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ORIGINAL ARTICLE

Dysregulation of mitochondrial sirtuin genes is associated with human male infertility

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Abstract

Mitochondrial sirtuins (SIRT3, SIRT4, SIRT5) are post-translational modifiers that regulate energy production, body homeostasis and mitochondrial activities via different substrates in response to environmental stressors. The present study aimed at assessing the expression of *SIRT3*, *SIRT4*, and *SIRT5* in the semen of infertile men. Expression analysis was performed using q-RT PCR. All mitochondrial sirtuin genes were significantly down-regulated in the semen of infertile men compared to fertile men. Mitochondrial sirtuin genes expression levels were correlated with mitochondrial *HSP90* expression. *HSP90* expression was positively correlated with *SIRT3*, *SIRT4* and *SIRT5* expression in the semen of fertile men, while a negative correlation was observed between *HSP90* in the semen of infertile men and mitochondrial sirtuin genes in the semen of fertile men. These data suggest that dysregulation of mitochondrial sirtuin genes causes mitochondrial dysfunction due to stress, which appears to be associated with human male infertility by compromising functional and structural sperm integrity.

KEYWORDS

human, infertility, male, mitochondria, semen, sirtuins

1 | INTRODUCTION

Infertility is the incapability of a couple that is sexually active without using any contraception, to achieve pregnancy spontaneously within a period of twelve months (Rowe et al., 2000). Around 13% and 17% of couples face problems to conceive first and subsequent child, respectively, while about 15% of couples fail to achieve pregnancy within a period of twelve months (Salonia et al., 2021). In about half of the infertile couples, ranging from 20% to 70% around the globe, a male-responsible factor is observed (Agarwal et al., 2015). In around one in three cases, no male factor is observed to explain impaired semen parameters (idiopathic male infertility) (Rowe et al., 2000; Salonia et al., 2021). Idiopathic male infertility may be associated with various non-identified pathological factors

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such as endocrine disruption (Amir et al., ; Amir et al., 2021; Kalliora et al., 2018; Mamoulakis et al., 2002, 2017; Petrakis et al., 2017; Tsiaoussis et al., 2018) and various genetic alterations (Cheung et al., 2019).

Sirtuins, the molecular sensors, comprise a family of NAD^+-dependent histone deacetylases and ADP-ribosyl transferases with seven members (SIRT1–7) phylogenetically grouped into four distinct classes: class I (SIRT1-3), class II (SIRT4), class III (SIRT5) and class IV (SIRT6-7) (Frye, 2000). SIRT1, SIRT6 and SIRT7 are located in the nucleus, SIRT2 in the cytoplasm and SIRT3-5 mainly in the mitochondria (mitochondrial sirtuins; MS), mediating among others homoeostasis; mitochondrial biogenesis; chromatin remodelling; and protection against oxidative stress (Kumar & Lombard, 2015; Tatone et al., 2018). Their role in reproduction has recently attracted attention with most studies focusing on SIRT1, mainly in female reproduction (Loganathan et al., 2021; Tatone et al., 2018). On the other hand, there is evidence supporting that SIRT1 is implicated in spermatogenesis as well by influencing functions of germ cells, Sertoli and Leydig cells (Loganathan et al., 2021; Tatone et al., 2018; Wahab et al., 2021). Nevertheless, the exact role of sirtuin members in male reproduction is still obscured (Loganathan et al., 2021) and supporting clinical data remain scarce (Mostafa et al., 2018, 2020; Nasiri et al., 2021). The present case–control study focuses on MS by assessing the expression of *SIRT3-5* in the semen of infertile men, with the aim to evaluate for the first time their potential role in human male reproduction.

2 | PATIENTS AND METHODS

The study was performed in line with the revised Helsinki Declaration (World Medical, 2013) and approved by the Ethical Review Board, COMSATS University Islamabad (CIIT/Bio/ERB/19/98). All participants provided written informed consent. Sixty male adult volunteers were included. Male partners of apparently fertile females, namely females without a history of fertility-associated diseases and a normal medical workup as confirmed at the infertility clinic at the National Institute of Health (NIH) Islamabad, Pakistan, in sexually active couples that were unable to achieve pregnancy spontaneously for over two years of marriage without using contraception were considered infertile subjects (n = 40). Apparently healthy fathers with no history of fertility-associated diseases were considered fertile subjects (n = 20). Semen samples were collected by masturbation three to five days following sexual abstinence. Examination/processing of all semen samples was performed at the NIH, Islamabad, Pakistan, following current guidelines (WHO, 2010).

Total RNA was extracted as previously reported (Shafeeque et al., 2014). Briefly, 1 ml of freshly ejaculated semen was centrifuged at 12,000 × g for 10 min at 4°C, re-suspended in 1 ml 1X PBS, 0.5 ml of trizol and incubated for 30 min at room temperature. After incubation, 0.2 ml of chloroform was added. Tubes were inverted 5 – 10 times, vortexed for 15 s and centrifuged at 12,000 × g for 15 min at 4°C. The upper aqueous was transferred to a clean Eppendorf tube containing 0.5 ml chilled isopropanol. The solution was incubated for 10 min at room temperature followed by centrifugation at 12,000 × g for 15 min at 4°C. RNA was washed with 0.75 ml chilled 70% ethanol and centrifuged under same conditions. The supernatant was discarded and RNA pellet was air-dried for 12 min, 40 µl DEPC treated water was added, and it was stored at −20°C for further analysis. Quality/quantity of extracted RNA was assessed using agarose gel electrophoresis and nanodrop spectrophotometer, respectively. Samples containing ≥100 ng of RNA were further processed. cDNA was synthesised from 1 µg total RNA, using RevertAid First Strand cDNA Synthesis Kit (Thermo-scientific™, USA). cDNA synthesis was confirmed by PCR using β-actin primers (*ACTB*; internal control).

To validate mitochondrial stress due to potential down-regulation of *SIRT3*, *SIRT4*, and *SIRT5*, the expression of mitochondrial *HSP90* known to be crucial in mitochondrial functions/protein folding quality control was assessed (Kang et al., 2007). The primers of target genes are presented in Table 1 and were designed using PrimerQuest™ Tool (Integrated DNA Technologies, USA). Specificity/quality of primers was assessed using the NCBI Primer-BLAST tool. In silico PCR was performed using UCSC Genome Browser. Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) was used for qPCR on StepOnePlus™ Thermal cycler (Applied Biosystem, USA). Reaction conditions included 5 min for denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 45 s and annealing/extension at specified temperature for 45 s per cycle. Relative expression was analysed using the $2^{-\Delta\Delta CT}$ method. All experiments were performed three times.

After assessing normality using Shapiro–Wilk test, data were processed with Mann Whitney U test, Kruskal–Wallis test, Spearman rank-order correlation test or one-way analysis of variance (ANOVA),

TABLE 1 Primers and respective amplicon size of target genes

Genes	Primer Sequence (5' – 3')		Amplicon Size (bP)	Tm (°C)
	Forward	Reverse		
SIRT3	GACTGGTAGGGCTGTGTTTAC	GAGGGTCACAGTCAGAAGAAAG	135	59.4
SIRT4	GAGCTTTGCGTTGACTTTCAG	GGACTTGCTGGCACAAATAAC	102	60.0
SIRT5	CTGCCATACAGGGTCATTCTC	AGCACTGAGTTAGCTGGTAAAG	134	60.0
HSP90	GGAGATAAACCCTGACCATTCC	GACAGGAGCGCAGTTTCATA	117	58.1
ACTB	GTTGGGTTACACCCTTTCTTG	ACCTTCACCGTTCCAGTTT	147	58.0

as appropriate. Post hoc comparisons were assessed with Dunn's multiple comparison test. Statistical analysis was performed using Origin (Pro), Version 2018. OriginLab Corp., Northampton, MA, USA and Graphpad Prism 8 (GraphPad Software, Inc. CA, USA). *p*-value ≤0.050 was considered significant.

3 | RESULTS

The infertile group included 10 normospermic (concentration: 92.9 ± 37.4 × 10^6 / ml; motility: 67.8 ± 6.3%), 16 asthenospermic (concentration: 77.1 ± 28.2 × 10^6 / ml; motility: 29.1 ± 8.9%) and 14 oligoasthenospermic men (concentration: 11.9 ± 8.4 × 10^6 / ml; motility: 22.0 ± 6.9%). Mean age was similar between groups (infertile versus. fertile: 32.1 ± 5.7 versus. 34.4 ± 7.5 years). The results of semen analysis are summarised in table 2. MS gene expressions in the semen were significantly reduced in the infertile group (median relative expressions of *SIRT3* and *SIRT4*: 1.5 times lower; of *SIRT5*: four times lower (Figure 1a)). *SIRT3* expression was significantly reduced in the infertile subgroups (normospermic/oligoasthenospermic men) compared to the fertile group and across the infertile subgroups (asthenospermic compared to normospermic/oligoasthenospermic men (Figure 1b)). *SIRT4* and *SIRT5* expression was significantly reduced in oligoasthenospermic and asthenospermic men compared to the fertile group, respectively (Figure 1b). Mitochondrial *HSP90* expression in semen was significantly reduced in the infertile group (median relative expression was 1.45 times lower; Figure 1c). *HSP90* expression was significantly reduced in the infertile subgroups (asthenospermic/oligoasthenospermic men) compared to fertile group (Figure 1c). Correlation among relative gene expressions and semen parameters are summarised in table 3.

4 | DISCUSSION

Sirtuins function as molecular metabolic sensors to maintain energy production/homeostasis, acting as post-translational modifiers via ribosylations/deacetylations to regulate several physiological processes such as fertilisation, by coordinating key processes in gametogenesis and cellular stress response (Tatone et al., 2018). SIRT3-5 are confined to the mitochondria regulating mitochondrial activities via various substrates in response to environmental stressors (Nakagawa & Guarente, 2011). SIRT3 influences and maintains important mitochondrial activities such as reactive oxygen species (ROS) level regulation and ATP production (Torrens-Mas et al., 2017). Studies using *SIRT3* knocked out models show increased ROS generation and chromosomal instability (Kim et al., 2010). Apart from ADP-ribosylation and energy metabolism, SIRT4 mediates DNA repair via nucleotide synthesis (Tatone et al., 2018). Similarly to SIRT4, SIRT5 lacks deacetylation activity but is involved in demalonylation, desuccinylation, and deglutarylation to regulate urea cycle and other metabolic pathways (Shi et al., 2019).

Male infertility is associated with several factors, including metabolic dysfunctions and oxidative stress (Agarwal et al., 2019; Alahmar, 2019; Rato et al., 2016). Lactate derived from Sertoli cells is the major substrate of germ cells, and therefore, the fact that sirtuins regulate testicular glycolytic metabolism suggests a potential role in male fertility (Rato et al., 2016). Oxidative species or a disturbance of redox cellular environment balance changes the sirtuin-dependent signalling via alteration of the expression of sirtuin genes or their post-translational modification patterns (Santos et al., 2016). Nevertheless, the exact role of sirtuins in male reproduction remains obscured (Loganathan et al., 2021) and supporting clinical data are still scarce (Mostafa et al., 2018, 2020; Nasiri et al., 2021).

Sperm quality is linked to mitochondrial function (Barbagallo et al., 2020). Defective mitochondria are related to oxidative stress, contributing to male infertility (de Almeida Chuffa et al., 2019), since they are crucial for several functions of the spermatozoa including motility, hyper-activation, capacitation, acrosome reaction, and fertilisation (Darr et al., 2016; Moraes & Meyers, 2018). Based on preclinical studies and knock-out models (Loganathan et al., 2021), SIRT3 is speculated to play a role in spermiogenesis by increasing ROS production; SIRT4 is speculated to have a significant role in steroidogenesis of Leydig cells; while SIRT5 appears to regulate the electron transport chain via oxidation of mitochondrial NADH oxidation ATP synthase activity (Shi et al., 2019) but its role in spermatogenesis is still obscured. To our knowledge, this case–control study is the first to focus on the expression of MS genes in the semen of infertile men.

We evaluated the expression of *SIRT3-5* in the semen of infertile compared to fertile men. *HSP90* expression was also evaluated to assess mitochondrial stress. Our findings show that the decrease in expression of MS genes is attributed to mitochondrial stress and may impair fertility. *SIRT3* expression was significantly reduced in the semen of infertile men and the gene dysregulation observed was more prominent in oligoasthenospermic and asthenospermic compared to normospermic men. *SIRT3* expression was also significantly reduced in infertile subgroups (normospermic/oligoasthenospermic men) compared to the fertile group and across the infertile subgroups (asthenospermic compared to normospermic/oligoasthenospermic men) in line with the sole relevant clinical

TABLE 2 Semen analysis

	Fertile men (*n* = 20)	Infertile men (*n* = 40)
Viscosity *n* (%)		
Thick	3 (15)	9 (23)
Thin	17 (85)	31 (78)
pH	7.40 ± 0.15	7.44 ± 0.17
Volume (ml)	4.12 ± 0.95	4.13 ± 1.30
Concentration (10^6 / ml)*	92.84 ± 30.29	60.75 ± 40.60
Motility (%)*	74.95 ± 8.25	36.28 ± 20.12
Morphology (%)	30.4 ± 17.7	24.2 ± 11.1

Note: Variables are expressed as mean ± standard deviation.
*Variables that differ significantly between groups.

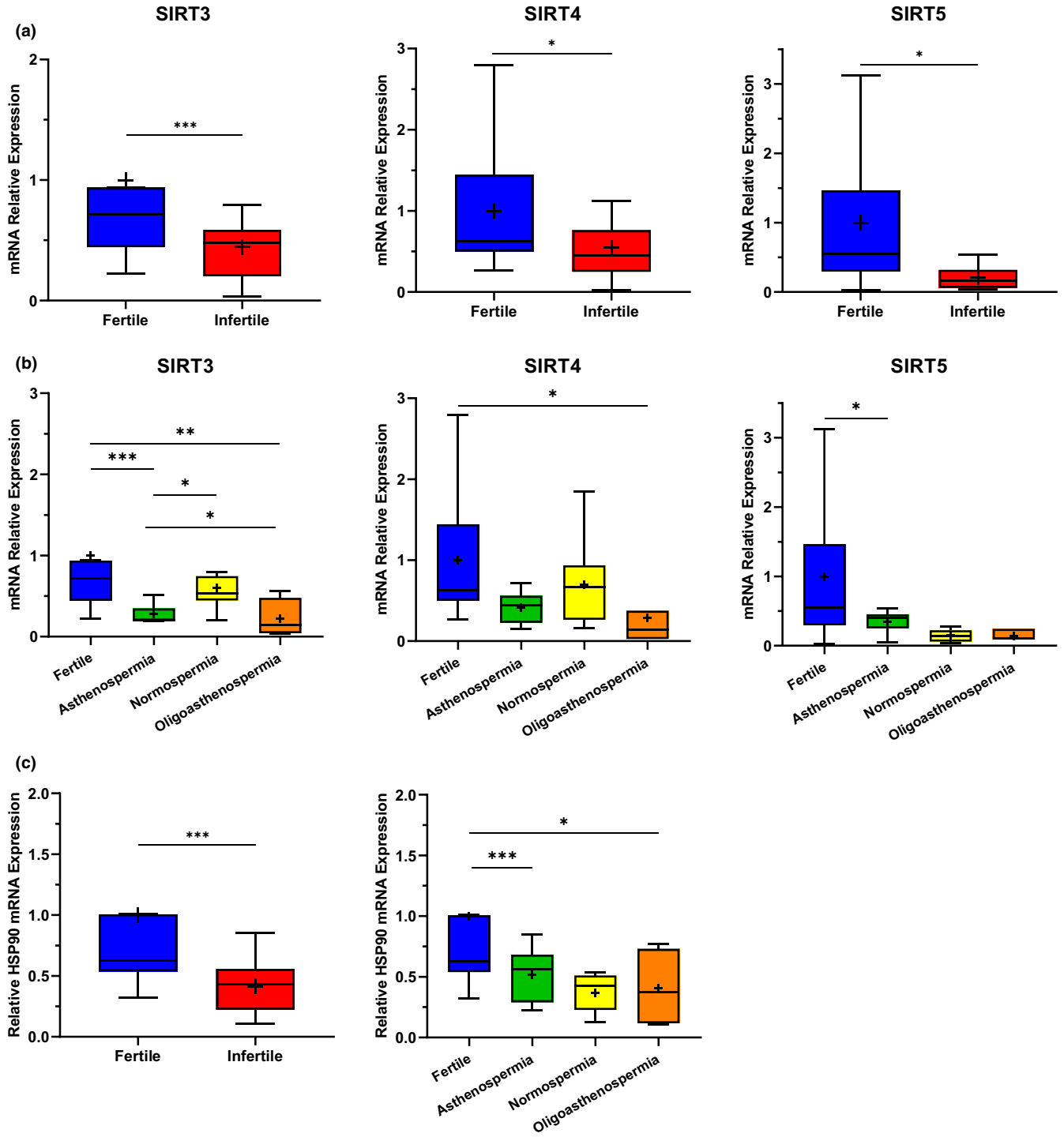

FIGURE 1 Comparative evaluation of SIRT3, SIRT 4, SIRT 5 expression (A-B) and mitochondrial HSP90 expression (C) in the semen of fertile/infertile group and infertile subgroups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

study on SIRT3 published to date (Nasiri et al., 2021). In addition, our data showed a significant down-regulation of *SIRT4-5* in infertile compared to fertile men. Therefore, our results showed that sperm motility decreases as *SIRT3-4* expression decreases and the decrease in their expression may affect sperm motility by increasing ROS levels. Our data showed a weak negative correlation between *SIRT5* expression and motility in infertile men, which may be attributed to the small sample size and should be further investigated in larger studies.

No clinical data exist on the potential role of SIRT4 and SIRT5 in male infertility, but a case–control study evaluated the correlation between SIRT1 and SIRT3 with antioxidants, oxidative stress biomarkers, and DNA fragmentation in the semen of asthenoteratozoospermic and normospermic men. The authors concluded that the levels of both proteins are negatively correlated with oxidative stress/DNA fragmentation in semen and that low levels of both proteins in asthenoteratozoospermic men may lead to increased oxidative stress, DNA fragmentation, and lipid peroxidation, eventually

TABLE 3 Correlation among relative gene expressions and semen parameters

Fertile group								Infertile group							
	Volume	Conc	Motility	*SIRT3*	*SIRT4*	*SIRT5*	*HSP90*		Volume	Conc	Motility	*SIRT3*	*SIRT4*	*SIRT5*	*HSP90*
Volume F	1.000														
Conc F	−0.264	1.000													
Motility F	0.323	**0.290**	1.000												
SIRT3 F	**0.295**	**0.253**	**0.416**	1.000											
SIRT4 F	0.187	**0.378**	−0.037	**0.864**	1.000										
SIRT5 F	−0.100	**0.872**	0.000	**0.400**	0.700	1.000									
HSP90 F	−0.119	−0.042	−0.056	**0.618**	**0.588**	**0.400**	1.000								
Volume IF	−0.305	0.337	−0.002	**−0.673**	**−0.448**	**0.447**	**−0.618**		1.000						
Conc IF	−0.389	0.047	−0.212	0.088	**0.351**	**0.205**	**0.389**		0.043	1.000					
Motility IF	0.019	0.017	−0.135	**0.393**	**0.606**	**−0.205**	**0.614**		**−0.254**	**0.401**	1.000				
SIRT3 IF	−0.163	0.029	**−0.284**	**0.322**	**0.673**	**−0.300**	**0.469**		**−0.186**	**0.604**	**0.313**	1.000			
SIRT4 IF	−0.298	**0.507**	−0.142	**0.392**	**0.709**	**0.300**	**0.329**		**−0.269**	**0.454**	**0.283**	**0.696**	1.000		
SIRT5 IF	0.165	−0.137	**−0.462**	**−0.455**	**−0.282**	**−0.500**	**−0.476**		0.096	−0.089	**−0.217**	−0.046	−0.032	1.000	
HSP90 IF	−0.013	**0.308**	0.095	−0.056	**−0.200**	**−0.700**	**−0.364**		−0.118	0.093	0.093	−0.180	0.089	**0.618**	1.000

Note: Abbreviations: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Conc, Concentration; F, Fertile; IF, Infertile.

The bold values indicate significant correlations.

resulting in asthenoteratozoospermia (Nasiri et al., 2021) in line with our results on SIRT3 and the results of two previously published clinical studies on SIRT1 (Mostafa et al., 2018, 2020).

The exact mechanism of how *SIRT3-5* expression potentially influence fertility still needs to be explored. Based on the results of preclinical studies, *SIRT3* ablation has been considered to play a role in spermiogenesis by increasing ROS level, and the absence of *SIRT1* and *SIRT3* has been speculated to affect spermatogenesis by leading to DNA damage, that might prevent the transition of histone to protamine (Loganathan et al., 2021). Several studies have reported that oxidative stress, namely the situation when ROS concentration overwhelms the antioxidant defense of the body, has a pivotal pathological implication on male fertility (Alahmar, 2019; Barati et al., 2020; Baskaran et al., 2021). Consequently, since SIRT3 appears to possess antioxidant properties as well as a role in energy homeostasis (Kim et al., 2010; Nogueiras et al., 2012; Tao et al., 2014; Watroba & Szukiewicz, 2016), a role of its reduced levels in male infertility appears to be plausible but warrants further testing in clinical studies (Loganathan et al., 2021; Rato et al., 2016). Similarly, it has been reported that SIRT4 and SIRT5 play a vital role in respiration, ROS detoxification and apoptosis (Alahmar, 2019; Liu et al., 2013). Consequently, decreased expression of *SIRT4* and *SIRT5* may ultimately affect reproductive processes such as spermatogenesis, sperm viability, maturation, capacitation, motility and acrosome reaction by affecting respiration and elevating ROS level.

In conclusion, the present study shows that dysregulation of MS genes causes mitochondrial dysfunction due to stress, which appears to be associated with human male infertility by compromising functional and structural sperm integrity.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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