Ovaries, the target site of toxicity following oral exposure to oxytocin: Mechanistic studies

6.1 Introduction

Oxytocin (OT), a biological hormone, has physiological role for performing specific functions at different stages of life. For example OT stimulates labor by uterine contraction and has been implicated as a drug during delivery cases (Chan et al., 1996). During lactation period of cattle including cows and buffaloes, there is natural release of OT in minute quantities (15 to 90 pg/ml) into the blood circulation which remains there for few minutes (Macuho et al., 2004). Under natural condition, OT causes contraction of the myoepithelial cells surrounding the milk alveoli in mammary gland for milk let-down (Bruckmaier and Blum, 1998).

In Indian subcontinent, OT ampules commercially known as pitocin or syntocinon, is indiscriminately used for enhanced milk production following intramuscular injections to cattle. The exogenous OT is absorbed in blood thereby increasing the concentration by 4-5 folds and this high level remained for at least two hours after OT injection to animals (Macuho et al., 2004). In dairy industry, exogenous OT is administered to those cattle having disturbed milk ejection caused by reduced internal OT release (Belo et al., 2009). It has been presumed that due to small size of OT (1Kd) there is a possibility that it may cross the blood-milk barrier and reaches into the milk as OT is known to cause changes in permeability of tight junctions of mammary gland (Nguyen and Neville, 1998). Our previous study has shown the presence of OT in milk samples which is substantiated by the work of Takeshita et al. (1986) where intraperitoneal injection of radiolabeled OT given to rat dams transferred OT to milk as radioactivity was detected in the plasma of neonates. It was also deduced from our previous study that OT present in milk is stable under high temperature, adverse pH and simulated gastric condition fluid (SGF) conditions. Studies in the past suggest that OT is not digestible in stomach, however, digestion in intestinal membrane is very low (Paulsen et al., 1995). Majority of peptides are absorbed with low bioavailability of about 0.5% to 1%, which may increase according to the conditions found in gastrointestinal (GI) tract and this may be one of the reason that oral OT demonstrates irregular effects when used as drug for parturition (Laudin et al., 1995; Florence, 1986). However, if 0.5 % to 1% OT is bioavailable
through contaminated milk having a range from 21 pg/ml to 11.9 ng/ml (data) which is consumed daily, it may have some toxicological manifestations as the exposure occurs since early childhood.

Being a hormone, small amount of OT is sufficient enough to perform physiological function in normal conditions while its continuous exposure through milk which occurs since childhood in non physiological manner, may have unwanted consequences as OT receptors have been found to be present in the epithelial cells of GI tract (Welch et al., 2009). Therefore, in the present study toxicity of OT has been investigated following oral administration to young immature rats.

6.2 Materials and methods

6.2.1 Chemicals

Standard OT was purchased from Sigma Chemicals Co. (St. Louis, MO). [\(^{3}H\) OT (oxytocin, tyrosyl-2,6-\(^{3}H\)) (30-60 Ci/mmol) was a product of PerkinElmer (Waltham, MA). Primary antibodies [phosphorylated epidermal growth factor receptor (pEGFR), epidermal growth factor receptor (EGFR), cyclooxygenase-2 (COX-2), phosphorylated extracellular signal regulated kinase 1/2 (pERK1/2), extracellular signal regulated kinase 1/2 (ERK1/2), growth differentiation factor-9 (GDF-9), hyaluronic acid synthase-2 (HAS-2), TNF stimulating gene-6 (TSG-6) and β-actin] and HRP-conjugated secondary antibodies (anti rabbit, anti mouse and anti goat) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [\(^{3}H\) thymidine (6.7 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). PGE-2, pERK inhibitor (PD98059) and PGE-2 inhibitor (Arachidonyl trifluoro methyl ketone) were procured from Cayman Chemicals (Ann Arbor, MI). OT antagonist [d (CH)\(^{2}\), 5'Tyr(Me)\(_{2}\)Arg\(_{8}\) vasopressin] (OTA) was purchased from Tocris Bioscience (Bristol, UK). All other chemicals and reagents used were of highest purity commercially available.

6.2.2 In vitro evaluation of OT internalization in IEC-6 cells

6.2.2.1 Cell culture

IEC-6 cells (NCCS, Pune, India) were routinely cultured in 25-cm\(^{2}\) flasks at 37°C under the influence of 5% CO\(_{2}\) and 95% humidified atmosphere as described in section 3.8.1 of material and method.
6.2.2.2  **Internalization assay**

For the OT internalization assay, cells were seeded in 12 well plates at a density of $1 \times 10^5$ cells/2ml. The IEC-6 cells after 24 h of plating were treated with 2 μci of $[^3]H$ OT for 15 min and 24 h, respectively. After completion of incubation, cells were gently scraped and harvested in glass fiber filters. These filters were transferred to scintillation cocktail W (Sisco Research Laboratories Pvt. Ltd. Mumbai, India) and counts were recorded on Hewlett-Packard β counter (Palo Alto, CA). To study the localization of internalized OT, cells were homogenized and cell fractionation was carried out by differential centrifugation (Das et al., 1982). The radioactivity was counted in subcellular fractions in β counter as described above.

6.2.3  **Animals and treatment protocol for oral administration**

Healthy female Wistar rat pups (8-10 day old) along with their mother were obtained from the animal breeding colony of Indian Institute of Toxicology Research (IITR) Lucknow, India. The animals were acclimatized under standard laboratory conditions for 3 days prior to the experiment. Animals were housed in air-conditioned room in plastic cages and maintained at $22 \pm 2 ^\circ C$ under standard laboratory conditions of light/dark cycle (12-12 h) and have free access to food and water *ad libitum*. Pups (15 ± 3 g) were randomly divided into four groups, of 15 each. Oral intubation of freshly prepared OT (0.1, 1 and 10 ng) was given daily for 25 days. For this treatment schedule pups were kept with respective dams for the first 7 days after which they were separated for the next 18 days treatment with OT. The doses of OT were given through soft plastic tube faced cannula to avoid damage in buccal cavity of pups. The animals of control group received daily oral intubation of vehicle. During the treatment schedule, the animals were observed for body weight twice weekly. After 25 days of treatment, rats were monitored daily for estrus stage through vaginal smear and sacrificed in the morning of estrus by cervical dislocation according to the guidelines for the care and use of laboratory animals of IITR. All the major organs including liver, lungs, kidney, heart, brain and ovaries from each animal were dissected out and weighed. Three animals from each group were sacrificed after 20 days of OT exposure (before puberty) and ovaries were dissected out to check the effects of oral OT on immature ovaries.

6.2.4  **Locomotive behavior study in rats**
Neurobehavioral studies in rats were undertaken prior to the termination of experimental schedule, using the fully computerized Actimot Monitor (TSE, Tubingen, Germany) housed in a quiet, temperature-controlled (21 ± 2 °C) room by using the protocol as described in section 3.12.

6.2.5 Specimen collection and estimation of serum markers

Blood was allowed to clot at room temperature for 5 min followed by keeping it on an ice bath for 30 min. Serum was separated by centrifugation at 3000g for 10 min and stored at -80°C until further analysis. Activities of alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST), creatine, creatine kinase and glucose were estimated in serum by using commercially available kits (Accurex Biomedical Pvt. Ltd., Mumbai, India) following the instructions given by the manufacturer.

6.2.6 Serum protein levels and profile

Total protein and albumin (A) contents in serum was assayed by kit (Accurex Biomedical Pvt Ltd, Mumbai, India). Globulins (G) and A/G ratio were calculated from these values and expressed as g/dl. Serum profiling of serum was carried out by electrophoresis using a pre-formed SDS-PAGE gel from Sebia (Gwinnett, GA) and densitometric scanning was performed on Phoresis software from Sebia (Gwinnett, GA).

6.2.7 Histopathological processing

All the organs were washed in cold saline, soak dried on filter paper and weighed. A portion of organ was fixed in 10% buffered formalin and embedded in paraffin. Sections of 5 μm thickness were cut and stained with hematoxylin and eosin for microscopic examination (Bogovski, 1978).

6.2.8 Prostaglandin E2 (PGE2) assay:

The level of PGE2 in ovaries was determined by enzyme-linked immunosassay according to the manufacturer's manual (Cayman Chemical Co, Ann Arbor, MI, USA). Total protein concentration in the ovary was determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). PGE2 values were expressed as picogram PGE2 per milligram tissue protein.

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6.2.9 Western Blot Analysis

Whole cell lysates were prepared from 6 pooled ovaries collected from control and 10 ng OT treated groups and western blotting was done as described in section 3.23 of material and methods.

6.2.10 Granulosa cell culture

Granulosa cells were harvested from estradiol primed intact immature (day 25) rats as described previously in section 3.82.

6.2.11 Measurement of $^{3}H$ thymidine incorporation in granulosa cells

To measure cell proliferative rate, $5 \times 10^4$ granulosa cells were seeded in each wells of 48 well plates in 0.5 ml of complete medium in triplicate with OT, OTA, pERK1/2 inhibitor, PGE-2 and PGE-2 inhibitor as mentioned above in section 2-10. $[^{3}H]$ thymidine (2 μCi) was added to each well, 18 h prior to the completion of 24 h of incubation time (Mishra et al. 2011; Yadav et al. 2011). The cells were collected with the help of harvester and incorporated radioactivity was measured in a liquid scintillation counter (Packard Bioscience Company, Meriden, CT).

6.2.12 Statistical analysis

All results were expressed as the mean ± standard error (SE), as indicated in the tables. Statistical analysis of variance was carried out using one way ANOVA (Snedecar and Cochran, 1967). A value of $P < 0.05$ was used as the level of significance.

6.3 Results

6.3.1 OT internalization in IEC-6 cells

The radioactivity of $[^{3}H]$ OT in IEC-6 cells was found to increase about 26 and 30 fold following 15 min and 24 h of incubation, respectively (Figure 6.1A). The internalization of $[^{3}H]$ OT was monitored in different fraction of IEC-6 cells incubated with OT for 15 min and 24 h. The results showed that maximum radioactivity was retained in plasma membrane (PM) fraction at 15 min while after 24 h the maximum radioactivity was found in mitochondrial and microsomal fraction when the data was expressed as counts /mg protein (Figure 6.1B). When the data was expressed as percent of total radioactive counts, 0.07 and 0.03% of radioactive OT uptake was observed in
Figure 6.1 Internalization of [3H] OT in IEC-6 cells
A - [3H] OT in whole IEC-6 cells following incubation for 15 min and 24 h
B - Internalization of [3H] OT (counts per mg protein) in different fractions of IEC-6 cells
C - Internalization of percent radioactivity of [3H] OT in different fractions of IEC-6 cells
Each value represents mean ± SE (n = 3).
*p<0.05, Significant when compared to respective control
respective mitochondrial and microsomal fraction after 24 h, while 0.002% of radioactivity was noticed in plasma membrane (Figure 6.1C).

6.3.2 Effect of oral intubation of OT to rat pups on body weight and ovarian weight

The trend in body weight of rats in control and OT exposure for 25 days is given in figure 6.2A. Significant reduction (25 and 31%) in body weight was observed in rats treated with 1 ng and 10 ng OT for 25 days, while no significant change was observed in animals treated with 0.1 ng OT.

There was no significant change in major organ weight like liver, lung, kidney, heart and brain following oral administration of OT at all the three doses. Weight of ovaries were significantly increased (10-18%) in 1 ng and 10 ng OT treated groups, however, no significant change was observed in 0.1 ng OT group after 25 days of oral exposure (Figure 6.2B).

6.3.3 Effect of oral intubation of OT to rat pups on locomotive behavior

OT (10 ng) intubation to rat pups for 25 days caused significant decrease in locomotive behavior in terms of total distance travelled (10%), and moving time (18%) along with increase in resting time (22%) (Figure 6.2C).

6.3.4 Effect of oral exposure of OT on serum biochemical markers

Serum ALP, ALT, and AST activities were not found to be significantly altered following oral intubation of OT (0.1, 1 and 10 ng) when compared to control group (Data not shown). Further, no significant change in serum creatine and creatine kinase and serum glucose levels were observed in OT treated (0.1, 1 and 10 ng) rats as compared to control group (Figure 6.2D).

6.3.5 Changes in serum protein levels and profile following OT intubation

Total protein content in serum was significantly increased (28 %) following 1 ng and 10 ng OT treatments to animals (Figure 6.3A). OT (1 and 10 ng) treatment to pups for 25 days resulted in significant increase (27-28%) in globulin content while albumin content was not altered in any of the OT treated groups (Figure 6.3B & C). A/G ratio was found to be decreased (25-30%) in OT (1 and 10 ng) treated animals (Figure 6.3D). Further there was no significant change in any of the parameters in 0.1 ng OT treated rats (Figure 6.3A-D).
Figure 6.2 Effect of OT intubation to rat pups on body weight, ovarian weight and locomotive behavior

A - Effect of OT on body weight, B - Effect of OT on ovary weight
Each value represents mean ± SE (n = 15).

C - Effect of OT on total distance travelled, D - moving time and resting time
Each value represents mean ± S.E. (n = 5).

*p<0.05, Significant when compared to respective control
Figure 6.2 D Effect of oral exposure of OT to young rats on serum biochemical markers
Figure 6.3 Changes in serum protein levels and profile following OT intubation to rat pups

(A) total protein, (B) globulin, (C) albumin, (D) A/G ratio,
Serum protein profiling through SDS-PAGE electrophoresis showed no change in albumin content while increase was observed in all the forms of globulins (α1, α2 and γ globulin) except β globulin in OT (1 and 10 ng) treated animals (Figure 6.3E & F).

6.3.6 Histopathological observation of liver, brain, heart, kidney, lung and ovary of OT treated rats

Microscopic examination of liver, brain, heart, kidney, lung and spleen of OT (10 ng) treated animals for 25 days showed normal histological structure similar to that of control animals (Figure 6.4A). Rat pups treated with OT for 20 days (1 and 10 ng) showed different stages of follicles and cystic dilation of ovarian bursa and profuse interstitial edema leading to enlargement of ovaries.

6.3.7 Effect of oral OT intubation to rat pups on ovarian PGE-2 level

OT treatment (1 and 10 ng) to rat pups for 25 days resulted in significant increase (41-74%) in ovarian PGE-2 levels (Figure 6.5A).

6.3.8 Effect of OT on p-EGF receptor, pERK 1/2, COX-2, GDF-9, HAS-2, and TSG-6 levels in ovaries of rats

The pEGFR level in ovary was found to be enhanced (2.1 fold) following OT (10 ng) treatment to rats for 25 days when compared to control (Figure 6.5B). Furthermore, pERK1/2, which is involved in ovulation process, was found to be enhanced (1.5 fold) following OT intubation to rats. OT treatment also led to significant up regulation of COX-2 (3.2 fold), GDF-9 (1.6 fold), HAS-2 (2.2 fold), and TSG-6 (2.4 fold) in the ovaries of rats (Figure 6.5B).

6.3.9 Effect of OT, OTA, pERK1/2 inhibitor, PGE-2 and PGE-2 inhibitor on OT induced granulosa cells proliferation

Morphological data on granulosa cells and thymidine uptake following treatment to OT, OTA, pERK1/2 inhibitor, PGE-2 and PGE-2 inhibitor is shown in figure 6.6A & B. Treatment of granulosa cells to OT caused increase in proliferation along with attachment to the surface. However treatment of OT induced granulosa cells with OTA or pERK1/2 inhibitor or PGE-2 inhibitor showed no proliferation activity (Figure 6.6A). These morphological results were substantiated with [3H] thymidine uptake experiments wherein OT treatment caused significant...
Figure 6.3 Changes in serum protein levels and profile following OT intubation to rat pups

(E) Densitometric scanning of SDS-PAGE electrophoresis of serum from control and OT treated rats.
(F) Levels of albumin and various globulins in serum of control and OT treated rats
Each value represents mean ± SE (n = 3).
*p<0.05, Significant when compared to control.
enhancement of thymidine as compared to control, while OTA treatment to OT induced cells resulted in significant reduction in radioactivity uptake when compared to cells treated with OT. Similarly, treatment of OT induced cells to pERK1/2 inhibitor or PGE-2 inhibitor resulted in significant decrease in thymidine uptake compared to OT treated cells (Figure 6.6B). Further, OT induced cells treated with (hypertrophied) along with greater number of follicles compared to control rats (Figure 6.4B). Animals treated with OT (1 and 10 ng) for 25 days showed greater number of CLs as compared to control animals (Figure 6.4C). However there was no significant morphological change in the ovaries of rats treated with 0.1 ng OT for 25 days.

pERK1/2 inhibitor along with PGE-2 resulted in significant increase in thymidine uptake compared to OT + pERK1/2 inhibitor treated cells (Figure 6.6C). Western blot analysis of pERK1/2 in granulosa cells in the presence of inhibitors is shown in figure 6.6C. Treatment of cells with OT resulted in enhanced expression of pERK1/2. However, OTA or pERK1/2 inhibitor treatment to OT induced cells caused decrease in pERK1/2 expression when compared to OT treated cells. Treatment of OT induced cells with pERK1/2 inhibitor in presence of PGE-2 resulted in decrease expression of pERK1/2 when compared to OT treated alone, while treatment with PGE-2 inhibitor to OT induced cells showed no change in pERK1/2 when compared to OT exposure alone (Figure 6.6C).
Figure 6.4 Histopathology of the organs

A- Histopathological observation of liver, kidney, heart, lung and brain of OT treated young rats
Figure 6.4 Histopathology of the organs
B- Histopathological observation of ovary of OT treated rats
(i) - H & E staining of ovarian sections of OT treated rats for 20 days
(ii) - H & E staining of ovarian sections of OT treated rats for 25 days
Figure 6.5 Effect of oral intubation of OT to rat pups on different ovulation markers
A - PGE-2 level
B - Western blots of pEGFR, pERK1/2, COX-2, GDF-9, HAS-2, and TSG-6 proteins
Figure 6.6 Effect of OT, OTA, pERK1/2 inhibitor, PGE-2 and PGE-2 inhibitor on granulosa cell proliferation and pERK1/2 expression

A - Morphological observation of granulosa cells following OTA, pERK1/2 inhibitor, pERK1/2 inhibitor + PGE-2 and PGE-2 inhibitor treatment for 24 h on OT induced proliferation.

* p<0.05, significant when compared to control group
** p<0.05, significant when compared to OT alone treated group
*** p<0.05, significant when compared to OT + pERK1/2 inhibitor treated group

B - [³H] Thymidine uptake of granulosa cells following OTA, pERK1/2 inhibitor, pERK1/2 inhibitor + PGE-2 and PGE-2 inhibitor for 24 h on OT induced proliferation.
Figure 6.6 Effect of OT, OTA, pERK1/2 inhibitor, PGE-2 and PGE-2 inhibitor on granulosa cell proliferation and pERK1/2 expression

C- Western blot of pERK1/2 in OT, OT + OTA, OT + pERK1/2 inhibitor, OT + pERK1/2 inhibitor + PGE-2 and OT + PGE-2 inhibitor treated (24 h) granulosa cells.

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Any protein or peptide consumed orally undergoes degradation by intestinal enzymes followed by absorption of amino acids. However, our prior studies have shown that OT is not digested by simulated gastric fluid or under adverse conditions of temperature and pH. This is not surprising as intestinal cells are equipped with OT receptors which may facilitate the absorption of OT in intestine (Martha et al. 2009). We therefore performed experiments related to internalization of radiolabeled OT in intestinal cells. In spite of known fact that during early life (till 21 postnatal days in rat) intestinal permeability is higher as compared to adult (Teichberg et al. 1992), the in vitro data provided evidence for internalization of [^3H] OT in 15 min which is further enhanced with incubation time (24 h). Francesca et al. (2009) showed that stimulation of G protein-coupled OT receptors (OTRs) leads to internalization of ligand-receptor inside intracellular compartments by endocytosis which further supports our present results. Cell fractionization studies on IEC-6 cells following incubation with [^3H] OT for 15 min and 24 h indicated that internalization is receptor mediated as radioactivity was found to be higher in the plasma membrane fraction after 15 min of[^3H] OT incubation and remained constant even after 24 h incubation time. Further, with increase in incubation time (24 h) concentration of OT in mitochondrial and microsomal fractions was enhanced by 24-30 fold suggesting that internalization of cell-surface bound radioactive OT begins immediately following the receptor ligand binding.

The female rat pups treated with oral OT for 25 days showed reduction in locomotive behavior and body weight. Studies of Arletti et al. (1990) and Gregory et al. (2011) showed that intracerebroventricular or intraperitoneal administration of OT caused inhibition in food intake thereby leading to weight loss. Since body weight and locomotive behavior are under the control of central nervous system (CNS) (Morton et al. 2006 and Morimoto et al. 1993) the results obtained on locomotive behavior in the present work is indicative of the effect of non physiological OT exposure on CNS.

OT treatment to female rat pups showed no effect on serum biochemical parameters which indicates normal physiology of major organs like brain, liver, lung, kidney and heart having normal histology. However, increase in total serum protein levels particularly α1, α2, and γ globulins in

6.4 Discussion

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OT exposed pups result in increase permeability in neuronal and epithelial cells of mammary gland (Konenenko & Osipenko, 1988; Nguyen & Neville, 1998).

Increase in ovarian weight of OT treated rat pups and enhanced number of follicles per ovary may be indicative of the effect of non physiological OT exposure. The increase in ovarian weight has also been reported by Corbin and Schottelius, (1961) following OT injection to central brain in postnatal rats. The histopathology of ovary showed greater number of CLs in OT treated female rats indicating increased ovulation due to the influence of oral administration of OT. The mechanism of increased ovulation following oral administration of OT to rat pups may be due to higher expression of pEGFR and its downstream pERK1/2 protein indicating its facilitatory effects toward follicular development and ovulation. Earlier studies have shown the role of pEGFR and pERK1/2 protein in ovulation process (Andric et al. 2010 and Panigone et al. 2008). However, the study of Rimoldi et al. (2003) suggests that OT receptors can activate EGFR and ERK1/2 sequentially using different signaling intermediates. Other functions of the ovary related to ovulation may be affected by oral OT as shown by increased expression of COX-2, which plays a crucial role in cumulus oocyte complex expansion and ovulation (Sirois et al. 2004; Takahashi et al. 2006). Other proteins like HAS-2 and TSG-6 are related to matrix deposition, which are responsible for ovulation process (Hess et al. 1999; Salustri et al. 1999). These proteins in ovaries were found to be substantially over expressed following OT exposure to rat pups suggesting enhanced matrix deposition in the ovaries. Increased GDF-9 protein (oocyte secreted factor) in the ovaries of OT treated rats suggests that follicular maturation may be enhanced as GDF-9 has been shown to be an important factor for folliculogenesis and ovulation (Moore et al. 2004).

To further validate the involvement of pERK1/2 protein in the process of enhanced ovulation by OT, we performed experiments in primary granulosa cells and investigated the effect of PGE-2 and inhibitors of OT, pERK1/2 and PGE-2 on proliferation and key protein pERK1/2 expression. The results indicate that the effect of OT on granulosa cells is mediated through pERK1/2, as pERK1/2 inhibitor in presence of OT caused decreased proliferation of granulosa cells. PGE-2, the main effector molecule, works downstream to pERK1/2, as PGE-2 in the presence of OT and pERK1/2 inhibitor was capable of cell proliferation. These results confirmed that OT through induction of
pERK1/2 caused the secretion of PGE-2, which ultimately led to granulosa cell proliferation and thereby increasing follicular growth. The increased level of ovarian PGE-2 following OT treatment to rat pups further supports the in vitro data. The putative mechanism of increased ovulation following OT exposure to female rat pups may be through activation of OT receptor which transactivate the pEGFR which thereby activates the ERK1/2, the key protein for ovulation which further regulate the expression of COX-2, GDF-9, HAS-2 and TSG-6 (Figure 6.7).

6.5 Conclusion

The minimum dose of OT responsible for producing ovarian toxicity is 1 ng through oral exposure while 0.1ng is the no observed adverse effect level (NOAEL) dose of OT in rats. Considering a safety factor of 100 for humans for toxic molecules (Rees and Hattis, 1994), the safe limit of OT can be implicated to be 0.001 ng (1 pg). However, minimum detectable limit of OT is 10 pg/ml in milk by EIA method (Prakash et al. 2009) and therefore, milk should be free from OT contamination.

The overall results suggest that oral OT exposure (through OT contaminated milk) since early childhood may not show alteration in classical toxicological parameters but its target organ, ovary, is a matter of concern as it enhances ovulation which may have unwanted consequences that needs to be explored further.
Figure 6.7 Putative mechanism of enhanced ovulation in the ovaries of rats treated with oxytocin