Introduction

Glucocorticoids are multitasking molecules (Julia, 2006) influencing almost all physiological functions including reproduction (Sapolsky et al., 2000). Dexamethasone (Dex), a commercially available synthetic glucocorticoid acts primarily as a potent anti-inflammatory (Barnes, 1998) and immunosuppressive (Franchimont, 2004) drug in clinical conditions namely chronic asthma (Barnes, 1998), rheumatoid arthritis (Laan et al., 1999; Kirwan, 1995) auto-immune diseases (Chatham and Kimberly, 2001) and prevention of graft rejection (Almawi et al., 1998). Exposure to glucocorticoid leads to varieties of unwanted physiological consequences including induction of oxidative stress by enhanced reactive oxygen species (ROS) production in many tissues and organs including vascular endothelial cells (Iuchi et al., 2003) and neural stem cells (Mutsaers and Tofighi, 2012). Although the cellular and molecular mechanisms involved in Dex induced oxidative injury in testis is not fully understood. Administration of glucocorticoid induces apoptosis in testicular germ cells (Yazawa et al., 2000) and Leydig cells (Gao et al., 2002) in a dose-dependent manner (Orazizadeh et al., 2010) and is mediated through glucocorticoid receptor (Yazawa et al., 2000). Glucocorticoids also known to inhibit Leydig cell steroidogenesis by influencing hypothalamo-hypophysial-gonadal (HPG) axis (Dong et al., 2004; Hardy et al., 2005) as well as by enhanced Leydig cell apoptosis (Gao et al., 2002). Thus, Dex induced germ cell apoptosis was regarded to be solely because of androgen deficiency (Woolveridge et al., 1999). Recent findings suggest that exogenous Dex administration causes germ cell apoptosis in both androgen dependent and independent stages of spermatogenic cycle and thus Dex induced germ cell apoptosis is not absolutely due to hormonal imbalance (Orazizadeh et al., 2010).

The golden hamster (Mesocricetus auratus) is a long-day seasonal breeder and, consequently, being studied for investigating seasonal regulation of reproductive functions employing experimental manipulation of circulatory level of melatonin (Bartke, 1985). Recent studies suggest golden hamster as a convenient model for investigating testicular toxicity (Ma et al., 2008; Imai and
Kitahashi, 2014; Kanimozhi et al., 2014). Melatonin is known to modulate reproductive behaviour in golden hamster by influencing the hypothalamo-hypophysial-gonadal (HPG) axis at multiple levels (Roy and Belsham, 2002; Balik et al., 2009). Further, the direct action of melatonin on hamster testes has been well documented (Frungieri et al., 2005). Melatonin, a chronobiotic neurohormone, primarily secreted from the pineal gland. Experimental studies suggest that melatonin is more potent than any known classical antioxidants for protection against oxidative and nitrosative stress-induced damage (Baydas et al., 2002b). It acts both as a direct free radical scavenger and indirect stimulator of antioxidant enzymes (Rodriguez et al., 2004; Galano et al., 2011). Being an amphiphilic molecule, melatonin can cross the blood–testes barrier to protect the germinal epithelium oxidative and nitrosative stress induced damages (Aitken and Roman, 2008). Further, the metabolites of melatonin have also been shown to scavenge free-radicals and thereby act as antioxidants (Galano et al., 2013).

Thus, we hypothesized that, whether increased ROS generation in testis could become a good companion of androgen deprivation culminating into germ cell apoptosis and whether melatonin could be a potent candidate in prevention of Dex induced germ cell apoptosis and testicular toxicity in male golden hamster, *M. auratus*. For this purpose, the activities of antioxidant enzymes; SOD, CAT and GSH-PX as well as MDA level as a marker of lipid peroxidation were assayed as indices of testicular oxidative damage. We have also checked the morphological analysis of apoptotic germ cells and the assessment of germ cell apoptotic markers Bax/Bcl-2 ratio and cleaved Caspase-3 expression. We have further extended our study to check the plasma level of melatonin and its high-affinity membrane receptor, MT1 in testis which has been reported to alter with progression of diseased condition (Zhang et al., 2013).

**Experimental plan**

Golden hamsters were procured from Central Drug Research Institute (CDRI), Lucknow, India and colony was developed and maintained in departmental animal house facility. Hamsters were kept under constant
temperature (25±2 °C) and light/dark cycle (Critical photoperiod; 12.5 h light, 11.5 h dark; i.e. lights on 07:00–19:30 h). Animals were maintained in polypropylene cages of equal sizes and provided with commercial rodent pellet and tap-water *ad libitum*. Adult male golden hamsters (average weight 125 ±5 g, 90-100 days old) were randomly selected and divided into four experimental groups (n=5/group): Group I: Control (ethanolic normal saline treated); Group II: Melatonin treated (10 mg kg⁻¹ day⁻¹); Group III: Dex treated (7 mg kg⁻¹ day⁻¹) and Group IV: Melatonin+ Dex treated (10 mg melatonin kg⁻¹ day⁻¹ + 7 mg Dex kg⁻¹ day⁻¹).

Melatonin and Dex were first dissolved in few drops (100 μl) of absolute ethanol and then working concentration was prepared as desired from the stock by diluting in 0.9% normal saline and injected. Treatment with melatonin was started 24 h before the first Dex injection. Both melatonin and Dex was given daily by i.p. injection at 10:00 h for 7 consecutive days. The volume of melatonin and Dex injected daily was 0.1 ml. The doses of melatonin and Dex used in the present study were chosen on the basis of previous reports of Oksay et al., (2014) and Yazawa et al., (2000) respectively. Control hamsters received i.p. injections of normal saline solution at an equal volume to that of melatonin and Dex. No alterations in general health status of the animals were noticed throughout the experimental period.

**Tissue collection**

At 1 day after the final injection, hamsters were weighed and sacrificed under deep ether anaesthesia. The trunk blood was collected in heparinized tubes and centrifuged immediately at 3000 rpm for 20 min. Serum was kept at −20°C for hormonal estimations. Right and left testes were immediately removed, blotted dry and weighed. Testes were then either quickly fixed in 10% neutral formalin for histological analyses or kept at -80°C for biochemical estimations. Germ cells were isolated immediately and processed for morphological analysis of germ cells apoptosis or kept at -80°C for western blot analyses.
Histological analyses

The testes tissues were fixed in 10% neutral formalin for 48 h, dehydrated and embedded in paraffin. 5µm sections were stretched on slides pre-coated with 1% gelatine and stained with hematoxylin and eosin (H&E) and finally observed and photographed under research microscope (Nikon, E 200, Tokyo, Japan). The details of the procedure have been provided in material and methods section.

Measurement of antioxidant status

Testis were homogenised in ice cold PBS (50 mmol, pH 7.4) to obtain 10% homogenate which was centrifuged at 12,000×g for 30 min to obtain the supernatant and protein levels were estimated using BSA as the standard (Bradford, 1976). The supernatant was used for measurement of antioxidant status.

Estimation of superoxide dismutase (SOD) activity

Estimation of SOD activity was performed following spectrophotometric method using nitrite formation by superoxide radicals (Das et al., 1999). The details of the procedure have been provided in the material and methods section.

Estimation of catalase (CAT) activity

CAT activity was measured following an indirect spectrophotometric method of Sinha (1972). The details of the procedure have been provided in the material and methods section.

Estimation of glutathione peroxidase (GSH-Px) activity

Glutathione peroxidase (GSH-Px) activity was assayed following the method of Mantha et al., (1993). The details of the procedure have been provided in the material and methods section.

Lipid peroxidation (LPO) level determination

Lipid peroxidation was measured by estimation of TBARS level following the method of Ohkawa et al., (1978). The details of the procedure have been provided in the material and methods section.
Isolation of germ cells

Testicular germ cells were isolated following the method of Ikeda et al., (1999), with few modifications. The details of the procedure have been provided in the material and methods section.

Western blot analyses for Bax, Bcl-2, caspase-3 and MT1R

The aliquots containing 100µg of protein was resolved on 12% SDS-polyacrylamide gel (PAGE). Immunodetection was carried out by using specific antibodies against Bcl-2 (Bcl-2, N-19; sc-492, Santa Cruz Biotech, USA, diluted 1:500); Bax (Bax, N-20; sc-493, Santa Cruz Biotech, USA, diluted 1:250), Caspase-3 (Caspase-3, H-277; sc-7148, Santa Cruz Biotech, USA, diluted 1:500), MT1R (MT1R, R-18, sc- 13186, Lot # L1812, Santa Cruz Biotech, USA, dilution 1:200) and β-actin antibody (A-2228, Sigma-Aldrich Chemicals, St. Louis, USA, diluted 1:5000). Further, horseradish peroxidase conjugated secondary antibodies (donkey anti goat IgG, for MT1R; donkey anti rabbit IgG for Bcl-2, Bax abd Caspase-3, diluted 1:1000 and donkey anti mouse IgG for β-actin, diluted 1:10,000) were used. The details of the procedure have been provided in material and methods section.

Morphological analysis of apoptotic germ cells

Apoptotic observation of germ cells was performed following Acridine orange (AO)-Ethidium bromide (EtBr) double staining method as described by Goswami et al., (2013). The details of the procedure have been provided in the material and methods section.

Hormonal estimations

Plasma level of melatonin was determined by using a commercial melatonin ELISA kit (RE54021, IBL, Hamburg, Germany) as per manufacturer’s instruction. Details of the assay procedure have been provided in materials and methods section.

Statistical analyses

All statistical analyses were performed using SPSS version 17.0. Statistical analysis of the data was performed with one way ANOVA followed by multiple
comparisons by the Duncan’s multiple range tests. Values are expressed as means ± SEM. The differences were considered statistically significant when value of \( p \leq 0.05 \).

**Results**

**Body weight**

Weight analysis is among the initial observations to check systemic toxicity by any chemical or drug used. In the present study, melatonin treatment showed no significant variation in body weight and relative testes weight when compared with saline treated control whereas administration of Dex presented significantly \( (p<0.01) \) decreased body weight and relative testes weight when compared with control (Fig. 1). Melatonin treatment along with Dex showed significant \( (p<0.01) \) increase in body weight and relative testes weight when compared with Dex treated hamsters (Fig. 1).

**Histological changes in testis**

Histopathological assessment showed normal histoarchitecture of seminiferous tubule and seminiferous epithelium and exhibit active spermatogenesis in the control group (Fig. 2a, b). Melatonin treatment (alone) showed no significant alterations in testicular histology as evident from the normal histoarchitecture of the seminiferous epithelium (Fig. 2c, d). Dex administration revealed varying degrees of germ cell degenerative changes characterized by presence of degenerating cells, germinal epithelium disruption and loosening, detachment of germinal epithelium (exfoliation) and vacuolisation in seminiferous tubule (Fig. 3a, b, c & d). Melatonin treatment along with Dex decreased the degenerative changes in the seminiferous tubule (Fig. 3e, f).

**Changes in antioxidant status and lipid peroxidation**

Significant \( (p<0.01) \) increase in testicular SOD, CAT and GSH-PX enzyme activities were noted in melatonin treated hamsters, while significantly \( (p<0.01) \) decreased in Dex treated hamsters when compared with control animals.
Dex treatment in combination with melatonin significantly ($p<0.01$) increased SOD, CAT and GSH-PX enzyme activities when compared with Dex treated group (Fig. 4a, b). Consistent with the increase in antioxidant enzyme activities, we observed a significant ($p<0.01$) decrease in MDA level, the end product of lipid peroxidation, in the melatonin treated group while Dex significantly ($p<0.01$) increased MDA level when compared with control. Dex along with melatonin treatment significantly ($p<0.01$) decreased MDA level when compared with only Dex treated hamsters (Fig. 4a, b).

**Plasma melatonin level**

Melatonin treatment (alone) showed non-significant ($p>0.05$) increase in plasma level of melatonin as compared to control. While, a significant ($p<0.01$) reduction in plasma melatonin level was observed in Dex treated group, whereas, melatonin pre-treatment significantly ($p<0.01$) rescued its own level in Dex plus melatonin treated group as compared to Dex treated group (Fig. 5).

**Germ cell apoptotic index**

Significant ($p<0.01$) increase in germ cell apoptotic index was observed in Dex treated group when compared with control (Fig. 6a, b). Whereas a significant ($p<0.01$) decrease was observed in germ cell apoptotic index in animals treated with Dex in combination with melatonin when compared with Dex treated group.

**Western blot analysis**

Treatment with Dex leads to increased germ cell death as indicated by significantly ($p<0.01$) increased Bax/Bcl-2 ratio (Fig. 7a) and expression of executioner caspase, caspase-3 (Fig. 7b) as compared to control group. Melatonin treatment significantly ($p<0.01$) decreased Bax/Bcl-2 ratio and caspase-3 expression and thereby rescued germ cells from Dex induced apoptosis. A significant ($p<0.01$) decrease in MT1R expression was noted in Dex treated group whereas melatonin pre-treatment significantly ($p<0.01$) rescued its own receptor in testis (Fig. 7b).
Discussion

Immunosuppressive and anti-inflammatory properties of acute and pharmacological doses of glucocorticoid have long been recognised (Guilpain and Le, 2012). Previous studies from our lab highlights the immunoenhancing activity of melatonin on Dex induced immune suppression (Vishwas et al., 2013b; Gupta and Haldar, 2013) but, the ability of melatonin to prevent oxidative stress to testis induced by Dex has never been reported. The present study establishes the involvement of Dex in testicular oxidative stress that induced germ cell apoptosis and also the ameliorative potentials of melatonin through its antioxidative (Rodriguez et al., 2004; Galano et al., 2011) and anti-apoptotic (Ji et al., 2012) attributes.

In the present study, we observed a significant decrease in body weight of hamsters treated with Dex that might be due to short-term administration of high dose of glucocorticoid as suggested in previous reports performed on rats (Eason et al., 2000). The observed recovery of body weight following melatonin plus Dex administration might be due to increased carcass lipid content and white/ brown adipose tissue mass by melatonin (Bartness and Wade, 1985).

As compared to control significant decrease in relative testes weight in Dex treated group suggest suppression of testicular function following Dex administration. Histologically, testis showed varying degrees of degenerative changes in Dex treated animals. Similar changes have also been reported in animals treated either with toxic chemicals (Kasahara et al., 2002) or endocrine disrupters (Ma et al., 2008). Administration of melatonin along with Dex restored weight and normal histoarchitecture of the testes by preventing the degenerative changes of the seminiferous tubules induced by Dex.

Oxidative stress is a consequence of the disturbed steady-state relationship between production of ROS and the body’s antioxidant defence capacity. Under normal physiological conditions, ROS are continuously generated by active metabolic processes. Tissues have virtually developed defence mechanisms against ROS by having elaborate array of antioxidant enzymes and free radical
scavengers and testis is not an exception in this regard (Aitken and Roman, 2008). Series of studies pointed out oxidative stress as a major factor for male infertility (Makker et al., 2009; Choudhary et al., 2010) as ROS is capable of disrupting the androgen producing Leydig cells and may cause increased lipid peroxidation and DNA fragmentation in germ cells. Antioxidant enzymes provide the first line of defence against the deleterious effects of ROS (Rodriguez et al., 2004). SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide (H$_2$O$_2$) and molecular oxygen whereas catalase and GSH-PX is known to be responsible for H$_2$O$_2$ detoxification (Calvin et al., 1981; Valko et al., 2006). In the present investigation, significantly decreased activity of testicular SOD, CAT and GSH-PX was observed in the Dex treated animals which further lead to significantly increased lipid peroxidation in the testis. Lipid peroxidation is one of the predominant processes resulting from oxidative stress. Administration of melatonin along with Dex resulted in enhanced antioxidative enzyme activities thereby suppressing lipid peroxidation and thus rescued testes from Dex induced oxidative load.

An increased level of ROS is considered as a probable signal for apoptosis (Mathur et al., 2011) which can be blocked by wide range of antioxidants (Poljsak et al., 2013). In the current investigation, significantly decreased antioxidant enzyme activity and increased lipid peroxidation in the Dex treated hamsters showed positive correlation with the increased germ cell apoptosis as evident from the significantly increased expression of caspase-3 and Bax/Bcl-2 ratio. Oxidative stress induced apoptosis is considered as the combinational effect of multiple pathways leading to membrane lipid preoxidation, loss of Ca$^{2+}$ homeostasis, DNA fragmentation and through activation of caspases and transcription factors. Among the transcription factors NF-kB, having mutual antagonism with glucocorticoid, is regarded as an anti-apoptotic agent in glucocorticoid induced apoptosis (Mathur et al., 2011). Melatonin has been proposed to enhance nuclear translocation of NF-kB via inactivation of calmodulin-dependent kinases thus might be leading to up-regulation of antioxidative gene expression and suppression of ROS induced apoptosis (Tomas-Zapico and Coto-Montes., 2005).
A significant decrease in Bax/Bcl-2 ratio and expression of caspase-3 in the melatonin and melatonin plus Dex treated groups as compared to control and Dex treated group supports the anti-apoptotic action of melatonin in Dex induced germ cell apoptosis. Our results got strong support from previous studies suggesting the anti-apoptotic actions of melatonin in somatic (Jang et al., 2009; Tunon et al., 2011; Zhang et al., 2013) as well as germ cells (Ji et al., 2012). The molecular mechanisms underlying anti-apoptotic actions of melatonin involves regulation of pro- and anti-apoptotic proteins, release of mitochondrial cytochrome c and activation of caspase dependent cell death pathways (Juknat et al., 2005; Baydas et al., 2005; Zhang et al., 2013). Melatonin is known to down-regulate the expression of testicular HO-1 (Heme oxygenase 1), a stress inducible form of HO, and thereby prevent oxidative stress induced germ cell apoptosis (Ji et al., 2012). A significant \((p<0.01)\) decrease in the germ cell apoptotic index in hamsters treated with Dex plus melatonin as compared to Dex treated animals is in accordance with the earlier studies (Ji et al., 2012).

In the present study, a significant \((p<0.01)\) decrease in circulatory melatonin level and expression of MT1 receptor in Dex treated hamsters was recorded. However, administration of melatonin along with Dex restored plasma melatonin and MT1 receptor expression in testis. Alteration in plasma level of melatonin and expression of MT1 has been suggested in neuro-denerative diseases as well as during normal ageing process (Sanchez-Hidalgo et al., 2009). Our results are in accordance to the previous studies suggesting loss of melatonin and its receptor with increased apoptosis and their recovery with melatonin administration delays disease progression. Thus it can be proposed that beside androgen deficiency, oxidative stress plays crucial role in Dex induced testicular toxicity and germ cell apoptosis which can be blocked by administration of neurohormone melatonin.

In conclusion, the present study highlights oxidative stress as a potent mediator of Dex induced testicular toxicity and germ cell apoptosis in male golden hamster. Melatonin, by the virtue of its antioxidant and anti-apoptotic attributes prevents Dex induced testicular damage by up regulating antioxidant defence.
mechanisms and inhibiting germ cell apoptosis. The results of the present study corroborate earlier studies suggesting beneficial role of melatonin in testicular oxidative stress and germ cell apoptosis (Oner-Iyidigan et al., 2001; Mogulkoc et al., 2005; Ji et al., 2012). Therefore, from the clinical perspectives, combinatorial therapy of melatonin along with glucocorticoid may show improved results for the management of male reproductive health. Since, the mechanism of Dex induced germ cell apoptosis is not fully understood, the present communication proposes enhanced ROS to be an important factor, beside hormonal imbalance, in mediating Dex induced germ cell apoptosis. However, deeper insights into the apoptotic pathways operating therein are required for a broader elucidation of the mechanism(s) by which ROS augments the Dex induced testicular toxicity and germ cell apoptosis.