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Influence of glucocorticoid-induced stress on reproductive function in female *Mus terricolor* during reproductively active phase

Sweta Arora[1, 2], Poonam Singh[1] and Chandana Haldar[2]

[1]Mahila Mahavidyalaya, Banaras Hindu University, Varanasi – 221005, India

[2]Pineal Research Laboratory, Department of Zoology, Centre of Advanced Study, Banaras Hindu University, Varanasi – 221005, India

Summary

Inhibitory effects of glucocorticoids have been reported in a number of species ranging from rodents to humans. No report till date exists demonstrating the effect of stress on reproduction in *Mus terricolor*, a tropical, wild, nocturnal, short day breeder. To replicate stress-like situation under experimental condition, dexamethasone (60µg/ 100g body weight) treatment was given to female *M. terricolor* during the reproductively active phase (RAP) of its breeding cycle. Suppressive effect of dexamethasone on reproductive functions of this tropical nocturnal rodent was inferred. There was a significant reduction in ovarian and uterine weight, which was accompanied by a significant reduction in uterine protein and plasma estradiol and progesterone levels while there was a significant increase in ovarian cholesterol. Histological observations revealed an inhibitory effect of dexamethasone on the reproductive tissues. The presence of glucocorticoid receptor in the ovary and uterus suggests that glucocorticoid might be directly involved in modulating reproductive functions of this rodent. Thus, exogenous glucocorticoid suppressed the reproductive function of this mouse suggesting a stressful condition.

Key words: Glucocorticoid, *M. terricolor*, reproduction, stress, tropical rodent

Introduction

Wild rodents face a multitude of stressors in their natural environment. Some of these environmental stressors are unpredictable harsh weather, non-static habitats, food shortage, water unavailability, social pressure, risk of predators and parasites, etc. (Gupta and Haldar, 2012) which lead to homeostatic, physiological and behavioral deterioration in an animal. Adrenal glands are known to play a key role in modulation of any kind of stressful condition. A number of corticosteroids, viz., mineralocorticoids (aldosterone), gonadocorticoids (androgens) and glucocorticoids (cortisol) are secreted from adrenal cortex (Alkass, 2009). Glucocorticoids play an important role during stress. The first line of defense for coping with any stressor/adverse situation in an organism is an increase in glucocorticoid secretion, which would lead to activation of hypothalmo-pituitary-adrenal (HPA) axis and consequent inhibition of not only reproductive functions (Rivier and Rivest, 1991) but also immunity (Haldar et al., 2004; Gupta and Haldar, 2012) of the rodents, ruminants and primates including humans (Maeda and Tsukamura, 2006).

Dexamethasone is a commercially available synthetic glucocorticoid. Soliman and Walker (1977) reported that dexamethasone administration following pregnant mare serum gonadotropin (PMSG) treatment inhibited ovulation and luteinization in rats. Inhibition of ovulation (Smith et al., 1971) and sexual maturation (Ramaley and Schwartz, 1980) has been observed in rats following glucocorticoid treatment. Chronic corticosteroid treatment has been reported to suppress LH secretion in monkeys as well as rats (Smith et al., 1971; Baldwin, 1979; Dubey and Plant, 1985; Rosen et al., 1988). Hagino et al. (1969) showed that dexamethasone injections, when given into the preoptic area of the hypothalamus of rats, inhibited ovulation. Presence of corticosteroid receptors has been demonstrated in ovarian granulose cells of rats (Schreiber et al., 1982).

Hsueh and Erickson (1983) have reported that glucocorticoid treatment suppressed activity of granulosa cell aromatase enzyme which caused estrogen deficiency. Campbell (1978) found diminished tissue uptake of estrogen following dexamethasone treatment. Bigsby (1993) reported that dexamethasone inhibited estrogen-induced synthesis of DNA in rat uterine epithelium. Glucocorticoid has been reported to inhibit protein synthesis in rat uterus (Sullivian et al., 1983) and blood flow in uterus of sheep (Monheit and Resnik, 1981). Delayed parturition and still births are also reported in rats due to excess glucocorticoid (Chatterjee et al., 1993). Glucocorticoid receptors have been localized in ovarian cells which suggest direct action of glucocorticoids on the ovary (Schreiber et al., 1982; Sugino et al., 1997; Gaytan et al., 2002).

Correspondence to be addressed to: Professor C. Haldar, Email: chaldar2001@yahoo.com

The Indian pygmy field mouse, *Mus terricolor,* is a small tropical wild rodent and an important pest of rice and wheat fields, found throughout South East Asia (Aplin et al., 2003). *M. terricolor* makes burrows in the earthen dykes rose for holding water in the cultivated fields (Singh et al., 2009). It has also been shown that these mice can bear hypoxic stress to a great extent, and this trait has probably evolved during the process of species-specific adaptation to the oxygen-depleted habitat of its burrow (Singh et al., 2009). *M. terricolor* shows subtle variations in typical morphological traits such as body size, shape and belly fur color, tail length, etc., that are important for taxonomic identification of this species. It has an earth-colored body and a grey belly with slightly longer tail in proportion to the head and body length (Sharma, 1996; Aplin et al., 2003; Singh et al., 2009; Basu and Singaravel, 2012; Basu et al., 2012). Singh et al. (2009) described seasonal influence on its abundance, and the authors speculated seasonal reproduction, but did not give any evidence in support of this view. This nocturnal mammal presents two peaks of annual gonadal cycle, one from October to January, and a second brief phase in April that is dependent on food availability (Data under communication). The evolutionary history and cytogenetics (Sharma, 1996; Singh et al., 2009) and circadian sensitivity (Basu and Singaravel, 2012; Basu et al., 2012) of *M. terricolor* have been explored in depth.

To date there is no report with experimental evidences demonstrating the effect of stress on reproduction of this wild rodent. To replicate stress-like situation under experimental condition, dexamethasone treatment was given to *M. terricolor.* Dexamethasone is a synthetic glucocorticoid which is available commercially. This glucocorticoid is used largely in treatment of hematological disorders, leukemia, cerebral edema, allergic reactions, chronic hepatitis (Alkass, 2009), asthma, rheumatoid arthritis (Kirkham et al., 1991), transplanted organ rejections (Scherer et al., 2007), chronic pulmonary disease and malignant neoplasm (Alkass, 2009).

Therefore, the aim of the present study has been to decipher the effects of experimentally induced stress by administration of dexamethasone on the reproductive functions of female *M. terricolor* during the reproductively active phase of its annual breeding cycle i.e., in the month of December.

Materials and Methods

Maintenance of animals

All the experiments were conducted in accordance with the Institutional practice and within the framework of revised Animal (Specific Procedure) Act of 2007 of Government of India on animal welfare. Experiment was performed during reproductively active phase of the animal. The mice were collected from the fields in the vicinity of Varanasi (Lat. 25º, 18' N; Long. 83º, 1'E), India, following the methods as described earlier (Bardhan and Sharma, 2000; Singh et al., 2009; Basu and Singaravel, 2012; Basu et al., 2012). In the laboratory the animals were acclimated to the ambient conditions for two weeks.

Experimental groups

Healthy young adult non-pregnant female mice of weight 11±1.0 g were randomly selected from the collection and assigned to two groups of six each. The animals were kept in commercial polypropylene cages during the experiments and maintained in a well-ventilated room exposed to ambient conditions (27 ± 2ºC, with gentle ventilation). Mice were fed with commercial food pellets along with wheat, paddy/rice and water *ad libitum*.

The mice in the first group (n=6) were treated with normal ethanolic saline (0.9%) and served as control. The mice in the second second group (n=6) were given intra-muscular injection of dexamethasone (Dex) at a dose of 60µg/100g body wt/day (Haldar et al., 2004; Gupta and Haldar, 2012) for 15 days during the afternoon. Dexamethasone was first dissolved in a few drops of ethanol and then diluted with normal saline to the desired concentration.

Sample collection

Twenty four hours after the last injection, mice were weighed and sacrificed by total body anesthesia. Blood was collected directly from the heart in heparinized tubes. Plasma was separated and frozen at -80ºC till the ELISA for estradiol and progesterone (Biotron Diagnostics Inc., Hemet, California, USA) was done. Ovaries and uteri were dissected out on ice, blotted free from blood, cleared from extra tissue, and weighed on an electronic balance (Denver Instruments, Gottingen, Germany). Ovary and uterine horn of left side were fixed in Bouin's fluid for histological and immunohistochemical analysis while these organs on the right side were kept for biochemical estimations of cholesterol and protein, respectively.

Histology

After fixation in Bouin's fluid, ovary and uterus were processed for routine histological procedure. The sections were stained with Ehrlich's hematoxylin and eosin. The stained sections were observed in a research microscope (Leica MPV-3, Germany) and documented.

Biochemical estimations

Ovarian cholesterol was estimated using commercial kit, and the manufacturer's protocol (Bio Lab Diagnostics, India) was strictly followed. The reagents in the supply were added in three sets of test tubes i.e., blank, standard and test. 1 ml of cholesterol reagent was added to all the three sets. 20µl of distilled water, standard reagent and test samples, the homogenate of the tissue, were added to blank, standard and test, respectively. After thorough mixing, all tubes were incubated at 37°C for 10 minutes and read at 510nm in a UV-Vis Spectrophotometer (UV-200-RS, mrc, Israel).

The protein content of the uterus was quantified adopting the Bradford (1976) method.

Hormone analysis

ELISA kits for estradiol and progesterone were purchased from Biotron Diagnostics Inc. Hemet, California, USA, and the plasma levels of both hormones were measured according to manufacturer's instructions. The standard, control and samples, 25 µl each, were added in each well of ELISA plate, followed by 100 µl of enzyme conjugate solution. The wells were then incubated under mild shaking at room temperature for two hours. The wells were then aspirated and washed three times with wash solution. Then, 100 µl of the TMB chromogenic solution (substrate) was added to each well and the plate was incubated at room temperature for 30 min in dark. Finally, 100 µl of stop solution was added to each well and the absorbance was recorded at 450 nm. Intra- and inter-assay variations were less than 5% and 14%, respectively for estradiol and progesterone. The assay was carried out in triplicate.

Immunohistochemical localization of glucocorticoid receptor (GR)

For immunohistochemical localization of GR, a few 6 µm thick transverse sections (TS) of ovary and uterus were mounted on gelatin-(1%)-coated slides and deparaffinized. After rehydration, endogenous peroxide activity was blocked using 0.3% H_2O_2 in methanol for 30 min at room temperature (RT). Sections were washed thrice with phosphate buffered saline (PBS; 0.1 M NaH_2PO_4, Na_2HPO_4, NaCl; pH 7.4) and pre-incubated with horse blocking serum (1:100 in PBS; PK-6200, Vector laboratories, Burlinghame, CA) for 2 h. Then the sections were incubated with primary antibody (GR, M-20, sc 1004, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200) overnight at 4ºC. The sections were washed thrice with PBS and incubated with biotinylated secondary antibody (Vectastain ABC Universal kit; PK-6200, Vector Laboratories, Burlinghame, CA, dilution 1:50). The sections were then washed with PBS and a pre-formed ABC reagent was conjugated to the free biotin of the secondary antibody. The antigens were visualized using the 0.03% peroxidase substrate 3, 3-diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, USA) in 0.01 M Tris–Cl (pH 7.6) and 0.1% H_2O_2. The sections were dehydrated and mounted with DPX mountant and observed and photographed in Leitz-MPV-3 microscope (Germany). The negative controls were obtained by omitting the primary antibody and incubating the sections with goat serum (Ahmad and Haldar, 2010).

Statistical analysis

Statistical analysis of the data was performed with one-way ANOVA followed by Student Newman–Keul's multiple range tests. The differences were considered significant when $P \leq 0.05$.

Results

Body weight

No significant difference in body weight was observed between ethanolic saline-treated control mice and dexamethasone-treated ones (Fig. 1).

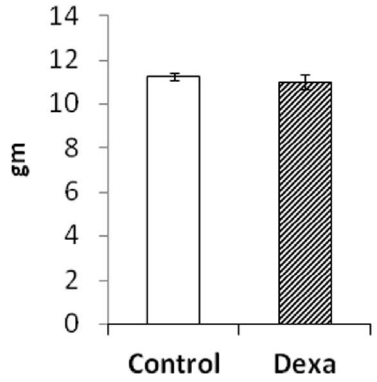

Fig. 1. Histogram representing effect of exogenous dexamethasone injection on body weight of *M. terricolor*. Values are expressed as mean ± SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

Weight of reproductive organs

Significant reduction was noticed in the relative weights of the ovary and uterus of dexamethasone-treated mice as compared with control (Figs. 2, 3) .

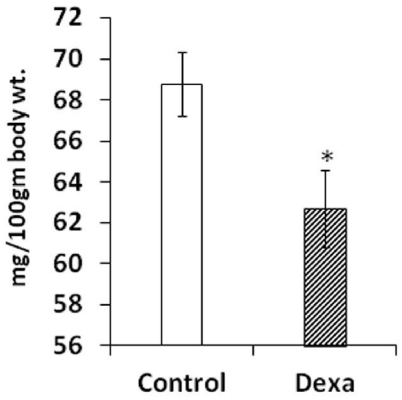

Fig. 2. Histogram representing effect of exogenous dexamethasone injection on relative ovary weight of *M. terricolor*. Values are expressed as mean ± SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

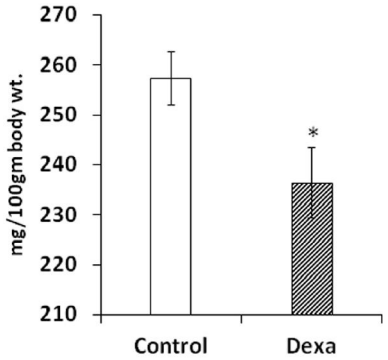

Fig. 3. Histogram representing effect of exogenous dexamethasone injection on relative uterus weight of *M. terricolor* .Values are expressed as mean ± SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

Biochemical estimations

A significant increase in the content of ovarian cholesterol and a significant decrease in the uterine protein were observed in dexamethasone-treated animals as compared with the control (Figs. 4, 5).

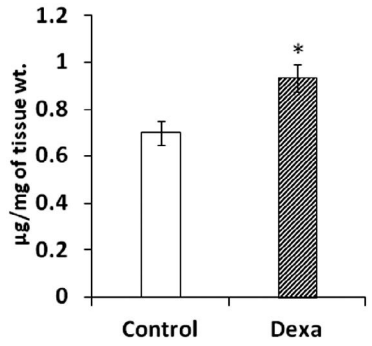

Fig. 4. Histogram representing effect of exogenous dexamethasone injection on cholesterol content in ovary of *M. terricolor*. Values are expressed as mean ± SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

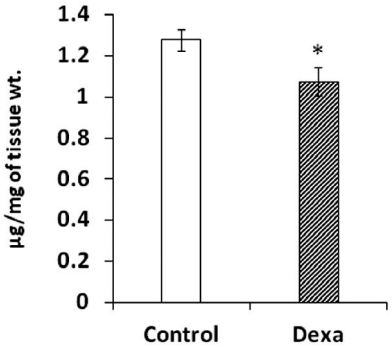

Fig. 5. Histogram representing effect of exogenous dexamethasone injection on protein content in uterus of *M. terricolor*. Values are expressed as mean ± SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

Hormonal analysis

Significantly decreased levels of peripheral plasma estradiol and progesterone were noticed in dexamethasone-treated group when compared with the control group (Figs. 6, 7).

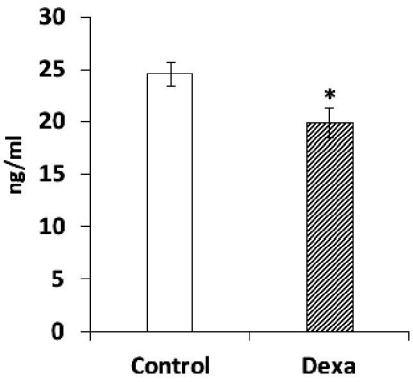

Fig. 6. Histogram representing effect of exogenous dexamethasone injection on plasma estradiol of *M. terricolor*. Values are expressed as mean ± SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

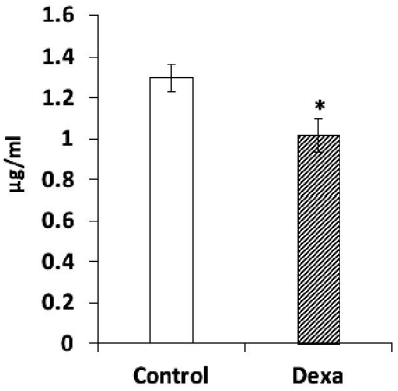

Fig. 7. Histogram representing effect of exogenous dexamethasone injection on plasma progesterone of *M.terricolor*. Values are expressed as mean ± SEM, N = 6. Significance of difference; *P < 0.05, **P < 0.01.

Histological observations

The uterus of vehicle-treated females had a well-developed endometrium, narrow lumen and prominent endometrial glands (Fig. 8) while the endometrium of uterus of dexamethasone-treated mice was regressed. The uterine lumen appeared wide and the endometrial glands did not show proliferation in the treated group (Fig. 9).

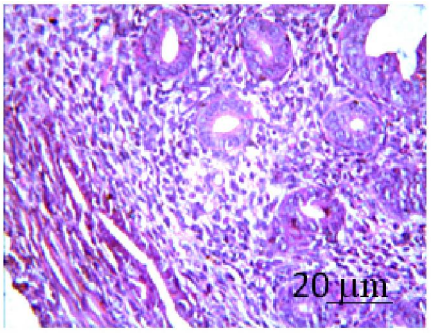

Fig. 8. Transverse section of the uterus (stained with haematoxylin-eosin) of *M. terricolor* following saline injection. Note the prominent endometrial glands.

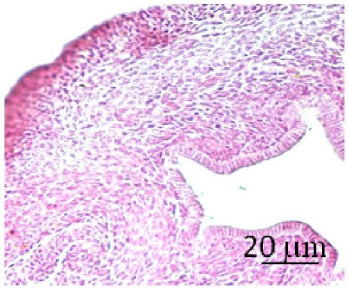

Fig. 9. Transverse section of the uterus (stained with hematoxylin-eosin) of *M. terricolor* showing histological changes following administration of dexamethasone. Note the non- proliferated endometrial glands.

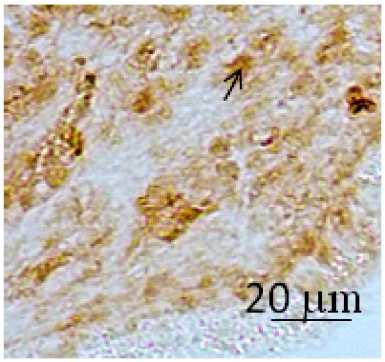

Fig. 10. Immunolocalization of glucocorticoid receptor (GR) in uterus of control mouse. Black arrows show immunopositivity for GR.

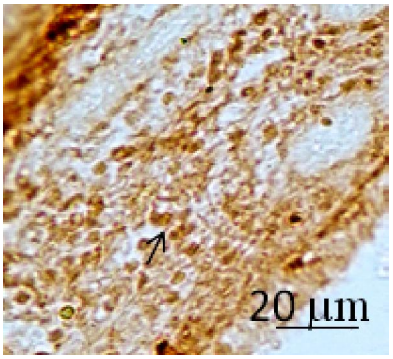

Fig. 11. Immunolocalization of GR in uterus of dexamethasone-treated mice. Black arrows show immunopositivity for GR.

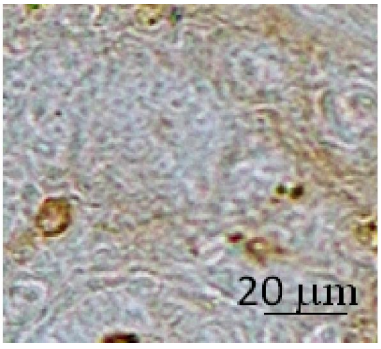

Fig. 12. Section of uterus showing no immunoreactivity for GR (Negative Control).

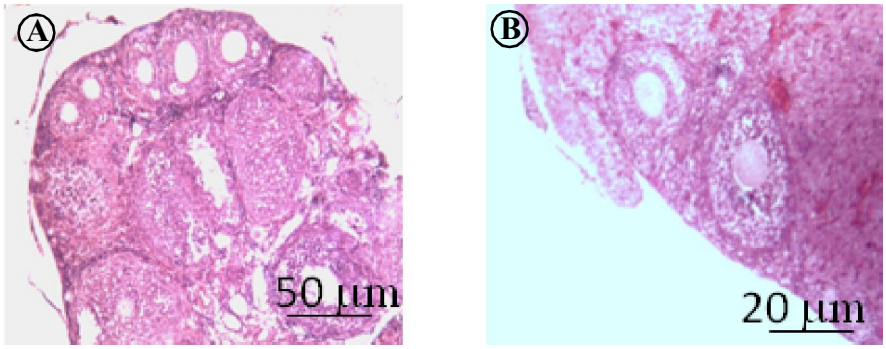

Fig. 13. TS of the ovaries (stained with haematoxylin-eosin) of *M. terricolor* following saline injection. (A) Low power micrograph (B) Magnified view showing normal antral follicles.

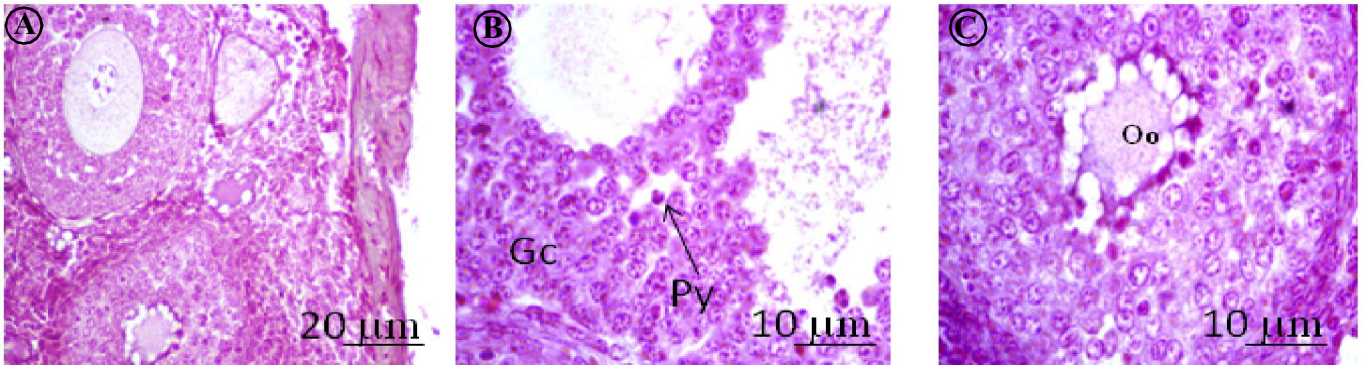

Fig. 14. TS of the ovaries (stained with haematoxylin-eosin) of *M. terricolor* showing histological changes following administration of dexamethasone. (A) Abnormal follicles; (B) Granulosa cells (GCs) (black arrow shows pyknotic nuclei (Py)); (C) Abnormal oocyte (Oo).

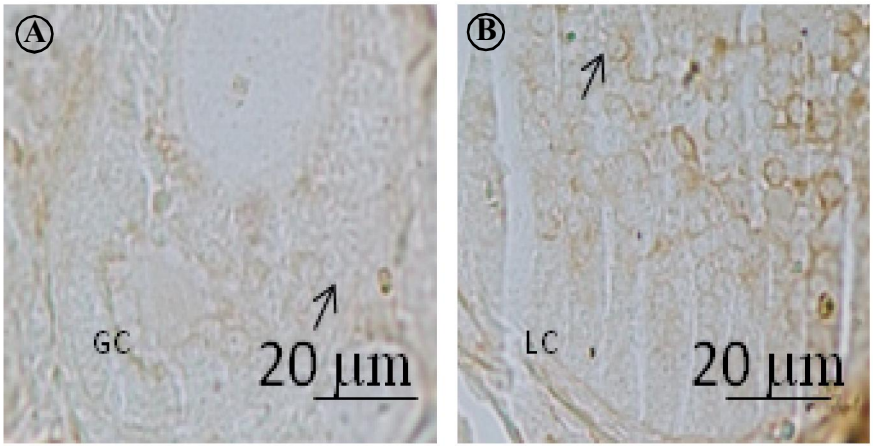

Fig.15. Immunolocalization of glucocorticoid receptor (GR) in ovary of control mice. Immunoreactivity of GR is mild in both (A) granulosa cells (GC) and (B) luteal cells (LC). Black arrows show immunopositivity for GR.

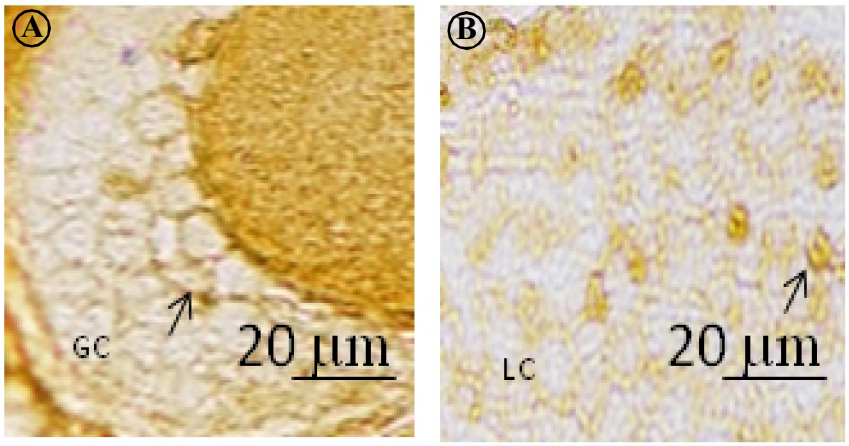

Fig. 16. Immunolocalization of GR in ovary of dexamethasone-treated mice. Strong staining was visualized in both (A) granulosa cells (GC) and (B) luteal cells (LC). Black arrows show immunopositivity for GR.

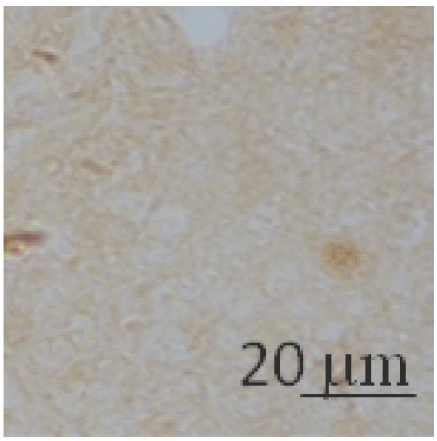

Fig. 17. Section of ovary showing no immunoreactivity for GR (Negative Control).

The ovary of the vehicle-treated control mice showed corpora lutea and numerous small-and medium-sized antral follicles (Fig. 13). On the other hand, the ovary of the mice treated with dexamethasone showed many abnormal follicles along with few normal follicles. The abnormal follicles showed marked regressive changes in the structure of granulosa cells such as condensed pyknotic nuclei. Some follicles showed presence of abnormal oocytes also (Fig. 14).

Immunohistochemical localization of GR

Immunohistochemistry was performed to localize glucocorticoid receptor (GR) in the ovarian and uterine tissues. GR was detected in the granulosa and luteal cells of ovary (Figs. 15, 16). Immunorectivity of uterus for GR was observed in the mucosal epithelium, glandular epithelium, stroma and myometrium (Figs. 10, 11).

Discussion

By and large, experimental studies on reproductive functions of seasonal wild female rodents are scanty. To work on a female animal model is challenging since the animals have different phases of reproductive cycle such as pre-pubertal, pubertal and pregnancy. We chose to work on a seasonally breeding wild female rodent model which has an active phase with estrous cycle of breeding and an inactive with no estrous phase. We studied the effects of synthetic glucocorticoid dexamethasone on the reproductive functions of female *M. terricolor* during its reproductively active phase to find the stress-induced inhibitory effect on reproduction reported previously in other animals (Smith et al., 1971; Soliman and Walker, 1976; Baldwin, 1979; Ramaley and Schwartz, 1980; Schreiber et al., 1982; Dubey and Plant, 1985; Rosen et al., 1988; Bigsby, 1993; Sugino et al., 1997). Suppressive effect of dexamethasone treatment on ovarian and uterine weight was accompanied by a significant reduction in uterine protein and plasma estradiol and progesterone while there was a significant increase in ovarian cholesterol. Increased ovarian cholesterol indicates low steroidogenesis as cholesterol is the raw material for steroidogenesis.

Glucocorticoid is regarded as the hallmark of stress and its level increases whenever an animal is confronted with a stressful condition and leads to hyperactivation of HPA axis. The different components of HPA axis, consequently, act on HPG axis to suppress the secretion of GnRH, LH, and gonadal steroids, thereby reproductive functions. The logic behind this suppression of reproduction is to give priority to survival of the individual rather than propagation of the species (Maeda and Tsukamura, 2006).

A plethora of reports suggest suppressive effects of glucocorticoid on female (mice and rats) reproduction (Smith et al., 1971; Soliman and Walker, 1976; Baldwin, 1979; Ramaley and Schwartz, 1980; Schreiber et al., 1982; Dubey and Plant, 1985; Rosen et al., 1988; Bigsby, 1993; Sugino et al., 1997). In the present study dexamethasone administration caused a significant reduction in ovarian weight and inhibited oogenesis. Our results concur with previous reports showing inhibition of ovulation and lutenization after dexamethasone treatment (Hagino et al., 1969; Smith et al., 1971; Soliman and Walker, 1976). Moreover dexamethasone affected steroidogenesis as indicated in reduced plasma estradiol (Campbell, 1978; Hsueh and Erickson, 1983) and progesterone levels, and increased ovarian cholesterol, a key component of steroidogenesis.

Different mechanisms are reported to be involved in the action of this synthetic glucocorticoid. It has been revealed by Rivier and Rivest (1991) that glucocorticoids can affect hypothalmo-pituitary-gonadal (HPG) axis at any of the three levels i.e., brain (affecting neurosecretion), pituitary (affecting gonadotropin secretion) and gonads (affecting gonadal steroid secretion). Glucocorticoid treatment has been reported to suppress LH secretion and consequently the gonadal action in many animals (Smith et al., 1971; Baldwin, 1979; Dubey and Plant, 1985; Rosen et al., 1988; Saketos et al., 1993). It could be possible that in this rodent also dexamethasone treatment might have inhibited LH secretion by decreasing responsiveness of the pituitary to GnRH or it might be a direct action on the pituitary itself. Corticosteroids are known to exert direct effect on gonadotropin release at the level of anterior pituitary (Baldwin et al., 1990; D'Agostino et al., 1990). It has been demonstrated by Suter and Schwartz (1985) and Kamel and Kubajak (1987) that corticosterone and cortisol can suppress GnRH as well as basal LH release from rat anterior pituitary cells incubated *in vitro*. It has been reviewed by Brann and Mahesh (1991) that modulation of opioid and catecholaminergic systems in the hypothalamus (Tong et al., 1990) and higher brain centers (Kizer et al., 1974) may also be one of the mechanisms by which corticosteroids inhibit gonadotropin. Kamel and Kubajak (1987) suggested a direct inhibitory effect of corticosteroids on coupling of GnRH receptors to second-messenger systems, leading to impaired release of LH in response to GnRH stimulus. This reduced

gonadotropin might have affected the secretion of gonadal steroids in the present study. Specific measurement of LH is required to support this inference but this measurement was not done due to lack of specific antibody.

Most of the biological actions of glucocorticoids are mediated via glucocorticoid receptor GR (known as nuclear receptor subfamily 3, group C, member 1 or NR3C1). GR is a member of the nuclear hormone receptor superfamily and is a ligand-dependent transcription factor (Silva et al., 2010; Gupta and Haldar, 2012). Schreiber et al. (1982), Sugino et al. (1997) and Gaytan et al. (2002) have reported presence of corticosteroid receptors in ovarian granulosa and luteal cells of rats. Gunin et al. (2002) and Rhen et al. (2003) immuno-localized GR in nuclei of luminal and glandular epithelia, endometrial stromal cells and smooth myocytes of the myometrium of mouse and rat. Since glucocorticoid receptors have been localized in gonad of rats and mouse and appear to be functional and capable of endocrine type regulatory activity in the gonads, a direct action of dexamethasone on gonads is also possible. For the first time we have immunolocalized GR receptors in ovarian and uterine tissues of *M. terricolor,* and so possible direct action of glucocorticoid on the gonad of this rodent cannot be ruled out.

In our study we have also documented negative effect of dexamethasone treatment on the uterine tissue of *M. terricolor*. We found a significant reduction in protein content of the uterus. The uterine protein might be required for preparation, impregnation and proliferation of uterus. Our observation is supported by Sullivian et al. (1983) who reported inhibition of protein synthesis in uterus of rat after glucocorticoid administration. A non-proliferative uterus with a broad lumen was observed following dexamethasone treatment which might be due to reduced gonadal steroids (estrogen/progesterone) as found in the present study (uterus is an active site of estrogen metabolism). Hsueh and Erickson (1983) reported suppressed activity of granulosa cell aromatase enzyme and, thus, estrogen deficiency due to action of glucocorticoid. Campbell (1978) has reported diminished tissue uptake of estrogen following dexamethasone treatment. Bigsby (1993) reported that dexamethasone inhibited estrogen induced synthesis of DNA and complement in rat uterine epithelium. Zamorano et al. (1992) and Monheit and Resnik (1981), working with rats and sheep, found the number of estrogen receptors and blood flow in uterus to be inhibited following glucocorticoid treatment. Delayed parturition and still births have also been reported in rats due to excess glucocorticoid (Chatterjee et al, 1993).

Thus, stress induced by administration of dexamethasone in *M. terricolor* led to suppression of reproductive functions. *M. terricolor* is a wild rodent and has to face various environmental challenges. Thus, it can be inferred from our findings that when confronted with stressful conditions, the HPA axis of this rodent becomes active and, consequently, inhibits the reproductive functions in order to give priority to survival of the individual.

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