche Molecular Biochemicals, Mannheim, Germany) and 200 U of Moloney-Murine Leukaemia Virus-Reverse Transcriptase (M-MLV-RT; Life Technologies, Inc., Paisley, Scotland, UK) in a total volume of 20 µl Tris-HCl-MgCl reaction buffer, supplemented with DTT, dNTPs (0.5 mmol l^{-1}; Roche Molecular Biochemicals) and RNase inhibitor (40 U; Roche Molecular Biochemicals). The RT reaction was performed for 1 h at 37 °C and stopped for 10 min at 75 °C. The volume of 20 µl was subsequently filled up to 60 µl with treatment water. Negative controls for the RT reaction were prepared in parallel, using the same reaction preparations with the same samples, without M-MLV. RT-PCR was performed using cDNA samples at a final dilution of 1 : 15 with two pairs of oligonucleotide primers (Sigma) (**Table 1**).

To assess the absence of genomic DNA contamination in RNA preparations and RT-PCR reactions, PCR was performed with negative controls of the RT reaction (RT-) and without cDNA (cDNA-). The PCR reactions were carried out on a Cycler II System Thermal Cycler (Ericomp, San Diego, CA, USA). Twenty microlitres of each PCR product was run on a 2% agarose gel that contained ethidium bromide and was photographed under UV light. The amount of mRNA used in each RT-PCR experiment was 50 ng.

Table 1 Reverse transcription (RT-) PCR primers

Stage	*Target*	*Primer*	*Sequence*	*T_m (°C)*	*Size (bp)*
Pre-meiotic	*Nanog*	Forward	3′-AGGGTCTGCTACTGAGATGCTCTG-5′	57	363
		Reverse	5′-CAACCACTGGTTTTTCTGCCACCG-3′		
	VASA	Forward	3′-GGTCCAAAAGTGACATATATACCC-5′	57	419
		Reverse	5′-TTGGTTGATCAGTTCTCGAGT-3′		
	OCT-4	Forward	3′-AGAAGGAGCTAGAACAGTTTGC-5′	57	416
		Reverse	5′-CGGTTACAGAACCATACTCG-3′		
	C-KIT	Forward	3′-GCATCACCATCAAAAACGTG-5′	57	331
		Reverse	5′-GATAGTCAGCGTCTCCTGGC-3′		
	GFR-α-1	Forward	3′-GGCCTACTCGGGACTGATTGG-5′	57	462
		Reverse	5′-GGGAGGAGCAGCCATTGATTT-3′		
	α-6-integrin	Forward	3′-AGGAGTCGCGGGATATCTTT-5′	57	502
		Reverse	5′-CAGGCCTTCTCCGTCAAATA-3′		
	CD9	Forward	3′-ATCTTCTGGCTCGCTGGCATT-5′	57	373
		Reverse	5′-ATGGCTTTGAGTGTTTCCCGCT-3′		
Meiotic	*Crem-1*	Forward	5′-TTCTTTCACGAAGACCCTCA-3′	57	114
		Reverse	5′-TGTTAGGTGGTGTCCCTTCT-3′		
	LDH	Forward	3′-GCACGGCAGTCTTTTCCTTAGC-5′	57	585
		Reverse	5′-TCGCGCCAGATCAGTCACAG-3′		
Post-meiotic	*Protamine*	Forward	3′-GGCCACCACCACCACAGACACAGGCG-5′	57	188
		Reverse	5′-TTAGTGATGGTGCCTCCTACATTTCC-3′		
Sertoli cells	*ABP*	Forward	3′-GGAGAAGAGAGACTCTGTGG-5′	57	900
		Reverse	5′-GCTCAAGACCACTTTGACTC-3′		
Peritubular cells	*α-Sm*	Forward	3′-CATCAGGCAGTTCGTAGCTC-5′	57	524
		Reverse	5′-CGATAGAACACGGCATCATC-3′		
Leydig cells	*LHR*	Forward	3′-AATACACAACTGTGCATTCAAC-5′	57	451
		Reverse	5′-ATTTGGATGAAGTTCAGAGGTT-3′		
Macrophages	*CD11-b*	Forward	3′-GTCAGTGGCATGGTG-5′	57	524
		Reverse	5′-CAAAGCTTCTGCTGT-3′		
House keeping gene control	*β-actin*	Forward	3′-AGAGGGAAATCGTGCGTGAC-5′	57	485
		Reverse	5′-GCCGGACTCATCGTACTCCT-3′		

Real-time PCR analysis

Real-time quantitative PCR amplification of total cDNA (500 ng per sample) used specific primers of the different sequences (**Table 2**).

The reactions were conducted following the protocol for the Absolute qPCR SYBR Green mix (ABgene House, Blenheim Road, Epsom, UK) containing modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mmol l^{-1} $MgCl_2$, dNTP mix and dUTP. The PCR reaction was performed using a real-time PCR machine (MyIQ; Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. The following PCR protocol repeated 45–50 times was used: denaturation (95 °C for 10 min), amplification and quantification (94 °C for 10 s), 72 °C for 30 s with a single fluorescence measurement, melting curve (60–95 °C with a heating rate of 0.5 °C per 30 s and a continuous fluorescence measurement) and a cooling step to 4 °C. PCR products were identified and distinguished by the generated melting curve. The 'threshold cycle' (C_t) values, which represented the cycle number at which the sample fluorescence rose statistically above background, and crossing points for each transcript were defined. The relative quantity of gene expression was analysed by the $2^{-\Delta\Delta C_t}$ method.

To ensure accurate products and the absence of contaminating DNA, all real-time PCR products were also examined in parallel by PCR analysis with the negative controls.

Immunofluorescence staining of cells from colonies developed in SACS

Cells were fixed in cold methanol for 10 min and stored at 4 °C until stained. Before the primary antibodies were applied, nonspecific background staining was blocked with PBS containing 4% FCS and/or antibodies against the relevant IgG isotype. Thereafter, polyclonal rabbit anti-mouse C-Kit (4 μg ml^{-1}), rabbit anti-mouse GFR-α-1 (4 μg ml^{-1}), rabbit anti-mouse CD9 (4 μg ml^{-1}), rabbit anti-mouse α-6-integrin (4 μg ml^{-1}), rabbit anti-ETQEDAQKILQEAEKLNYK-DKKLN peptide (1 : 300), rabbit anti-mouse Crem-1 (4 μg ml^{-1}), goat anti-mouse lactate dehydrogenase (LDH) (4 μg ml^{-1}), goat anti-mouse Protamine (4 μg ml^{-1}), goat anti-mouse Acrosin (4 μg ml^{-1}), goat anti-mouse Dazl (4 μg ml^{-1}) and rabbit anti-mouse Vasa (4 μg ml^{-1}) antibodies were used as primary antibodies. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The primary antibodies were incubated over night at 4 °C in PBS containing 4% FCS. PBS was used for all subsequent washing steps. Fluorescein-conjugated antibodies (Cy3; donkey anti-rabbit or donkey anti-goat antibodies; 4 μg ml^{-1}; Jackson ImmunoResearch, West Grove, PA, USA) were used for visualisation of the signal according to the suppliers' directions. After 1 h of incubation, the slides were washed in PBS and subsequently subjected to DAPI staining (Santa Cruz Biotechnology, Inc). Negative controls were included for each specimen using PBS containing FCS/BSA instead of the primary antibodies.

Immunofluorescence staining of mouse testicular tissue

Four-micron thick sections from formalin-fixed, paraffin-embedded testicular tissue blocks were mounted on saline-coated slides, dried at 37 °C for 48 h and stored at room temperature. Before the primary antibodies were applied, nonspecific background staining was blocked with PBS containing 0.05% casein and/or relevant antibodies for the

Table 2 Real-time PCR primers

Stage	Target	Primer	Sequence	T_m (°C)	Size (bp)
Pre-meiotic	*Vasa*	Forward	5′-GTATTCATGGTGATCGGGAGCAG-3′	60	88
		Reverse	5′-CAACAAGAACTGGGCACTTTCCA-3′		
	Dazl	Forward	5′-GCACTCAGTCTTCATCAGCAACCA-3′	60	187
		Reverse	5′-CTTCGACACACCAGTTCGATCAGT-3′		
	OCT-4	Forward	5′-GAAGTTGGAGAAGGTGGACCA-3′	60	91
		Reverse	5′-GCTTCAGCAGCTTGGCAAA-3′		
	C-Kit	Forward	5′-TGATTGTGCTGGATGATGGATGG-3′	60	106
		Reverse	5′-ATCTGCTCTGCGTCTGTTGGT-3′		
	GFR-α-1	Forward	5′-CATATCAGATGTTTTCCAGCA-3′	60	127
		Reverse	5′-TGGTACAGGGGGTGATGTAGG-3′		
	CD9	Forward	5′-ATGGCTTTGAGTGTTTCCCGCT-3′	60	372
		Reverse	5′- ATCTTCTGGCTCGCTGGCATT -3′		
	α-6-integrin	Forward	5′-CCGGCCAGTGATTAACATTCT-3′	60	62
		Reverse	5′-TGAGCCACACATGGACTTCT-3′		
Meiotic	*Crem-1*	Forward	5′-TTCTTTCACGAAGACCCTCA-3′	60	114
		Reverse	5′-TGTTAGGTGGTGTCCCTTCT-3′		
	LDH	Forward	5′-GAGTCAGCAGTAAGCTCAACA-3′	60	111
		Reverse	5′-ATTTCCAACTCGACACAG-3′		
Post- meiotic	*Protamine*	Forward	5′-ACACAGGCGCTGCTTCGTAA-3′	60	169
		Reverse	5′-GTGATGGTGCCTCCACATTTCCT-3′		
	SP-10	Forward	5′-ATCTGAAGGGTTTGGAGTGAGAG-3′	60	134
		Reverse	5′-TGGGTCTTTATCTGGTTGGATCTGCC-3′		
	Acrosin	Forward	5′-TGTCCGTGGTTGCCAGGATAACA-3′	60	85
		Reverse	5′-AATCCGGGTACCTGTTGTGAGTT-3′		
House keeping gene control	*β-actin*	Forward	5′-AGAGGGAAATGTGCGTGAC-3′	60	99
		Reverse	5′-CAATAGTGATGACCTGGCCGT-3′		

relevant of IgG isotype. This solution was also used for incubation with primary antibodies. For antigen retrieval, sections were boiled in 6 mol l^{-1} urea for 10 min.[19] Thereafter, tissues were stained with the different primary and secondary antibodies using methods similar to those described for staining cells above. Negative controls were included for each specimen using PBS/casein/relevant IgG isotype instead of the primary antibodies.

Identification of spermatozoa in SACS

The entire agar well (1 ml, with all colonies and cells) was transferred to a cassette for fixation in formalin (250 ml of 4% formalin) for 24 h. After fixation, the agar was washed in different concentrations of alcohol (30%, 50%, 70%, 85%, 95% and 100%), for 30 min in each concentration.

At the end of the washing process, the agar containing colonies (around 0.2 ml of loosely agar) was transferred from the cassette into an Eppendorf tube. One millilitre of saline was added to the tube and was repeatedly pipetted to destroy the agar. The suspension was transferred to a slide and left overnight in a laminar flow hood to evaporate the water while most of the residual agar reverted to powder. Thereafter, the slide was fixed in cold methanol for 15 min and dried at room temperature before staining with haematoxylin and eosin to identify cells.

Differentiation of male germ cells to spermatozoa in SACS

Differentiation of tubular cells to spermatozoa was determined in SACS after 30 days of culture (in 11 different experiments using 32 samples).

Acrosome identification in sperm cells

The presence of acrosomes was confirmed in the spermatozoa that developed after 30 days of culture in SACS (in four different experiments using four samples).

The presence of acrosomes in spermatozoa was determined using a fluorescence microscope. Sperm cells isolated from SACS were smeared on microscope slides at room temperature for 10 min. After air drying, sperm smears were fixed in cold absolute methanol for 15 min, washed once in Tris-buffered saline (TBS) and twice in distilled water at 5 min intervals, air dried, incubated with lectin from Archis hypogaea (peanut)-conjugated FITC (PNA-FITC) (Sigma)[20] (25 μg ml^{-1}) in Tris-buffered saline for 30 min, washed with distilled water and mounted with FluoroGuard Antifade (BioRad Laboratories).

Spermatozoa with green staining over the acrosomal cap were considered to be cells with intact acrosome.

Microscopy

Samples were observed with an Olympus IX70 microscope (Olympus, Novato, CA, USA). Digital images and signal intensity charts were prepared using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA), Microsoft Excel and Adobe Photoshop 7.0 software.

Data analysis and statistical evaluation

Each culture condition was tested in 4–6 wells. The plotted data are means calculated from 3–10 repeats of the experiment. The standard deviation represents the variability between independent experiments. For quantitative data on RNA expression and colony counts, the single data points obtained in independent experiments from each well with identical culture conditions were combined to calculate mean±s.d. One-way ANOVA and Bonferroni's multiple comparison test were used to estimate statistical significance, and P values <0.05 were considered significant.

RESULTS

Isolated tubular cells from 7-day-old and mature (8-week-old) mice were examined by RT-PCR (**Figure 2a**) and immunofluorescence analysis (for tubular cells from 7-day-old mice; **Figure 2b**) to

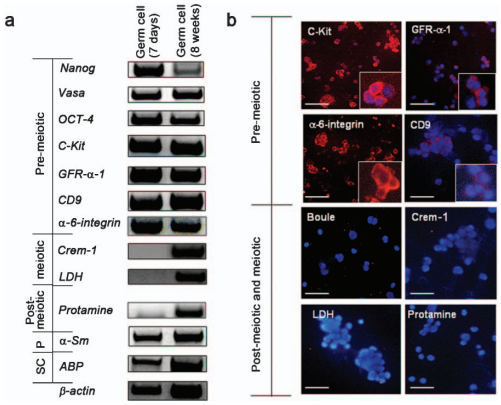

Figure 2 Characterisation of isolated tubular cells before culture in SACS. Isolated tubular cells were examined by RT-PCR analysis using specific markers for pre-meiotic (*Nanog*, *Vasa*, *OCT-4*, *C-Kit*, *GFR-α-1*, *CD9* and *α-6-integrin*), meiotic (*Crem-1* and *LDH*) and post-meiotic stages (*Protamine*) and also for Sertoli cells (*ABP*) and peritubular cells (P) (*α-Sm*) (**a**). Immunofluorescence analysis for testicular tubular cells from 7-day-old mice was used to identify cells positive for pre-meiotic (*C-Kit*, *GFR-α-1*, *α-6-integrin* and *CD9*), meiotic (*Boule*, *Crem-1* and *LDH*) and post-meiotic markers (*Protamine*) (**b**). Scale bars=10 μm. LDH, lactate dehydrogenase; SACS, Soft Agar Culture System; SC, Sertoli cell.

characterize the isolated cells and their differentiation stages before culturing in SACS. Our results showed expression of the pre-meiotic genes *Nanog*, *Vasa*, *OCT-4*, *C-Kit*, *GFR-α-1*, *CD9* and *α-6-integrin* in tubular cells from 7-day-old mice. Meiotic and post-meiotic gene expression (*Crem-1*, *LDH* and *Protamine*) could not be observed. In addition, Sertoli, peritubular, macrophages and Leydig cells were also

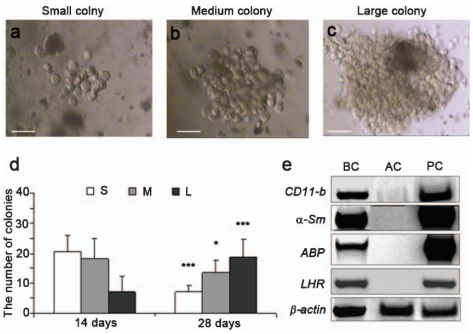

Figure 3 Tubular cell colonies development in the SACS. Tubular cells (10^6 cells per well) were cultured in the upper layer of the SACS. The lower layer of the SACS consisted of RPMI containing 20% FCS. The size of the colonies in the upper layer was evaluated after 14 and 28 days of culture. (**a**) Colonies were designated as small (S) when they contained around 50 cells; (**b**) medium (M) when they contained around 150 cells; and (**c**) large (L) when they contained more than 300 cells. (**d**) The capacity of tubular cells to form S, M or L colonies in SACS was examined after 14 and 28 days of culture. *P<0.05, ***P<0.001, compared with 14 days. (**e**) The expression of markers for Sertoli, peritubular, macrophage and Leydig cells (*ABP*, *α-Sm*, *CD11-b* and *LHR* respectively) was examined by PCR analysis using specific primers for each marker. Scale bars=10 μm. BC, before culture; AC, after culture (colonies); PC, positive control (RNA from the testis of an 8-week-old mouse). FCS, fetal calf serum; SACS, Soft Agar Culture System.

present when examined by specific markers (*ABP* and *α-Sm*; **Figure 2a and 3e**) and *CD11-b* and *LHR* (*LH receptor*) (**Figure 3e**) before culture, respectively). Thus, the isolated cells contained only pre-meiotic germ cells in addition to somatic cells.

Isolated tubular cells were cultured in the upper layer of SACS (**Figure 1**). Distinct colonies in the upper layer were classified according to their size, as presented in **Figure 3a–c**. Colonies of different sizes were encountered after 14 and 28 days of culture in SACS (**Figure 3d**). The number of small and medium colonies was greater than the number of large colonies after 14 days in SACS. A significant decline in the number of small (P<0.001) and medium (P<0.05) colonies was detected after 28 days of culture compared to 14 days in SACS. In contrast, the number of large colonies increased after 28 days compared to 14 days in SACS (P<0.001) (**Figure 3d**).

The handpicked colonies did not contain RNA indicating the presence of Sertoli, peritubular, macrophages or Leydig cells as examined by PCR analysis using specific markers for those cells (*ABP*, *α-Sm*, *CD11-b* or *LHR*, respectively) (**Figure 3e**).

We examined testicular cells freshly isolated from 7-, 14-, 28- and 40-day-old mice for pre-meiotic (*OCT-4*, *GFR-α-1*, *C-Kit*, *CD9* and *α-6-integrin*), meiotic (*LDH* and *Crem-1*) and post-meiotic (*Protamine*, *Acrosin* and *SP-10*) markers (**Table 3**). The results show that meiotic and post-meiotic genes were expressed in 28- and 40-day-old mice but not at the earlier time points. Determination of cell type-specific marker expression in colonies was performed by real-time PCR analysis (**Table 4**) of 14- and 30-day cultures (six independent experiments for each) when each sample run in one to five wells Each data point represents the results from colonies collected from a single well. In general, analyses conducted prior to culturing the cells in SACS indicated that only pre-meiotic markers (*OCT-4*, *GFR-α-1*, *C-Kit*, *CD9* and *α-6-integrin*) were present. After 14 and 30 days of culture in SACS, the expression of the pre-meiotic markers became variable. This variability existed between different experiments and also between individual colonies within the same experiment. After 2 weeks of culture, meiotic and post-meiotic markers were occasionally detectable irrespective of the presence or depletion of pre-meiotic markers (**Table 4**). After 30 days of culture, meiotic and post-meiotic markers were consistently expressed (**Table 4**). With the exception of *CD9* and *OCT-4*, the average expression of pre-meiotic markers in tubular cells cultured in SACS for 14 days and 30 days (respectively) was significantly reduced (**Table 4**). These results correspond to the *in vivo* situation, with the exception that the expression levels of *CD9* decreased with age (**Table 3**). Meiotic and post-meiotic markers significantly increased with time in culture in SACS (except for *LDH*, *Acrosin* and *SP-10* after 14 days) (**Table 4**). This increase in meiotic and post-meiotic markers in SACS cultures corresponded to the situation *in vivo* (**Table 3**).

In general, the composition of markers did not depend on the size of the colony. We confirmed the RT-PCR data on germ cell maturation by immunohistochemical detection of proteins specific for pre-meiotic, meiotic and post-meiotic germ cells in colonies isolated after 28 days of culture in SACS (**Figure 4a and 4b**). The protamine signal was localized differently in the cultured cells compared to cells in the sections. This difference could be related to the *in vitro* culture conditions of the cells in the agar system. We confirmed the validity of these markers in testicular tissue from 7-day-old and 8-week-old mice (**Figure 4c and 4d**), and for 'boule' staining in our previous study[11]).

We also confirmed the presence of post-meiotic germ cells by a series of micrographs depicting progressive stages of differentiating spermatids up to morphologically normal spermatozoa (**Figure 5a**

Table 3 Expression of spermatogenesis genes in cells from mouse tubular cells of different ages

Age		*Pre-meiotic*					*Meiotic*		*Post-meiotic*		
		OCT-4	GFR-α-1	C-Kit	CD9	α−6-integrin	Crem-1	LDH	Protamine	Acrosin	SP-10
7 days	AVG	8.04±0.16	4.94±0.53	12.59±1.00	4.74±1.00	127.77±21.00	0	0	0	0	0
14 days	AVG	2.96±0.18	2.95±0.57	12.64±3.70	8.25±1.00	63.45±10.00	0	0	0	0	0
	P	***	***	n.s.	***	***	n.d.	n.d.	n.d.	n.d.	n.d.
28 days	AVG	1.60±0.46	1.50±0.62	6.23±0.20	0.95±0.50	4.90±0.18	35.83±5.00	181.66±7.00	114.43±5.00	35.83±5.00	51.10±15.00
	P	***	***	n.s.	**	***	***	***	***	*	**
40 days	AVG	0.75±0.37	0.62±0.30	2.93±1.00	0.32±0.20	2.19±0.53	60.06±10.00	590.00±55.00	162.00±6.90	54.91±20.00	196.71±15.00
	P	***	***	**	***	***	***	***	***	**	***

Abbreviations: n.d., not determined; n.s., not significant.

Isolated tubular cells from 7-, 14-, 28- and 40-day-old mice were examined by real-time PCR analysis using specific markers for pre-meiotic, meiotic and post-meiotic stages as described in **Table 2**.

The values in the table represent the ratio of expression levels of the related marker to *β-actin* of the same sample (as an internal housekeeping control).

Averages (AVG) of RNA levels from three different experiments using the same age cells are presented. Comparisons between RNA levels from tubular cells from 14-, 28-, 40-day-old mice and 7-day-old mice were performed.

*$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with 7-day-old age.

and b). Lectin-staining revealed that the spermatozoa had normal acrosomes (**Figure 5c**).

Spermatozoa were present in SACS after 30 days of culture. We were able to detect spermatozoa in 11 out of 16 independent experiments. When analysing microscopic smears from 16 wells after 30 days of SACS, we detected normal-looking spermatozoa in 11 wells. We counted the number of spermatozoa whenever they were present and determined an average count of 15.9±5.7 sperm per well.

DISCUSSION

This study is the first original report revealing the generation of morphologically normal spermatozoa from mouse testicular germ cells in SACS and confirms our recent studies describing a continuous maturation of pre-meiotic germ cells in such a culture system.[11,17] In our recent study, we have shown that the addition of testicular somatic cells to the lower phase of the agar system resulted in more extensive colony formation and improved spermatogenic differentiation of MACS-enriched *GFR-α-1*-positive cells in the upper phase.[11] This result was consistent with other studies using conventional culture systems.[5,21] In the present study, we cultivated a crude single cell suspension of day 7 seminiferous tubules containing all tubular somatic cells and pre-meiotic germ cells. These cells reconstituted into distinct colonies, which expanded during the culture period. Our results indicate that factors present in FCS affect the growth and proliferation of pre-meiotic germ cells. Indeed, FCS stimulates proliferation of mouse gonocytes and bovine type A spermatogonia *in vitro*.[4,5]

The variability in the detection of markers for pre-meiotic, meiotic and post-meiotic germ cells reveals that the reconstituted and expanding colonies are heterogeneous and that individual colonies may consist of expanding clones of distinct developmental stages of germ cells. However, the detection of markers using RT-PCR and immunohistochemistry revealed the continuous maturation of germ cells into post-meiotic stages. Many colonies contained cells that expressed and stained for meiosis markers such as *Crem-1*/Crem-1 and Boule. We therefore assume that SACS supports the development of differentiating germ cells from pre-meiotic stages into meiosis, a critical step which is usually blocked under *in vitro* conditions. Transition into post-meiotic stages was detected again with high variability after longer culture periods. In some experiments, the meiotic and post-meiotic markers were expressed irrespective of the absence or presence of pre-meiotic markers. In our previous studies, we observed that the expansion of diploid germ cells is hormone-dependent, while the meiotic and post-meiotic development appeared to occur independently of hormones.[11] This observation indicates that expansion of germ cell colonies occurs without the synchronized development of less mature germ cells as would usually occur in the seminiferous epithelium. We assume that the handpicked meiotic and post-meiotic germ cell colonies represent an expansion of pre-meiotic germ cells that were present either in small isolated fragments after enzymatic digestion or re-aggregated when the single-cell suspension was embedded into the agar.

Interestingly, *CD9* showed a different expression pattern compared to other markers. Recently, it was reported that CD9 is present in spermatids.[22] Therefore, it may not be surprising that *CD9* expression increases in SACS with time and supports our finding that spermatids and mature sperm are generated *in vitro*. We could not detect CD9 at the protein level in our system using a polyclonal rabbit anti-mouse antibody (Santa Cruz Biotechnology). We did also not detect CD9 protein in spermatozoa from mature mice.

Our results revealed that prior to culturing (**Figure 3e**; BC), the cell preparation consisted of germ cells and Sertoli cells, peritubular cells, macrophages and Leydig cells as somatic components. However, the differentiating colonies in SACS (**Figure 3e**; AC) appeared to be free of somatic cells. However, we observed adherent cells in the bottoms of the culture wells. Thus, FCS components may affect germ cells in SACS directly or may be acting indirectly by causing adherent cells to produce factors that affect the germ cells. In our recent study, we previously showed that the addition of testicular somatic cells to the lower phase of the agar system resulted in more extensive colony formation and improved spermatogenic differentiation of MACS-enriched *GFR-α-1*-positive cells in the upper phase,[11] which was consistent with other studies using conventional culture systems.[5,21]

While the post-meiotic progression was already shown in our previous study using SACS,[11] here, we present the first original study on the detection of spermatozoa using SACS, which confirms a preliminary analysis that was published in a recent review.[17] Possible reasons that we failed to detect sperm in our previous manuscript may include difficulty in microscopically detecting sperm in the thick agar layer or the limited number of sperm in the wells. Here, we attempted to quantify the sperm created in the SACS by analysing smears prepared

Table 4 Expression of spermatogenesis genes in cells from colonies developed in the SACS

		Pre-meiotic					*Meiotic*		*Post-meiotic*		
	Exp	OCT-4	GFR-α-1	C-Kit	CD9	α-6-integrin	Crem-1	LDH	Protamine	Acrosin	SP-10
Before culture	1	5.38	2.00	12.00	6.04	132.18	0.00	0.00	0.00	0.00	0.00
	2	9.77	1.60	10.85	5.47	110.09	0.00	0.00	0.00	0.00	0.00
	3	10.19	2.02	12.59	7.15	120.22	0.00	0.00	0.00	0.00	0.00
	4	0.07	6.94	3.62	10.45	21.49	0.00	0.00	0.00	0.00	0.00
	5	0.00	6.48	27.02	19.24	34.43	0.00	0.00	0.00	0.00	0.00
	6	3.75	7.87	29.16	13.60	103.66	0.00	0.00	0.00	0.00	0.00
	Avg	4.90±4.00	4.90±3.00	15.90±10.00	10.30±5.00	87.01±47.00	0.00	0.00	0.00	0.00	0.00
After 14 days	1	0.01	0.25	0.00	79.66	12.01	0.00	0.00	0.00	0.00	0.00
	1	0.00	0.14	0.00	12.69	4.27	0.00	0.00	0.00	0.00	0.00
	2	0.14	0.00	0.24	22.88	4.49	0.00	0.00	0.00	0.00	0.00
	2	0.00	0.07	0.00	12.26	2.27	0.00	0.00	0.00	0.00	0.00
	3	0.00	0.07	1.74	24.69	7.60	1.63	4.98	21.94	0.00	0.00
	3	0.61	0.42	3.59	15.41	0.90	0.03	0.36	21.20	0.00	0.00
	4	0.00	0.00	0.11	18.07	5.80	0.00	0.00	0.00	0.00	0.00
	5	0.01	0.00	0.52	7.87	2.69	0.31	0.08	14.99	0.00	0.00
	5	0.00	0.00	0.76	6.05	14.28	1.06	0.57	5.01	0.00	0.00
	5	0.09	0.01	1.58	20.33	14.78	1.01	0.66	5.34	0.00	0.00
	6	0.00	0.25	0.00	0.65	0.17	ND	0.00	0.00	0.00	0.00
	6	0.00	0.15	0.00	0.65	0.19	ND	0.00	0.00	0.00	0.00
	6	0.00	0.15	0.00	0.54	0.11	ND	0.00	0.00	0.00	0.00
	6	0.00	0.08	0.00	0.35	0.16	ND	0.00	0.00	0.00	0.00
	6	0.00	0.05	0.00	0.60	0.19	ND	0.00	0.00	0.00	0.00
	Avg	0.06±0.20	0.11±0.10	0.57±1.00	14.90±19.00	4.70±5.00	0.40±0.60	0.40±1.30	4.60±8.00	0.00	0.00
	P	**	***	***	ns	***	ns	ns	ns	ns	ns
After 30 days	1	5.72	0.13	0.10	243.28	61.21	12.19	0.42	55.92	264.25	242.29
	1	8.43	1.48	6.83	397.77	45.75	15.73	0.76	55.55	248.70	250.29
	2	8.43	0.47	5.30	234.67	40.95	16.91	0.41	59.54	334.48	0.00
	2	0.00	1.48	0.00	136.79	40.95	0.00	0.00	0.00	0.00	0.00
	3	0.45	0.00	1.32	21.64	9.49	2.39	6.43	47.37	0.00	0.00
	3	0.00	0.00	1.27	14.78	54.79	2.29	2.40	24.69	0.00	0.00
	3	0.00	0.00	1.46	34.20	51.12	6.85	7.98	47.37	0.00	0.00
	4	0.00	0.47	0.00	136.79	21.58	0.00	0.00	0.00	0.00	0.00
	4	0.00	0.11	0.00	106.98	23.85	0.00	0.00	0.00	0.00	0.00
	5	0.00	0.00	2.56	37.68	145.59	19.10	5.76	138.70	0.00	0.00
	5	0.00	0.15	1.75	55.94	18.58	2.72	0.00	0.00	0.00	0.00
	5	0.00	0.94	1.00	69.83	30.19	9.69	1.46	0.00	0.00	0.00
	6	0.00	0.00	0.00	157.11	60.87	0.00	0.00	0.00	0.00	0.00
	6	0.00	0.00	0.00	212.97	0.00	0.00	0.00	0.00	0.00	0.00
	Avg	1.70±3.20	0.37±0.50	1.50±2.10	132.00±109.00	43.20±35.00	6.28±7.00	1.83±2.80	30.70±40.00	60.60±121.00	35.10±89.00
	P	ns	###	###	##	#	#	ns	ns	ns	ns
	P	ns	ns	ns	$$$	$$	$	ns	$	ns	ns

Abbreviations: ns, not significant.

Colonies that had developed after 14 or 30 days of culture, as described in **Figure 3**, were examined for expression of genes related to spermatogonial cells (*Oct-4*, *GFR-α-1*, *C-Kit*, *CD9* and *α-6-integrin*) and meiotic (*LDH* and *Crem-1*) or post-meiotic markers (*Protamine*, *Acrosin* and *SP-10*).

Gene expression was evaluated by real-time PCR analysis using specific primers for each gene (**Table 2**). The values represent the ratio of expression levels of the related marker to β-actin within the same sample (as an internal housekeeping gene control).

This table presents the results of six different experiments before culture, six experiments after 14 days of culture in SACS and six experiments after 30 days of culture in SACS. One to five wells from each experiment were examined using real-time PCR analysis.

Averages (AVG) of RNA levels of the different experiments for specific markers (pre-meiotic, meiotic and post-meiotic) were compared after 14 and 30 days of culture in SACS with the RNA levels of the examined marker from tubular cells before culture. RNA levels of the different markers were compared between 14 and 30 days of culture in SACS.

*Comparison between the 14-day culture in SACS and before culture.

Comparison between the 30-day culture in SACS and before culture.

$ Comparison between the 14- and 30-day cultures in SACS.

*,#,$ $P<0.05$. **,##,$$ $P<0.01$. ***,###,$$$ $P<0.001$.

from individual wells. We found that the number of normal-looking sperm was low (about 16 per well per 10^6 cells seeded), although their detection was not a rare event (11 out of 16 experiments). Around 6% of the cells in the colonies expressed acrosin, as detected by immunofluorescent staining, indicating the presence of spermatids. Because a vast majority of these cells were round, we considered most of these cells to be round spermatids. Although the efficiency of sperm generation appears to be low, the SACS system provides an opportunity to generate a limited number of spermatozoa showing normal sperm morphology and acrosome development under *in vitro* conditions. We are looking for opportunities to improve the efficiency of the system. In a clinical context, implementing intracytoplasmic sperm

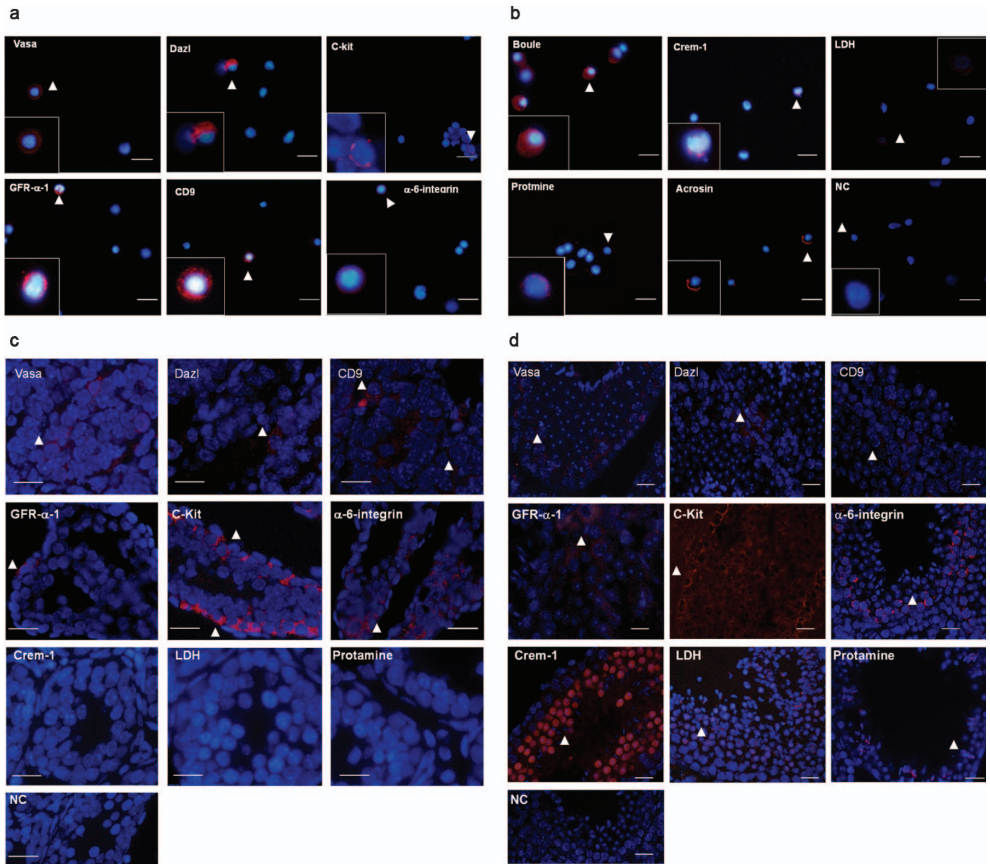

Figure 4 Immunofluorescence staining of cells from colonies developed in the SACS and testicular tissue from immature and mature mice. Colonies that developed within 28 days in culture in SACS were isolated and stained by specific antibodies for different markers of germ cell development by immunofluorescence, including *Vasa*, *Dazl*, *C-Kit*, *GFR-α-1*, *CD9*, *α-6-integrin* (**a**), *Boule*, *Crem-1*, *LDH*, *Protamine*, *Acrosin*, and the negative control (NC) (**b**). The presence of *Vasa*, *Dazl*, *CD9*, *GFR-α-1*, *C-Kit*, *α-6-integrin*, *Crem-1*, *LDH* and *Protamine*, was examined in parallel in testicular tissue from 7-day-old (**c**) and 8-week-old mice (**d**). Scale bars=10 μm. LDH, lactate dehydrogenase; SACS, Soft Agar Culture System.

injection in assisted reproductive techniques renders even low numbers of sperm sufficient to achieve fertilisation and pregnancy. The low efficiency of the sperm generation of SACS is in agreement with other systems using mouse embryonic stem cells to generate haploid germ cells (<0.01%).[23,24] However, in contrast to the rather complex strategies used to derive sperm from embryonic stem-like cells, the SACS approach is very simple and none of the ethical concerns that may be associated with the use of embryonic stem cells can be raised.

We only detected sperm after mounting the agar and fixing the cells to slides, which will create a significant problem when these cells are prepared for assisted reproductive technique procedures. A strategy to isolate sperm from the culture system will be an important prerequisite for future development of SACS as a basic research and clinical tool.

Because we have not yet been able to isolate live spermatozoa from SACS, we could not test their fertilisation abilities. In conclusion, our study confirms that SACS can be used as a novel *in vitro* system for the expansion and maturation of pre-meiotic male germ cells into meiotic and post-meiotic stages. For the first time, we show *in vitro* generation of morphologically normal spermatozoa with intact acrosomes using SACS. This unique system could lead to new strategies for the study of spermatogenesis and to new therapies for male infertility.

AUTHOR CONTRIBUTIONS

MAE designed the experiments; carried out the SACS, qPCR analysis and immunofluorescence staining; performed the statistical analysis; and participated in drafting and revising the paper. EL participated in designing the study, interpreting the data and critically revising the paper for key intellectual content. SS made substantial contributions to the conception and design of the study and interpretation of the data and participated in drafting and critically revising the paper for key intellectual content. MH pioneered the use of SACS for spermatogenesis *in vitro*, made substantial contributions to the conception and design of the study and the interpretation of the data, and participated in drafting and critically revising the paper for key intellectual content. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they do not have any competing financial interests.

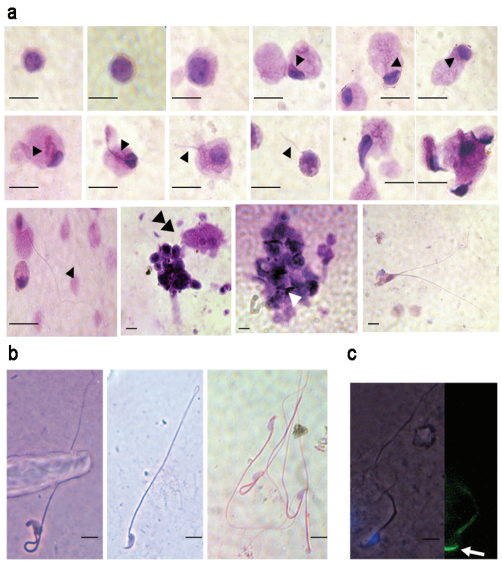

Figure 5 Differentiation of tubular cells to spermatozoa in SACS. Tubular cells were cultured in the SACS as described in **Figures 1 and 2** and evaluated as described in the section on 'Materials and methods'. The presence of differentiated germ cells (**a**), including spermatozoa (**b**), in the SACS was examined under the microscope after H&E staining. More than 10 spermatozoa were determined in each slide (each well). The presence (**c**) of acrosomes was examined by PNA-FITC staining (green-colour acrosomes). DAPI (blue) staining indicated the heads of the sperm (**c**). Scale bars=3–5 μm. Arrowheads in the upper panel indicate the developing flagellum. H&E, haematoxylin and eosin; SACS, Soft Agar Culture System.

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ORIGINAL ARTICLE

Comparison of metformin plus myoinositol vs metformin alone in PCOS women undergoing ovulation induction cycles: randomized controlled trial

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ABSTRACT

The present study was planned to evaluate the benefit of synergetic effect of Metformin plus Myo-inositol versus Metformin alone in infertile polycystic ovarian syndrome (PCOS) women undergoing ovulation induction. One hundred and twenty infertile PCOS women were randomized: Group I ($n = 60$) received Metformin (500 mg) plus Myoinositol(600 mg) three times a day; Group II received Metformin 500 mg three times a day. Subjects were advised to try for spontaneous conception. Those who did not conceive after 3 months, were given three cycles of ovulation induction + intrauterine insemination. Hormonal and biochemical profile parameters were done at baseline and after 3 months of therapy. Primary outcome measure was live birth rate. Secondary outcomes were improvement in menstrual cycle, hormonal and biochemical parameters, spontaneous conception, abortions, multiple pregnancy, and ovarian hyperstimulation syndrome. Baseline demographic, hormonal and biochemical parameters were comparable in two groups. There was a significant improvement in menstrual cycles (cycle length and bleeding days) in Group I as compared to Group II. Improvement in HOMA-IR was significantly higher in Group I ($p = .03$) as compared to Group II after 3 months. Live birth rate was significantly higher in the Group I as compared to Group II [55% (33/60); 26.67% (16/60); $p = .002$]. The study concluded significantly higher live birth rate in women receiving the combination as compared to metformin alone.

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Infertility; PCOS; metformin; myoinositol; OHSS

Introduction

Polycystic ovarian syndrome (PCOS) is the most common cause of anovulatory infertility and prevalence among infertile women is 15–20% [1,2]. Hyperinsulinemia due to insulin resistance occurs in approximately 80% of women with PCOS and central obesity, as well as in 30–40% of lean women diagnosed with PCOS [3,4].

Main treatment modalities for anovulatory infertile PCOS women are dietary and lifestyle modifications, oral ovulation induction agents, gonadotropins, laparoscopic ovarian drilling, and insulin sensitizers. Among insulin sensitizers, metformin has been studied most extensively and there is evidence that it may have metabolic and reproductive benefits [5]. Inspite of being used for decades, metformin has not been able to show results in terms of improved live birth rates in infertile PCOS women though there is evidence of improved clinical pregnancy rate when given along with clomiphene (CC) as compared to CC alone [6].

Recent addition to insulin sensitizers are inositols, among which myo-inositol is the most extensively studied. It acts by a membrane-associated sodium-dependent inositol co-transporter GLUT4 as a post-receptor mediator (second messenger) of insulin signal and decreases hyperinsulinemia. Myoinositol improves ovarian function, decreases leutinising hormone/follicle stimulating hormone (LH/FSH) ratio, reduces serum androgens, increases sex hormone binding globulin (SHBG) and decreases serum total and free testosterone [7].

As the two insulin sensitizers act through different mechanisms, these may be combined to act synergistically to improve metabolic and reproductive outcomes simultaneously in infertile PCOS women. Till now there is no study published to evaluate whether the combination of metformin and myo-inositol can act better than metformin alone in infertile PCOS women. The present study was planned to study the effect of combined metformin and myo-inositol as compared to metformin alone in terms of reproductive outcome and improvement in metabolic and hormonal parameters in infertile PCOS women undergoing ovulation induction cycles.

Materials and methods

A randomized controlled trial was conducted at outpatient department of a tertiary referral center between January 2016 and May 2017. The study was started after approval by the Clinical Research Ethics Committee of the institute. A total of 250 infertile PCOS women (according to Rotterdam criteria) attending infertility clinic were screened for the study. Inclusion criteria were: age between 20 and 38 years who failed to conceive for >12 months, BMI <30kg/m^2 and bilateral patent tubes on hysterosalpingography/laparoscopy. According to inclusion and exclusion criteria, 120 patients were finally recruited for the study. Male factor infertility and uncontrolled hypo/hyperthyroidism couples were excluded. All the participants were randomized into two groups according to computer-generated randomization table. Informed written consent was taken from

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the couple after explaining the detailed plan, purpose, and duration of the study in their own language.

After taking detailed history pertaining to PCOS and infertility, detailed physical examination including weight, height, BMI (Kg/m^2), hip circumference (widest part of hip), waist circumference (horizontal to umbilicus), hirsutism scoring, (modified Ferriman Galway (mFG) scoring), Acne scoring, (Global Acne Grading System) and secondary sexual characteristics were recorded.

Baseline investigations included complete blood count, liver function tests and kidney function tests, lipid profile, Blood sugar fasting, fasting serum insulin and HOMA-IR (mmol/L) (Homeostatic Model Assessment of insulin resistance). Hormonal analysis included serum FSH, LH, TSH, Prolactin, Testosterone, SHBG, and AMH (anti-mullerian hormone) done on day 2–3 of the previous menstrual cycle. Antral follicle count for ovarian reserve and ovarian volume was done on day 2–3 of the cycle.

The subjects were randomized into two groups and received the treatment according to the protocol. Group I ($n = 60$) received Metformin 500 mg + Myoinositol 600 mg three times a day for 6 months. Group II ($n = 60$) received Metformin 500 mg three times a day for 6 months. Patients were advised to keep a record of menstrual cycles and weight and were advised to try for natural conception.

All patients were followed up first at 1 month and then at 3 months of drug therapy and then during their ovulation induction cycles. After 3 months of drug therapy, improvement in clinical parameters weight (kg), BMI (kg/m^2) and menstrual cycle length and flow during periods were reassessed. All the biochemical and hormonal parameters were repeated after 3 months of therapy to see the improvement. Those who conceived spontaneously in initial 3 months were documented and were excluded from subsequent analysis. Rest of the patients were continued on the same drug according to group allocated and, in addition, were given ovulation induction. For ovulation induction, tablet Clomiphene citrate 50 mg was started from day 2 to day 6 of the cycle. Transvaginal ultrasound (TVS) was done from day 9 of cycle and patients were followed with serial TVS till a mature follicle (>18 mm) was documented. Highly purified gonadotropins injections at the dose of 75 IU was administered accordingly depending upon the size of follicle from the eighth day of the cycle of OVI and the dose was increased till follicle of 14 mm size documented if the patient did not ovulate in the first cycle. Inj HCG 5000 IU was given when 1–2 follicles of diameter >18 mm were documented. IUI was done about 36 h following ovulation trigger. Urine pregnancy test was done 16 days after IUI. The drug was continued for first 3 months of conception and then stopped, and patients were then followed till delivery. Those with negative urine pregnancy test and inadequate response were recruited for second cycle of OVI with Clomiphene citrate 100 mg maximum dose in addition of gonadotropins if the patient did not ovulate in first cycle till a maximum of three cycles. The maximum dose of gonadotropins used per day was 225 IU. After every successive cycle, patients who conceived were excluded from subsequent analysis. A record of dropouts and premature terminations from the study was maintained.

Primary outcome measure was live birth rate. Secondary outcome included improvement in clinical, metabolic and hormonal parameters after drug therapy, clinical pregnancy rate, incidence of abortions, multiple pregnancies and ovarian hyperstimulation syndrome (OHSS) cases. Data analysis was carried out using STATA version 12.0. The level of significance was accepted when $p < .05$.

Table 1. Baseline demographic and clinical characteristics between two groups.

Characteristics	Group I ($n = 60$)	Group II ($n = 60$)	p Value*
Age (years)	28.35 ± 2.74	28.12 ± 3.34	0.06
BMI (kg/m^2)	27.71 ± 3.60	27.38 ± 3.92	0.69
Duration of infertility (years)	3.75 ± 2.96	4.52 ± 2.66	0.11
Menstrual Cycle length (months)	2.04 ± 0.80	2.15 ± 0.87	0.44
Bleeding per cycle (days)	4.40 ± 1.42	4.83 ± 1.75	0.46

*$p < .05$ is statistically significant.

Results

According to inclusion and exclusion criteria, 120 infertile PCOS women were recruited for the study and were randomized. Group I ($n = 60$) received metformin + myo-inositol and Group II ($n = 60$) received metformin alone. Table 1 shows the baseline demographic and clinical characteristics of the study subjects.

After 3 months, there was a significant improvement in menstrual cycles (both length and bleeding per cycle) in Group I as compared to Group II ($p = .03$ and .01, respectively). Improvement in HOMA-IR was significantly higher in Group I after 3 months of drug treatment as compared to Group II ($p = .03$). Although, the improvement in fasting blood sugar and insulin levels were statistically insignificant, improvement in HOMA-IR could be due to the difference in standard deviation. Spontaneous resumption of menstrual cycle was considered as a sign of ovulation although serum progesterone level was not done in this study. The clinical parameters, that is, BMI, acne score, and modified Ferriman Gallaway score and hormonal parameters improved in both the groups and the levels were comparable after 3 months (Table 2).

The number of patients who conceived spontaneously in initial 3 months was higher in Group I (23.3%, 14/60) as compared to Group II (13.3%, 8/60) but the difference was not statistically significant ($p = .15$).

Rest of the patients were given ovulation induction + IUI. Ovulation/pregnancy was documented in each cycle and those who conceived were excluded from subsequent analysis. Total number of ovulation induction cycles given per group (OVI ± IUI): Group I: 63; Group II: 70. Clinical pregnancy rate after three cycles of ovulation induction was significantly higher in Group I as compared to Group II.

In Group I, the total clinical pregnancy rate was 63.3% and in Group II, it was 33.3%, respectively at the end of 6 months and the difference was statistically significant ($p = .001$). In Group I and Group II, the live birth rate was 55% (33/60) and 26.67% (16/60), respectively and the difference was statistically significant ($p = .002$) (Table 3). The incidence of gestational diabetes and hypertension was comparable among the two study groups. Mean birth weight was 2908.6 ± 329.8 in Group I and 2861.6 ± 393.5 in Group II and the difference was not statistically significant (p values .66).

Total five patients in Group I suffered from OHSS after ovulation induction with clomiphene citrate in three patients and gonadotropins use in two patients. Out of five cases, one case was early onset OHSS due to exogenous HCG trigger and four cases were late onset OHSS due to endogenous HCG due to pregnancy (twins), There was no case of OHSS reported in Group II.

Table 2. Improvement in clinical, hormonal and biochemical parameters.

Parameter	Baseline			After 3 months		
	Group I	Group II	*p* value	Group I	Group II	*p* Value
Clinical parameters						
Body mass index (kg/m^2)	27.71 ± 3.60	27.38 ± 3.92	.69	25.77 ± 3.48	25.45 ± 3.22	.60
Cycle length (months)	2.04 ± 0.80	2.15 ± 0.87	.44	1.13 ± 0.28	1.25 ± 0.36	.03
Bleeding (days)	4.40 ± 1.42	4.83 ± 1.75	.46	4.34 ± 0.64	4.57 ± 0.84	.01
Modified FerrimanGallaway score	12 (0.5–18)	12 (6–15.75)	.854	5.0 (0–8)	5.5 (2–8)	.71
Global acne score	4 (0–8.75)	0 (0–7.75)	.361	0 (0–6)	0 (0–4)	.09
Hormonal parameters						
Sr. LH (mIU/ml)	11.53 ± 5.34	10.32 ± 4.43	.99	7.36 ± 2.88	7.20 ± 2.59	.51
Sr. FSH(mIU/ml)	5.64 ± 1.73	5.51 ± 2.32	.85	5.81 ± 1.09	5.69 ± 2.06	.82
LH/FSH ratio	2.1 ± 0.87	2.03 ± 0.98	.87	1.25 ± 0.40	1.30 ± 0.46	.38
Sr. Testosterone (ng/dl)	0.50 ± 0.16	0.52 ± 0.16	.48	0.36 ± 0.10	0.37 ± 0.10	.75
Sr. SHBG(nmol/L)	10.91 ± 3.21	11.82 ± 3.29	.45	29.76 ± 9.52	28.54 ± 8.64	.41
Sr. AMH (ng/ml)	12.22 ± 5.16	11.23 ± 4.26	0.09	7.91 ± 2.97	7.23 ± 2.23	.10
Biochemical parameters						
Blood sugar fasting (mg/dl)	92.73 ± 9.41	93.75 ± 11.46	0.40	81.725 ± 7.47	83.350 ± 7.80	.92
Blood sugar PP (mg/dl)	118.95 ± 20.79	120.07 ± 26.18	0.10	103.57 ± 15.63	108.18 ± 15.79	.48
Insulin fasting (uIU/dl)	12.03 ± 6.13	12.03 ± 4.39	0.70	7.29 ± 2.57	7.85 ± 2.65	.91
HOMA-IR index	2.78 ± 1.6	2.83 ± 1.29	0.85	1.46 ± 0.51	1.62 ± 0.59	.03
Total cholesterol (mg/dl)	162 ± 25.57	160 ± 34.68	0.164	136.56 ± 21.49	137.79 ± 21.12	.93
LDL(mg/dl)	99.04 ± 21.31	100.40 ± 16.70	0.416	85.59 ± 11.42	84.84 ± 12.17	.93
HDL(mg/dl)	42.52 ± 5.96	41.55 ± 4.86	0.104	48.97 ± 5.21	48.99 ± 5.05	.54

$p < .05$ is statistically significant.

Table 3. Per cycle analysis of ovulation induction cycles between two groups.

Ovulation induction cycle	Parameters	Group I ($n = 60$)	Group II ($n = 60$)	*p* Value
Spontaneous conception		14 (23.3%)	8(13.3%)	.15
First cycle	No of patients	46	52	
	Ovulation rate	16/46 (34.8%)	7/52 (13.5%)	.013
	Conception rate	9/46 (19.6%)	4/52 (7.7%)	.08
Second cycle	No of patients	37	48	
	Ovulation rate	29/37 (78.4%)	29/48 (60.4%)	.07
	Conception rate	13/37 (35.1%)	3/48 (6.2%)	.001
Third cycle	No of patients	24	45	
	Ovulation rate	18/24 (75%)	34/45 (75.6%)	.95
	Conception rate	2/24 (8.3%)	5/45 (11.1%)	.71
Conception after ovulation induction		24/46 (52.17%)	12/52 (23.07%)	.003
Clinical pregnancy rate		38/60 (63.3%)	20/60 (33.3%)	.001

Discussion

The present study shows significantly higher live birth rate in women who received the combination of metformin and myo-inositol as compared to metformin alone.

Though both metformin and myo-inositol are insulin sensitizers, the mechanism of action of the two is different. While metformin is the classical and most frequently used molecule for the treatment of PCOS [8,9], the focus on myo-inositol is comparatively recent [10]. The studies have shown improved metabolic and reproductive functions with myo-inositol without any gastro-intestinal side effects of metformin [11,12].

Fruzzetti et al. compared metformin and myo-inositol for treatment of clinical and metabolic aspects of PCOS. The study concluded that two insulin-sensitizers lower BMI and ameliorate insulin sensitivity and improve menstrual cycle without any significant differences between the two treatments [13]. About 50% of women resumed spontaneous menstrual cycles in this study though serum progesterone levels were not checked. The present study showed improved menstrual cyclicity in both the groups but the improvement was significantly higher in the combination group. Improved ovulation may be the reason for spontaneous menses and higher pregnancy rate in the group who received the combination. The comparable improvement in hormonal and biochemical parameters in two groups of present study may be attributed to metformin which has been labeled to improve metabolic mileu in PCOS women [14].

In a recent Cochrane review, authors have concluded that metformin may increase the live birth rate among women undergoing ovulation induction with gonadotrophins and can be started before giving ovulation induction [15].

Raffone et al. compared the two insulin sensitizers in infertile POCS women. Though the absolute number of women resuming spontaneous ovulation and total conception rate was higher in myo-inositol group, the study failed to show any statistically significant difference between the two groups [16].

In a small three arm study, metformin (1500 mg), myo-inositol (1g/day), or the combination were compared in infertile PCOS women. There was a significantly higher improvement in symptom profile, weight loss, and hormonal parameters in myo-inositol and the combination group. The study concluded that myo-inositol may be used for improving the ovarian function and hormonal parameters in PCOS women [17].

The ideal dose of myo-inositol has not yet been defined. The studies have used 1–4 g per day in different settings [16–18]. As it acts at ovarian level, myo-inositol corrects the insulin resistance and hormonal disturbances at ovarian level thus reducing the dose of gonadotrophins and risk of ovarian hyperstimulation syndrome in PCOS women during ovulation induction and IVF [18,19].

Emekçi Özay et al. compared Myoinositol and folic acid with myoinositol alone in infertile PCOS women and documented significantly higher spontaneous conceptions and clinical pregnancy rate in myo-inositol group. [20].

As myo-inositol has no GI side effects with similar clinical and hormonal benefits as metformin, it may be considered as first line option in PCOS women with insulin resistance without pre-diabetes or diabetes [21].

The inference may be made that the two drugs acting synergistically, have more hormonal, clinical, and reproductive benefits as compared to when one drug is given alone.

Main limitation of the present study was that serum progesterone levels were not checked to document ovulation.

Among metformin and myo-inositol, which molecule is more beneficial is still a debate. But literature is inclining toward more benefits of myo-inositol and choosing it first line option in PCOS women with or without insulin resistance though its ideal dose is yet to be defined. Further randomized controlled trials are needed comparing the two molecules separately with the combination to prove or disprove the benefits of combinations.

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Disclosure statement

No financial relationship with any organization. Authors have full control of all primary data. Informed written consent was obtained from the patients for publication. The authors report no conflicts of interest.

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REVIEW ARTICLE

Short-term effects of metformin and myo-inositol in women with polycystic ovarian syndrome (PCOS): a meta-analysis of randomized clinical trials

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ABSTRACT

Metformin (MET), the most commonly used insulin sensitizer, is the reference off-label drug for the treatment of polycystic ovary syndrome (PCOS), worldwide. However, its use may be limited mainly by gastrointestinal adverse effects. Myo-inositol (MI), a well-recognized food supplement, also represents an evidence-based treatment for PCOS women, popular in many countries. Our aim is to provide a systematic review of the literature and a meta-analysis which compares these two treatments, for their short-term efficacy and safety in PCOS patients. Systematic review and meta-analysis of randomized clinical trials (RCTs). RCTs were identified from 1994 through 2017 using MEDLINE, Cochrane Library, PubMed, and ResearchGate. Included studies were limited to those one directly comparing MET to MI on several hormones changes. Standardized mean difference (SMD) or risk ratios (RRs) with 95% CIs were calculated. Changes in fasting insulin was the main outcome of measure. Six trials with a total of 355 patients were included. At the end of treatment, no difference between MET and MI was found on fasting insulin (SMD = 0.08 μU/ml, 95% CI: −0.31–0.46, $p = .697$), HOMA index (SMD = 0.17, 95% CI: −0.53–0.88, $p = .635$), testosterone (SMD = −0.01, 95% CI: −0.24–0.21, $p = .922$), SHBG levels (SMD = −0.50 nmol/l, 95% CI: −1.39–0.38, $p = .263$) and body mass index (BMI) (SMD = −0.22, 95% CI: −0.60–0.16, $p = .265$). There was strong evidence of an increased risk of adverse events among women receiving MET compared to those receiving MI (RR = 5.17, 95% CI: 2.91–9.17, $p < .001$). No differences were found in the effect of MET and MI on short-term hormone changes. The better tolerability of MI makes it more acceptable for the recovery of androgenic and metabolic profile in PCOS women.

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PCOS; metformin; myo-inositol; fasting insulin; HOMA index; testosterone; androstenedione; SHBG; BMI; side effects

Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder presenting with several complaints including ovarian dysfunction, hyperandrogenism, menstrual irregularity, insulin resistance (IR), and obesity [1]. Hyperinsulinemia is one of the main factors in PCOS causing hyperandrogenism [2], as it directly induces both ovarian and adrenal androgen release and, increasing glucose concentrations, restrains liver sex hormone binding globulin (SHBG) synthesis, as well as production of insulin-like growth factor binding protein 1 (IGFBP-1). The increased androgen signaling causes premature follicular atresia and anovulation [1, 3]. IR and reactive hyperinsulinemia are further stimulated by adipose tissue, being enhanced in obese patients [4].

Due to the pathophysiological link between IR and PCOS aberrations, insulin sensitizers have been used to counteract the above described clinical and metabolic signs. Metformin (MET) is the most common insulin sensitizer, used over the past 50 years for type 2 diabetes in many countries [5], as well as an off-label drug in nondiabetic women with PCOS. Existing evidence shows that MET may have metabolic and reproductive benefits, including weight reduction, decreasing IR, and androgen levels, besides restoration of normal menstrual cyclicity and ovulation [6, 7]. However, its use may be limited by significant side effects such as nausea, vomiting, and gastrointestinal discomfort [8]. The poor compliance observed with MET motivated clinicians worldwide to find novel approaches for PCOS.

Myo-inositol (MI), a naturally-occurring compound, has been investigated in the last decade because of its insulin-sensitizing effects [9]. Accordingly, several clinical trials have been carried out for the evaluation of the efficacy of MI in the treatment of metabolic and reproductive complaints of PCOS women [10, 11], also in view of its safety profile [12].

More recently, different authors performed head to head comparisons of MET and MI. For this reason, we have decided to systematically review those randomized studies and to perform a meta-analysis in order to compare these two treatments, MET and MI, for their short-term efficacy and safety in PCOS patients.

Materials and methods

Search strategy and data extraction

A systematic review of studies that compared MET to MI treatment in patients with PCOS was carried out. The database of MEDLINE, the Cochrane Library, PubMed, ResearchGate, and bibliographies were searched with the following medical subject headings (MeSH): 'Myo-inositol,' 'Metformin,' 'PCOS,' 'randomized clinical trials (RCTs).' No language restriction was imposed. The search

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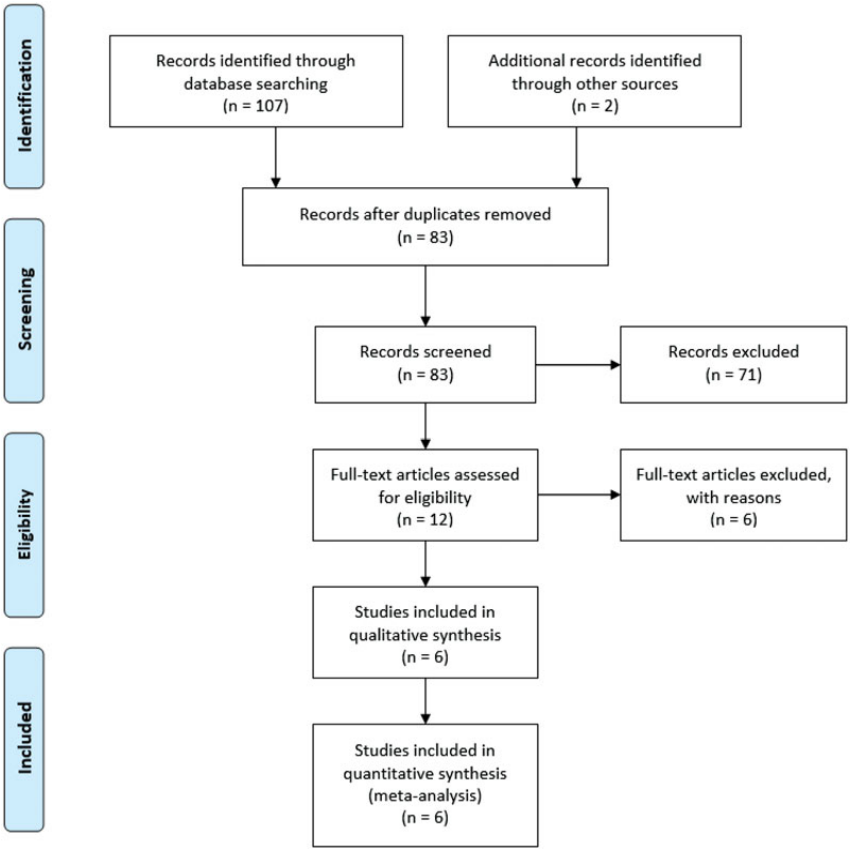


Figure 1. Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram of study selection and inclusion [14].

included literature published until December 2017. Article titles and abstracts were first reviewed and then the full-texts were obtained to assess study eligibility. Two review authors (F.F. and V.U.) independently evaluated and classified studies for inclusion and trial quality and extracted data. Any disagreement among reviewers was resolved by discussion.

The meta-analysis was performed according to the Cochrane Collaboration recommendations [13]. The analysis of results was reported according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement [14]. The characteristics of each study were extracted from the article full-text including: study's first author and year of publication, Country where the study was performed, study design, number (N°) of subjects, inclusion and exclusion criteria, lifestyle change, intervention, and duration of treatment expressed in weeks. Data [means ± standard deviation (SD) or ± standard error of the mean (SEM)] for each outcome pre- and post-treatment were extracted and, if required, converted accordingly for homogeneity. Missing outcome data in the original article were asked directly to the authors.

Including and excluding criteria

Studies were included in the analysis if they met the following conditions: (a) designed as RCT; (b) use of MET versus MI; (c) population is represented by patients with PCOS diagnosed according Rotterdam Criteria [15] or Androgen Excess Society (AES) Guidelines [16]; (d) outcomes include at least one among the following, fasting insulin, homeostasis model assessment (HOMA) index, testosterone, androstenedione, SHBG, body mass index (BMI); and (f) side effects related to treatments are described. Studies were excluded if: (a) selected treatments were combined with other drugs or supplements (excluding folic acid), (b) duplicate publications, and duplicates on different database, (c) review papers, and (d) animal or cell culture studies.

Quality assessment

For the risk of bias the Cochrane recommendations were followed, considering random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective outcome reporting [13]. Categories were assessed as low, unclear or high risk of bias and summarized in a table with a plus, question mark or minus, respectively. A debate over the risk of bias was undertaken for the studies in order to find unanimity between the review authors.

Statistical analysis

The effect size was measured as the standardized mean difference (SMD) obtained as Hedges' adjusted *g*, for continuous outcomes and the risk ratios (RR_s) with 95% confidence intervals (CIs) for the dichotomous outcome. The heterogeneity among the

Table 1. Characteristics of the included studies.

Study's first author	Country	Study design	N° of subjects (Mean BMI)	Inclusion criteria	Exclusion criteria	Life style change	Intervention	Duration (weeks)
[26]	India	RCT	(N° = 71) MI: 35 MET: 36	Age: 15–45 years PCOS according to AES/2006 criteria, hyperandrogenism, oligo or anovulation, PCOM	Pregnancy/nursing, neoplastic disease, Cushing's disease, Hypo- hyperthyroidism, Hyperprolactinemia. Active liver disease, renal impairment, type 1/2 diabetes mellitus, antidiabetic/estrogen/ progesterone, any treatment taken in last 3 months, Smokers/ alcoholic subjects	*N*	MI 2 g/d vs. MET 1.5 g/d	12
[25]	India	RCT	(N° = 100) MI: 50 (24.1) MET: 50 (23.2)	Age: 15–40 years PCOS according to Rotterdam's criteria	Undergoing other drug treatment for PCOS (like oral contraceptive pills); Deranged kidney or liver function tests; Thyroid disorders; Known hypersensitivity to MI	*N*	MI 2 g/d vs. MET 2g/d	24
[27]	Iran	RCT	(N° = 60) MI: 30 (25.8) MET: 30 (27.1)	Age: 18–40 years PCOS according to Rotterdam's criteria	Pregnancy during intervention, adrenal hyperplasia, androgen-secreting tumors, hyperprolactinemia, thyroid dysfunction, diabetes impaired glucose tolerance	*N*	MI 4 g/d vs. MET 1.5 g/d	12
[30]	Italy	RCT	(N° = 34) MI: 17 (31.5) MET: 17 (29.7)	PCOS according to Rotterdam's criteria	Adrenal enzyme defect, neoplasm, pregnancy/ nursing, Liver/renal impairment, cardiovascular disease, and other hormonal dysfunctions	*N*	MI 2 g/d vs. MET 1.7 g/d	24
[28]	Italy	RCT	(N° = 50) MI: 25 (27.3) MET: 25 (28.4)	Age: 18–28 years PCOS according to Rotterdam's criteria, oligomenorrhea hyperandrogenism	Hyperprolactinemia, Cushing's syndrome, androgen-secreting tumors, hypo-hyperthyroidism, congenital adrenal hyperplasia	*N*	MI 4 g/d vs. MET 1.5 g/d	24
[29]	Italy	RCT	(N° = 40) MI: 20 (28.2) MET: 20 (28.8)	Age: 24–32 years, PCOS according to Rotterdam's criteria, IR	Other endocrinopathies	*N*	MI 3 g/d vs. MET 1.7 g/d	24

RCT: randomized controlled trial; BMI: body mass index; MI: myo-inositol; MET: metformin; Y: yes; N: no; AES: androgen excess society; PCOM: polycystic ovarian morphology; IR: insulin resistance

included studies was tested using the Cochran's Q test and the I^2 statistic, with a p value=.10. A fixed-effect model (Mantel–Haenszel method) [17] and a random-effects model (Der Simonian–Laird method) [18] were used to obtain the pooled estimates as appropriate. No differences of baseline values between the two groups were found. Comparison among studies was carried out on parameter values post-treatments. Forest plots showed the results of the analyses performed. Meta-analysis was evaluated by use of Stata Statistical Software: Release 12 (College Station, TX: StataCorp LP). Results were considered statistically significant when the two-sided p value $< .05$.

Results

Description of the studies

Literature search yielded a total of 109 studies. After removing of duplicates, 83 articles remained and were reviewed by the titles and abstracts. A total of 12 full-texts had been carefully evaluated for eligibility and 6 left for the quality assessment (Figure 1). One study was excluded because it has a retrospective design [19] and another one was an observational non-RCT [20]. In one study, differences of baseline values between the two groups were found, particularly reporting mistaken results in the insulin parameter. The corresponding author was required twice to explain, but a reply was never received and study was then excluded [21]. Three further studies were excluded because they reported outcomes not considered in this meta-analysis [22–24]. The characteristics of the included studies are summarized in Table 1. The six RCTs were published between 2013 and 2017 and originated from three countries, i.e. India [25, 26], Iran [27], and Italy [28–30] (Table 1). A total of 355 patients had been randomized into treatment with MET ($n = 178$) or MI ($n = 177$). The specific doses for MET (ranging between 1.5 and 2 g/d and MI (ranging between 2 and 4 g/d used are reported in Table 1. Their mean age was 25.4 ± 4.1 years in MET group and 25.7 ± 4.2 years in MI group. Treatments duration ranged between 12 and 24 weeks. All studies but one [26] reported BMI.

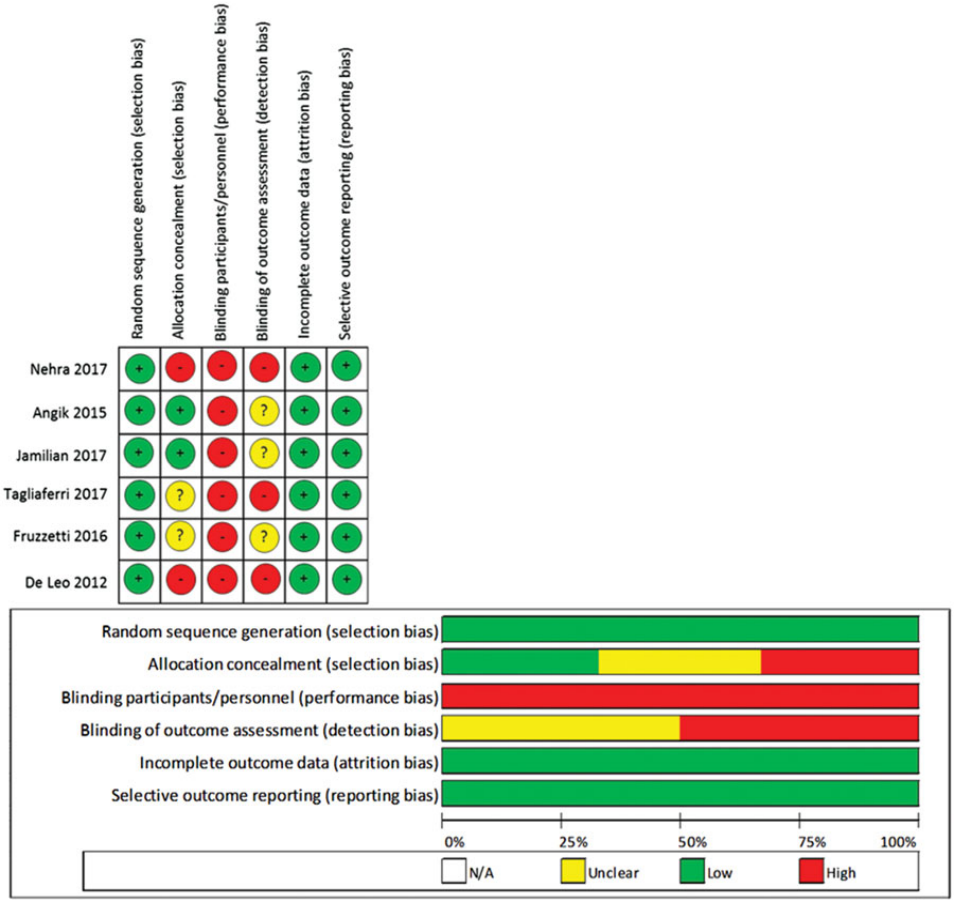


Figure 2. Assessment of risk of bias for included studies. Upper part: Risk of bias summary for each RCT assessed according to the methods recommended by the Cochrane Collaboration. In green: positive sign, low risk of bias; in red: negative sign, high risk of bias; in yellow: question mark, unclear risk of bias; Risk of bias graph about each risk of bias item illustrated as percentage across all selected RCTs.

The mean BMI of the subjects treated falls into the overweight range (>25) (Table 1) for all trials considered with the exception of a study including normal-weight subjects [25]. No study reported the contemporary prescription of lifestyle changes. Data of the selected outcomes were provided in most of the studies. Insulin was reported in 4/6 articles, HOMA index in 4/6, testosterone in 5/6, androstenedione in 2/6, SHBG in 3/6, BMI in 5/6, and side effects in 4/6 articles.

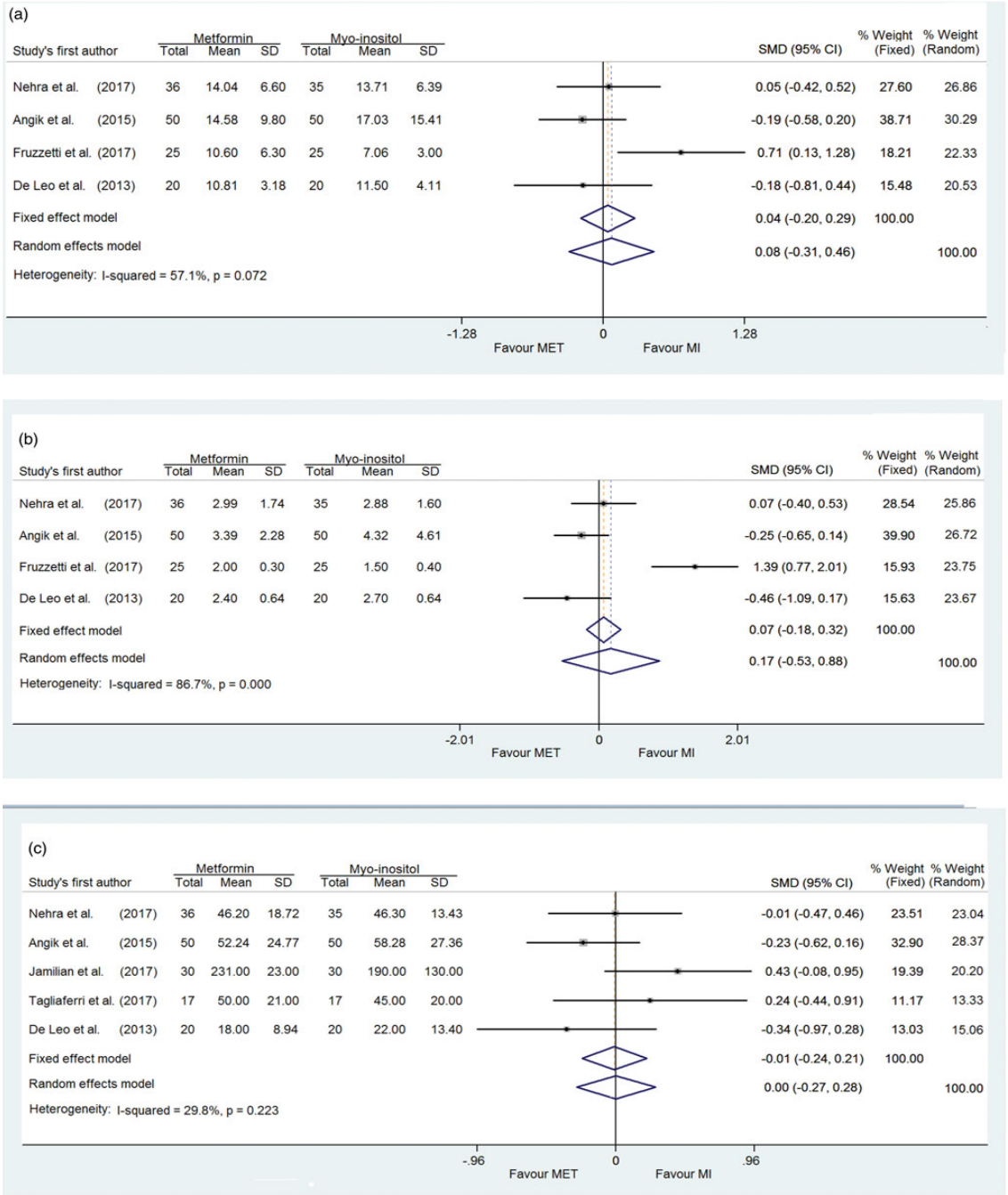
Quality assessment

Overall, the risk of all types of bias in the RCTs was mainly low to unclear (Figure 2). All the studies clearly reported the random sequence generation. Only two reported the allocation concealment, while in two other studies it was not specified, leaving risk of bias unclear. The most evident risk of bias was the lack of blinding procedures. All RCTs had no adequate description of blinding of outcome assessment thus having an unclear risk of bias. The doses of the two treatments groups (MET and MI) are variables between different trials. Outcomes were well reported in most of the studies.

Meta-analysis

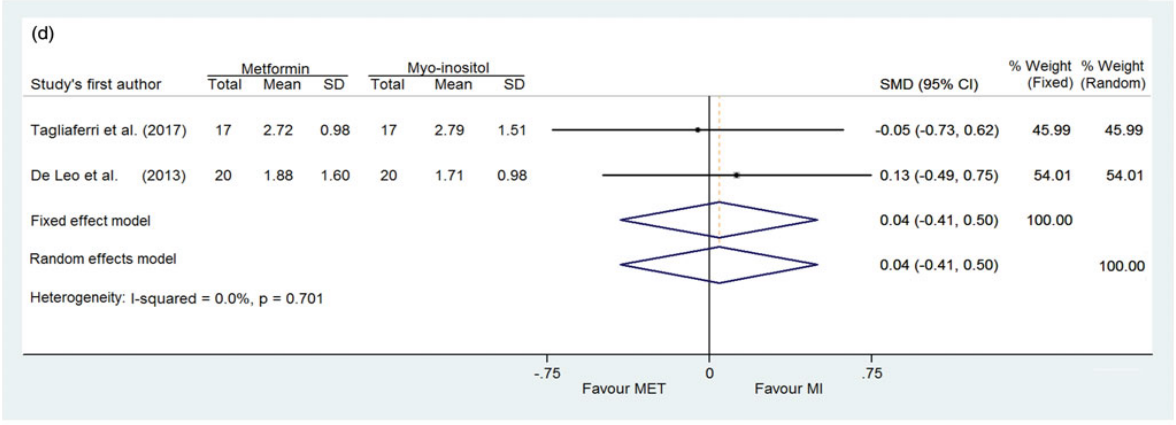
In the six selected studies, a total of 178 women received MET and 177 women received MI alone or combined with folic acid. The random model showed no difference in fasting insulin between women receiving MET and those receiving MI (SMD=0.08 μU/ml, 95% CI: −0.31–0.46, p=.697) (Figure 3(A)). A moderate heterogeneity among studies was found ($Q = 6.99$, df= 3, I^2 =57.1%, p=.072).

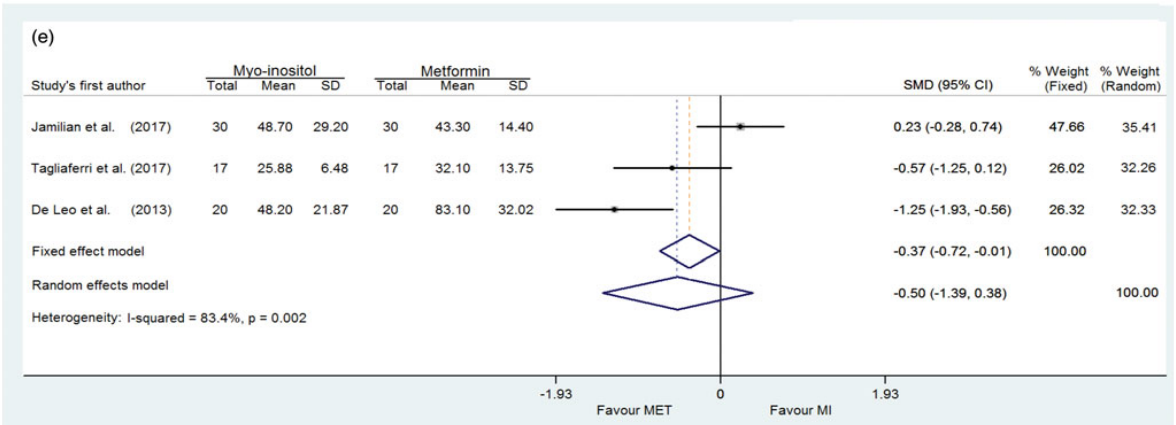
No evidence of a difference in the effect on HOMA was found between the MET and MI group (SMD =0.17, 95% CI: −0.53–0.88, p=.635). For this outcome, considerable heterogeneity across studies was found ($Q = 22.62$, df =3, I^2 =86.7%, $p < .001$) (Figure 3(B)). On the contrary, five trials reporting the effect of MET and MI on serum testosterone revealed no heterogeneity across studies ($Q = 5.69$, df =4, I^2 =29.8%, p=.223). There were no differences in the changes of testosterone concentrations between MET and MI treatments (SMD= −0.01, 95% CI: −0.24–0.21, p=.922) (Figure 3(C)). As well, for the androstenedione outcome, heterogeneity across studies was not found ($Q = 0.15$, df =1, I^2 =0.0%, p=.701). No differences were observed between treatments on androstenedione concentrations

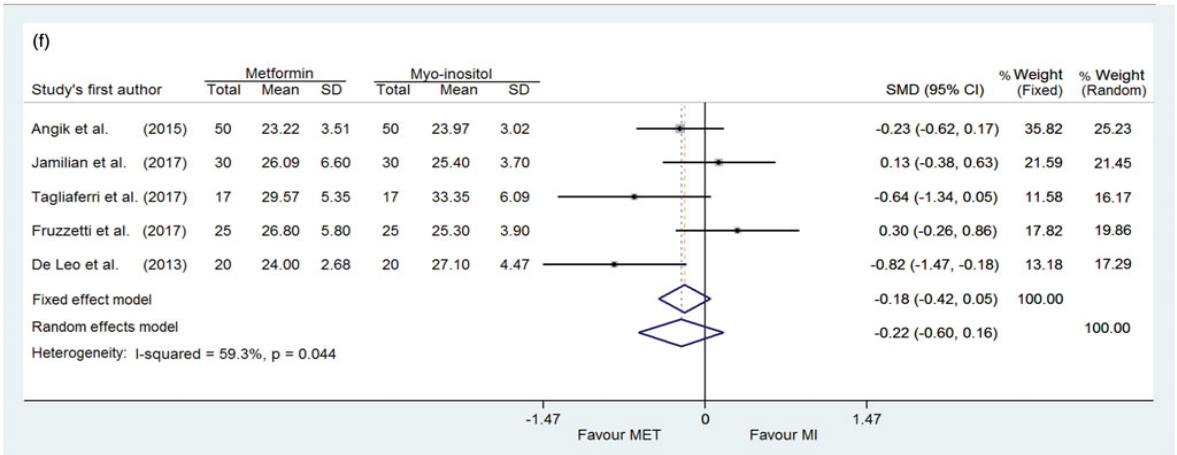


3. Forest plots of comparison metformin (MET) vs. myo-inositol (MI) on fasting insulin (A), HOMA index (B), testosterone (C), androstenedione (D), SHBG (E),), and side effects (G).

=0.04, 95% CI: −0.41–0.50, p=.853) (Figure 3(E)). The om effects model showed no difference between MET and effect on SHBG levels (SMD= −0.50 nmol/l, 95% CI: 9–0.38, p=.263). Considerable heterogeneity was observed (Q = 12.03, df =2, I^2 =83.4%, p=.002) (Figure 3(D)). No evidence of a difference in the effect on BMI was found between the MET and MI group (SMD= −0.22, 95% CI: −0.60–0.16, p=.265). Substantial heterogeneity across studies was found







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= 9.82, df =4, I^2 =59.3%, p=.044) (Figure 3(E)). There was a risk of side effects among women who received MET compd to those administered with MI (RR =5.17, 95% CI: -9.17, $p < .001$); women in MET group were almost five times more likely to have side effects than those in MI gr No heterogeneity among studies was found ($Q = 2.28$, df I^2 =0.0%, p=.517) (Figure 3(F)). Most reported side effects MET were nausea, diarrhea, in some cases also of severe ei

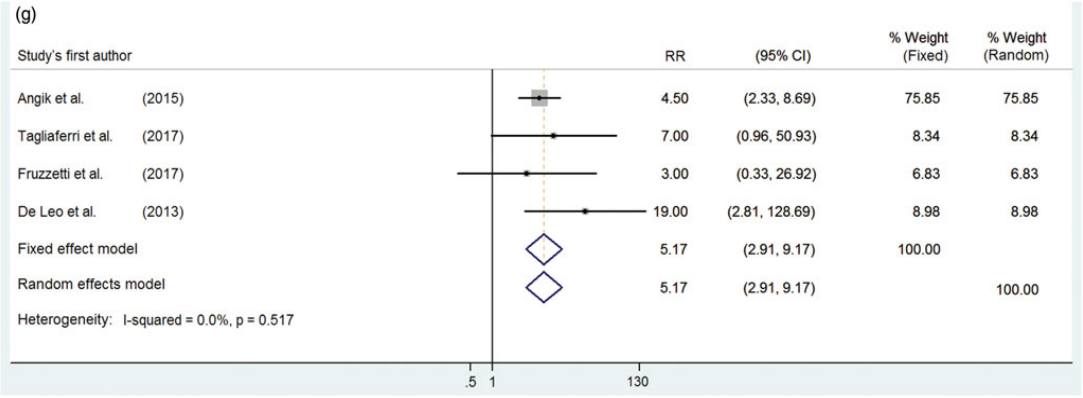

Figure 3. Continued.

abdominal pain, lactic acidosis, and generalized weakness [25, 28–30], while with MI were nausea, mild diarrhea, and menorrhagia [25, 29].

Discussion

These meta-analyses demonstrate that in PCOS patients there is no difference in the short-term effect of MET versus MI as far as fasting insulin, HOMA index, testosterone, androstenedione, SHBG, and BMI are concerned. However, a statistically significant heterogeneity among studies was found for HOMA, SHBG, BMI changes. The main difference observed was the absence of adverse reactions in patients treated with MI compared to those reported in women treated with MET. This seems remarkable as a natural molecule such as MI can be used effectively as treatment for PCOS women, while assuring a great patients' compliance. Avoiding discontinuation of the treatment, due to any intolerable side effect, is quite recommendable as this disorder is associated with severe consequences such as infertility [31, 32].

MET is a complex drug with multiple sites of action and multiple molecular mechanisms. It acts directly or indirectly on the liver to lower glucose production and on the gut to increase glucose utilization and glucagon-like peptide 1 production and alter the microbiome. At the molecular level, MET inhibits the mitochondrial respiratory chain in the liver, enhancing insulin sensitivity with effects on fat metabolism and reducing the expression of gluconeogenic enzymes [33].

MET has proven to reduce glucose absorption and hepatic glucose synthesis and increase insulin sensitivity by increasing peripheral glucose uptake with no significant direct effect on pancreatic insulin production [7, 34, 35]. It has long been studied alone or in combination with other agents to restore ovulation [36]. MET has also been shown to reduce the risk for hyperstimulation during *in vitro* fertilization, but insufficient evidence reporting an increased live-birth rate [37, 38]. However, according to the new guidelines [39], MET alone offers little advantage and therefore, is not recommended as a first-line agent for correcting infertility in patients with PCOS. Moreover, a review of RCTs about MET treatment in PCOS could not confirm any weight-reducing effect [36].

MI is one of the nine stereoisomers of inositol, a physiological compound belonging to the sugar family, contained in foods such as legumes, nuts, fruits, whole grains. Furthermore, it is synthesized endogenously from glucose 6-phosphate [40, 41], and it is found at the level of cell membranes in the form of phosphatidylinositol, bound to membrane phospholipids. MI is the most abundant form among inositol(s) family, accounting for about 99% of the intracellular inositol in ovaries and testis [42, 43]. Its effect starts when it is incorporated into cell membranes as phosphatidyl-MI, the precursor of inositol triphosphate that acts as second messenger regulating the activities of several hormones such as thyroid-stimulating hormone, stimulating follicle hormone, and insulin, improving their signals [44, 45]. MI has been shown to improve insulin sensitivity and oocyte quality, to reduce hyperandrogenism and regulate menstrual cycles ovulation and hirsutism [10, 46].

This meta-analysis clearly shows that MI is associated with a lower risk of adverse events in comparison to MET: for this reason its use could be safer or possible also in association with lower levels of MET in subjects that do not tolerate higher MET therapeutic dose.

Much more studies are available on MET respect with MI, the former being used since a longer period of time. Indeed, in subjects at risk for developing diabetes including PCOS women, MET administration was associated with an improvement of lipid profile and IR while reducing new onset diabetes, respect with placebo or no treatment [47]. On the contrary, the long-term effects of MI on the above health-related parameters are still unknown, in particular its long-term effect on the onset of type II diabetes and cardiovascular diseases in users.

Strength and weaknesses

To our knowledge, this is the first meta-analysis providing quantitative estimates of the comparison of MET versus MI treatment in PCOS women. A comprehensive search was carried out to avoid missing any relevant information. Subjects included in the studies were from different ethnic groups allowing the findings to have a wide transferability. Only RCTs were included to remove potential bias although all the studies lack of blinding. However, a double-blind design was objectively difficult because of the diverse pharmaceutical presentation of treatments (sachet versus pill) and poor reliability due to different adverse reactions associated with treatments.

Another weakness was that every single outcome was not reported in every study. Moreover, in some case the main

outcome, we choose was not the primary outcome in the primary study.

The specific doses of treatments (MET versus MI) are variable between different trials and this issue could be a source of heterogeneity, especially for the dose of MI that has the biggest range.

The BMI of the included trials ranges between normal and over weight: the relationship between the efficacy of these treatments and the specific BMI of subjects treated has to be evaluated in future studies.

The short-term length of follow up of all the studies included (between 12 and 24 weeks) is another important limitation of this meta-analysis: RCTs with a longer follow up must be performed in order to confirm these short-term comparable effects.

Conclusion

This meta-analysis demonstrates no differences in the effect of MET and MI on short-term hormone changes in subjects with PCOS. The better tolerability of MI makes it more acceptable for the recovery of androgen and metabolic profile in PCOS women.

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Disclosure statement

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Potential roles of carnitine in patients with polycystic ovary syndrome: a systematic review

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ORIGINAL ARTICLE

Potential roles of carnitine in patients with polycystic ovary syndrome: a systematic review

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ABSTRACT

Polycystic ovary syndrome (PCOS) is recognized as the most prevalent endocrinopathy in reproductive-aged women. This systematic review was performed with focus on the current knowledge on carnitine concerning metabolic variables in PCOS. PubMed, Scopus, Embase, ClinicalTrials.gov and Google Scholar databases were searched from inception until May 2018. All clinical trials and observational studies published in English-language journals were eligible. Studies that provided insufficient outcomes, animal and *in vitro* studies were excluded. Out of 451 articles identified in our search, only six articles were eligible for analysis. Two observational studies evaluated the association of serum carnitine levels with metabolic variables, and four clinical trials examined the effect of carnitine supplementation in patients with PCOS. Serum carnitine levels had inverse relationship with glycemic status, body mass index (BMI) and waist circumference. Also, carnitine supplementation resulted in improved weight loss, glycemic status, oxidative stress, follicles and size of ovarian cells; no significant effects were reported on sex hormones and lipid profile. According to the current evidence, carnitine might improve weight loss, glycemic status and oxidative stress. However, to explore the exact mechanisms of carnitine role in patients with PCOS, further studies are recommended.

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Carnitine; polycystic ovary syndrome; glycemic status; oxidative stress

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrinological abnormality in reproductive-aged women, with a prevalence of approximately 2–20% worldwide [1]. It is marked by hyperandrogenic anovulation and oligo-amenorrhea, leading to symptoms of hirsutism, acne, alopecia, increased androgens, irregular menstruation and infertility [2]. Although various factors including genetic, behavioral and environmental factors have been emerged in the pathophysiology of PCOS, the exact pathogenesis of the disease remains unexplored [3]. Hyperandrogenism caused by excess androgen production from ovaries is the key feature in PCOS [4]. Hyperandrogenism and insulin resistance have potential roles in evoking metabolic abnormalities, inflammation and oxidative stress. These lead to the development of obesity, type 2 diabetes (T2DM) and cardiovascular diseases in these patients [4,5]. Carnitine or 'b-hydroxy-g-N-trimethylamino-butyric acid' as an essential nutrient in β-oxidation of fatty acids, is the carrier of fatty acids across the inner mitochondrial membrane [6]. Animal foods including meat, fish, milk and dairy products are rich sources of carnitine; carnitine can also be synthesized from lysine and methionine in the liver and kidney [6,7]. Previously published studies have reported that carnitine supplementation may improve insulin sensitivity by increasing the rate of fatty acids oxidation, glucose metabolism and improvement of oxidative stress in patients with T2DM [8,9]. Carnitine as a shuttle, transfers acetyl groups from outside to inside of the mitochondrial membrane and decreases acyl-CoA to acetyl-CoA ratio, leading to improved lipid-induced insulin resistance through altering cell metabolism of glucose and lipids (Figure 1) [8,10]. Furthermore, recent studies have addressed carnitine insufficiency as a cause of developing insulin resistance during states of chronic metabolic stresses, such as T2DM and obesity [11,12]. In addition, sex steroids and their precursors are known to play a modulating role in carnitine turnover [13]. Also, there is an inverse correlation between estrogen and free carnitine levels in women [14]. Although several studies have investigated the role of carnitine in patients with PCOS, no comprehensive study has summarized the findings to increase our knowledge in this regard, thus far. The purpose of this systematic review was to highlight the available information on the correlation between carnitine and metabolic as well as hormonal status, and the effect of its supplementation in PCOS. Furthermore, exploring knowledge gaps and providing suggestions for future studies were addressed.

Methods

Search strategy

PubMed, Scopus, Embase, ClinicalTrials.gov and Google Scholar were searched using keywords including 'carnitine' or

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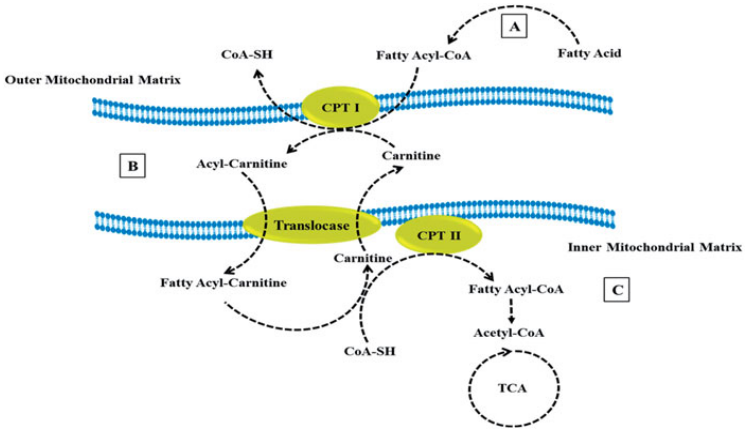

Figure 1. (A) Activation of fatty acid to acyl-coA and (B) carnitine transferase and (C) fatty acid oxidation. CPT I: Carnitine palmitoyltransferase I; CPT II: Carnitine palmitoyltransferase II;CoA-SH: Coenzyme A.

'L-carnitine' or 'Acetyl-L-carnitine' or 'propionyl-L-carnitine' or 'Levocarnitine' and 'polycystic ovary syndrome' or 'PCOS' or 'sclerocystic ovary syndrome' or 'dysmetabolic Syndrome'. The search was limited to studies published in the English language, and up to March 2018. Guideline of the Preferred Reporting for Systematic Reviews (PRISMA) was used for designing this systematic review, and the review protocol was registered at PROSPERO database of Systematic Reviews (registration number: CRD42018090881).

Inclusion and exclusion criteria

Two researchers independently performed the screening of titles or abstracts. Studies were eligible only if it complied withthe following criteria: (1) clinical trials (2) observational studies (3) published in English-language journals and (4) reporting the dosage as well as duration of carnitine administration; studies that (1) provided insufficient information and (2) involved animals and in vitro models were excluded.

Data extraction and assessment of study quality

Two researchers reviewed independently the full text of the studies screened for data extraction and analyzed them according to a checklist of aims. Studies with insufficient information were excluded from the review. Then, a third reviewer assessed the accuracy and quality of the included data.

Results

Figure 2 presents a summarized flowchart of the process of selecting studies for the systematic review. In total, 451 potentially eligible articles were identified by the search strategy, which decreased to 443 after removal of duplicate records; titles and abstracts were then screened. Of these, 437 were excluded based on their title or abstract, because they did not meet the inclusion criteria, and seven full-text documents were retrieved and reviewed. After reading the full texts of articles, one study was removed for it met the exclusion criteria. Only six articles had the inclusion criteria for qualitative synthesis (Table 1).

Carnitine and weight changes in PCOS

About 50% of patients with PCOS suffer from obesity, which can exacerbate symptoms of the disease [15]. Serum carnitine levels in patients with PCOS and the effects of carnitine supplementation on their weight loss have been examined in some studies. In a cross-sectional study [16], plasma concentration of L-carnitine had a negative and significant correlation with BMI in patients with PCOS. But, in a study by Fenkci et al., no such association was observed [17]. In the study by Jamilian et al. carnitine supplementation (250 mg/day) for 12 weeks in PCOS patients resulted in a significant reduction of BMI and weight compared to placebo group [18]. In another study, the same amount of carnitine for the same intervention period did not reveal any significant decrease in weight, BMI and WC in comparison with the control group [19]. Also, in a study by Ismail et al. among clomiphene resistant PCOS women, receiving clomiphene citrate combined with 3000 mg per day of L-carnitine for 12 weeks, significantly decreased BMI in the L-carnitine group [20].

Carnitine and glycemic control in PCOS

Insulin resistance and metabolic disorders are key features in PCOS. The effects of carnitine on these parameters have been studied in several studies [21]. In a cross-sectional study, plasma concentrations of L-carnitine had a negative and significant correlation with HOMA-IR-index in PCOS patients [16]. Moreover, 250 mg of oral carnitine supplement for 12 weeks resulted in a significant decrease in HOMA-IR, levels of fasting blood glucose and insulin levels compared to the control group [19]. In a similar study, a significant reduction was observed in glucose by carnitine supplementation [20].

Carnitine and ovarian hormones, androgens and anovulation in PCOS

Insulin resistance and hyperinsulinemia are caused by increased androgens in patient with PCOS [22]. Also, insulin resistance and hyperinsulinemia may increment LH/FSH ratio and production of androgens [2]. The effect of carnitine on ovarian hormones has been shown in the previous studies [23,24].

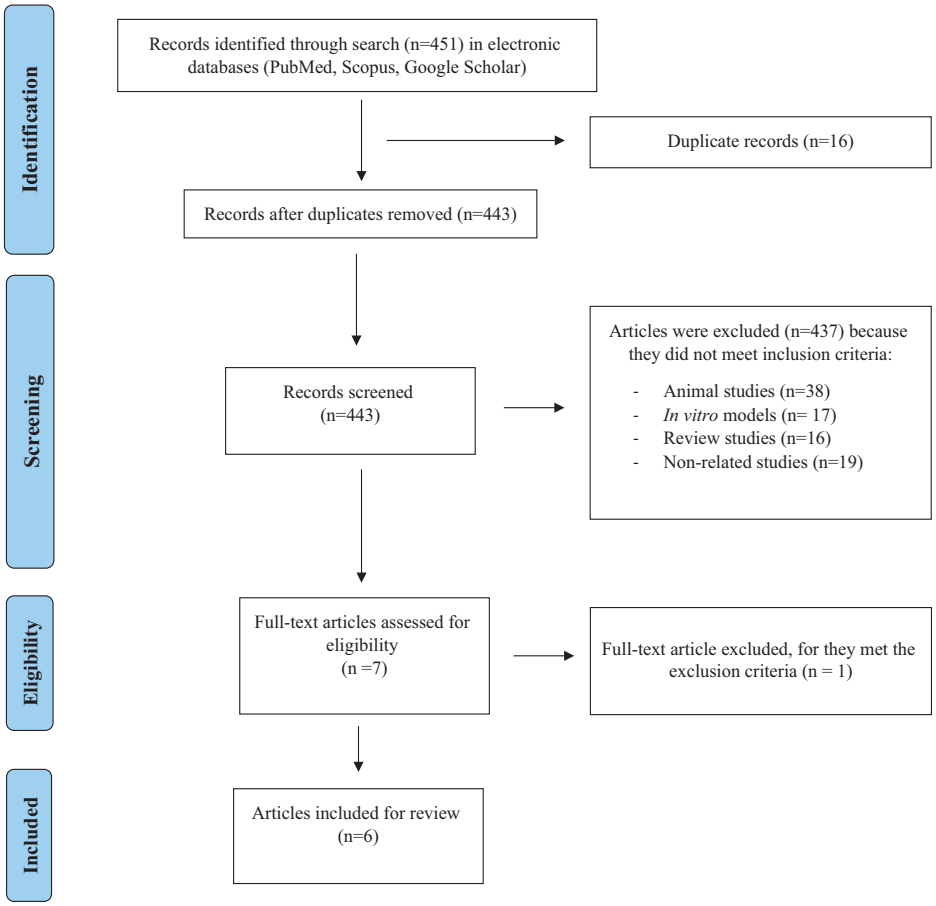

Figure 2. Flowchart of the studies search and selection process.

Accordingly, it appears that carnitine improves insulin sensitivity which in turn affects androgens and ovarian hormones levels [25]. Association between serum levels of carnitine and hormonal status such as estrogen and testosterone has been investigated in some studies. In one study, there was an inverse relationship between SHBG and total as wells as free carnitine in obese PCOS women; but, there was no relationship between carnitine and androgens levels [26]. According to the results of a study by Ismail et al. [20], combination of L-carnitine and clomiphene citrate for 12 weeks in patient with clomiphene-resistant PCOS significantly improved ovulation, pregnancy rates, and significantly increased ovulation rate, number of pre-ovulatory follicles, estradiol and progesterone. Latifian et al. [27] examined the effects of carnitine in PCOS infertile women who were both gonadotropin and clomiphene resistant; the results showed that carnitine lead to the growth of dominant follicles, rise the mean thickness of endometrium and inculcate positive variations in the size of left ovarian follicles. Fenkci et al. [17] showed that there was a significant and negative correlation between carnitine levels and free androgen index (FAI), and a positive and significant relationship with SHBG; they concluded that attenuated levels of carnitine may be due to hyperandrogenism and/or insulin resistance in nonobese women with PCOS. In another study, 250 mg oral carnitine supplementation for 12 weeks did not significantly alter free testosterone levels compared to control group [19].

Carnitine and dyslipidemia in PCOS

Dyslipidemia is one of the co-existing features in patients with PCOS [28]. The relationship between serum carnitine and lipid profile has been assessed in some studies [29,30]. Fenkci et al. showed an inverse association between carnitine levels and high serum LDL-C levels; an insignificant relationship was observed between carnitine levels and other components of lipid profile as well [16]. The result of 3000 mg/day of oral carnitine supplementation for 12 weeks was a significant reduction in total cholesterol, triglyceride and LDL-C, and an increase in HDL-C [20]. But Samimi et al. indicated that 250 mg daily oral carnitine supplementation for 12 weeks had no effect on lipid profile [19].

Discussion

Hyperandrogenism and insulin resistance are the most important features of PCOS, which is associated with decreased total serum carnitine levels [16,17]. Generally, the increase in LH/FSH ratio

Table 1. Summary of included publications.

Author, year and place	Type of study	Sample size and age	Daily dose	Duration	Main outcomes
a. Clinical trials					
Jamilian et al. Iran [18]	Randomized controlled study	$N=60$ 18–40 years	250 mg carnitine supplement	12 weeks	Significant: reduction in weight and BMI, improvement of TAC, MDA and MDA/TAC ratio. Insignificant: GSH levels
Samimi et al. Iran [19]	Randomized controlled study	$N=60$ 18–40 years	250 mg carnitine supplement	12 weeks	Significant: reduction in weight, BMI, WC, FPG, insulin, HOMA-IR and DHEAS Insignificant: lipid profile and free testosterone.
Latifian et al. Iran [27]	Randomized controlled study	$N=50$ 20–30 years	2 g carnitine orally every 12 h	third day	Significant: mean of left ovary follicles size, mean endometrial thickness
Ismail et al. Egypt [20]	Randomized controlled study	$N=170$ <35 years	Carnitine 3 g daily and 250 mg clomiphene citrate	12 weeks	Significant: improvement of the ovulation, cholesterol, TG, LDL-C, HbA1c, glucose, BMI, increase of HDL-C, insulin, improvement of ovulation rate, endometrial thickness, mean number of pre-ovulatory follicles, estradiol and progesterone levels. Insignificant: FBS
b. Observational studies					
Fenkci et al. Turkey [17]	Case-control study	27 non-obese women with PCOS and 30 healthy 16–37 years			Significant: total carnitine and SHBG levels were lower; carnitine level was negatively correlated with FAI and positively with SHBG, LDL-C, LH/FSH ratio, FAI and HOMA-IR in PCOS group. Insignificant: waist measurements, WHR, serum FSH, TC, TG, HDL-C
Celik et al. Turkey [16]	Cross-sectional study	60 PCOS and 28 healthy 17–50 years			Significant: lower carnitine level negatively correlated with BMI and HOMA-IR index in PCOS group.

SHBG: sex hormone-binding globulin; LH: luteinizing hormone; FAI: free androgen index; DHEA-S: dehydroepiandrosterone sulfate; HOMA-IR: homeostasis model assessment; GSH: glutathione; FPG: fasting plasma glucose; WC: waist circumference; FSH: follicle-stimulating hormone; MDA: malondialdehyde; TAC: total antioxidant capacity; IR: insulin resistance.

leads to stimulation of the ovarian theca cells followed by an increased production of androgens and reduced secretion of estrogen. Androgens with a negative feedback effect due to secretion of GnRH hormone, which result in augmented LH/FSH ratio. Moreover, insulin resistance and hyperinsulinemia can be affected by overproduction of androgens [31,32] which in turn affects the liver cells resulting in decreased production of SHBG and an increase in androgens [31,33]. In addition, both hyperandrogenism and insulin resistance are related to obesity, dyslipidemia and subsequently risk factors for cardiovascular diseases [22]. Studies have shown that serum carnitine levels in obesity and metabolic syndrome decrease following insulin resistance [34]. Also, carnitine supplementation leads to reduction in weight, BMI, WC, waist to hip ratio, body fat mass (FM) and increased lean body mass (LBM), and basal metabolism [8,35–37]. The findings from the observational studies reviewed indicated that relationship between serum level of carnitine and anthropometric status are inconsistent [16,17]. However, clinical trials using 250 mg and 3000 mg carnitine supplements revealed a significant decrease in weight, BMI and WC [18–20]. Due to the limited studies, the exact effect of carnitine on anthropometric indices would require further studies.

Previous studies have shown the useful effects of carnitine supplementation on parameters of glucose homeostasis [38,39]. Disorders of glycemic status are the most frequent complication following insulin resistance in PCOS. In these patients, there is a negative correlation between carnitine levels and FBS, HOMA-IR, insulin and HbA1c [16,20]. Also carnitine supplementation with daily doses 250 and 3000 mg showed a significant decrease in HbA1c [20], glucose, insulin [19,20] and HOMA-IR [19]. Probably, carnitine improves factors of insulin metabolism by moderating the expression of gluconeogenic and glycolytic enzymes [40], improving glucose oxidation in mitochondria, and acting as an acetyl-group donor in high-energy metabolism situation, or a transport molecule for free fatty acids [41] leading to increased insulin sensitivity and improvement in glycemic status. Molecular studies have elucidated some possible mechanisms involved in carnitine action on cellular glucose uptake. It has been reported that carnitine deficiency attenuates insulin sensitivity. Furthermore, carnitine can improve glucose metabolism by enhancement of mitochondrial oxidation of acyl-CoA, which further induces insulin resistance and alters the activity of gluconeogenesis enzymes via modifying expression of genes associated with insulin signaling [42].

Patients with PCOS are susceptible to dyslipidemia [43] leading to increased TC, TG, LDL-C levels and decreased HDL-C, which is mainly attributed to insulin resistance in these patients [44]. It has been revealed that carnitine has a hypo-triglyceridemic effect [45]. Moreover, carnitine supplementation may relieve overload of lipid [46]. Few studies have examined the association between the levels of carnitine and lipid profile, as well as its complementary effect on patients with PCOS. Only one study [17] showed a significant relationship between carnitine levels and lipid profile. A dose of 3000 mg carnitine for 12 weeks significantly improved lipid profile [20]. But at a dose of 250 mg for the same duration, no significant effects were observed [19]. A meta-analysis study showed that carnitine supplementation improves lipid profile in patients with T2D [38]. In a similar meta-analysis study, carnitine supplementation led to improved LDL-C but did not affect triglyceride, total cholesterol and HDL-C in hemodialysis patients [47]. Carnitine in mitochondria transfers acetyl groups to the cytosol [48]. Also,

through the carnitine palmitoyltransferase system, carnitine mediates the transport of activated acyl residues into mitochondria for β-oxidation [49]. Thus, it helps maintain adequate amounts of free CoA for improved function and protection of mitochondria by decreasing the accumulation of space long-chain acyls in mitochondria [50]. Also, carnitine reduction may restrict the availability of fatty acids in the mitochondria to produce ATP [42].

Chronic low-grade inflammation has been observed in patient with PCOS [51]. Serum levels of IL-6, TNF-α and CRP increase in these patients [52]. There is a significant relationship between circulating oxidative stress and inflammatory biomarkers, as well as androgen levels [53]. These findings suggest that in PCOS, hyperandrogenism can induce inflammation and enhance oxidative stress through hyperglycemia and insulin resistance, and/or conversely inflammation stimulated with hyperglycemia may promote excess ovarian androgen production. Furthermore, oxidative stress and inflammatory markers are associated with insulin resistance [54–56]. Therefore, the interaction of oxidative stress and inflammation with insulin resistance and hyperglycemia can lead to exacerbations in hyperandrogenism. The antioxidant effects of carnitine are partly related to free radical scavenging and prevention of free radical formation, maintaining of the integrity of electron-transport chain in mitochondria leading to decreased secretion of ROS under stress conditions, and affecting redox-signaling via inhibition of NF-κB resulting in additional synthesis of antioxidant enzymes and molecules [57]. Carnitine has been reported to maintain cellular energy by improving the mitochondria or by eliminating elements which may cause lipotoxicity [58]. It also promotes cellular proliferation and decreases apoptosis by inhibiting TNF-α and other anti-proliferative agents [59]. Furthermore, carnitine may decrease apoptosis induced by inflammatory cytokines such as TNF-α [59]. Also, previous studies have reported that carnitine may lead to down-regulation of cytokines such as IL-6, IL-1 and TNF-α [60]. In the study of Jamilian et al., supplementation of 250 mg of carnitine for 12 weeks in PCOS patients resulted in a significant improvement in total antioxidant capacity (TAC), malondialdehyde (MAD) and MDA/TAC ratio; no significant changes were found in glutathione (GSH) levels, though [18]. Latifian et al. [27] showed that carnitine supplementation in PCOS infertile women led to growth of dominant follicles, a rise in mean thickness of endometrium and inculcated positive variations in the size of left ovarian follicles. Also, in another study [17], there was a significant and negative correlation between L-carnitine and FAI, and a positive and significant relationship between SHBG and the levels of carnitine, due to hyperandrogenism and/or insulin resistance in PCOS. However, in a clinical trial, supplementation with 250 mg of carnitine did not significantly alter free testosterone levels compared to the control group [19]. More studies with different doses and durations are required to better understand the effect of carnitine on sex hormones in PCOS patients.

Knowledge gaps and future directions

PCOS patients are different in body compositions and distribution of fat mass (FM) and lean body mass (LBM) from their non-PCOS peers. There is a significant relationship between android obesity and the risk of diabetes, arthrosclerosis and other chronic metabolic diseases [61–63]. Also, it seems that body composition influences glycemic status, lipid profile and hormone levels. Therefore, the reported effects of carnitine administration on body composition, FM and LBM in patients with PCOS inspire future studies.

Adipose tissue which secretes adipokines has an important role in regulating hyperandrogenism and insulin resistance in PCOS, and increased secretion of inflammatory adipokines leads to exacerbations of chronic inflammation and oxidative stress [64,65]. Future clinical trials on the effects of carnitine on adipokines in PCOS can increase our knowledge and understanding of its anti-inflammatory and antioxidant effects.

Studies have shown that the development and activation of brown adipose tissue can lead to increased thermogenesis, body metabolism, weight loss, improved insulin sensitivity, fatty acid metabolism and ultimately reduced oxidative stress and inflammation [66–69]. It is postulated that increasing the activity of this tissue may reduce the complications of PCOS [70,71]. On the other hand, studies have shown that carnitine can enhance the function of this tissue [72]. Therefore, studying the effects of carnitine on brown adipose tissue can reveal new pathways and attitudes to control the complications of this disease.

Conclusions

As a whole, the results of this systematic review study showed that carnitine can lead to weight loss and improved glycemic status, and may reduce insulin resistance in patients with PCOS. However, the effects of carnitine on lipid profile were contradictory. Also, in observational studies, low serum levels of carnitine had a reverse association with glycemic status, BMI and WC; no association was reported between carnitine levels and lipid profile. Carnitine improves function of follicles and the size of ovarian cells, but has no significant effect on sex hormones. However, according to the gaps in knowledge and the future direction in this area, more studies are needed to determine the exact mechanisms of the effects of carnitine in patients with PCOS.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Impact of antioxidative supplementation on semen quality according to MSOME criteria

Improvement of semen parameters by dietary supplement

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ABSTRACT

Purpose: To investigate the influence of an oral antioxidative and micronutrient supplementation on semen quality assessed by MSOME (motile sperm organelle morphology examination) criteria in a collective of in vitro fertilization (IVF) patients.

Methods: Sperm analysis of 160 IVF patients was evaluated 2-12 months before undergoing IVF/IMSI (intracytoplasmic morphologically selected sperm injection) cycle. The following parameters were analyzed: semen volume, sperm concentration, motility and morphology according to MSOME criteria. Patients were grouped according to the WHO criteria into normozoospermic, oligoasthenoteratozoospermic (OAT) oligo- and asthenozoospermic men, respectively. Between first and second semen analysis, patients were treated orally with a dietary antioxidative supplement. The Student's t-test was used to evaluate the significance of data.

Results: In the total number of patients we found a significant reduction in the percentage of immotile sperms and a highly significant increase of total sperm motility after antioxidative therapy. We also observed substantial improvement of motility, the sperm concentration as well as sperm morphology (according to MSOME criteria) in the semen of oligozoospermic, asthenozoospermic and OAT patients. For normozoospermic patients, we found almost no significant increase in sperm parameters.

Conclusions: The results display a considerable improvement in semen quality, such as motility, sperm concentration and morphology after dietary supplementation, notably in IVF patients with poor semen quality. Amelioration of sperm quality of IVF patients can have a crucial impact in assisted reproductive technology (ART). Our findings suggest that micronutrient supplementation might be generally beneficial for semen quality in men undergoing ART, but mostly in those with a higher grade of alterations in their sperm parameters. The suggested mode of action is probably due to a decline of oxidative stress.

Keywords: semen quality, spermatozoa, morphology, MSOME, IMSI, oxidative stress, antioxidative supplementation, male infertility, assisted reproductive technologies, IVF, ART

RESUMO

Objetivo: investigar a influência de uma suplementação oral de antioxidantes e micronutrientes na qualidade do sêmen avaliado por MSOME em pacientes de fertilização in vitro (FIV) .

Métodos: análise de esperma de 160 pacientes de FIV foi realizada de 2-12 meses antes do ciclo FIV / IMSI (injeção intracitoplasmática de espermatozóides morfologicamente selecionados).Foram analisados os seguintes parâmetros: volume seminal, a concentração de espermatozóides, motilidade e morfologia de acordo com critérios MSOME. De acordo com os critérios da OMS, os pacientes foram agrupados em normozoospérmicos, oligoastenoteratozoospérmicos (OAT) e oligo-astenozoospérmicos, respectivamente. Entre a primeira e a segunda análise seminal, os pacientes foram tratados oralmente com um suplemento antioxidante. O teste t de Student foi utilizado para avaliar a significância dos dados.

Resultados: No total de pacientes houve uma redução significativa na porcentagem de espermatozóides imóveis e um aumento muito significativo da motilidade espermática total após terapia antioxidante. Observamos, também melhoria substancial da motilidade e na concentração de espermatozóides, bem como na morfologia (de acordo com critérios MSOME) dos pacientes astenozoospérmicos, oligozoospérmicos e OAT. Nos pacientes normozoospérmicos, encontramos quase nenhum aumento significativo nos parâmetros seminais.

Conclusões: Os resultados exibem uma melhoria significativa na qualidade do esperma, tais como a motilidade, concentração espermática e morfologia após a suplementação dietética em pacientes de fertilização in vitro com semen de baixa qualidade. A melhora da qualidade do esperma dos pacientes de FIV pode ter um impacto crucial na tecnologia de reprodução assistida (ART). Nossos resultados sugerem que a suplementação de micronutrientes pode ser benéfica para a qualidade do esperma em em homens submetidos a ART, mas principalmente naqueles com maior grau de alterações nos parâmetros seminais. O modo de ação sugerido é provavelmente devido a uma diminuição do estresse oxidativo.

Palavras-chave: qualidade seminal, espermatozoides, morfologia, MSOME, IMSI, estresse oxidativo, suplementação antioxidante, infertilidade masculina, reprodução assistida, FIV

INTRODUCTION

According to the World Health Organization (WHO), the total proportion of male-factor-related infertility comes to 46%. Not surprisingly, numerous publications have suggested a worldwide decline in sperm count and other semen parameters (Nelson & Bunge, 1974; Carlsen et al., 1992).In contrast to these findings, other studies observed no, or only decline of a few parameters

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(Rasmussen et al., 1997; Acacio et al., 2000). However, there is strong evidence that several subpopulations or certain countries in the world do suffer from decreased human fertility. Even though reasons for this are numerous and varying, one of the contributing factors clearly is environmental pollution (Oliva et al., 2001; Multiger & Olivan, 2002). This is particularly true for countries with fast economic and industrial growth, such as the BRICS states (Brazil, Russia, China, South Africa). Here environmental protection and work safety policy is still permissive, cities are growing unrestrainedly and/or the agricultural sector is the main branch of the economy. According to the annual list of the world's worst polluted places presented by the Blacksmith institute, seven of the top ten of the world's worst polluted regions in 2007 could be found in China, India, Russia and South America (Blacksmith Institute, 2007). There are reasons to assume that this increasing environmental pollution and the broad usage of biocides not only affects human health in general, but also fertility in particular, partly due to endocrine disruption but also due to increased oxidative stress. Several reviews and studies from animal model systems as well as population studies in areas of heavy environmental pollution in South America and China support suggestions that human fertility declines in these regions (Mohallem et al.,
2005; Lichtenfels et al., 2007; Koifman et al., 2002; Huang et al., 2010). Regarding male fertility, reproduction does not only require normal sperm parameters in terms of sperm concentration and motility, but also the integrity of the genetic constitution of the spermatozoa. Although assisted reproductive technology (ART) has helped to overcome certain types of human infertility, the reproductive capacity of the gametes remains largely unaffected by the ART procedure. Given that the cellular repair mechanisms are restricted in the chromatin condensed mature sperm, intense exposure to ROS or other noxious substances does not only impair sperm membrane integrity and therefore motility and fertilization, but also affects the genetic constitution of the sperm. Even though it is not unequivocally clear to which extend oocytes can overcome sperm DNA damage after fertilization, several studies point out that a pro-oxidative state or diminished DNA integrity in the semen affects the developmental competence of the embryo (Silva et al., 2007; Agarwal & Allamaneni, 2011). Exposure to toxic substances is widely known to affect sperm DNA integrity substantially. Furthermore, it was even shown that sperm DNA was hypermethylated in mice when exposed to particulate air pollution (Yauk et al., 2008). Although, epigenetic research is still a young topic in science and only few data concerning epigenetic modifications of sperm are available, broad alterations in DNA methylation are associated with infertility and consequences for the offspring (Jenkins & Carrel, 2012). Failures in genome and/or imprinting failures in the epigenome are reflected by low semen quality. On the other hand, numerous studies have clearly demonstrated that improved sperm quality, in particular sperm's fine morphology and DNA integrity, contributes to a better outcome for IVF patients in terms of better fertilization- and implantation-, as well as a lower miscarriage rate and to the health of the offspring (Vanderzwalmen et al., 2008; Lewis and Simon, 2010; Bartoov et al., 2003). Conversely, these results indicate that impaired sperm quality affects fertilization, pregnancy and baby take home rate in a negative manner.
Furthermore, beside all progress in the ART, it is of course still eligible to allow human reproduction done the natural way. For this reasons, it would be preferable to have adequate instruments to circumvent certain problems of infertility in the first place.
Even though it is still under debate, more and more studies from animal model systems and even clinical data implicate that lifestyle factors such as nutrition have a considerable effect on sperm quality, in particular with regard to the total antioxidative capacity of semen (Omu et al., 2008; Balercia et al., 2009). In accordance with this, studies have demonstrated a benefit of antioxidative and/or micronutritional supplementation (Omu et al., 2008; Balercia et al., 2009;Cifti et al., 2009). Furthermore, vitamin and or micronutrient intake was demonstrated to attenuate detrimental influences on semen quality in animal model systems such as exposure to toxic substances (Cifti et al., 2011). In concordance to these findings, infertile men were shown to have increased level of reactive oxygen species (ROS) in seminal plasma and spermatozoa,reduced antioxidative capacity (Zini et al., 1993) and increased number of mitochondrial DNA mutations or nuclear DNA fragmentation (Mahfouz et al., 2010; Kumar et al., 2009). Therefore, antioxidative nutrition is mostly regarded as a helpful tool to quench ROS and thereby improving sperm quality in humans or in laboratory animals, but not without controversy. This might be due to the - still - little clinical data, the effectiveness of different antioxidatives, the administered doses, the analyzed semen parameter(s), types of infertility and, probably, due to the heterogeneity of the studied subpopulations.
The study presented here aimed to examine whether semen quality (in particular the sperm morphology according to MSOME criteria and with regard to differences of male fertility) of IVF patients can be improved by a dietary supplementation comprising defined antioxidants.

MATERIAL AND METHODS

In the period from January 2008 to July 2011 a total of 160 patients from our IVF clinic in Bregenz (Austria) were included in this study. The semen samples of patients undergoing (intracytoplasmic morphologically selected sperm injection) IMSI cycles were analyzed with respect to several sperm parameters such as ejaculation volume, sperm concentration and sperm motility according to WHO criteria (WHO, 2010). Additionally, semen quality was assessed according to MSOME criteria (modified from Vanderzwalmen et al., 2008). Selection of spermatozoa was done at 6000x magnification under a Nomarski interferential Leica AM 6000 inverted microscope (Leica, Germany). Grade I sperm was defined by normal shape and size, no vacuoles or only small vacuole(s) <4% of the sperm's head, grade II: normal shape and size but large vacuoles >4% of the sperm's head, grade III: large vacuoles >4% of the sperm's head and,additionally, abnormal shape and/or size of spermatozoa.
For our study patients were recruited according to foll wing criteria: They consented to have a dietary supplementation, they had no indication of azoospermia and did not have any known genetic reasons for impaired spermatogenesis (such as chromosomal aberrations or other genetic defects). Additionally, they were not exposed to irradiation or chemotherapy. After the first semen analysis patients were treated orally with a dietary supplement (Fertilovit® Mplus) for at least 2 month, one capsule twice daily (substances of content are given in Table 1). The different components of the preparation were described as beneficial for protecting sperms from oxidative

Table 1. Substances of content for Fertilovit® M^{plus}.

Content	Daily dose/ 2 capsules
Vitamin C	100 mg
Vitamin E	100 mg
Folic acid	500 µg
Zinc	25 mg
Selenium	100 µg
N-acetyl-L-cysteine	50 mg
L-carnitine	300 mg
Citrulline	300 mg
Glutathione red.	50 mg
Lycopene	4 mg
Coenzyme Q10	15 mg

A daily intake of 2 capsules was recommended.

damage or having other supporting effects, respectively. The semen samples were examined by MSOME again after a 2-12 months course of antioxidative therapy (mean 3.5 months). No undesired side effects of the supplementary intake were noted so far.

The total patients' age ranged from 28 to 61 years (mean 39.2). Sperm concentration and percentage of motility were assessed according to the WHO criteria (WHO, 2010). According to these criteria patients were class-divided into normozoospermia (sperm concentration 15Mio/ml and progressive motility 32%), OAT (sperm concentration <15 Mio/ml and progressive motility <32%) or patients, which revealed either oligozoospermia (sperm concentration <15 Mio/ml) or asthenozoospermia (progressive motility <32%). Data was expressed as mean ± standard deviation for parametric variables and analyzed by Student's t-test.

RESULTS

According to these criteria, 65 patients were normozoospermic (NO), 42 men were referred to the OAT group, 20 patients had restrictions in sperm count (oligozoospermia) and 33 were referred as asthenozoospermic (see Table 2).

The mean age of the normozoospermic patients was 39.2, for OAT patients 39.2, oligozoospermic and asthenozoospermic men 38.7 and 39.2 years, respectively. By comparing the semen parameters of the first semen analysis, OAT patients revealed considerable lower total sperm motility in contrast to the other groups (total motility for OAT patients 21.8 ± 21.1 vs. 63.4 ± 12.0 of normozoospermic patients and 52.9 ± 15.6 for oligoand 36.1 ± 16.9 for asthenozoospermic patients (Table 2a).

The second semen analysis was performed within a period of 2 to 12 months of antioxidative supplementation in an IVF/IMSI cycle (mean: 3.2 month for OAT, 3.5 for normozoospermic patients and 3.4 month for oligo- and asthenozoospermic patients). After the dietary supplementation, we found remarkably less immotile sperm (grade d) and a significant increase in total motility in the total number of patients (see Table 2a).

Additionally, a highly significant increase in the progressive and total motility in the OAT group in the second semen analysis could be observed. The sperm concentration of the OAT patients also significantly raised (2.6 Mio ± 4.9 vs. 6.7 Mio ± 9.6).

Moreover, a significant increase in grade a and grade b sperm was observed, while the percentage of grade d sperm (immotile sperm) was significantly decreased. Other alterations of semen parameters analyzed according to the WHO criteria in this subgroup with exception of the semen volume were not significant. Oligo- as well as asthenozoospermic patients revealed a significant higher percentage of class a sperm in the second semen analysis. Both subgroups also display an improvement of progressive and total motility, although not significant for the oligozoospermic patients. Nevertheless, sperm concentration in the oligozoospermic patient group has risen significantly. In contrast to this, alterations of semen parameters from normozoospermic patients showed a trend but without significance.

Regarding the sperm morphology according to MSOME criteria, we observed a significant increase of sperm with a low vacuolisation rate (grade I) and decrease of sperm with high vacuolisation rate and/or malformations (grade III) sperm in the total number of patients (Table 2b). A significant increase of grade I sperm was also found in the semen of oligo- or asthenozoospermic patients. For OAT patients we observed a significant decline in the percentage of grade III sperm. In contrast the percentage of grade I and grade II sperm was increased, although not significant for grade I. These results indicate that dietary supplementation might be beneficial not only for sperm motility, but also for sperm morphology and integrity (MSOME criteria) in distinct IVF patient groups.

Tabela 2b. Routine semen assessment before and after antioxidative supplementation.

		All patients			Normozoospermic patients			Oligozoospermic patients			Asthenozoospermic patients			OAT patients		
		Before treatment	After antioxidative intake	*p*-value	Before treatment	After antioxidative intake	*p*-value	Before treatment	After antioxidative intake	*p*-value	Before treatment	After antioxidative intake	*p*-value	Before treatment	After antioxidative intake	*p*-value
MSOME criteria	Grade I	5.1 ± 5.4	6.6 ± 6.7	*	7.2 ± 6.1	8.3 ± 6.3	n.s.	3.4 ± 3.2	10.8 ± 7.5	*	5.1 ± 5.2	8.4 ± 7.0	*	2.6 ± 4.0	5.0 ± 7.2	n.s.
	Grade II	39.7± 15.5	42.9 ± 14.6	n.s.	44.4± 13.0	47.1 ± 12.5	n.s.	47.1 ± 12.2	43.9 ± 9.0	n.s.	42.5± 11.7	41.8 ± 11.6	n.s.	26.9± 15.7	37.4 ± 15.8	**
	Grade III	55.2± 18.1	50.5 ± 18.2	*	48.4 ± 15.6	44.6 ± 14.5	n.s.	49.5.0 ± 14.4	45.3 ± 10.2	*	52.4 ± 13.1	49.8 ± 14.8	*	70.5± 17.7	57.6 ± 20.1	**

Oligoasthenoteratozoospermia was defined according to WHO criteria. OAT= oligoasthenoteratozoospermia; n.s. = not significant; * = p< 0.05; **= p< 0.01; *** = p< 0.001

DISCUSSION

The study presented demonstrates the amelioration of sperm morphology under MSOME criteria as well as improved motility and sperm concentration of IVF patients after oral supplementation with antioxidants and other micronutrients. The most remarkable benefit was observed for IVF patients with impaired semen parameters. Various publications have already suggested that restricted sperm parameters such as low sperm count, poor morphology or immotility of sperms are caused by a rise of reactive oxygen species (ROS) or ineffective ROS scavenging including malnutrition, inflammation, advanced age or exposure to toxic substances and endocrine disruptors (Eskenazi et al., 2005). Antioxidants, such as ascorbic acid, tocopheroles, carotenoides and micronutrients such as zinc, which is known as cofactor of glutathione peroxidases and superoxide dismutases (SOD1 and SOD3), have been demonstrated to be critically important for normal semen quality and reproductive function in a number of studies in both animals and humans (Eskenazi et al., 2005). They are thought to counteract the detrimental effects of oxidative stress on the spermatozoa (Ross et al., 2010). A number of studies have described beneficial effects of oral antioxidative treatment on sperm quality (reviewed by Ross et al., 2010).

These effects are thought to be brought about by protecting developing sperms from detrimental oxidative stress. In spite of the low number of IVF patients within the subgroups, this study clearly demonstrated the effects of a dietary supplement not only on conventional criteria of sperm parameters (according to WHO) but also on MSOME criteria and depicts the correlation of population subgroup and the outcome in terms of improved semen parameters. While oligo-, astheno or OAT patients experienced the most benefit of the dietary intake, the normozoospermic patients showed only slight but not significant improvements in the morphological sperm parameters.

These findings indicate that severe restriction in semen parameters might reflect more imbalances of the ROS/antioxidant levels, possibly caused by environmental, lifestyle or genetic reasons. For example, it is known, that aberrant expression of the antioxidative-acting enzyme GPX4 in spermatozoa is considered as one of the causes of oligozoospermia in infertile men (Schneider et al., 2009; Chabory et al., 2010). Another crucial factor is the environmental pollution, which has increased continuously during the last decades, less in the industrialized, but in particular in the emerging economies. Most environmental toxicants have been shown to impair testicular spermatogenesis by inducing reactive oxygen species (Mathur & D'Cruz, 2011). This is important as a decrease of sperm parameters and increase of DNA damage in the sperm as a result of oxidative stress has been linked to poor reproductive outcomes.

CONCLUSION

Consumption of antioxidative foodstuff such as fruits, minerals or essential amino acids was found to have a positive correlation with semen quality (Braga et al., 2012). During the last decade, it has become more and more clear, that not only personal factors like lifestyle, nutrition and genetic disposition have an impact on sperm quality but also increasing pollution and environmental factors impact on human fertility and the offspring. Therefore, couples who desire having children, in particular IVF patients, should be counselled to be aware of putative detrimental influences of environmental and lifestyle factors and the impact of their nutritional behaviour and intake of dietary supplements might have supportive effect, especially in men with poor sperm quality.

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Dietary Supplementation of Antioxidants Improves Semen Quality of IVF Patients in Terms of Motility, Sperm Count, and Nuclear Vacuolization

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Abstract: *Background:* This study aimed to investigate the influence of an oral antioxidative supplementation on sperm quality of in vitro fertilization (IVF) patients, as analyzed by sperm motility according to the WHO criteria and motile sperm organelle morphology examination (MSOME). Methods: Semen samples were collected from 147 patients before undergoing an IVF/intracytoplasmic morphologically-selected sperm injection (IMSI) cycle and 2–12 months after an antioxidative supplementation. Semen analysis was evaluated according to WHO and MSOME criteria. Spermatozoa were grouped according to the size of nuclear vacuoles within the sperm's heads. Patients were divided into oligoasthenoteratozoospermic (OAT) and non-OAT men. Between first and second semen analysis, patients were supplemented orally with an antioxidative preparation. *Results:* After the antioxidative therapy we observed a significant reduction in the percentage of immotile sperm cells in the patients. Additionally, the percentage of class I spermatozoa according to MSOME criteria was significantly higher after antioxidative supplementation. In OAT patients the percentage of class I sperm was found to be increased, although not significantly. However, we observed a drastic improvement in sperm motility as well as in total sperm count in this group. *Conclusion:* The results demonstrated a considerable improvement in semen quality, notably in OAT patients. Considering the putative relationship between semen quality on the one hand and reactive oxygen species on the other, the observed changes in the sperm parameters indicate that a decline in semen quality, and even subtle morphological changes, might be associated with oxidative stress. Our findings suggest that an antioxidative and micronutrient supplementation has a remarkable benefit for IVF patients having restricted sperm parameters, in particular.

Key words: IVF, sperm quality, IMSI, MSOME, nuclear vacuoles, oligoasthenoteratozoospermia, oxidative stress, antioxidative supplementation

Introduction

Assisted reproductive technology (ART) has helped overcome human infertility for more than three decades. Nevertheless, bypassing the natural barriers of reproduction still raises questions about the danger of possible negative effects in terms of implantation failure, abortion, or congenital malformations of the offspring. By comparing ART cycles and natural births, different studies observed an increased risk for children conceived after *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI). Thereby the occurrence of chromosomal aneuploidies [1], imprinting disorders [2], or other defects [3, 4] were elevated. Even though it is not unequivocally clear whether these anomalies are related to the ART procedures, it is reasonable that sperm quality can affect pregnancy and birth in terms of fertilization, implantation, and abortion rates as well as health of the offspring [5]. Therefore selecting spermatozoa for ICSI is a delicate and important step, which is primarily done according to morphological criteria.

In fact, several publications indicate that morphology is a crucial parameter for sperm quality. Poor sperm morphology was associated with acrosome anomalies, abnormalities in chromosome number, alterations in chromatin packaging, or increased DNA damage [6–8]. Moreover, spermatozoa from infertile men were shown to have higher incidence of DNA damage such as DNA fragmentation as compared to sperm from fertile men [9]. These observations are of particular importance for ART outcome. Sperm DNA damage was found to impair reproductive outcomes [9–11].

A detailed examination of subtle sperm morphology by MSOME was first introduced by Bartoov *et al.* 10 years ago [10]. It allows the examination of the sperm's fine morphology *in vivo* at very high magnification (6000–12,500x), thus providing the possibility of detailed sperm analysis, in particular assessment of the sperm head. MSOME enables observation of so-called nuclear vacuoles, which cannot be detected by lower magnifications. MSOME was subsequently applied to complement ICSI, and subsequently intracytoplasmic morphologically-selected sperm injection (IMSI) was successfully established in ART. Using the MSOME technique, spermatozoa can be categorized according to the presence and size of nuclear vacuoles within the sperm head. It has been clearly demonstrated that the size and number of these vacuoles in human sperm impede the outcome of ART considerably [12, 13]. In accordance with these findings, a large number of studies that used the stringent MSOME criteria in ART cycles displayed a significant improvement in implantation and pregnancy rates and a statistically significant reduction in miscarriage rates [10, 14–18].

Within the last decade it has become more and more accepted that environmental as well as certain individual factors can have an impact on human sperm quality. Thereby, life style factors such as smoking, physical training, the state of nutrition, and other personal and environmental factors were postulated to influence sperm quality according to WHO criteria [19–21]. Additionally, it was recently demonstrated that the subtle sperm morphology defined by MSOME criteria declines during aging [22]. These findings indicate that several sperm parameters such as morphology or motility are not inalterable in perpetuity but can be influenced in a positive or negative manner by a multitude of intrinsic and extrinsic factors.

Beside genetic reasons, oxidative stress (OS) has been considered as a major contributory factor to reduced male fertility [23]. Oxidative stress occurs when the formation or presence of oxidants such as reactive oxygen species (ROS) overshoots the pool of antioxidants, molecules that are able to scavenge these reactive species. Although the production of ROS as by-products of our life is a physiological process and low amounts of ROS are crucial to mediate signals between cells and also act as intracellular signal transduction factors, e. g., in sperm-oocyte interaction [24], an imbalance might lead to damage of the sperm's membrane, DNA, or enzymes. It has been shown in animal model systems and confirmed in human male infertility patients that inadequate ROS quenching or rise in ROS production in the seminal plasma caused by inflammation, aging, malnutrition, genetic reasons, or excessive pollution might impair sperm's motility and morphology, and in consequence its function [24].

The objective of this study was to evaluate the effect of antioxidative supplementation on sperm parameters in IVF patients. Not only semen quality in

terms of sperm concentration and motility according to WHO was taken into account, but also vacuolization of the sperm head, as detected by MSOME. We evaluated the semen samples of 147 men undergoing ART before and after a 2–12 month treatment period with an oral antioxidant supplement.

Material and methods

In the period from January 2008 to July 2011 a total of 147 patients from our IVF clinic in Bregenz (Austria) were included in this study. The semen samples of patients undergoing IMSI cycles were analyzed with respect to their morphology by MSOME and motility and sperm count by WHO criteria (WHO Laboratory Manual for the Examination and Processing of Human Semen, Fifth Edition, 2010).

For the study, patients were recruited according to following criteria: They consented to an antioxidative supplementation, they had no or were not planning to have a testicular sperm extraction (TESE), they exhibited no indication of azoospermia and did not have any known genetic reasons for an oligoasthenoteratozoospermia (OAT) syndrome (such as chromosomal aberrations, e.g., Klinefelter syndrome or other genetic defects). Moreover, they had had no chemotherapy or exposure to other noxious agents in the past and did not have any other medication with a known influence on sperm quality. After the first semen analysis all patients were treated orally with an antioxidant supplement (Fertilovit® M^{plus}) for at least 2 months, twice daily. Content of the supplement is given in Table I. The different components of Fertilovit® M^{plus} were found to be suitable substances for protecting sperm from oxidative stress, or to have other supportive effects. The semen samples were examined during IMSI again after a 2–12 month period of antioxidative therapy (mean duration, 3.5 months). No side effects of the antioxidative intake were noted.

The patients' ages ranged from 28 to 61 years (mean 39.3, 25th and 75th percentile between 35 and 43 years). Sperm concentration and percentage of motility were assessed according to the WHO criteria. According to this assessment patients were grouped into OAT and non-OAT patients. A sperm-washing procedure was performed after centrifugation on a three-layer gradient of pure sperm, as previously described [5]. Briefly, post-ejaculated liquefied semen was gently placed onto the gradient and centrifuged at 375 x g for 20 minutes at room temperature. The sperm cell pellet was suspended in human tubular fluid (HTF) medium supplemented with human serum albumin (HSA, Life-Global, Ontario, Canada) and centrifuged for 10 minutes. After this washing step, the samples were kept at room temperature. For MSOME analysis, 1 µL of the sperm suspension was transferred in glass bottom dish (WPI, Berlin, Germany) in a micro-droplet with polyvinyl pyrrolidone (PVP). Analysis of MSOME criteria was performed under 6000x magnification on a Nomarski interferential Leica AM 6000 inverted microscope (Leica, Germany). MSOME classification was determined in three categories according to the shape [25] and the presence of nuclear vacuoles as modified from Vanderzwalmen *et al.* [5]. Spermatozoa were classified as grade I when showing normal shape, size, and no vacuoles or only small vacuole(s) (<4 % of the sperm's head). Grade II were spermatozoa with normal shape and size, but with one or more vacuoles (>4 % of the sperm's head). Finally, grade III included spermatozoa with abnormal shape and/or size with or without vacuoles.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 17.0 for Windows (SPSS Inc., USA). For analysis of the statistical significance of differences between-groups [sample volume, total motile sperm count (TMSC) and MSOME criteria], the non-parametric Wilcoxon test was applied.

Table I: Substances of content for Fertilovit® M^{plus}

Content	Daily dose/ 2 capsules
Vitamin C	100 mg
Vitamin E	100 mg
Folic acid	500 µg
Zinc	25 mg
Selenium	100 µg
N-acetyl-L-cysteine	50 mg
L-carnitine	300 mg
Citrulline	300 mg
Glutathione red.	50 mg
Lycopene	4 mg
Coenzyme Q10	15 mg

For all patients a daily intake of 2 capsules was recommended.

Results

The study population comprised a total of 147 IVF patients. Patients were grouped into OAT (sperm concentration < 15 Mio/mL and progressive motility < 32 %) and non-OAT (sperm concentration ≥ 15 Mio/mL and/or progressive motility ≥ 32 %). According to these criteria 39 men were classified as OAT and 108 as non-OAT (see Table II). From these 108 non-OAT patients, 57 were found to have normozoospermia and 51 had either restrictions in sperm motility or concentration (data not shown). The mean age at the onset of the therapy for OAT patients was 40.1 years and for non-OAT men 39.0 years. By comparing the semen parameters of the first semen analysis, OAT patients revealed lower sperm motility (total motility for OAT patients 20.9 % vs. 54.0 %) and sperm concentration (2.5 Mio/mL vs. 20.9 Mio/mL), as expected. Additionally, we observed smaller ejaculation volume (2.5 vs. 3.0 mL) and, interestingly, a lower percentage of the MSOME class I spermatozoa (2.6 % vs. 5.9 %) in the OAT group.

The second semen analysis was performed in a period of 2 to 12 months after an antioxidative supplementation (mean: 3.2 months for OAT, 3.5 for non-OAT patients). We found significantly more progressive motile sperm in the OAT group in the second semen analysis (0.4 % vs. 2.4 %, $p < 0.05$), Table II. The total sperm number of the OAT group was significantly increased (2.5 Mio ± 5.0 vs. 6.7 Mio ± 9.8, $p < .0.05$), but not in the non-OAT group.

Additionally, a highly significant increase in non-linear motility was observed (8.0 % vs. 28.6 %), $p < 0.001$. In concordance to this observation the percentage of immotile sperm was dramatically decreased (79.1 % vs. 49.5 %, $p < 0.001$). In the non-OAT group we observed no significant increase of progressive motility (4.2 % vs. 5.0 %) or in total motility, (54.0 vs. 56.7 %).

Regarding the subtle sperm morphology, we observed a tendency toward a higher number of MSOME class I spermatozoa in OAT patients (2.6 % vs. 5.0 %), and a significant rise of MSOME class I spermatozoa in the total patient number (5.0 vs. 6.6 %); $p < 0.05$. There were also more class I spermatozoa in the non-OAT patients semen in the second spermiogram (7.2 % vs. 5.9 %), although the rise was not significant.

Discussion

Spermatozoa are particularly sensitive to oxidative stress. First, the sperm's cell membrane contains a multitude of unsaturated fatty acids, which are prone to ROS attack. Second, the sperm's genetic material is highly condensed, which might protect the DNA from ROS, but on the other hand, due to condensation, the sperm DNA is transcriptionally inactive and therefore unable to scavenge elevated ROS concentrations via transcription of ROS-inactivating enzymes.

An imbalance between ROS and antioxidative substances has been hypothesized as a cause for several clinical pictures of infertility. In fact, there are several associations of ineffective ROS scavenging and human disease [26]. In addition, poor sperm quality caused by several intrinsic or extrinsic factors, such as inflammation, irradiation, or malnutrition can be explained by an increase of ROS levels [27]. Advanced male age is also associated with increased ROS levels [28] and might therefore offer an explanation for the decline of sperm quality in advanced age. A number of publications showed a beneficial effect of the intake of antioxidants such as ascorbic acid, tocopherol, glutathione, N-acetyl-L-cysteine, lycopene, and coenzyme Q.

Akmal and colleagues observed a significant improvement in sperm motility in oligozoospermic men after 2 months of oral supplementation with ascorbic acid [29]. Moslemi and Tavanbakhsh reported an amelioration of semen parameters in asthenozoospermia patients with vitamin E and selenium [30]. A placebo-controlled, double-blind randomized study revealed a positive effect of coenzyme Q on sperm motility and number in patients affected by idiopathic asthenozoospermia [31]. In a preliminary report including 30 infertile men, Gupta and Kumar demonstrated an improvement in sperm concentration and motility with the carotene lycopene, which has antioxidative and anti-inflammatory properties [32]. Folate was shown to reduce the probability of aneuploidy and disomy in sperm [33]. Zinc has antioxidative properties and is a cofactor of the ROS-scavenging enzyme, glutathione peroxidase. Decreased zinc concentrations in seminal plasma were found in infertile men [34]. N-acetylcysteine (NAC) was reported to prevent oxidative stress in animal model systems and humans, thus preserving male fertility [35, 36]. Moreover, NAC is a precursor of the ROS-scavenging tripeptide, glutathione. Some studies show a positive effect of nitric oxide (NO)-donating amino acids like citrulline or arginine, attributed to improved blood circulation and alleged reduction of oxidative stress [36]. Although several studies implicate that the redox balance and the intake of antioxidative drugs have an impact on semen quality according to WHO criteria in terms of cell number or motility [38–41], there is still an ongoing discussion

Table II: Routine semen assessment before and after antioxidative supplementation.

		All patients			Non-OAT patients			OAT patients		
		Before treatment	After anti-oxidative intake	p-value	Before treatment	After anti-oxidative intake	p-value	Before treatment	After anti-oxidative intake	p-value
Number of patients		147			108			39		
Mean age at onset of therapy (years)		39.3			39			40.1		
Sample volume (ml)		2.9 ± 1.5	2.4 ± 1.4	**	3.0 ± 1.3	2.4 ± 1.2	***	2.5 ± 1.8	2.5 ± 1.7	n.s.
Sperm count (Mio/ml)		16.0 ± 19.7	19.1 ± 22.7	n.s.	20.9 ± 20.7	23.6 ± 24.3	n.s.	2.5 ± 5.0	6.7 ± 9.8	*
Total sperm count (TSC)		43.8 ± 54.6	38.4 ± 42.8	n.s	58.4 ± 56.9	47.9 ± 44.4	n.s.	3.3 ± 3.5	12.2 ± 17.5	*
Sperm motility	Total motile sperm count (TMSC)	45.2 ± 24.4	55.1 ± 22.2	***	54.0 ± 18.7	56.7 ± 19.6	n.s.	20.9 ± 21.6	50.5 ± 27.8	***
	Grade a	3.1 ± 5.9	4.3 ± 6.6	n.s.	4.2 ± 6.5	5.0 ± 6.8	n.s.	0.4 ± 1.4	2.4 ± 5.3	*
	Grade b	27.7 ± 19.1	30.5 ± 20.4	n.s.	34.8 ± 16.4	31.2 ± 18.5	n.s.	8.0 ± 10.4	28.6 ± 24.9	***
	Grade c	14.4 ± 14.6	20.3 ± 18.8	**	15.0 ± 13.0	20.6 ± 16.5	**	12.5 ± 18.1	19.6 ± 24.1	n.s.
	Grade d	54.8 ± 24.4	44.9 ± 22.2	***	46.0 ± 18.7	43.3 ± 19.6	n.s.	79.1 ± 21.6	49.5 ± 27.8	***
MSOME criteria	Grade I	5.0 ± 5.5	6.6 ± 6.7	*	5.9 ± 5.7	7.2 ± 6.4	n.s.	2.6 ± 4.0	5.0 ± 7.2	n.s.
	Grade II	39.5 ± 15.7	42.8 ± 14.7	n.s.	44.1 ± 12.9	44.7 ± 13.7	n.s.	26.8 ± 16.0	37.4 ± 15.8	**
	Grade III	55.5 ± 18.4	50.6 ± 18.3	*	50.0 ± 15.2	48.0 ± 16.9	n.s.	70.6± 18.1	57.7 ± 20.1	**

Oligoasthenoteratozoospermia was defined according to WHO criteria. MSOME classification was done with modifications according to Vanderzwalmen *et al.,* 2008. OAT= oligoasthenoteratozoospermia; n.s. = not significant; * = $p<0.05$; **= $p<0.01$; *** = $p<0.001$.

about these effects. Our results are well in line with these findings. We found a substantial improvement in sperm quality after a dietary supplementation in terms of motility, number, and moreover the sperm's morphology.

In the context of the importance of antioxidants to maintain redox balance and prevent oxidative stress, nutrition has to be mentioned as a crucial aspect. In a recent questionnaire in our center, the included 1499 male IVF patients reported their mean fruit and vegetable intake to be 1.3 portions/day [42]. This is far below the recommended intake as given in the Joint FAO/WHO Expert Consultation on diet recommending a minimum of 3–5 servings/day. Men attending a fertility clinic should be reminded of the importance of healthy nutrition habits, might be advised to reconsider their foodstuffs, alcohol, and tobacco consumption; men who feel they will not be able to change their dietary habits might be advised to take a suitable antioxidative supplement.

Another important focus of this study was the question of whether an oral antioxidative treatment also has an effect on the appearance of nuclear vacuoles in the sperm head. We observed that the percentage of sperm with no vacuoles (Class I) was significantly increased after the antioxidant supplementation. Although it is not unequivocally clear whether nuclear vacuoles reflect an increase of DNA damage [6,7], failure in chromatin condensation [8], or other subcellular events, it is quite obvious that nuclear vacuoles do not present a physiological, but a pathological state. Given that vacuoles might reflect defective DNA-packaging, epigenetic alternations, or DNA fragmentation on a morphological level and considering the fact that nuclear vacuoles affect IVF outcome, our results provide further evidence for the effectiveness of an oral antioxidative therapy on sperm quality. As long as the origin of the nuclear vacuolization is not fully understood, the pathways of influencing the vacuolization rate are only hypothetical. Different pathways can be discussed such as improvement of sperm condensation by better protamination, influenced by improved blood circulation and nutritive support, reduced DNA fragmentation by reduced oxidative stress or decreased inflammation inside the urogenital tract, or improved intracellular processes by improved mitochondrial activity. Further studies are needed to address this topic.

Our data show an improvement of sperm parameters after antioxidant supplementation. In addition to a higher percentage of motile sperm and an increased sperm count, a positive effect on sperm morphology, in terms of vacuole formation, was observed. To our knowledge, this is the first time that a reduction of the number of sperm with vacuoles due to antioxidant supplementation has been reported. Given that sperm quality is one major determinant for the success of any fertility treatment, and that sperm morphology has been found to reflect sperm health, it can be concluded that these findings should influence counseling of male patients prior to fertility treatment.

Footnotes

ART: assisted reproductive technology
FAO: Food and Agriculture Organization
HSA: Human serum albumin
HTF: Human tubular fluid
ICSI: intracytoplasmic sperm injection
IMSI: intracytoplasmic morphologically selected sperm injection
IVF: in vitro fertilization
MSOME: motile sperm organelle morphology examination
NAC: N-acetylcysteine
OAT: oligoasthenoteratozoospermia
OS: Oxidative stress
PVP: Polyvinyl pyrrolidone
ROS: reactive oxygen species
SPSS: Statistical Package for Social Sciences
TESE: testicular sperm extraction

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Antioxidant Supplementation of Subfertile Men Improves Top-Blastocyst Rate in Couples Undergoing IVF/IMSI

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Abstract

In recent years, oxidative stress (OS) has been identified as important factor in male infertility. Thus, the intake of antioxidants to improve semen quality (in subfertile men) has been widely discussed. Improvements of semen quality after supplementation have been reported. However, this issue is still underevaluated. Critics complain a lack of data regarding firstly the use of antioxidants due to the heterogeneity between patient groups, nutritional supplements and treatment effect and secondly rare data in regard to the impact of supplementation on assisted reproduction technique (ART) outcome. In this study the effect of an antioxidant supplementation (AOS) on semen quality and therapy outcome of 92 couples undergoing fertility treatment was assessed. Semen analysis was performed during a first treatment cycle and data regarding treatment outcome were recorded. These were compared to a second treatment cycle of the same couples, prior to which the male partners had received AOS (Fertilovit® Mplus for 3 - 6 months). Semen samples were assessed according to WHO and MSOME (motile sperm organelle morphology examination) criteria. Parameters chosen for evaluation of treatment outcome were fertilization-, (top-)blastocyst rate, pregnancy- and clinical pregnancy rate. After AOS, we found a slight improvement of semen quality according to WHO – and a significant improvement according to MSOME criteria ($p < 0.01$). Even though – naturally – the age of the female partner had increased, we observed a rise of blastocyst rate after AOS. Top-blastocyst rate even improved significantly ($p < 0.05$). In addition to this, pregnancy- and clinical pregnancy rate showed a marked improvement. In summary, the use of a concomitant AOS might be discussed not only for men with impaired semen quality, but also for men with normozoospermia undergoing ART as it may contribute to improved semen parameters and an increased success of the treatment.

Keywords ART; Sperm; MSOME; Blastocyst quality; Antioxidant; Nutritional supplementation

Abbreviations

AOS: Antioxidant Supplementation; ART: Assisted Reproductive Techniques; BR: Blastocyst Rate; cPR: Clinical Pregnancy Rate; FR: Fertilization Rate; ICSI: Intracytoplasmic Sperm Injection; IMSI: Intracytoplasmic Morphologically Selected Sperm Injection; IVF: In Vitro Fertilization; MSOME: Motile Sperm Organelle Morphology Examination; OAF: Oocyte Activation Factor; OS: Oxidative Stress; PR: Pregnancy Rate; ROS: Reactive Oxygen Species; tBR: top-Blastocyst Rate; TUNEL: TdT-mediated dUTP-biotin Nick End Labelling; WHO: World Health Organization

Introduction

It is estimated that in the industrial nations an average of one in every 10 couples have problems with reproduction and stay childless without an appropriate fertility treatment. According to the World Health Organization (WHO) the estimated total proportion of male-factor-related infertility comes to approximately 46%.

Sperm quality is generally considered to be a proxy measure of male fertility. The initial semen analysis to evaluate the number of sperm, motility and morphology is mostly performed according to the current WHO criteria [1]. In addition to this, a multitude of other tests are meanwhile available to analyze semen quality in more detail e.g. TUNEL-assay, Comet assay), acritin orange, hyaluron-binding assay and, most importantly, motile sperm organelle morphology examination (MSOME) [2]. MSOME allows the investigation of subtle sperm morphology in vivo. It enables in particular the observation of nuclear vacuoles, which cannot be detected by lower magnifications. The origin of these vacuoles and their impact on fertility is still somewhat debated. However, the majority of studies substantiate that vacuoles represent pathologic conditions [3-5]. In accordance with this, the combination of MSOME and ICSI, also designated as intracytoplasmic morphologically selected sperm injection (IMSI) results in a significant improvement in implantation- and pregnancy rates and a statistically significant reduction in miscarriage rates [4-10].

Reasons for male subfertility are numerous and next to purely medical reasons such as infections, genetic or chromosomal disorders, use of drugs as during chemotherapy, radiation and environmental pollution are held responsible. In addition to this a man's age and his lifestyle have been found to have an impact on sperm quality as well [11-16]. Interestingly, according to various publications the presence of seminal oxidative stress (OS) plays a key role in male infertility. Normally, the controlled generation of reactive oxygen species (ROS) is associated with normal physiological functions. However, uncontrolled and excessive ROS might be an important factor in the pathophysiology of infertility. In fact, infertile men were shown to have increased levels of ROS in seminal plasma and spermatozoa, reduced antioxidative capacity, and increased number of mitochondrial DNA mutations or nuclear DNA fragmentations [17-19]. This raises the question whether semen quality can be positively influenced by an oral AOS. According to a large number of studies, AOS is mostly regarded as a helpful tool to quench ROS and thereby improve sperm quality in humans or in laboratory animals [20-23], but not without controversy. This might be due to the - still - little clinical data, the effectiveness of different antioxidants, the administered doses, the analyzed semen parameter(s), types of infertility and, probably, due to the heterogeneity of the studied

subpopulations. Additionally, critics have particularly emphasized the fact that only few of the clinical studies presented to date have evaluated the impact on outcome of fertility treatment [24]. For example, to date there is only limited data on the impact of antioxidant treatment on sperm head vacuolization. Yet nuclear vacuolization has been reported to have in turn a negative correlation to blastocyst- and top blastocyst rates [25,26,8]. Currently - to our knowledge – there is no data available assessing directly the association between antioxidant intake, vacuolization rate and the outcome of fertility treatment.

In this study we present data on the treatment outcome of IVF patients undergoing IVF/IMSI. Semen samples were collected during a first treatment cycle without AOS (control). Second sample was taken during the second cycle after the men had taken an AOS for a continuous period. Semen samples were evaluated according to WHO and MSOME criteria. Additionally, fertilization rate (FR), blastocyst rate (BR), top-blastocyst rate (tBR) as well as pregnancy- (PR) and clinical pregnancy- (cPR) rates were recorded and the results of both cycles were compared.

Material and Methods

In the period from January 2008 to July 2011 a total of 92 patients from our IVF clinic in Bregenz (Austria) were included in this study. Couples were recruited according to the following criteria: Male patients consented to have a dietary supplementation. Indications of azoospermia and patients with known genetic reasons for impaired spermatogenesis (such as chromosomal aberrations or other genetic defects) were excluded. Additionally, exposition to irradiation or chemotherapy in the past were further exclusion criteria. Only females at the age of ≤43 years at the start of the 1st cycle were included. Male size and weight and smoking were asked by questionnaire. Female BMI was assessed at the beginning of the first cycle.

Before starting the second cycle male patients were treated orally with a dietary supplement (Fertilovit® Mplus) for at least 3 months (one capsule twice daily, substances of content are given in Table 1).

Content	Daily dose/ 2 capsules
Vitamin C	100 mg
Vitamin E	100 mg
Folic acid	500 µg
Zinc	25 mg
Selenium	100 µg
N-acetyl-L-cysteine	50 mg
L-carnitine	300 mg
Citrulline	300 mg
Glutathione red.	50 mg
Lycopene	4 mg
Coenzyme Q10	15 mg

Table 1: Supplement facts of Fertilovit® Mplus. Dosages were given in micro- or milligram respectively.

The different components of the preparation were described as beneficial for protecting sperms from oxidative damage or having other supporting effects, respectively. The semen samples were examined by MSOME again after a 2-12 months course of antioxidative therapy. No undesired side effects of the supplementary intake were noted.

Analysis of semen samples included several sperm parameters such as ejaculation volume, sperm concentration and sperm motility according to WHO criteria [1]. Additionally, semen quality was assessed according to MSOME criteria (modified from Vanderzwalmen et al., 2008) [8]. Selection of spermatozoa was performed at 6000x magnification under a Nomarski interferential Leica AM 6000 inverted microscope (Leica, Germany). Grade I sperm was defined by normal shape and size, no vacuoles or only small vacuole(s) <4% of the sperm's head, grade II: normal shape and size but large vacuoles >4% of the sperm's head, grade III: large vacuoles >4% of the sperm's head and, additionally, abnormal shape and/or size of spermatozoa. The GnRH long protocol was applied for all cycles with daily injections of triptorelin (Decapeptyl®, Ferring Arzneimittel, Vienna, Austria) 0.1 mg/day, beginning in the mid-luteal phase of the preceding cycle for down-regulation of the pituitary gland. HMG (Merional®, IBSA, Lugano, Switzerland) 2-4x 75 IU/day was used for follicle stimulation.

To evaluate impact on treatment outcome, data on the following parameters were collected: Number of two-pronuclear stage (2PN), number of blastocysts, top-blastocyst rate (tBR), fertilization- (FR), pregnancy- (PR) and finally clinical pregnancy rate (cPR) were determined. Blastocysts with a degree of expansion of 2, 3, 4 and 5 and with A-grading for inner cell mass and trophectoderm, or a combination of A- and B-grading, were classified as top-blastocysts. (Top-) blastocyst rate was calculated by the number of (top-) blastocysts divided by the number of 2PN stages. Pregnancy rate (PR) was determined by urinary ß-hCG level 14 days after transfer, clinical pregnancy rate (cPR) was defined as observation of fetal heartbeat(s) by ultrasound 6-8 weeks after ET.

Data was expressed as mean ± standard deviation for parametric variables and analyzed by Student's t-test and chi-square test to evaluate the significance of data.

Results

The mean male age at the onset of the first IVF cycle was 39.2 years. Male patient cohort revealed moderate overweight (BMI: 26.0 at the first and BMI 26.1 at the second cycle). Twenty patients stated occasional or heavy smoking (19 patients at the 2nd cycle). According to the WHO criteria 32 patients were found to have normozoospermia, 29 men were classified as OAT, while 31 were found to have either asthenozoospermia or oligozoospermia. Lapse of time between the IVF attempts was 1.4 years (mean +/- 1.5)

By comparing the semen parameters By comparing the semen parameters before and after supplementation a highly significant increase in class I sperm could be observed after supplementation (6.0 +/- 5.8 versus 3.8 +/- 4.9 , $p < 0.01$, see Table 2). the patients revealed a highly significant increase in class I sperm according to MSOME criteria (6.0 +/- 5.8 versus 3.8 +/- 4.9 before supplementation, $p<0.01$, see table 2). However, no significant improvement of semen parameters in regard to sperm count and motility could be observed.

In a further step we evaluated and compared IVF outcome of both cycles (Table 3). Female ageing is normally associated with deterioration in oocyte number and quality, and subsequent in embryo quality. The mean female age at the onset of the first therapy was 36.8 years. Mean BMI was 23.0. Although the average age of the female patients had increased until the second cycle (38.1 years, p =0.03) and number of oocytes retrieved were marginally lower, we observed an increase in the blastocyst rate in the second IVF cycle after the men had been supplemented orally with the antioxidative preparation. Although this increase was not significant, the number of top-blastocysts augmented significantly ($p<0.05$). We also found a rise in PR and cPR, however this rise was not significant, due to the fact that that the total number of patients included in this study was low.

	First cycle without Supplementation	Second cycle with Supplementation	p-value
Male characteristics			
Male age (years)	39.2 +/- 8.5	40.6 +/-8.5	n.s
Male BMI (kg/m^2)	26.0+/-3.0	26.1+/-3.1	n.s
Semen assessment			
Sample volume (ml)	2.9 +/- 1.5	2.3 +/- 1.4	< 0.01
Total sperm count (TSC)	44.3 +/- 49.5	49.4 +/- 41.5	n.s
Concentration (Mio/ml)	16.7 +/- 17.6	20.8 +/- 22.5	n.s
Sperm motility (%)			
Grade a	3.9 +/- 6.3	4.0 +/- 6.5	n.s
Grade b	30.6 +/- 18.7	29.0 +/- 19.6	n.s
Grade c	14.9 +/- 14.7	21.4+/- 18.1	< 0.05
Grade d	50.6 +/- 24.2	45.6 +/- 22.1	n.s
Progressive Motility (%)	34.5 +/- 21.6	32.6 +/- 21.3	n.s
MSOME criteria (%)			
Class I	3.8 +/- 4.9	6.0 +/- 5.8	< 0.01
Class II	38.9 +/- 16.7	41.9 +/- 14.5	n.s
Class III	57.3 +/- 19.3	52.1 +/- 18.0	n.s

Table 2: Patients characteristics and results of semen analysis before and after supplementation.

Discussion

It is well known that spermatozoa are highly prone to OS. Enhanced OS hinders motility and development of normal sperm cells. It accelerates apoptosis, thus lowering sperm number, and in addition to this, ROS attack sperms' DNA integrity by base modification and strain breaks. Moreover, ROS can alter epigenetic modifications. This aspect is most important as modified genetic signatures can lead to diseases in the offspring [27].

Elevated levels of ROS are meanwhile even suggested to be a major cause of idiopathic male factor infertility, which is an increasingly common problem today. One promising approach is the reduction of OS in the male reproductive tract by application of oral antioxidants and a variety of studies have shown that this indeed can provide a means of ameliorating sperm quality and quantity as assessed by standard WHO criteria [22].

Female characteristics	First cycle without Supplementation	Second cycle with Supplementation	p-value
Female Age (years)	36.8 +/- 4.2	38.1 +/- 3.9	n.s
Female BMI (kg/m^2) (mean)	23.0+/-3.5	n.d.	
Stimulation dose (IU)	2451 +/- 745	2647 +/- 764	n.s.
Number of oocytes retrieved (total)	1127	1092	n.s.
Oocytes (mean)	12.4 +/- 5.9	12.1 +/- 5.7	
Number of 2PN (total)	672	659	
2PN (mean)	7.3+/- 3.9	7.3 +/- 4.3	
FR (%)	59.6	60.4	n.s.
Number of blastocysts (total)	267	288	
Blastocysts (mean)	2.9 +/- 2.4	3.1 +/- 2.7	
Blastocyst Rate (%)	39.7	43.7	n.s.
Top-Blastocysts (mean)	0.4 +/- 1.1	0.6 +/- 1.0	
tBR (%) (Nb. of top-blastocysts)	5.5 (n= 37)	8.5 (n= 56)	< 0.05
Number of embryos transferred (mean)	1.9+/- 0.4	1.9+/- 0.3	n.s.
PR	34.8	44.5	n.s.
cPR	32.8	39.1	n.s.

Table 3: Patients characteristics and comparison of treatment outcome of the same couples. First cycle without supplementation (left) and in a second cycle after supplementation (right).

Non-enzymatic antioxidants include vitamins A, E, C, and B complex, glutathione and co-enzymes or co-factors of antioxidative enzymes such as coenzyme Q10, zinc or selenium. Various studies have documented a positive impact of such micronutritional and antioxidative supplementation for sperm quality. However, only a few studies established current medical evidence on the effect of oral antioxidants on sperm quality as evaluated according to MSOME criteria [25].

In addition, there has been a lot of criticism. Most studies investigated several sperm parameters, however, there is lack of studies evaluating the main outcome of any fertility-related study, namely pregnancy [22].

Therefore, after confirming the positive impact of an antioxidative preparation on semen parameters [25,26]. We examined the impact of

the same preparation on the treatment outcome of 92 couples undergoing IMSI. In the course of the study, we compared the therapy outcome of these couples who first underwent fertility treatment without any supplementation of the male partner with a second treatment cycle, during which adjuvant supplementation of the male partner was used. By analyzing the same cohort of patients we circumvent the problem of comparing populations with heterogeneity of medical history. Even though the age of the female partner therefore was higher during the second cycle, we were able to find a significant beneficial impact on the top-blastocyst rate as well as a marked improvement of PR and cPR. The improvement observed in spite of increased female age hints strongly at the male impact.

Historically, at first, it was thought that the success rates of ICSI are not even related to basic semen parameters [28-30]. Sperm were considered "mere vectors that carry the paternal genetic component to the oocyte" [31]. However, in several cases of recurrent negative IVF results in conventional IVF and ICSI attempts the influence of the paternal effect on early embryogenesis is suggested as a reason for IVF failure [32-34].

Today it is well known that various components of the spermatozoa actively participate in early embryonic development [35-37]. So-called early paternal effects are visible from day 1 to day 3 of development and include transfer of oocyte activation factor (OAF), which is critical for successful fertilization, centrosomes, which are crucial for cell division [38], as well as a population of RNAs with developmental importance [35,39].

Late paternal effects, however, are effective from day 3 onwards of embryonic development. Impaired development at this stage is usually linked to defects within the sperm nucleus. These include chromosomal aberrations, DNA fragmentation, as well as faulty epigenetic modifications of the DNA (such as DNA-methylation, histone tail modifications, telomere-shortening, targeted histone retention and protamine incorporation into the chromatin) [31].

The fact that we found no significant changes in FR but effects in top-blastocyst rate might therefore be seen as a hint to the fact that AOS is most effective in improving aspects of late paternal effects, that is, nuclear factors. This is consistent with previous findings that AOS can attenuate DNA fragmentation of spermatozoa [40]. Sperm DNA damage is thought to be induced by several mechanisms, including apoptosis during spermatogenesis, induction of DNA strand breaks during remodeling of sperm chromatin, DNA fragmentation induced by endogenous caspases and endonucleases, radiotherapy, chemotherapy, environmental toxicants and xenobiotics and DNA damage induced by oxidative stress [31].

Sites within the sperm genome particularly sensitive to OS include the telomeres as well as the peripheral nuclear compartment which contains histone-bound DNA (5-15%) [31]. Susceptibility to oxidative damage is particularly high in spermatozoa as compared to other cells because sperm lose the majority of cytosolic antioxidants at the time of spermiogenesis and at the same time have lower levels of DNA repair enzymes [31].

We therefore propose that AOS in subfertile male may help to quench ROS, thus contributing to protecting spermatozoa from OS. This is beneficial for success of fertility treatment by supporting DNA integrity and therefore late paternal effects on embryonic development.

This study might also hint at the fact that, given the correlation between ameliorating vacuolization and blastocyst formation rate through late paternal effects, that vacuolization, though still not fully understood, might develop in association with nuclear defects. For the clinician, the here presented data not only confirms previous studies [22,41] but directly emphasizes the correlation between sperm head vacuolization and success of fertility treatment and gives the opportunity to reduce the vacuolization grade and to improve the IVF outcome by nutritional supplementation. We therefore suggest, that the issue of sufficient AOS should be addressed when counseling and treating ART-patients, predominantly if morphology according to MSOME criteria is impaired. The same applies, if patients feel they will not be able to comply with recommendations regarding fruit and vegetable intake or if semen quality is strongly impaired with respect to WHO criteria, supplementation might be considered.

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Research Article

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Dietary Supplementation Improves Blastocyst Number and Ongoing Pregnancy Rate of IVF Patients with Hashimoto Thyroiditis

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Abstract

In Assisted Reproduction Techniques (ART), autoimmune disorders of the thyroid gland present as common concomitant diseases. Hypothyroidism caused by autoimmune thyroiditis can impair fertility and pregnancy. Hashimoto thyroiditis (HT) is the most common autoimmune thyroid disease (AITD). Patients with HT undergoing IVF/ICSI using the long protocol are thought to benefit from a broad therapeutic concept. We compared the outcome of two different therapeutic schemes for HT patients presenting at our fertility clinic and compared the outcome to ART patients without thyroiditis. TSH level was adjusted to under 2 µIU/mL using L-thyroxine, as required. Concurrent medication from the time of oocyte puncture included daily administration of fragmin (dalteparin) and acetylsalicylic acid (ASA), as well as prednisolone in increasing dosage. One group of these HT patients (group1, n=56) had additionally highly-dosed folic acid, another group (group 2, n=50, referred to as the supplemented group) was alternatively supplemented with a micronutrient preparation containing selenium, high-dose folic acid, B-vitamins, antioxidants and iron. We compared the number of oocytes, fertilization rate, blastocyst formation rate, pregnancy- and ongoing pregnancy rate between the two groups. Also, the ART outcomes of both groups were compared to ART results of non-HT patients within the same age group. We observed a significant increase in the blastocyst rate and demonstrated a substantial rise in ongoing pregnancy rate of the supplemented patients. These also needed less L-thyroxine to achieve optimal TSH level. The outcome of the micronutrient supplemented patients corresponded to the average of healthy IVF patients without HT at our clinic.

Keywords

Autoimmune thyroiditis; IVF/IMSI; Miscarriage; Blastocyst; Ongoing pregnancy; Antioxidative dietary supplement

Introduction

Hashimoto's thyroiditis (HT) is an autoimmune thyroid disease (AITD), which results in chronic inflammation due to the occurrence of auto-antibodies directed against thyroperoxidase (TPO) (sometimes also against thyroglobulin (Tg) and TSH receptor (TSH-R)), with subsequent destruction of thyroid tissue by T-lymphocytes. HT is the commonest autoimmune disorder in humans. It affects an estimated 5-10% of the general population, with women being affected 2 to 5 times more often than males. The incidence of HT is even higher among female patients attending fertility clinics (up to 25%). HT is remarkably the most frequent cause of primary hypothyroidism in women of reproductive age [1]. At the onset of disease, there may be a period of hyperthyroidism due to the progressive destruction of thyroid tissue, causing damage to the integrity of thyroid follicle storage. However, the final state of disease is hypothyroidism, which is highly associated with female infertility.

The reasons for the disorder are various environmental factors which trigger the autoimmune response in genetically susceptible individuals, though the exact mechanisms remain unclear [2]. Severe viral infections have been reported to be involved in the onset of the disease. Besides, other autoimmune diseases or endocrine disorders, as well as environmental pollution, pesticides, xenohormones and many other chemicals with a putative impact on the immune system are suggested to trigger the outburst of the disease [2-4]. Moreover, a few studies proposed increased oxidative stress (OS) in HT, as assessed by elevated lipid peroxidation, and/or decreased antioxidant status [5].

Many women with thyroid dysfunction experience subfertility and increased miscarriage rates [6,7]. Given the fact that for conception, ongoing pregnancy and healthy delivery, various subtle interactions of hormones and components of the immune system are required, this is not surprising. HT, in fact, impairs fertility and reproduction in a multitude of ways.

Recently, Monteleone et al. [8] demonstrated that fertilization, embryo quality and pregnancy rate was lower for female IVF patients with thyroid autoimmunity, and suggested that the presence of anti-thyroid antibodies in ovarian follicles might play a critical role in female infertility. The zona pellucida and thyroid tissue seem to share similar antigens and anti-thyroid antibodies were suggested to alter fertility by targeting zona pellucida, human chorionic gonadotropin receptors and other placental antigens [9].

Patients with primary hypothyroidism often develop hyperprolactinemia, which is detrimental for oocyte maturation. Hyperprolactinemia is triggered in HT by various mechanisms. First, in response to the hypothyroid state, there is a compensatory increase in the discharge of central hypothalamic thyrotropin-releasing hormone, resulting in stimulation of prolactin (PRL) secretion. In addition to this, PRL elimination from the systemic circulation is reduced [10,11], and other reasons for increasing PRL levels in hypothyroidism individuals are also well known [12].

Menstrual irregularities are frequently found in female patients with hypothyroism [13], and its severity is linked to the serum TSH levels [14]. These irregularities are thought to be caused by a change in pulsatile gonadotropin release on the one hand, and by the lack of thyroxin leading to limited luteinizing on the other hand.

Furthermore, a number of other biochemical characteristics associated with HT have been identified. First, patients frequently exhibit elevated homocysteine levels (hyperhomoscysteinemia),

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caused by a reduction of hepatic remethylation of homocysteine into methionine [15,16]. Second, patients have an increased risk of vitamin D deficiency [17]. Also, HT is frequently associated with decreased levels of iron, vitamin B12 and folic acid, even when patients are euthyroid [16,18]. The frequent deficiency of selenium [19-21] or zinc [22] increases the vulnerability to OS, impairing oocyte maturation. All of these specifics have independently been identified as important risk factors for impaired fertility.

Another factor contributing to fertility problems in patients suffering from HT is the fact that HT patients have a higher incidence of endometriosis [23,24], polycystic ovary (PCO) syndrome [6,25], as well as an increased risk for premature ovarian failure (POF) and early miscarriage [23]. POF might be up to the immune system itself. Using a mouse model system, Matalon et al. [26] found that autoimmunity with its hyperactive immune function leads to early reproductive failure.

Miscarriage might be influenced by hypercoagulation and endothelial vascular damage.

Systemic inflammation, as observed in autoimmune diseases, modulates thrombotic responses by suppressing fibrinolysis, up-regulating pro-coagulant and down regulating anti-coagulants [27]. Some of the central features of the hyper-coaguability induced by inflammation are cytokine induction of tissue factor (TF) expression, endothelial dysfunction, suppression of the protein C signal cascade and inhibition of fibrinolysis [27-32].

Finally, there is the risk of hypothyroidism during later pregnancy, even when patients show euthyroidism in early gestation. This is due to the fact that maternal thyroid requirements increase as gestation progresses, but cannot be met [33]. Together with an alteration in renal clearance associated with increased iodine excretion, the rise of the placental type 3 Iodothyronine Deiodinase (D III) and the increase of TBG (Thyroxin Binding Globulin) with subsequent lower levels of free T4 can lead to severe hypothyroidism. This can cause obstetric complications during pregnancy, such as miscarriage, anemia, gestational hypertension, placental abruption, premature delivery and postpartum hemorrhage [6]. Hypothyroidism during pregnancy also elevates the risk for the neonate to be admitted to intensive care, mainly for respiratory stress syndromes [34], and the children are at the risk of poor neurodevelopmental outcome [35].

Data regarding fertilization-, implantation- and pregnancy rates are still inconsistent for women with thyroid autoimmunity [8,36,37]. However, there is a strong association between miscarriage and the presence of auto-antibodies [6].

For these reasons, we hypothesized that HT patients undergoing IVF/ICSI may benefit from a broad therapeutic approach, addressing the wide variety of Hashimoto-associated factors impairing fertility and early pregnancy.

This includes above-normal TSH (<2 µU/mL, optimal TSH: 0.5-1 µU/mL) and PRL adjustment before the start of stimulation, wide anti-coagulation (heparin and ASA) from the day of ovarian pickup (OPU) onwards, immune-modulation using selenium (100-200 µg) and steroids as required (up to 15 mg/day).

Interestingly, within recent years, the application of multivitamin/mineral supplements in otherwise healthy female patients for improving the outcome of IVF/ICSI treatment has been widely discussed and several promising studies demonstrated that this can have a positive impact [38-40]. Nevertheless, discussion is not without controversy. So far - to our knowledge - this is the first study analyzing the effects of a dietary supplementation on the IVF outcome of patients with autoimmune thyroiditis.

Given that HT patients have an increased risk for a number of micronutrient deficiencies as described above and that OS has been discussed to be involved in the progress of the disease, we supposed that in addition to a broad therapeutic concept as outlined above, supplementing a suitable dietary antioxidative preparation (multivitamin, folic acid, iron, zinc, selenium without iodine) to the diets of women with autoimmune thyroiditis undergoing ART treatment may have a positive impact on treatment outcome.

Materials and Methods

Patients

From January to July 2011, a total of 106 women with a history of HT disease attending the IVF center Prof. Zech were recruited for this study. Participants were randomized and 50 women received a micronutrient containing dietary preparation (Fertilovit®F^{THY}, Table 1) containing selenium, high-dose folic acid, B-vitamins, vitamin D, antioxidants and iron instead of folic acid only. Written consent was obtained from all participants of this study. The control group of 56 women received folic acid only (5 mg/day). Patient's mean age in the supplemented group was 36.1 years and 36.7 years in the non-supplemented group.

Hormonal stimulation schedule and medication

Stimulation was performed using the long protocol [41]. TSH level was adjusted before stimulation to under 2 µIU/mL, using L-thyroxine, as required. Dietary supplementation was started with the beginning of hormonal stimulation. Concurrent medication for HT patients from the time of oocyte pickup included daily administration of dalteparin (2500 IU/day) and ASA (100 mg/day), as well as prednisolone in increasing dosage (7.5 mg-15 mg/day).

Table 1: Substances of content for Fertilovit® F^{THY}. For all patients a daily intake of one capsule was recommended.

Content	Per capsule	Per 100 g	% RDA*
Caloric value	389 kJ (0.94 kcal)	903 kJ (218 kcal)	
Proteins	0.041 g	9.5 g	
Fats	0.035 g	8.6 g	
Carbohydrates	0.06 g	13.86 g	
Vitamin C	100 mg	23.203 g	125
Vitamin E	15 mg	3.48 g	125
Vitamin B1	4 mg	9.28 g	364
Vitamin B2	4.5 mg	1.044 g	321
Pantothenic acid	18 mg	4.177 g	300
Vitamin B6	5.4 mg	1.253 g	386
Vitamin B12	9 µg	2 mg	360
Folic acid	800 µg	186 mg	400
Vitamin D	5 µg	1.16 mg	100
Niacin	17 mg	3.944 g	106
Biotin	180 µg	42 mg	360
Zinc	2.25 mg	522 mg	50
Magnesium	100 mg	23.203 g	26
Iron	7.5 mg	1.740 g	54
Selenium	100 µg	23 mg	181
Coenzyme Q10	20 mg	4.641 g	-

*% of recommended daily allowance (according to EU-guideline)

Oocyte retrieval, fertilization, embryo culture and evaluation of embryo quality

Oocytes were retrieved by ovarian puncture in sedo-analgesia thirty-six hours post human chorionic gonadotropin (hCG) administration, and were fertilized using intracytoplasmic sperm injection (ICSI) or intracytoplasmic morphologically selected sperm injection (IMSI) [42]. Fertilized embryo was identified by the presence of two pronuclei (2PN). Embryo culture was performed in Global medium (LifeGlobal, Ontario, Canada) supplemented with human serum albumin (HSA LifeGlobal, Ontario, Canada) in four-well dishes (Nunc A/S, Roskilde, Denmark) and incubated (Incubator Hera Cell Incubator 240 CO_2) for 5 days, until embryo transfer. On day 5, the embryo quality was evaluated according to Gardner et al. [43]. Blastocysts with a degree of expansion of 2, 3, 4 and 5 and with A-grading for inner cell mass and trophectoderm, or a combination of A- and B-grading, were classified as top blastocysts.

For evaluation of treatment outcome, we compared the number of oocytes, fertilization rate, blastocyst formation rate, pregnancy rate and ongoing pregnancy rate between the two groups and the average results of healthy IVF-patients. Pregnancy rate (PR) was determined by urinary ß-hCG level 14 days after ET. Ongoing pregnancy rate (oPR) was defined as observation of foetal heartbeat(s) by ultrasound 6-8 weeks after ET. Differences in the fertilization-, blastocyst- and pregnancy rates were evaluated using Pearson's chi-squared test. Between-group comparisons of normally distributed variables were assessed using Student's t-test.

Results

The results of this study are summarized in Tables 2 and 3. While there was no difference between the two groups regarding the number of retrieved oocytes and fertilization rate, there was a significant increase in the blastocyst rate (Table 2), within the supplemented group. In addition, patients of the supplemented group required less L-thyroxine to achieve the TSH value aimed for ($p<0.01$). Moreover, the supplemented HT-patient group also demonstrated a substantial increase in the ongoing pregnancy rate detected by foetal heart beat (Table 2) after ET. Pregnancy rate was also slightly, yet not significantly elevated. This data of supplemented patients corresponds to the IVF outcome of non-HT patients (Table 3).

Discussion

Despite the low number of patients, this study demonstrates that IVF patients with chronic lymphocytic thyroiditis benefit significantly from a broader therapeutic concept within ART therapy. Dietary supplementation with an iodine-free micronutrient combination, including selenium and vitamins, seems to be markedly beneficial in terms of the number of blastocysts at day 5 of embryo culture. Considering the similar number of retrieved oocytes and the higher rate of top-blastocysts, these findings indicate that the micronutrient supplementation increases the competence of oocytes to develop to the blastocyst stage.

Another crucial aspect is the finding of a higher ongoing pregnancy rate following embryo transfer of the supplemented patients, although there was no significant difference in pregnancy rate detected. We, therefore, postulate that this is due to either

Table 2: Comparison of data from dietary supplemented and non-supplemented (control group) HT-patients.

	Supplemented group	Non-supplemented group	p-value
Number of patients	50	56	
BMI (kg/m²) (mean) +/- s.d.	22.3 +/-4.2	22.7 +/-3.7	n.s.
Age at the begin of stimulation (years) (mean) +/- s.d.	36.1 +/- 3.5	36.7 +/- 4.2	n.s.
L-Thyroxine Dosage needed for adjustment (µg) (mean) +/- s.d.	66.2 +/- 31.9	86.5 +/- 38.5	**<0.01 ****
Stimulation period (days) (mean) +/- s.d.	11.6 +/-1.41	11.4 +/-1.8	n.s.
Stimulation dose (IU)	2484 +/-891.5	2592+/-924	n.s.
Number of Oocytes retrieved (mean) +/- s.d.	11.1 +/- 6.7	10.9 +/- 6.2	n.s.
Number of Mature Oocytes M II oocytes (mean) +/- s.d.	9.08 +/- 5.6	8.78 +/- 6.1	n.s.
Fertilization rate %	73.7	76.3	n.s.
Blastocyst rate %	48.6	38.5	**<0.01 ****
Top Blastocysts rate %	27.2	30.5	n.s.
Embryos transferred (mean) +/- s.d.	1.72 +/- 0.53	1.68 +/-0.60	n.s.
Pregnancy rate (PR) %	48.0	39.3	n.s.
Ongoing pregnancy rate (oPR) %	44.0	32.1	**<0.05 ***

s.d: Standard Deviation
*p-value<0.05; **p-value<0.01; ***p-value<0.001

Table 3: Comparison of IVF outcome from supplemented HT patients and non- HT patients from the year 2011 within the same age class.

	Non-HT patients (control group)	Supplemented patients	p-value
Number of patients	274	50	n.s.
Age at stimulation start (years) +/- s.d.	36.1 +/- 3.1	36.1 +/- 3.5	n.s.
Number of oocyte retrieved (mean) +/- s.d.	11.6 +/- 6.0	11.1 +/- 6.7	n.s.
% MII oocytes	81.0	83.0	n.s.
Fertilization rate %	72.0	73.7	n.s.
Blastocyt rate %	49.9	48.6	n.s.
Pregnancy rate (PR) %	51.2	48.0	n.s.
Ongoing pregnancy rate % (oPR) %	42.9	44.0	n.s.

s.d: Standard Deviation.
*p-value<0.05; **p-value<0.005; ***p-value<0.001

an improved embryo competence or improved intrauterine environment, both of which may reduce the risk of early abortion. In fact, as mentioned before, there is a strong association of HT, implantation failure and (recurrent) foetal loss [6].

As a secondary effect, dietary supplemented patients obviously require less L-thyroxine to achieve the TSH levels aimed for, which is desirable, as it lowers the patient's risk for side effects, such as cardiac arrhythmias, insomnia or hypertension.

How these changes are brought about is currently not known and beyond the scope of this study. However, a few crucial aspects of micronutrients should be mentioned that might hint to possible mechanisms of action. One micronutrient with a substantial impact on thyroid function is undeniably iodine. Whereas a sufficient intake has been shown to be critical for normal thyroid function, for women with autoimmune thyroiditis, it has been observed that higher iodine concentrations may have detrimental effects, as well. Autoimmune thyroiditis worsens with iodine excess [44], whereas improvements have been reported in several studies when the iodine content of patients' diet was low [45-47].

Several other trace elements have been identified to be essential for normal thyroid hormone metabolism. Among these, iron and selenium are most worth to be mentioned. Deficiencies of these elements can restrain thyroid function markedly. Iron deficiency impairs thyroid hormone synthesis by reducing the activity of heme-dependent thyroid peroxidase [48]. Erdal et al. [49] demonstrated that basal levels of iron in patients with subclinical hypothyroidism were significantly lower when compared to a control group.

Under physiological conditions, thyroid gland retains high selenium concentrations and expresses many known seleno-cysteine containing proteins, which are needed for the (in)activation of thyroid hormones, responsible for normal development, growth and metabolism. Furthermore, seleno-cysteine can be often found in the catalytic center of enzymes protecting the thyroid from OS [50]. Adequate selenium-rich nutrition has been shown to be important for efficient thyroid hormone synthesis and metabolism, and protects the thyroid gland from damage by excessive iodide exposure. Various studies indicate other beneficial effects of selenium supplementation in patients with HT, such as modulation of the immune system by reducing thyroid antibody titers [19,51]. Positive effects of selenium supplementation during pregnancy in terms of reduced thyroid inflammatory activity have also been reported [52].

Recently, it was demonstrated that the GSH levels in HT patients are markedly lower compared to healthy controls. Even though the pathways of the autoimmune thyroiditis are not fully understood, OS has been found to be an additional important contributing factor [53,54], and the imbalance of ROS/antioxidants might be a responsible factor for a spectrum of HT symptoms.

Meanwhile, high susceptibility of oocytes to ROS is well established. ROS impair the membrane and interfere with protein synthesis, as well as energy production, and have also been associated with various problems throughout pregnancy [54]. Several studies revealed that patients with autoimmune thyroiditis have increased OS levels and a decrease in antioxidants [5]. The present study indicates that supplementation of antioxidants would be beneficial to the diets of HT-patients. In addition, a study comprising 115 patients with autoimmune thyroid disease demonstrated that this patient collective also has an increased risk of having a vitamin B12 deficiency [18]. Together with folic acid and vitamin B6, vitamin B12 is vitally needed for proper homocysteine metabolism.

Vitamin D deficiency was also suggested as a pre-disposing factor for autoimmune diseases and can also be observed in autoimmune thyroiditis patients [17]. Given that vitamin D deficiency has been linked to infertility and pregnancy loss [55], this is an important issue.

Conclusion

Although the detailed mechanisms in HT disease are still unclear, patients with HT undergoing fertility treatment (IVF/ICSI) obviously benefit from taking a dietary supplement, including selenium, iron, vitamins, as well as antioxidants, in addition to other supportive medication, such as ASA, dalteparin and prednisolone. This therapeutic regimen results in a marked increase of ongoing pregnancy rate, while requiring less L-thyroxine.

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