Oxytocin (OT), a peptide hormone, is produced by neurons in the paraventricular nuclei (PVN) and supraoptic nucleus (SON) of the hypothalamus and stored in the posterior pituitary for secretion in blood. The hormone is packaged into granules and secreted along with carrier proteins called neurophysins. OT is mainly secreted from brain as well as few other tissues including the ovaries and testes, in both male and female. OT consists of nine amino acids linked with (1-6) disulphide bond and a carboxyamidated tail. The level of OT in plasma varies between 1 and 10 nmol/ml, whereas in cerebrospinal fluid it is 5 to 10 fold higher than plasma. The half life of OT is in minutes which changes according to physiology of the organism. This hormone once thought to have limited functions like female smooth muscle physiology at parturition, milk ejection and establishment of maternal behavior. Recently, OT has been shown to posses other functions such as neurotransmitter, involved in social sexual behavior and in male reproductive physiology and a few studies indicates its role in breast cancer. OT receptors have also been reported in bone cells, myoblasts, cardiomycocytes and endothelial cells, which indicate that it may have other roles at different sites which are still not explored.

For decades OT was identified as a key hormone in milk production because of its ability to induce milk ejection. This neuropeptide hormone OT is released by the posterior pituitary gland after tactile teat stimulation by the sucking calf, hand or machine milking. OT causes the contraction of myoepithelial cells surrounding the alveoli forcing the milk into the milk ducts and cisternal cavities. Many dairies use synthetically manufactured OT to facilitate milk ejection, and also as a treatment for mastitis. OT is a schedule 'H' drug in India that means that it cannot be bought or sold without a prescription. It is specifically banned under section 12 of the Prevention of Cruelty to Animals Act, 1960. OT drug have been used to stimulate breastfeeding, labor induction and to support labor in case of non-progression of parturition. Despite their importance, long-term effects of OT have only recently been investigated and showed that exposures during developmental periods could result in even higher CNS levels of OT, due to the leaky nature of the infant blood brain barrier. These high central levels of hormone could cause organizational changes later manifesting as altered behavior or growth patterns that persist into adulthood. OT has also been
shown to cause long-term changes in the expression and distribution of receptors. It has been also observed that OT exposure leads to early LH surge and GnRH release.

Although detailed information is available regarding the oxytocin system, the underlying mechanisms of reported side-effects are not always clear. In addition to peripheral effects, OT also acts as a neurotransmitter in the brain influencing many aspects of social behaviour. The last two decades have witnessed a surge in research investigating the application of OT as a method of enhancing psychological function in humans. Research involving healthy adults has linked OT with a range of effects such as reducing levels of anxiety, and increasing levels of trust, gaze to the eyes, and accurate emotion processing. Current safety information regarding the use of OT in humans is largely derived from usage by mothers to promote lactation. In India, OT injection ampules, commercially known as pitocin or syntocinon, are indiscriminately used for milking cows and buffaloes. This unrestricted use of OT may increase the level of hormone in plasma and other organs of milk consumers in the non-physiological manner. Since milk is an essential part of our daily diet, it is hypothesized that long term exposure of OT since childhood in non physiological manner may be a cause of concern.

Due to unavailability of sufficient data regarding side effects and safety of daily exposure of oral OT (through OT contaminated milk), there is a controversy regarding use of OT in cattle and thereby in milk. For this reason, the present work was designed to have a detailed study on every aspects of OT used in dairy industry and there likelihood effects due to consumption of OT contaminated milk. This study will go in a long way in helping regulatory agencies to frame new norms regarding OT. To achieve the above mentioned goal the study was carried out with following objectives.

1. Detection and quantification of oxytocin in milk.

2. Evaluation of toxicity during nonphysiological exposure levels of oxytocin using rat as an animal model.

3. Investigation of possible mechanism of action of hormone for early puberty mechanism.

Different methodologies were used in this dissertation to achieve these objectives.
The prescribed level of OT in milk was not determined by regulatory bodies. So, the first step of this dissertation was to quantitate the levels of OT in marketed milk samples. For this, we developed an efficient extraction method based on TCA precipitation and SPE process, further EIA, HPLC and LC-MS methods were used for quantitative estimation of OT in milk. General serum toxicity parameters, OT internalization assay in IEC-6 cells, behavioral changes, and histopathological studies were done for toxicity analysis and target organ evaluation. Observation of vaginal opening and vaginal smear were used for puberty evaluation. Further *in vitro* granulosa cell culture, ovulation induction, western blot, PCR, immune florescence, number of follicles and CLs counts, germinal vesicles break down counts and hormonal analysis were done for elucidation of OT role and mechanism in the process of puberty and follicular development.

The study design of the entire study is shown in Figure below.
<table>
<thead>
<tr>
<th>DETECTION</th>
<th>STABILITY</th>
<th>GENERAL TOXICITY</th>
<th>MECHANISM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method for OT detection in milk</td>
<td>Stability analysis</td>
<td>Internalization in IEC-G cells</td>
<td>Effect of oral OT on puberty and ovarian maturation</td>
</tr>
<tr>
<td>Quantization of OT in milk</td>
<td>At temperature</td>
<td>Body weight</td>
<td>Elucidation of Mechanism/mechanisms of action of OT</td>
</tr>
<tr>
<td>Surveillance for OT</td>
<td>pH and</td>
<td>General serum parameters</td>
<td></td>
</tr>
<tr>
<td>Exposure through milk</td>
<td>Simulated gastric fluid condition</td>
<td>Serum proteins</td>
<td></td>
</tr>
<tr>
<td>Enzyme immuno assay</td>
<td>HPLC</td>
<td>Behavioral parameters</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>Western Blot</td>
<td>Histopathology</td>
<td></td>
</tr>
<tr>
<td>LC-MS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in vitro culture</td>
<td></td>
<td>ovulation induction</td>
<td></td>
</tr>
<tr>
<td>Spectrometry</td>
<td></td>
<td>Western Blot</td>
<td></td>
</tr>
<tr>
<td>Serum protein electrophoresis</td>
<td></td>
<td>Immunofluorescence analysis</td>
<td></td>
</tr>
<tr>
<td>Histopathology</td>
<td></td>
<td>Histopathology</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td></td>
<td>PGE-2 analysis</td>
<td></td>
</tr>
<tr>
<td>PGE-2 analysis</td>
<td></td>
<td>Hormone analysis</td>
<td></td>
</tr>
<tr>
<td>Florescence HPLC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure: Systematic representation of work plan
Quantitative method for the determination of oxytocin in milk by enzyme immunoassay or HPLC: Validation by LC-MS

There has been a controversy regarding the use of exogenous oxytocin (OT) in milking cattle which may have toxicological consequences during non physiological exposure, if milk gets contaminated with OT. Till now there is no study available in the literature regarding surveillance of OT in marketed milk samples. In the present study a new sensitive extraction method for OT was developed followed by enzyme immunoassay (EIA) or HPLC analysis. The prerequisite extraction process of OT in milk involves two steps: (i) TCA precipitation of milk proteins followed by washing of the precipitate (ii) Solid phase extraction (SPE) cleanup process of the aqueous extract of step (i). Without these extraction steps analysis of OT in milk was not possible either by EIA or HPLC. Utilizing EIA as a quantitative tool the limit of detection (LOD) and limit of quantitation (LOQ) were found to be 7.74 pg/ml and 10.3 pg/ml, precision in terms of intra and inter day coefficient of variation was below 13% (%RSD, N=6), while % recoveries were between 85-92%. When UV-HPLC was used the LOD, LOQ, precision and recovery values were found to be 4.1 ng/ml, 9.8 ng/ml, 2-10% and 84-91%, respectively. OT was found to be stable against adverse temperature (up to 100°C) and pH (2 to 10) and stimulated gastric fluid (SGF) digestibility assay re-emphasizing that there may exposure risk under non-physiological conditions. Four milk samples collected from the market were analyzed by EIA and HPLC, which showed that TCA precipitation and SPE steps are mandatory for the quantitation of OT and the results were validated by LC-MS showing mass ion peak at 1Kd. The newly developed method shall be useful for the detection of OT in milk samples.

Extraction method for OT in milk based on TCA precipitation and SPE cleanup was developed which is suitable for analysis by EIA, HPLC and LC-MS. The OT present in milk was also found to be stable against temperature (up to 100 °C) and pH (2 to 10) and stimulated gastric fluid (SGF) digestibility assay.

Surveillance of oxytocin in milk samples and intake pattern in different age groups of Indian population

We have developed an extraction method for the analysis of oxytocin (OT) in milk samples by EIA and HPLC. OT injections have been indiscremately used to milk cattle. In the present study
a survey of the occurrence of OT in milk samples (milkman and branded) collected from
different locations of Lucknow, Uttar Pradesh (India) has been conducted. A total of 55 milk
samples (39 milkman and 16 branded) were extracted and analyzed for OT by EIA and UV-
HPLC. OT was detected in all the milk samples, ranging from 21 pg/ml to 18.9 ng/ml with a
mean value of 4.3 ng/ml. OT contamination in milkman samples was found to be 21 pg/ml to
12.1 ng/ml with mean value of 2.5 ng/ml and 90th percentile 11.4 ng/ml, whereas in branded
milk samples the contamination was 1.3-18.9 ng/ml with the mean value of 8.9 ng/ml and 90th
percentile 17.1 ng/ml. The levels of OT in 12 milk samples detected by both EIA and UV-
HPLC were found to be comparable with % RSD ranging between 0.2-7%. For analysis of
intake pattern of OT through milk, a survey of 255 subjects belonging to 150 families was
conducted. The surveyed population contains 3% infant (0-1 year), 66% children (1-12 year)
and 31% of adolescence (13-15 year). Average milk consumption was found to be highest
(542.9 ml/day/person) in 1-3 year age children group followed by 420.7 ml/day/person in
10-12 year age group children. The average and 90th percentile daily intake in terms of
µg/day/person was maximum (2.4 µg/day/person and 5.2 µg/day/person) in 1-3 year age
group. This study suggests that milk (milkman as well as branded) were contaminated with OT
and the levels were higher in branded milk as compared to milkman samples. Since, there is no
prescribed level of OT in milk and the intake of OT through this commodity is quite high there
is need to implement regulatory laws so that non-physiological OT exposure may not occur in
children which may have deleterious effects.

**OT was found to present in milk ranging 21 pg to 18.9 ng/ml and the daily intake was
found to be maximum (2.4 µg/day/person) in 1-3 year children.**

Ovaries, the target site of toxicity following oral exposure to oxytocin under non
physiological condition

Toxic potential of oral oxytocin (OT) in rat pups was undertaken since OT injections are
illegally used for milk let down in cattle thereby causing exposure to population at early age. *In
vitro* studies showed internalization of [³H] OT in IEC-6 cells following 15 min and 24 h of
incubation with maximum retention in mitochondria and microsomes. Daily oral intubation of
OT (0.1, 1 and 10 ng/100 [µl] saline) to female Wistar rat pups (10 days old) for 25 days
resulted in significant decrease in body weight while ovarian weight was significantly enhanced
at 1 and 10 ng doses. Although, serum biochemical parameters and histopathology of major
organs showed no alterations, animals treated with OT (10 ng) showed significant decrease in locomotive behavior. OT (1 and 10 ng) treatment for 25 days to rat pups showed significant increase in total protein and α1, α2 and γ globulins. Histopathology of ovary showed greater number of follicles following 20 days of OT (1 and 10 ng) exposure while enhanced number of corpus luteum was observed after 25 days of exposure, indicating maturation of ovaries with enhanced ovulation. The mechanism of enhanced ovulation by OT may involve over expression of pEGFR followed by downstream pERK1/2 and subsequently COX-2 along with enhanced PGE-2, HAS-2 & TSG-6 (matrix deposition proteins) and GDF-9 (oocyte factor) level, which was further confirmed using inhibitors of OT, pERK1/2 and PGE-2 in granulosa cells, suggesting that OT (1 and 10 ng) may affect the physiology and function of the ovary.

Oral intubation of OT for 25 days (d10-d35) showed no significant changes in general toxicity parameters but changes in histopathology and markers protein in ovary showed that ovary is the target organ.

Effect of early age oral OT on Puberty, follicular development and 1st ovulation:

Role of PGE-2, pAKT and pERK

Precocious puberty is a significant child health problem, especially in girls, because 95% of cases are idiopathic. It has been earlier proposed that oxytocin (OT) could play a facilitatory role in the preovulatory LH surge in rats and human and also facilitates sexual maturation in female rats. Here we provide the first evidence that exogenous oral OT at an early age also facilitates sexual development and caused precocious puberty in female rats. The administration of an oral OT (1ng and 10ng/day) for 25d (d10-d35) to immature female rats decreased the age at vaginal opening (VO) and first estrus. Increased level of PGE-2 found in median basal hypothalamus (MBH) of OT treated animals suggests that exogenous oral OT facilitate the pubertal process via PGE-2. To have a clearer onset we had also observed the effect of early age oral OT on the process of follicular development and 1st ovulation. For this after 15 days (d10-d25) of oral OT intubation the rats were induced for ovulation by PMSG and HCG and several proteins (oocyte maturation, cells survival, matrix deposition and cumulus expansion) and processes (ovarian PGE-2 level, GVBD, number of follicles, different stage follicles, estradiol, progesterone concentration and cumulus expansion) related to ovulation were analyzed. The early age oral OT leads to higher initial recruitment process via activation of pAKT and further earlier shift from proliferation to differentiation via earlier activation of the key protein pERK1/2. The
activation of AKT and ERK leads to earlier follicular maturation indicated by higher serum estradiol level, number of follicles, survival signals (pAKT, cJNK, BCL-2 and PARP); earlier germinal vesicles break down (GVBD), matrix deposition (HAS-2 and TSG-6) and cumulus expansion (pERK1/2, GDF-9, PR, COX-2 p-P38, pNFkB, cumulus oocyte complex area measurement) on the influence of early age oral OT. So these findings collectively suggest that early age oral OT causes the early activation of hypothalamic pituitary gonadal axis which leads to early puberty via PGE-2 and stimulates the follicular development through activation of AKT and ERK1/2 proteins in the ovary.

*Early age oral OT causes the early activation of hypothalamic pituitary gonadal axis which leads to early puberty via PGE-2 and stimulates the follicular development through activation of AKT and ERK1/2 proteins in the ovary.*
Figure: Systematic representation of the effect of early oral exposure of oxytocin on puberty, follicular development and 1st ovulation

The above studies have elucidated the molecular mechanism of OT induced early puberty which involves the early activation of hypothalamic pituitary gonadal axis which leads to early puberty via PGE-2 and stimulate the follicular development through activation of AKT and ERK1/2 proteins in the ovary which pose health risks to humans. The study also developed a sensitive method for detection of OT in milk samples and elucidates that milk should be free from OT for human use. The studies will go in a long way in helping the regulatory agencies to frame new emission norms regarding OT.
References


............... List of Publications


