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— SHORT COMMUNICATION —

Alteration in uterine environment by adrenal feedback mechanism during estrous cycle

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Changes in superoxide anion radical and superoxide dismutase in the uterus and ovaries of normal mice (*Mus musculus*) with respect to the change of the estrus cycle, have previously been studied. Furthermore, earlier studies have suggested that adrenalectomy does not interfere with the normal physiological functioning of the reproductive system in mice. In this study, the change in oxyradical-antioxidant balance in the uterus and ovaries of normal cycling mice has been compared with that of adrenalectomized (ADX) mice showing normal cyclicity. Normal cycling Balb/c female mice were chosen based on the stage of estrus cycle, namely proestrus, estrus, metestrus and diestrus. Animals were sacrificed and lipid peroxidation (LPO) and superoxide dismutase (SOD) estimations were carried out in the ovaries and uterus. Furthermore, a similar group of mice was subjected to bilateral adrenalectomy, after one-week normal cyclicity was reascertained, and LPO-SOD estimations were performed as earlier. Results showed a peak in LPO and a fall in SOD at the proestrus stage in uterus of intact animals which was not mimicked in the ADX animals where the levels of LPO and SOD were maintained stable during all the cyclic stages. Similarly, a peak in SOD level was noted at the proestrus stage in ADX ovaries along with the LPO peak in both intact and ADX mice ovaries. This suggests that actually adrenalectomy does affect the ovarian and uterine physiology and environment.

Key words: adrenalectomy, estrus cycle, superoxide dismutase, lipid peroxidation.

INTRODUCTION

External factors such as noise, diet, light and population density play an important role in reproduction, and directly or indirectly influence the hypothalamic-pituitary-adrenal axis for hormonal control of ovarian and testicular functions (Meer & Raber, 2005). The gonadotropic hormones secreted and regulated via the Hypothalamus-Pituitary-Adrenal (HPA) axis, are involved in the regulation of gonadal functions (including spermatogenesis, ovulation and implantation) and also in the development of secondary sexual characteristics.

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Earlier researchers have reported that adrenalectomized (ADX) rats had regular estrus cycle for a week or so after adrenalectomy after which the cycle became irregular and remained so until death (Tobin, 1941). Furthermore, it has been noted that the number of rats which were mated and ovulated was not affected by adrenalectomy (Thoman *et al.*, 1970). However, Anderson & Turner (1963) have presented conflicting results that rats did not ovulate after adrenalectomy. The response to pregnant mare serum gonadotropin comprised a delayed ovulation in ADX animals when compared to intact controls (Ramaley & Bartosik, 1975). Adrenalectomy did not seem to affect the *corpora lutea* or the number of *corpora* formed in rats, but it abolished the ovarian hypertrophy and ovulatory compensation that followed hemi-

ovarectomy (Edgren & Peterson, 1964; D'Souza & Rao, 1973). White *et al.* (1980) were the first to observe that adrenalectomized mice mated within four days of exposure to male mice, a fact indicating that the adrenals of the mouse may be not of major influence on the timing of the ovulatory LH surge, as suggested by earlier researchers in experiments conducted also in rats (Feder *et al*., 1971; Nequin & Schwartz, 1971; White *et al*., 1980).

It has been noted that the dynamic oxyradical-antioxidant balance serves as a good marker for the biophysical and biochemical changes occurring in any biological system (Tiwari, 2001). Superoxide anion radical and superoxide dismutase have been used as markers in the study of physiology of the reproductive system (Nivsarkar *et al*., 2001, 2002, 2005, 2006). They have been also considered to play a significant role in luteal steroidogenesis of the ovaries (Laloraya *et al*., 1988) and in oedema and cell proliferation of the uterus during proestrus (Laloraya *et al*., 1991). Hence, there is a need to elucidate the role of adrenals in regulating a normal estrus cycle and maintaining a normal physiological environment in the uterus and ovaries with respect to their oxyradical-antioxidant status. In this paper, we discuss the biophysical and biochemical changes occurring in the uterus and ovaries of adrenalectomized normal cycling female mice.

MATERIALS AND METHODS

Mature inbred female mice (Balb/c strain, 2-3 months old) housed in temperature controlled rooms (27 ± 1°C) at light: dark regimen of 14:10 hrs, were used. The experimental protocol was approved by the Institutional Animal House Ethics Committee, constituted by the Ministry of Social Justice and Empowerment, Government of India. Only those females which showed a regular 4-5 day estrus cycle were used. Vaginal smears were examined daily (Stockard & Papanicolaou, 1917).

Intact animals

Normal cycling females were taken, and the stages of the estrus cycle i.e. proestrus, estrus, metestrus and diestrus, were ascertained every morning at 10:00 am. These animals were immediately sacrificed and intact uterus and ovaries were excised into normal saline, cleaned to remove adhering fat, and processed further for LPO (lipid peroxidation) and SOD (superoxide dismutase) estimations (n = 6/stage).

Adrenalectomized animals

Bilateral adrenalectomy was performed through a dorsal incision under ketamine:xylazine anaesthesia (80:10 mg kg^{-1}). After surgery, the animals were returned to their cages with free access to food and normal saline (instead of drinking water) to maintain the normal body homeostasis even in the absence of the adrenals (Waynforth & Flecknell, 1992). All the adrenalectomized animals were monitored for their health conditions and those found to be active and healthy were used for further study. The females were checked for normal cyclicity, one week after surgery. As in intact animals, the adrenalectomized females were taken according to the stages of the estrus cycle (n = 6/stage). The animals were sacrificed and intact uterus and ovaries were processed as above for LPO and SOD estimations.

Estimation of lipid peroxidation (LPO)

The ovarian and uterine tissues were weighed, immersed in 5 ml of Hank's balanced salt solution (HBSS, pH 7.4) and homogenized at 5000 rpm by using a Polytron homogenizer (Kinematica, Switzerland) (3 cycles of 30 sec each). The homogenate was then centrifuged at 3500 rpm (500g) for 10 min. The pellet was resuspended in 0.1 ml of HBSS that was then used for estimation of lipid peroxidation.

Lipid peroxidation was measured in terms of malonaldehyde (MDA): thiobarbituric acid (TBA) reaction (Okahwa *et al*., 1979). The reaction mixture contained 0.1 ml of tissue homogenate (as described above), 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with 1M NaOH), and 1.5 ml of 0.8% aqueous solution of TBA. The reaction mixture was made up to 4 ml with the addition of 0.7 ml of double distilled water and heated at 95°C for 1 h, in water bath. After cooling, 1 ml of double distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and the mixture was shaken vigorously on a vortex mixer for 5 min. This mixture was centrifuged at 3000 rpm for 7 min, the upper organic layer was separated, and the amount of MDA formed in this layer (extinction coefficient of MDA is 1.45×10^{-5}/min/cm) was measured at 532 nm using an ultra violet/visible spectrophotometer (Systronics, India). Appropriate controls were used at different steps during this estimation.

Assay of superoxide dismutase (SOD) activity

The ovarian and uterine tissues were weighed, immersed in 4 ml of chilled Tris buffer 50 mM (pH 8.2) and homogenized at 13000 rpm (3 cycles of 30 sec each; intermittently placed on ice) using a Polytron homogenizer (Kinematica, Switzerland). The homogenate was treated with 1 ml of 0.1% Triton-X 100 (v/v) for 20 min at 4˚C, and then it was centrifuged at 15000 rpm at 4˚C for 30 min using a Sorval high-speed centrifuge (Sorval, USA) with fixed angle rotor (SS34). The supernatant was used for the assay of superoxide dismutase (SOD) activity (Marklund & Marklund, 1974). All calculations were made as per gram fresh weight.

Statistical analysis

All results have been presented as mean ± MSE (Mean Standard Error). Statistical analysis has been performed using paired t-test and $p < 0.05$ has been considered significant.

RESULTS AND DISCUSSION

Figure 1 shows the changes in superoxide dismutase activity and lipid peroxidation during the estrus cycle in the uterus of intact mice. Superoxide dismutase activity was found to be high during metestrus and low during proestrus ($p < 0.05$). However, low levels of lipid peroxidation were observed during metestrus and high levels during proestrus. These data agree with previous findings in *Rattus norvegicus* wherein it had been proved that the uterus undergoes luteal steroidogenesis during proestrus along with a superoxide anion radical peak which makes the membrane fluid to receive the blastocyst in the event of a conception (Laloraya *et al*., 1991). The presence of superoxide radical would cause oedema and cell proliferation, while the superoxide dismutase at metestrus and diestrus would be using this radical, which would in turn be responsible for the lack of oedema and cell proliferation of the uterus at metestrus and diestrus.

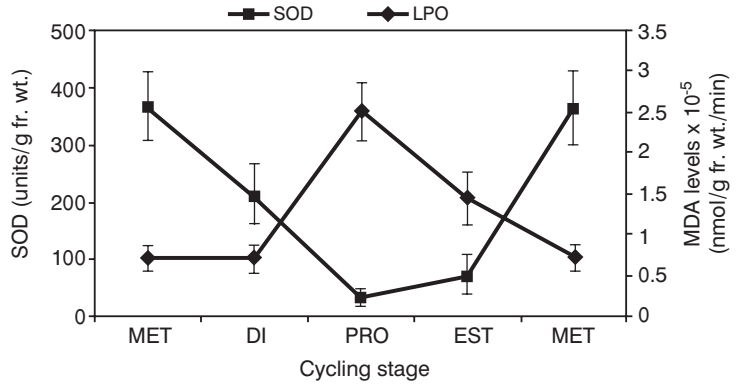

FIG. 1. Superoxide dismutase activity and superoxide anion radical in the uterus at different stages of oestrus cycle in intact mice. (Met – Metestrus, Pro – Proestrus, Est – Estrus, Di – Diestrus). Bars indicate MSE.

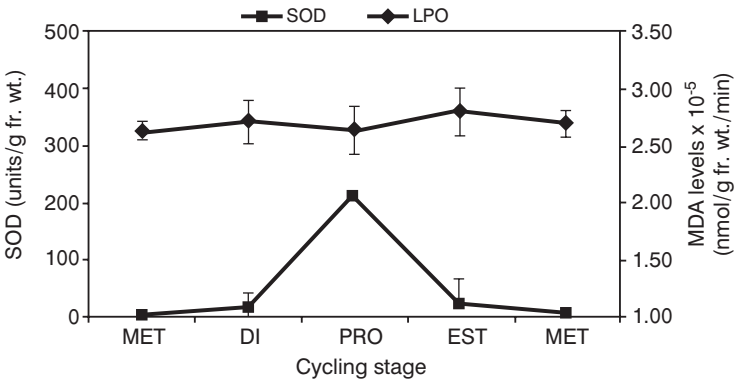

FIG. 2. Superoxide dismutase activity and superoxide anion radical in the ovaries at different stages of oestrus cycle in intact mice. (Met – Metestrus, Pro – Proestrus, Est – Estrus, Di – Diestrus). Bars indicate MSE.

Figure 2 depicts superoxide dismutase activity and lipid peroxidation in the ovaries of intact mice during various stages of the estrus cycle. These data suggest that high superoxide dismutase activity during proestrus ($p < 0.05$) supports the hypothesis by Oberley *et al*. (1981), that hydrogen peroxide may be involved in the regulation of normal cell division. This supports the consideration that superoxide dismutase in conjunction with peroxidase in a sequential development process may be involved in the regulation of follicular development, ovulation and luteal functions and lead to the production of hydrogen peroxide (Laloraya *et al*., 1989). Lipid peroxidation in the ovaries did not show any significant change during any of the cycling phases. Thus, we have confirmed that the radical-antioxidant profile in the mice uterus and ovaries mimics the pattern observed in rats by other investigators (Laloraya *et al*., 1991).

The influence of bilateral adrenalectomy on normal estrus cyclicity in rats has been a matter of debate in the past. Earlier researchers have suggested that though mating and ovulation patterns were not affected, the estrus cycle became irregular after one week of adrenalectomy in rats (Tobin, 1941). In our experiments, all mice had regained their normal cyclicity within one week of adrenalectomy. Animals were maintained up to one month after adrenalectomy and the normal cyclic pattern was ascertained thereafter.

In the case of adrenalectomized mice, superoxide dismutase activity and lipid peroxidation levels in the uterus are depicted in Fig. 3. In contrast to the results obtained in intact mice, the levels of superoxide dismutase and lipid peroxidation did not show any remarkable decrease or increase. This is in contradiction with the hypothesis that adrenalectomy does not change the cycling and pregnancy pattern in adrenalectomized mice (White *et al*., 1980). As seen here, SOD activity does not show a significant fall at the proestrus stage, a fact correlated with a lack of lipid peroxidation at this stage. Hence, it can be hypothesized that adrenal feedback plays a role in the regulation of membrane fluidity in the uterus. In this condi-

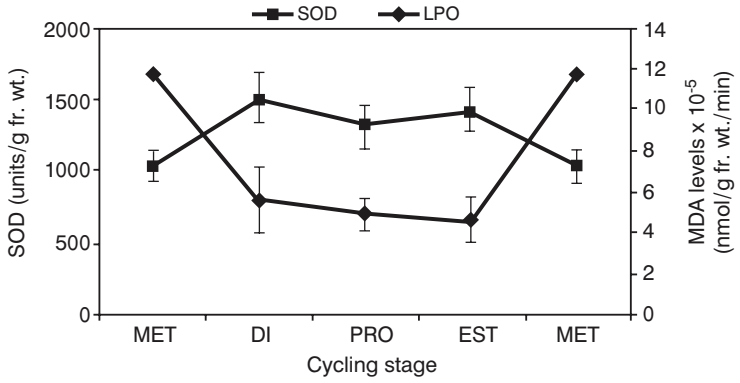

FIG. 3. Superoxide dismutase activity and superoxide anion radical in the uterus at different stages of oestrus cycle in adrenolectomized mice. (Met – Metestrus, Pro – Proestrus, Est – Estrus, Di – Diestrus). Bars indicate MSE.

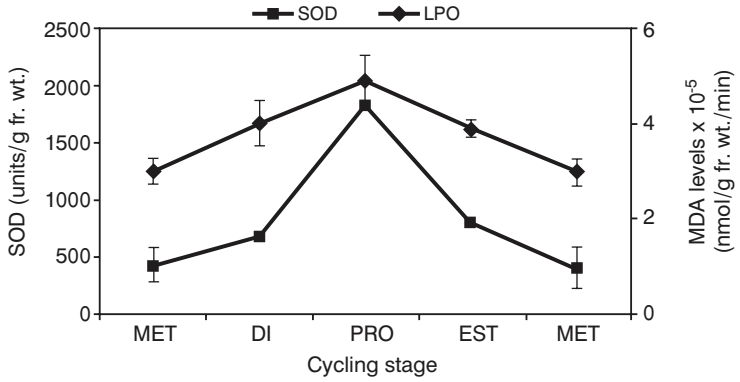

FIG. 4. Superoxide dismutase activity and superoxide anion radical in the ovaries at different stages of oestrus cycle in adrenolectomized mice. (Met – Metestrus, Pro – Proestrus, Est – Estrus, Di – Diestrus). Bars indicate MSE.

tion, it can be assumed that there maybe a failure in proper implantation of the blastocyst in the case of pregnancy. Further research pertaining to the adrenal regulation of the implantation process needs to be conducted. A high level of superoxide dismutase activity was seen during the proestrus in the ovaries of adrenalectomized mice, as compared to intact mice. This may be due to the high LPO levels during this stage (Fig. 4).

Thus, we may suggest that initially follicular functioning and ovulation may be insignificantly affected by adrenalectomy. However, removal of the adrenals may lead to a higher oxidative stress and over a period of time ovarian atresia may result, leading to further loss of ovarian functioning, as suggested earlier. This theory might also explain the varied duration of normal cyclicity observed by earlier researchers in different adrenalectomized rodent species.

Thus, this study presents a concept regarding the adrenal feedback regulation of ovarian and uterine milieu by regulating its oxyradical-antioxidant balance. The mechanism may provide an insight into the reproductive failures in conditions of adrenal hypertrophy or associated disorders in humans.

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