Corticosterone-Induced Oxidative Stress Alters Epididymal Sperm Fertility in Rats

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Elevated glucocorticoid levels during stressed conditions have been demonstrated to impair reproductive function in rats. In our previous study investigating the dose-related effects of corticosterone (CORT) on the fertilising capacity of epididymal sperm in surgically-manipulated rats, we found that 25 mg/kg/day of CORT given subcutaneously for seven consecutive days significantly decreased the number of implantation sites and increased intrauterine embryonic loss compared to controls. Based on these findings, the current study aims to elucidate the possible mechanisms of action of CORT-induced stress on impaired sperm fertility in rats. Results of the present study showed that compared to controls, 25 mg/kg/day of CORT given subcutaneously for 7 consecutive days significantly increased the level of plasma malondialdehyde (MDA) with corresponding attenuated levels of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities. Plasma adrenocorticotropin (ACTH) and testosterone levels were also found to be decreased in CORT-treated rats. These findings suggest that CORT-induced oxidative stress and exert an inhibitory effect at the hypothalamic-pituitary-gonadal (HPG) axis, as evidenced by increased lipid peroxidation, reduced enzymatic antioxidant activities, and decreased testosterone production. These subsequently result in decreased fertilising capacity of epididymal sperm leading to poor pregnancy outcomes.

Key words: Corticosterone; oxidative stress; HPG axis activity; blastocyst implantation; fetal outcome

Activation of the hypothalamic-pituitary-adrenal (HPA) axis releases glucocorticoids, which play an important role in the homeostatic control of the body and the organism's response to stress (Lin *et al.* 2004). Corticosterone (CORT) is the predominant circulating glucocorticoid in rodents and its excess levels in the circulation is a major indicator of stress (Lerman *et al*. 1997, Hardy *et al*. 2005). Administration of CORT in rats has been shown to impair the nervous system (Nitta, *et al*. 1997, Gregus *et al*. 2005, Zafir & Banu 2009) and reproductive function (Lerman *et al*. 1997, Gao *et al*. 2003, Nasibah *et al*. 2012). Studies show that high levels of exogenous CORT are capable of inducing oxidative stress in mammals, which is reflected by an increase in lipid peroxidation (Lin *et al*. 2004, Zafir & Banu 2009), decreased enzymatic antioxidant activities (Zafir & Banu 2009), impaired steroidogenesis (Damsson *et al*. 2009, Dhanabalan *et al.* 2013) and increased apoptosis in the Leydig cells of the rat (Gao *et al*. 2003, Hardy *et al*. 2005).

Reactive oxygen species (ROS) plays a crucial role in the physiological processes of the reproductive system in both males and females. Physiological levels of ROS facilitate spermatogenesis, capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion (Aitken *et al*. 2004, Kothari *et al*. 2010) in the male, as well as aids in oocyte maturation and regression of corpus luteum in the female (Nenkova & Alexandrova 2013). The production of ROS is kept in check by the presence of antioxidants, which quench the activity of these free radicals. However, increased generation of ROS overwhelms the body's antioxidant defence mechanism, creating a condition called oxidative stress (Sharma & Agarwal 1996, Nenkova & Alexandrova 2013). Oxidative stress (OS) arises from an imbalance between the production of ROS and the body's antioxidant defence mechanisms (Makker *et al.* 2009). This could be due either to excessive ROS production or inadequate scavenging capacity of the antioxidants (Lanzafame *et al*. 2009; Agarwal & Sekhon 2010). OS causes damage to proteins, lipids, and nucleic acids resulting in lipid peroxidation, DNA damage and apoptosis (Makker *et al*. 2009). DNA damage in spermatozoa has shown a significant correlation with apoptosis in patients with male factor infertility (Wang *et al*. 2003) which may

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affect embryo development (Sakkas *et al*. 1999), and in severe cases, can lead to embryo death, miscarriage and congenital malformations (Agarwal *et al*. 2006).

There are enzymatic and non-enzymatic antioxidant systems which act as free radical scavengers. The three main enzymatic antioxidants in seminal plasma are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Nenkova & Alexandrova 2013). SOD and CAT eliminate superoxide anion and peroxide radicals by converting them into oxygen and water. GPx consists of a family of antioxidants that are involved in the reduction of hydroperoxides using glutathione as an electron donor. Reduction in the enzymatic antioxidant levels has been linked with male factor infertility (Tremellen 2008). Examples of non-enzymatic antioxidants include vitamin C, vitamin E, glutathione and albumin (Agarwal *et al*. 2004).

In an earlier study, we had showed that exogenous CORT of 25mg/kg/day given subcutaneously for seven consecutive days attenuated the fertilising capacity of surgically-manipulated epididymal sperm in rats. The fetal outcomes of the pregnant rats mated with these rats were found to be severely affected. The number of blastocyst implantation and live foetuses at parturition were significantly reduced along with increased post-implantation fetal loss (Abd-Aziz *et al*. 2012). In this follow up study, we aim to determine the possible mechanisms involved in the reduction of sperm fertility in rats, by studying the response of the antioxidant enzymes activities and the level of lipid peroxidation marker following exogenous CORT administration.

MATERIALS AND METHOD

Treatment of Animals

Thirty-four male Sprague Dawley rats (220-260 g) with proven fertility and 4-day cyclic females (180-220 g) were used for experimentation. The animals were maintained under standard laboratory conditions at 27ºC with a 12-h light/dark cycle and had free access to food and drinking water. The animal care and handling practices employed throughout the study were approved by the Animal Care and Use Committee (ACUC) of the Faculty of Medicine, Universiti Teknologi MARA, Malaysia. The experimental males were divided into 4 groups. Under anaesthesia, the epididymides were surgically separated at the testis-caput junction, leaving the blood vasculature supplying the testis and epididymis intact (Chatterjee 1976). Twenty-four hours after surgical manipulation, these animals were subjected to subcutaneous injection of either corn oil (MP Biomedicals, USA) (Group 1, control) or CORT (Sigma-Aldrich Inc., USA) at doses of 5, 10, and 25 mg/kg/day, respectively for seven consecutive days (Groups 2, 3 and 4).

Pregnancy Outcomes

Following seven consecutive days of treatment, each male rat was cohabitated overnight with a cyclic proestrus female on day 8 post-surgery. The following morning (day 9 post-surgery), a sperm-positive vaginal smear confirmed the day 1 of pregnancy (post-coital, p.c.). On day 8 p.c., laparotomy was performed to count the number of implantation sites along bilateral horns of the uterus, following which the incision site was immediately sutured and pregnancy was continued until term. During parturition, the number of pups delivered were counted and compared with the number of implantation sites of the same animal. Fetal loss was determined by inclusion of the following: presence of resorption sites during laparotomy on day 8 p.c., discrepancies in litter size when compared with the number of blastocyst implantations during laparotomy on day 8 p.c., and the number of dead foetus delivered during parturition.

Sample Collection

Once pregnancy was confirmed, the experimental males were anaesthetised and cardiac puncture was performed to collect blood samples. The blood samples were centrifuged (3000 rpm for 15 minutes at 4ºC), and the plasma layer was pipetted off and frozen at -80ºC until analysed.

Protein Assay

Protein concentration was estimated using the Protein Determination Kit (Cayman Chemical Company, USA), which is based on the well-known Bradford's method (Bradford 1976, Erejuwa *et al.* 2010). The absorbance was measured at 595 nm and the concentration was expressed as mg/ml.

Lipid Peroxidation Assay

The level of lipid peroxidation was determined as the concentration of thiobarbituric acid reactive substances (TBARS) using an assay kit (Cayman Chemical Company, USA) according to manufacturer's instructions. The amount of malondialdehyde (MDA) formed was measured colorimetrically at 532 nm and the concentration was expressed as nmol/mg protein.

Superoxide Dismutase (SOD) Assay

Superoxide dismutase activity was measured using an assay kit (Cayman Chemical Company, USA) according to manufacturer's instructions (Erejuwa *et al.* 2010). The SOD assay measured all three types of SOD

(Cu/ZnSOD, MnSOD, and FeSOD). The kit utilises a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The absorbance was read at 450 nm and the activity was expressed as U/mg protein.

Glutathione Peroxidase (GPx) Assay

Glutathione peroxidase activity was measured using an assay kit (Cayman Chemical Company, USA) according to manufacturer's instructions (Erejuwa *et al.* 2010). The assay kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). The oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. One unit of GPx is defined as the amount of enzyme that causes the oxidation of 1.0 nmol of NADPH to NADP+ per minute at 25ºC. The absorbance was read once every minute at 340 nm to obtain 5 time points. The activity was expressed as U/mg protein.

Catalase (CAT) Assay

Catalase activity was measured using an assay kit (Cayman Chemical Company, USA), according to manufacturer's instructions. The assay utilises the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide (H_2O_2). One unit of catalase is defined as the amount of enzyme that causes the formation of 1.0 nmol of formaldehyde per minute at 25ºC. The absorbance was read at 540 nm and the activity was expressed as nmol/mg protein.

Adrenocorticotropin (ACTH) and Testosterone Assays

ACTH and testosterone levels were analysed by using electrochemiluminescence immunoassay (ECLIA) on an automated Elecsys 2010 Cobas analyser (Roche Diagnostics, USA), and the concentrations were expressed as pmol/L and nmol/L, respectively.

Corticosterone (CORT) Assay

Corticosterone level was analysed using assay kit (IBL, USA), according to the manufacturer's instructions. Absorbance was read at 450 nm and the concentration was expressed as nmol/L.

Statistical Analysis

Data were analysed using the statistical package for social sciences (SPSS) program version 19.0. All variables were expressed as mean ± standard error of the mean (SEM) and assessed by one-way ANOVA analysis, followed by the Bonferroni test (post-hoc analysis) for differences between control and treatment groups. P values of <0.05 were considered as statistically significant.

RESULTS

Figures 1 and 2 show plasma concentration of MDA (Figure 1a), activities of the antioxidant enzymes (Figure 1b-d), ACTH (Figure 2a), testosterone (Figure 2b) and CORT (Figure 2c). When compared to controls, CORT of 25 mg/kg/day for 7 consecutive days showed a significant increase in plasma MDA level (539.32 ± 58.67 nmol/mg vs 814.66 ± 45.16 nmol/mg) with corresponding decreases in SOD activity (119.64 ± 6.3 vs 77.49 ± 4.06 U/mg) and GPx activity (2125.29 ± 74.60 vs 1595.37 ± 56.16 U/mg). Similarly, ACTH levels were decreased (22.91 ± 2.27 vs 11.58 ± 1.01 pmol/L), along with a subsequent decline in testosterone level (22.47 ± 2.49 vs 9.33 ± 1.51) as compared to that of controls. Plasma CORT levels were found to be increased in a dose-dependent manner and its concentration in the group treated with 25 mg/kg body weight was significantly higher as compared to that of controls (252.69 ± 4.70 vs 283.60 ± 2.93).

DISCUSSION

Our study aimed to determine the possible mechanisms involved in the reduction of fertility of surgically-manipulated epididymal sperm in rats following exogenous CORT administration. This was done by studying antioxidant enzymes activities and levels of the lipid peroxidation marker, MDA in the treated rats. As maturation of spermatozoa takes place along the length of the epididymis, surgical separation of both epididymides from the testes at the testis-caput junction confines the fully-formed and mature sperm within the detached epididymis (Chatterjee 1976). Following surgical separation of epididymides and testes, the males are able to maintain intact sexual activity to have two successive matings within a span of three weeks (Chatterjee 1976, Chatterjee *et al*. 1977).

In our previous study, we used different doses of CORT and showed that exogenous CORT of 25 mg/kg/day given subcutaneously for seven consecutive days attenuated the fertilising capacity of surgically-manipulated epididymal sperm in rats. The fetal outcomes of the pregnant rats mated with these rats were found to be severely reduced as evidenced by the decreased number of blastocyst
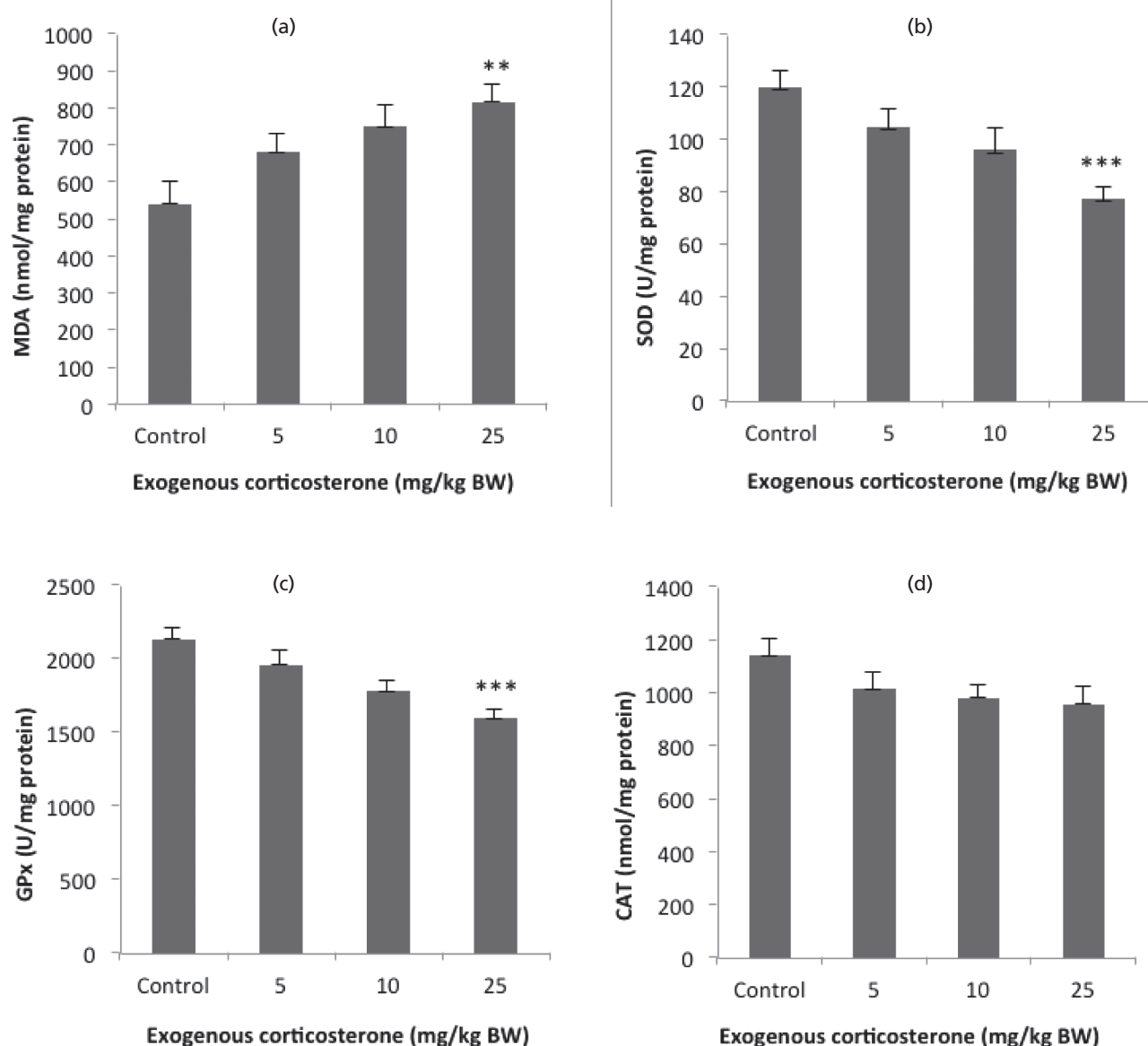
*Figure 1. Effects of exogenous corticosterone on (a) concentration of malondialdehyde (MDA),
(b) activity of superoxide dismutase (SOD), (c) activity of glutathione peroxidase (GPx) and
(d) activity of catalase (CAT) in plasma of control and corticosterone-treated rats.*

Note: Each group consisted of seven to ten animals. Data are expressed as mean ± SEM. Values are statistically significant
at **$p<0.01$, ***$p<0.001$ compared with control group.

implantation and litter size together with increased post-implantation loss (Abd-Aziz *et al*. 2012). In this follow up study, our goal was to determine the possible mechanisms involved in the reduction of sperm viability in rats following exogenous administration of CORT.

The present study revealed an increase in plasma MDA concentration with a corresponding decrease in enzymatic antioxidant (SOD, GPx and CAT) activities following administration of a high dose of CORT (25 mg/kg BW). Similar findings were also reported by other studies using different stress modalities, namely chronic restraint stress (Dhanabalan *et al*. 2010), restraint stress and forced swimming stress (Nirupama *et al*. 2013), as well as chronic CORT administration in broiler chickens (Lin *et al.* 2004) and in male rats (Zafir & Banu 2009, Dhanabalan *et al*. 2013). These studies ad collectively reported of increased plasma CORT and MDA levels with a corresponding decrease in antioxidant enzymes activities in the treated group.

MDA is a stable by-product of lipid peroxidation (Agarwal & Prabakaran 2005), which is a well-known mechanism of cellular injury in animals. It has been
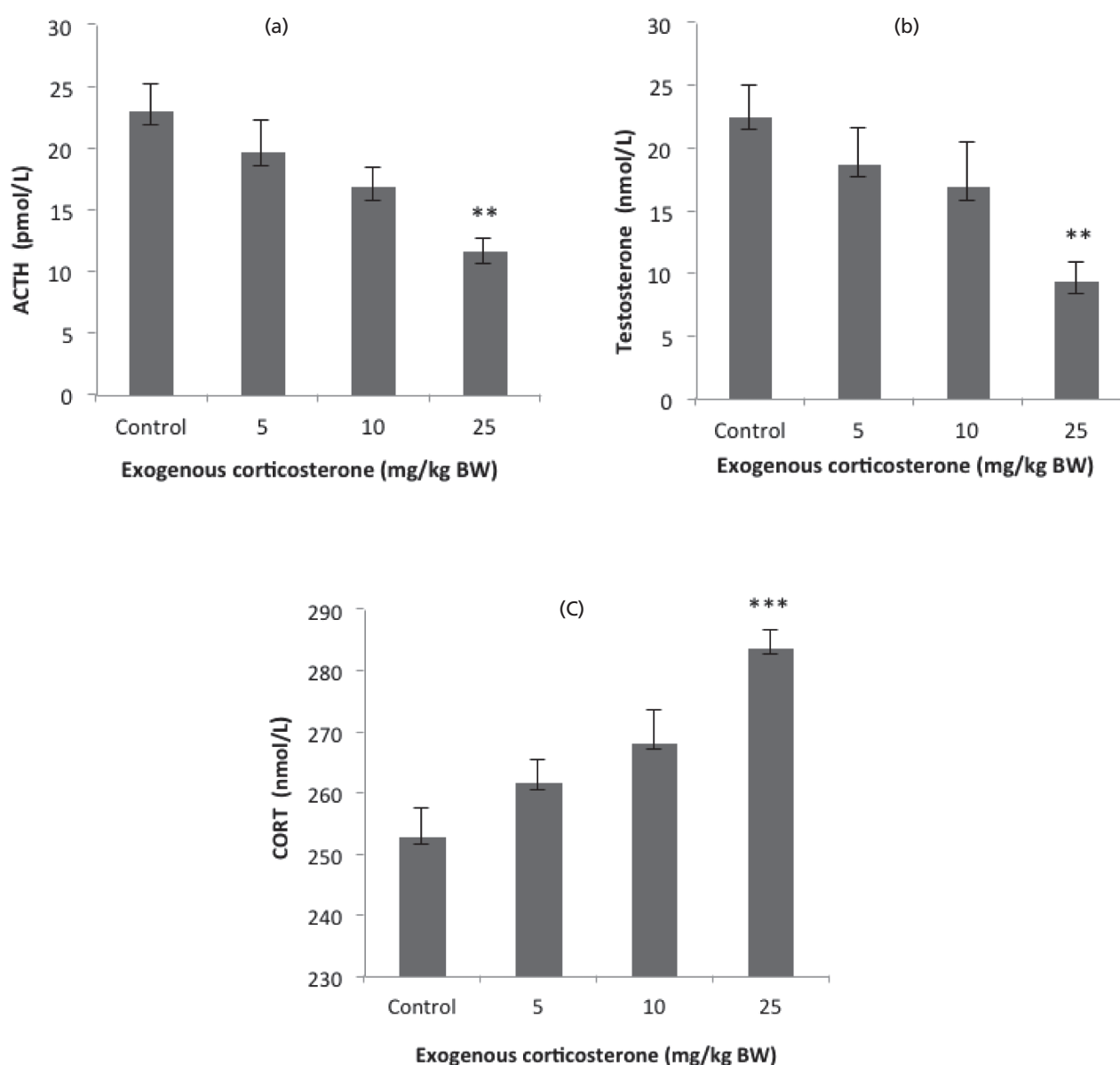
Figure 2. Effects of exogenous corticosterone on (a) level of adrenocorticotropin (ACTH), (b) level of testosterone, and (c) level of corticosterone in plasma of control and corticosterone-treated rats.
Each group consisted of seven to ten animals. Data are expressed as mean ± SEM.

Note: Values are statistically significant at **$p<0.01$, ***$p<0.001$ compared with control group.

widely used to quantify lipid peroxidation damage, and is measured using the spectrophotometric TBARS test. Levels of the lipid peroxidation marker, MDA has been shown to be increased in patients with abnormal semen parameters (Khosrowbeygi & Zarghami 2007).

SOD, GPx and CAT are the main enzymes that act as oxidant scavengers in the seminal plasma (Tremellen 2008, Nenkova & Alexandrova 2013). SOD acts by catalysing the conversion of superoxide to form oxygen and hydrogen peroxide, while CAT degrades hydrogen peroxide to oxygen and water (Tremellen 2008). GPx on the other hand,

scavenges hydrogen peroxide along with glutathione, and becomes oxidised and reduced by glutathione reductase (Kefer *et al*. 2009).

Increased plasma MDA level in the present study indicates an increase in lipid peroxidation, which is suggestive of oxidative stress. At the same time, the enzymatic antioxidants (SOD, GPx and CAT) activities were found to be reduced in our study. The decline in endogenous antioxidant status with corresponding increase in MDA level (an oxidative stress biomarker) in rats treated with 25 mg/kg BW CORT are a reflection of insufficient

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antioxidant defence mechanisms. The resultant imbalance of high pro-oxidant–low antioxidant levels lead to a state of oxidative stress.

In the present study, plasma corticosterone level increased in a dose-dependent manner whereas adrenocorticotropin (ACTH) and testosterone levels were found to be significantly decreased in the male rats treated with 25 mg/kg BW CORT compared to controls. The decrease in ACTH level measured in the treated rats was probably due to negative feedback on the HPA axis. Increased level of CORT inhibits the anterior pituitary from secreting ACTH, thus reducing ACTH levels in the circulation.

High levels of glucocorticoids decrease testosterone levels in rats under various stressful conditions (Gao et al. 2002, Dhanabalan et al. 2010). Stress-induced activation of hypothalamic-pituitary-adrenal (HPA) axis may affect reproductive functions at all three levels of the hypothalamic-pituitary-testicular axis (Rivier & Rivest 1991, Tsigos & Chrousos 2002). These include suppression of gonadotropin-releasing hormone (GnRH) secretion by the hypothalamus and interference of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release by the anterior pituitary, which consequently will lower testosterone production (Rivier & Rivest 1991).

Previous studies have reported that CORT administration decrease the number of Leydig cells in the interstitium and initiate apoptosis in Leydig cells, leading to a decline in testosterone secretion (Gao et al. 2002, Hardy et al. 2005). Leydig cells are the primary source of testosterone in males and the levels of testosterone in circulation are determined by the steroidogenic capacity of Leydig cells. Stress-induced increase in serum glucocorticoids decrease the rate of testosterone secretion by inhibiting testosterone-biosynthetic activity (Hardy et al. 2005), which is evident by the reduced activities of testicular steroidogenic enzymes 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase (Dhanabalan et al. 2010, Nirupama et al. 2013).

In general, CORT-induced impairment of testosterone secretion could occur either directly at the testicular level or indirectly via suppression of the HPG axis. At the testis, excess CORT induces Leydig cell apoptosis causing a decline in Leydig cell numbers. This reduction contributes to inhibition of steroidogenic enzyme activity and lowers the production of testosterone at Leydig cells. At the HPG axis, high levels of CORT causes inhibition at the levels of hypothalamus, pituitary and testis causing a reduction in GnRH, LH, FSH which consequently will lower testosterone production. Decreased testosterone levels have been shown to reduce testicular sperm production and epididymal sperm motility (Almeida et al. 2000, Dhanabalan et al. 2010). As testosterone is essential for normal spermatogenesis to occur, reduction in testosterone levels would impair spermatogenesis and sperm quality.

In conclusion, excess CORT suppresses surgically-manipulated epididymal sperm fertility through the induction of oxidative stress with a corresponding inhibition of HPG axis. These subsequently result in poor pregnancy outcomes in females mated with the stressed rats. Therefore, optimal maintenance of physiological scavenging of free radicals together with appropriate levels of sex steroids are crucial for sustainable pregnancy and better quality of reproductive health.

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