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## Oxidative stress and male infertility: A cross sectional study

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## Oxidative stress and male infertility: a cross sectional study

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### Abstract

**Objective:** To compare stress markers and antioxidants in fertile and infertile males, and to explore their effects on reproductive hormones and fertility.

**Methods:** The cross-sectional case-control study was conducted from July 2017 to July 2018 at the Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan, and comprised male subjects aged 25-55 years. Infertile subjects were the cases, while healthy fertile males acted as the controls. Stress hormones cortisol and adrenaline and antioxidants glutathione peroxidase and superoxide dismutase were measured using enzyme-linked immunosorbent assay. Data was analysed using SPSS 22.

**Results:** Of the 376 subjects, 241 (64%) were cases and 135 (36%) were controls. Median cortisol, adrenaline, superoxide dismutase and glutathione levels were significantly higher among the cases compared to the controls ( $p < 0.05$ ). Follicle stimulating hormone and luteinizing hormone levels were higher in cases compared to the controls ( $p = 0.05$ ). Mean testosterone level was higher among the controls than the cases ( $p < 0.001$ ). After adjusting for other covariates, every increase of 7 units in cortisol increased the prevalence of infertility by 3% ( $p = 0.001$ ). There was significant interaction between luteinizing hormone and testosterone in the final model ( $p < 0.05$ ).

**Conclusion:** Stress together with decrease in antioxidants was found to play a significant role in reducing the fertilising potential of male infertile subjects.

**Keywords:** Oxidative stress, Male infertility, Cortisol, Glutathione peroxidase.

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### Introduction

Male infertility is reported to affect 5-7% of the general male population and it accounts for 20% of the cause of infertility and 50% cases of male infertility are idiopathic in nature.<sup>1</sup> It has been reported that oxidative stress (OS) is one of the important causes of male infertility (30-80%) as it has adverse effects on both the structural and functional integrity of the sperm, and causes loss of sperm function.<sup>2</sup> Spermatozoa have antioxidant defence mechanisms, to reduce reactive oxygen species (ROS) and protect gonadal cells and mature spermatozoa from oxidative damage, but this defence system has limited capacity. (2) OS is produced if the uncontrolled production of ROS exceeds the total antioxidant capacity (TAC) of the seminal plasma.<sup>3</sup> Both fluidity and permeability of the sperm plasma membrane as well as integrity of

deoxyribonucleic acid (DNA) in the sperm nucleus are affected by OS, thus causing apoptosis and increased risk of male infertility.<sup>4</sup> Unfortunately, spermatozoa, due to lack of the necessary cytoplasmic-enzyme repair systems, are not capable to restore the OS-induced damage.<sup>5</sup> OS can cause a number of clinical manifestations which include reduced fertilisation rates, failure of implantation, impaired embryonic development, recurrent pregnancy loss and poor assisted reproductive technology (ART) outcomes.<sup>6</sup>

Stress and infertility are closely related to each other.<sup>7</sup> Long-term stress produces elevated cortisol levels which is found to be associated with male infertility owing to decreased conversion of androstenedione to testosterone, thereby reducing sperm volume and concentration.<sup>8</sup> Glucocorticoids have both central and peripheral effects on reproductive function.<sup>9</sup> Glucocorticoids affect the hypothalamic pituitary axis (HPA) by exerting suppressive effects on the release of gonadotrophin Releasing Hormone (GnRH) at the level of the hypothalamus, the release of luteinizing hormone (LH) and follicle stimulating

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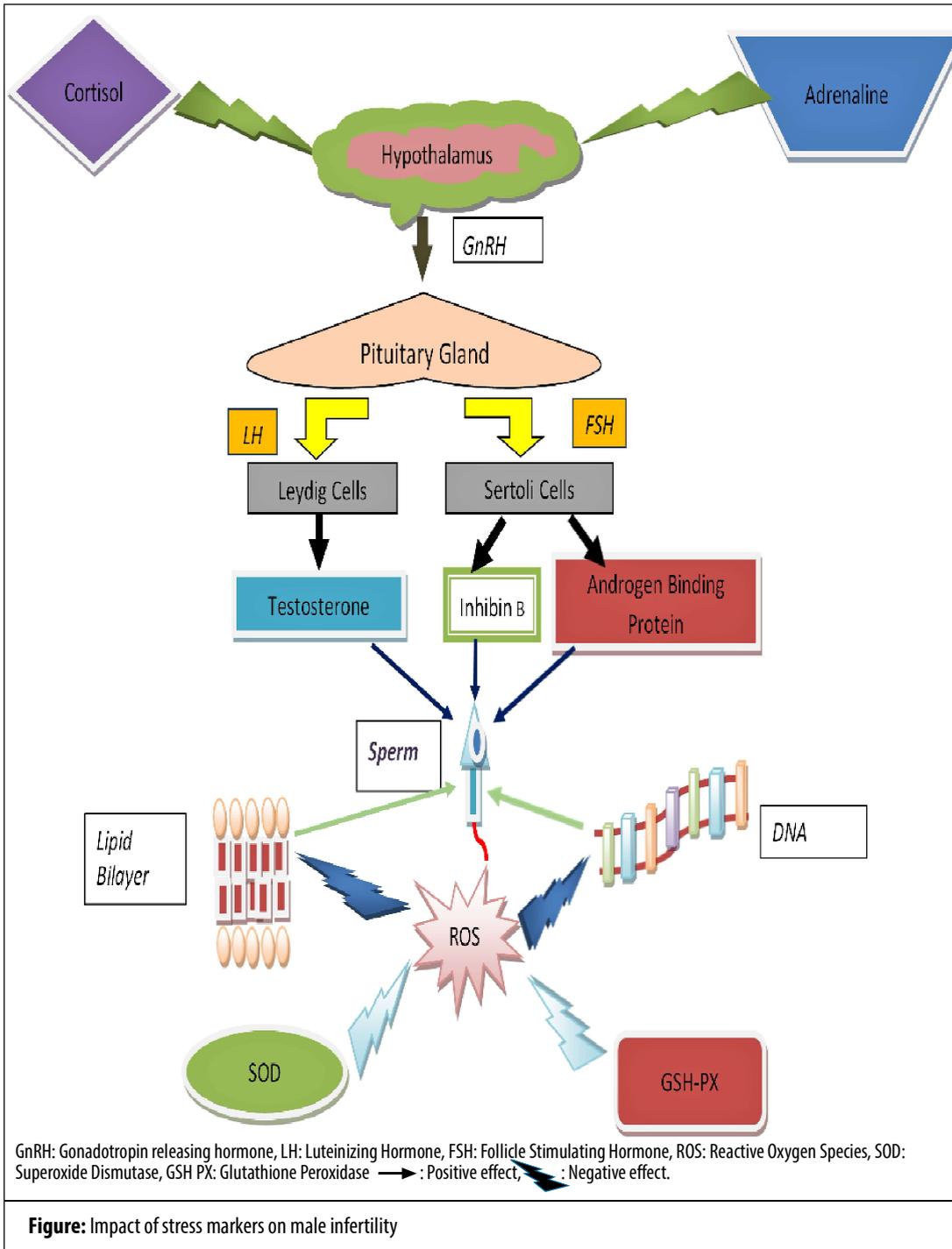
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hormone (FSH) at the level of anterior pituitary gland and on the testes.<sup>9</sup> The direct effect of glucocorticoids causes inhibition of steroid hormone production by repressing the expression of steroidogenic enzymes, reduction of the testicular response to gonadotropins, or glucocorticoid-induced apoptosis of Leydig and germ

cells. Glucocorticoids may also have control of Leydig cell number through arrest of cell cycle.<sup>9</sup> Short-term stress affects male fertility by releasing catecholamines. Adrenaline is reported to decrease testosterone levels in the body by inhibiting testosterone production at HPA as well as by blocking retrovirus-associated DNA sequences (RAS) pathway.<sup>10</sup>

Superoxide dismutase (SOD) is also an important marker of male infertility as studies have shown the positive association between SOD and sperm quality in terms of sperm motility and concentration.<sup>11</sup> SOD, an antioxidising enzyme normally produced and secreted in seminal plasma, is found to be high in Sertoli cells and germ cells to maintain spermatogenesis in developing and maturing spermatozoa.<sup>12</sup> This enzyme protects sperm from OS, lipid peroxidation, DNA damage, especially mitochondrial DNA, which leads to decreased adenosine triphosphate (ATP) production and energy availability, impeding forward movement of sperm and thus affecting sperm motility.<sup>12</sup> Hence, low SOD activity is associated with infertility.

Another antioxidant enzyme, glutathione peroxidase (GPX), essential for normal sperm function due to its defensive action against



**Figure:** Impact of stress markers on male infertility

ROS, is considered to be a useful marker for male infertility.<sup>12</sup> All the three isoforms of GPX, which are cytosolic, mitochondrial and nuclear, are considered to be important for spermatogenesis. The cytosolic isoform is essential for embryonic spermatogenesis whereas the mitochondrial isoform GPX is essential for sperm quality, motility and mitochondrial membrane potential.<sup>13</sup>

The current study was planned to compare the stress markers and antioxidants in fertile and infertile males and to explore their effects on male infertility. The hypothesis was that stress, as estimated by increase in adrenaline and cortisol, with impairment of antioxidants, disturbs FSH, LH and testosterone levels required for normal reproductive functions (Figure).

### Patients and Method

The cross-sectional case-control study was conducted from July 2017 to July 2018 at the Islamabad Clinic Serving Infertile Couples (ICS), Islamabad, Pakistan. After acquiring approval from the institutional ethics review committee, the sample size was calculated using Open Source Epidemiologic Statistics for Public Health<sup>14</sup> to compare the mean cortisol, GPX, SOD and adrenaline levels in fertile and infertile men adjusting for 10% non-response rate. Level of significance was set at 5%, power for detecting the true effect 80% and anticipated difference in mean levels of the stress markers at -0.27.<sup>15</sup> Against the required sample size of 398, the final sample had 22(5.5%) fewer individuals on account of a number of refusals to provide semen samples.

Those included were male subjects aged 25-55 years. The control group comprised healthy fertile males without any history of fertility problems and whose spouses conceived within one year of unprotected intercourse.<sup>16</sup> The cases comprised male subjects referred to the infertility clinic and were from couples who had failed to conceive after one year of regular unprotected intercourse. Those excluded were individuals who had female cause of infertility, suffered from secondary infertility, had cryptorchidism, testicular trauma, orchitis or testicular hypotrophy. Subjects having diabetes, hypertension, arthritis, malignancy, epilepsy, tuberculosis, endocrinal disorders, liver/renal diseases, and those on drugs and had conditions known to influence OS and serum cortisol level were also excluded and so were those receiving testosterone or thyroxin replacement therapy or steroids. After taking consent from each subject, general physical

examination, assessment of height and weight. The calculation of body mass index (BMI) and estimation of body fat (%) was done by bioelectrical impedance analyzer (BIA). BIA, a safe, non-invasive and portable device was used to assess the percentage of body fat composition of the participants from the estimate of entire body water. Foot-to-foot (FF) BIA scales is more acceptable and convenient as the subject only needs to stand without shoes on the scale for simultaneous measurements of BW (body weight) and impedance across the lower limbs. By adding the gender, age and height manually into the system, the fat mass (FM) of the subject or percentage fat mass (% FM) is displayed immediately.<sup>17</sup> The BMI of the study participants was measured and categorized according to the Asian criteria, the normal weight (18–22.9 kg/m<sup>2</sup>), overweight (23–24.9 kg/m<sup>2</sup>) and obesity ( $\geq 25$  kg/m<sup>2</sup>) (WHO, 2004). The cut-offs used for BF% were normal weight (12%–22%), overweight (22.1%–27%) and obese (>27.1%).<sup>18</sup> Blood samples were collected from the subjects at 9 am and the serum from each was separated, centrifuged and analysed. Cortisol was analysed using the cortisol enzyme-linked immunosorbent assay (ELISA) kit (catalogue number: CO103S, CALBIOTECH) with a detection range of 50-230 ng/mL from 8am to 11 am and 30-150 ng/mL at 4 pm. Adrenaline was analysed using Human Epinephrine/Adrenaline (EPI) ELISA kit (catalogue number: 95362, Glory Science Co., Ltd), with a detection range of 5ng/L-1000ng/L. SOD was analysed using Human SOD ELISA kit (catalogue number: 11086, Glory Science Co., Ltd) according to the the manufacturer's protocol. GPX was analysed using Human GPX ELISA kit (catalogue number: 10800, Glory Science Co., Ltd), with a detection range of 30-700 U/L.

Data was analysed using SPSS 22. Descriptive statistics were computed for categorical variables by computing their frequencies and percentages, assessed by chi-square/Fishers exact test. The distribution of quantitative variables was computed by their means and standard deviation (SD) / median and interquartile range (IQR) depending on the normality of data and assessed by independent t test / Mann Whitney test. Unadjusted and adjusted prevalence ratios (PRs) with their 95% confidence intervals (CIs) were calculated using simple and multiple Cox Proportional hazard algorithm. Statistical significance was set at  $p < 0.05$ .

**Table-1:** Demographic, Biochemical characteristics of infertile and fertile males.

Factors	Infertile (n=241)	Fertile (n=135)	p-value
Age (in years) Mean ± SD	33.90 ± 5.97	37.65 ± 6.13	<0.001*
BMI (in kg/m <sup>2</sup> ) (Mean ± SD)	26.77 ± 3.58	24.76 ± 3.085	<0.001*
<b>BMI (in)</b>			<0.001*
≤24 kg/m <sup>2</sup>	56 (23.2%)	57(42.2%)	
>24 kg/m <sup>2</sup>	185(76.8%)	78(57.8%)	
<b>Body fat %</b> (Mean ± SD)	34.53 ± 4.67	32.97 ± 4.06	<0.01*
<b>Body fat %</b>			0.32
<25%	3 (1.2%)	4 (3.0%)	
25-29.99%	39 (16.2%)	26(19.3%)	
>29.99%	199(82.6%)	105(77.8%)	
<b>Smoking status</b>			0.002*
Smoker	187 (77.6%)	85 (63.0%)	
Non smoker	54 (22.4%)	50 (37.0%)	
<b>SHBG</b> (nmol/L) Median (IQR)	16.40 (13-16.39)	30.71 (25.6-41.7)	<0.001*^
<b>Cortisol</b> ng/mL Median (IQR)	168.16 (122.3-212.3)	82.89 (77.5-123.3)	<0.001*^
<b>Adrenaline</b> (ng/L)			
<b>Median</b> (IQR)	33.72 (20-49.67)	19.53 (11-36.6)	0.03*^
<b>SOD</b> (ng/L) Median (IQR)	22.36 (10.2-37.14)	10.80(0.5-42.7)	0.02
<b>Glutathione peroxidase</b> (U/L)			
<b>Median</b> (IQR)	60.66 (44.5-114.52)	73.05 (42.8-160.7)	0.005*^
<b>FSH</b> (mIU/ml) Mean ± SD	9.61 ± 1.39	9.30 ± 1.31	0.03*
<b>LH</b> (mIU/ml) Mean ± SD	10.91 ± 5.98	6.97 ± 1.81	<0.001*
<b>Testosterone</b> (ng/ml) Mean ± SD	3.54 ± 1.57	6.05 ± 0.82	<0.001*

\* Significant at p value <0.05 (Independent t test/ Mann Whitney test was used),

\*^ Significant at p value <0.05 (Chi-square test of independence/ Fishers Exact test was used), SD: Standard deviation; BMI: Body mass index; SHBG: Sex hormone-binding globulin; IQR: Interquartile range; SOD: Superoxide dismutase; FSH: Follicle stimulating hormone; LH: Luteinizing hormone.

## Results

Of the 376 subjects, 241(64%) were cases and 135(36%) were controls. The mean age of the cases was significantly lower compared to the controls ( $p < 0.001$ ). The mean BMI and body fat (%) were significantly higher among the cases compared to the controls ( $p < 0.05$ ).

The median sex hormone-binding globulin (SHBG) and GPX were significantly higher in controls ( $p < 0.05$ ). However, median cortisol, adrenaline levels and SOD were significantly higher in the cases ( $p < 0.05$ ).

Mean FSH and LH levels were higher in cases ( $p < 0.05$ ), and mean testosterone was higher in controls ( $p < 0.001$ ). There was a significant weak positive correlation between cortisol and FSH ( $r = 0.110$ ,  $p = 0.03$ ), and between cortisol and LH ( $r = 0.342$ ,  $p < 0.001$ ). However, there was a significant moderate negative correlation between cortisol and testosterone ( $r = -0.472$ ,  $p < 0.001$ ). A significant weak positive correlation was evident between adrenaline and LH ( $r = 0.147$ ,  $p = 0.004$ ), a significant weak negative correlation between adrenaline and testosterone

**Table-2:** Univariate and Multivariable Analysis for factors associated with male infertility.

Factors	Unadjusted Prevalence Ratio (95% CI)	Adjusted Prevalence Ratio (95% CI)
Cortisol ng/mL	1.005 (1.003-1.007) *	1.021 (1.008-1.034) **
Luteinizing Hormone (mIU/ml)	1.044 (1.023- 1.065)**	0.796 (0.650-0.97) *
Testosterone (ng/ml)	0.78(0.938-0.967)**	0.478 (0.380-0.602) **
LH*Testosterone		0.970 (0.590-0.990) #
Adrenaline(ng/L)	1.001 (0.999-1.003)	
Superoxide Dismutase (ng/L)	0.996(0.992-0.999) *	
Glutathione peroxidase (U/L)	0.999 (0.998-1.000) *	
Median (IQR)		
SHBG (nmol/L)	0.952 (0.938-0.967) *	
Age (in years)	0.964 (0.944-0.985) *	
BMI (in kg/m <sup>2</sup> )	1.059 (1.022-1.096) *	
Normal weight (18- 22.9) (ref)	1	
Over weight (23- 24.9)	1.419 (1.053-1.914) *	
Obese (≥ 25)	1.28 ( 0.848-1.920)	-
Body fat (in %)	1.028 (1.000-1.057) *	
Normal weight (12-22)	1	
Over weight (22.1-27)	1.400 (0.433-4.530)	
Obese (> 27)	1.527 (0.488-4.776)	-
Smoking Status		
Non smoker	1	
Smoker	1.324(0.978-1.792)*	-
Total Count (10 6 in ml) Mean ± SD	0.997 (0.995-0.999)*	
Motility Median (IQR)	0.992(0.987-0.997)*	
Morphology Mean ± SD	0.960 (0.952-1. 024)*	-
Rapid Linear Progression Median (IQR)	0.987(0.952-1.024)*	
FSH (mIU/ml) Mean ± SD	1.060 (0.969-1.161)*	-

\*Significant at p value < 0.25 by cox proportional hazard algorithm, # Significant at p value < 0.05 by cox proportional hazard algorithm after adjusting, CI: Confidence interval; BMI: Body mass index; SHBG: Sex hormone-binding globulin; IQR: Interquartile range; FSH: Follicle stimulating hormone.

( $r = -0.173$ ,  $p = 0.001$ ), and a significant weak positive correlation between GPX and testosterone ( $r = 0.139$ ,  $p < 0.007$ ) (Table 1).

Univariate analysis showed that with every 1 year increase in age, the prevalence of infertility decreased by 4% ( $p = 0.001$ ). However, with every 1 unit increase in BMI and body fat (%) the prevalence of infertility was increased by 5.9% ( $p = 0.001$ ) and 2.8% ( $p = 0.054$ ) respectively.

With every 1 unit increase in FSH and LH, the prevalence of infertility increased by 6% ( $p = 0.2$ ) and 4.4% ( $p < 0.001$ ) respectively. However, with every 1 unit increase in testosterone levels and SHBG, the prevalence of infertility decreased by 20% ( $p < 0.001$ ) and 4.8% ( $p < 0.001$ ) respectively.

Among the OS markers, with every 1 unit increase in cortisol, the prevalence of infertility increased by 0.5% ( $p = 0.001$ ), but with every 1 unit increase in SOD and GPX,

the prevalence of infertility decreased by 0.4% ( $p=0.008$ ) and 0.1% ( $p=0.003$ ) respectively. The effect of adrenaline level was not significant ( $p<0.25$ ).

In the final Cox regression algorithm, after adjusting for covariates LH and testosterone, with every increase of 7 units in cortisol, the prevalence of infertility increased by 2.1% ( $p=0.003$ ). There was significant interaction between LH and testosterone in the final model (Table 2). Among patients with testosterone levels 0, the prevalence of infertility for LH=4.4 was 20% lower compared to patients with LH=0. However, among patients with testosterone=2.17, the prevalence of infertility for LH=4.4 was 3% lower compared to LH=0 indicating that LH interacted with testosterone in its effect on infertility.

## Discussion

The increased prevalence of infertility with exacerbation of psychological and economic burden of developing countries has motivated researchers to look for the relationship of infertility with various causative factors.<sup>19</sup> OS is a potential contributor to the aetiology of male infertility due to the impairment of both the structural and functional integrity of spermatozoa.<sup>12</sup> The current study, conducted to compare the stress markers and antioxidants in male fertile and infertile subjects, showed that cortisol and adrenaline levels were significantly higher in the infertile group compared to the fertile group. These results are consistent with literature.<sup>9</sup> It has also been reported that persistent stressful conditions may lead to sustained increase in cortisol levels and if at the same time reduced GPX activity is also present, then this fails to counterbalance the disturbed microenvironment.<sup>12</sup> Both physical and emotional stress cause activation of the neurons that secrete corticotropin-releasing hormone and ultimately result in higher plasma cortisol levels.<sup>18</sup> Excess cortisol in turn diminishes the production of testosterone, thereby decreasing sperm volume and sperm parameters.<sup>21</sup> Cortisol can also directly reduce testosterone production by blocking the transcription of genes that code for enzymes essential for testosterone synthesis.<sup>21</sup> The significant finding of increased adrenaline levels in the infertile group is due to the decreased production of testosterone that causes infertility.<sup>10</sup>

The current study also showed that antioxidant GPX was significantly lower in infertile males. OS results from

disequilibrium between the pro-oxidants and the antioxidant defence system in the body. The antioxidant defence system comprises scavenger system formed by enzymes such as GPX and SOD which protect the structure and function of the sperm from oxidative damage.<sup>22</sup> GPX has a role in preserving the integrity of the sperm membrane that is rich in polyunsaturated fats, thereby preserving sperm motility from lipid peroxidation caused by ROS.<sup>23</sup>

However, SOD was found to be significantly higher in the infertile group. This is contrary to the findings of literature.<sup>24</sup> This might be due to increased levels of SOD in the local population and due to the low number of fertile patients. SOD is another important part of the antioxidant scavenger system which has an established role in protecting the polyunsaturated fatty acid of sperm membrane from OS and DNA fragmentation.<sup>12</sup> ROS can cause germ cell apoptosis, thereby leading to decreased sperm count. As such, by combating ROS oxidative damage, SOD preserves sperm count, volume and motility, thereby serving as a positive indicator of male fertility.<sup>24</sup>

The current study showed significantly high adrenaline levels and low testosterone in infertile males. This negative association between adrenaline and testosterone has also been reported by other studies.<sup>25,26</sup> This is because of the inhibitory effect of adrenaline and dopamine on testosterone production, both central through HPA, and peripheral by blocking testosterone release.<sup>10</sup>

The current study has shown significant increase in FSH, LH and decrease in SHBG levels among infertile males compared to the fertile ones. These results have also been reported by other studies.<sup>27</sup> It is due to involvement of SHBG in the regulation of Sertoli cell function and spermatogenesis.

The hypothesis of defective scavenging systems as a causative factor for male infertility needs to be further explored by estimation of TAC in seminal fluid in a diverse population of infertile subjects. By identifying the cause, targeted therapy may be helpful in treating male infertility.

In terms of limitations, the current study did not take seminal plasma even though semen analysis is essential to diagnose infertility and basic seminal parameters are required to compare fertile and infertile subjects.

## Conclusion

Increased stress hormones and decreased antioxidant

enzymes were observed in infertile groups. Stress, as indicated by increase in cortisol and adrenaline hormone secretion, together with decrease in antioxidants, was found to play a significant role in reducing the fertilising potential of male infertile subjects.

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**Conflict of Interest:** None.

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