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Effect of oxytocin injection to milching buffaloes on its content & stability in milk

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***Background & objectives*: Oxytocin (OT) injections to milch cattle for milk letdown have become a common practice amongst dairy farmers in India. Although there is no reported evidence, it is widely presumed that long term consumption of such milk leads to adverse health consequences. However, there is no information on the effect of exogenous OT injections on milk OT content and its stability during heating and gastrointestinal digestion. This study was carried out to determine the OT content in milk samples given by buffaloes with and without OT injections and to assess the stability of OT in the milk.**

***Methods*: Milk samples from milch buffaloes (*Murrah buffalo*) were collected from local farmers with (n=121) or without (n=120) exogenous OT injections during 3 to 5 months of lactation period. The OT content of milk samples was estimated by competitive enzyme immunoassay (EIA). The thermal and digestive stability of OT was assessed by *in silico* and *in vitro* digestion methods.**

***Results*: The OT content of the milk samples was similar regardless of OT injections used. Further, OT was found to be stable to heat treatment and gastric pepsin digestion while it was rapidly digested during the simulated intestinal digestion. Reduced OT was digested by pepsin, implying that internal disulphide bridge of OT rendered the peptide resistant to peptic digestion. On the other hand, phenylmethylsulphonyl fluoride (PMSF), a serine protease inhibitor, abrogated the pancreatin induced digestion of OT.**

***Interpretation & conclusions*: These findings suggest that exogenous OT injections do not influence its content in milk. Further, OT present in milk is rapidly degraded during intestinal digestion, ruling out its intestinal absorption and associated adverse health consequences, if any.**

Key words Digestion - disulphide bonds - *in silico* - *in vitro* - milk - oxytocin - proteases - toxicity

Oxytocin (OT), a neuropeptide, is first synthesized in the hypothalamus of the brain as 125 amino acid precursor, and is transferred to the posterior pituitary after proteolytic processing and disulphide bond assembly[1-4]. OT is secreted into the blood stream in response to various physiological stimuli. Apart from brain, OT is also synthesized in various other tissues and organs, including the uterine epithelium, ovary, testis, vascular endothelium and heart[5]. It is now known that OT elicits its biological actions by binding to G-protein coupled receptor[4]. In structural terms, OT is a nona-peptide wherein the first cysteine residue is disulphide

bonded to the 6th cysteine, thus creating partial cyclic peptide[6-8]. The disulphide bridge in OT is essential for its interaction with the receptor and thus for biological activity[1,8].

It has been demonstrated that the plasma OT concentrations increase in response to infant suckling to induce milk let down[9,10]. In mice lacking oxytocin, milk ejection was specifically blocked and is reversed by oxytocin injections[1,11]. Therefore, synthetic OT is a drug of choice for facilitating lactation after the child birth[12]. Injections of oxytocin in dairy animals have been reported to induce milk let down[13-17]. Since OT administration rapidly induces milk let down, it is being used indiscriminately by dairy farmers in India[18]. There is paucity of information on the OT content of milk, its modulation by OT injection, and the fate of milk derived OT during milk boiling and gastrointestinal digestion. To address some of these concerns, the present study was undertaken to record the OT content of buffalo milk samples produced with and without OT injections and also to assess its stability during boiling and gastrointestinal digestion using *in silico* and simulated *in vitro* digestion methods.

Material & Methods

Standard oxytocin and all other chemicals were procured from Sigma Chemical Co., Bangalore, India unless otherwise specified. The study was conducted in the Food and Drug Toxicology Division, National Institute of Nutrition, Hyderabad, India, during 2011-2012. The Hyderabad city, Andhra Pradesh, India was divided in to four zones (South, North, East & West) and from each zone 30 samples each of milk from control and treated buffaloes were collected. A total of 241 milk samples (50 ml each) were randomly collected from milch buffaloes (*Murrah buffaloes)* during mid lactation period (between 3-5 months) from 43 different farmers. Among these 121 samples were collected from buffaloes given exogenous oxytocin injections and the remaining 120 samples were collected without oxytocin injections. The samples were immediately placed on ice and brought to the Food and Drug Toxicology Division, National Institute of Nutrition, Hyderabad and kept frozen at -20 ^{o}C until analysis. Ten ml milk samples were centrifuged in 15 ml tubes at 10000 *g* for 30 min at 4 ^{o}C to separate the fat and whey fractions. The whey fractions were collected by puncturing the tubes at the bottom, of which 50 µl aliquots were used directly for OT measurement using competitive enzyme immuno assay (EIA) as described by the manufacturer (Phoenix Pharmaceuticals, Burlingame CA, USA).

In silico studies: OT amino acid sequence was fed to the MS-Digest software *(http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest)* and digested *in silico* with various gastric and intestinal proteases to identify the possible proteolytic sites.

Reverse phase (RP) - high performance liquid chromatography (HPLC) analysis of oxytocin: The HPLC (Agilent Technologies, Model#1100, Pala Alto, CA, USA) system equipped with an auto-sampler injector and a ultraviolet-visible detector controlled by Chemstation software (Agilent, USA) was used. Separation was carried out using a analytical scale C-18 reversed-phase column (Thermo-Hypersil ODS, 5µ, 250 × 4.6 mm) protected by a C-18 guard column (20 mm × 3.9 mm) at ambient temperature and a flow rate of 1.0 ml/min. OT (100 µl injections) was eluted from the column using a gradient of 80 per cent solvent A [water with 0.1% (v/v) triflouroacetic acid (TFA)] for 8 min, followed by 20 - 40 per cent solvent B [100% acetonitrile containing 0.1 % (v/v) triflouroacetic acid] from 8 to 30 min, and 80 per cent solvent A from 30 to 40 min. OT was identified by the retention time and quantified by comparing the peak areas with pure standard.

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry: To further confirm the identity and integrity of disulphide bond of OT, the HPLC peak fractions were subjected to MALDI-TOF analysis before and after reduction with dithiothreitol (DTT). Briefly, the major HPLC peak fraction was collected and aliquoted in two portions. One of these aliquots was boiled in the presence of 10 mM DTT to achieve reduction of the disulphide bond. The peptide was then desalted and enriched by C-18 zip-tips (Millipore, India) as described by the manufacturer. The samples were mixed with equal volume of 50 per cent acetonitrile, 0.1 per cent TFA containing 4 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA), spotted on a stainless steel target plate and dried under the air. The MALDI mass spectrometer was an ABI 5800 TOF-TOF (Applied Biosystems, USA). The instrument was equipped with a nitrogen laser and operated in a positive-ion delayed extraction reflector mode. External calibration was performed with a standard peptide/protein mixture. Usually, 250 individual spectra of each spot were averaged to produce a mass spectrum.

Thermal stability: Ten ml of milk samples (spiked with 500 µg of standard OT) taken in 15 ml conical tubes were boiled for a period of 60 min. At 0, 10, 30, and 60 min time points, 1 ml samples were withdrawn, cooled immediately on ice. The samples were subjected to ultrafiltration through 3 kDa cut-off centrifugal filters (Amicon Ultra, Millipore, India) at 5000 *g* for 30 min at 4 °C. The filtrate containing low molecular mass components (<3kDa) were collected and 50 µl aliquots of these samples were immediately analyzed by HPLC.

Digestion with simulated gastric fluid: The protocol described by Astwood *et al*[19], was followed with minor modifications in accordance with the United States Pharmacopeia[20]. Simulated gastric fluid (SGF) consisted of 3.2 mg/ml pepsin in 0.03 M NaCl at *p*H 2. Aliquots of SGF (200 µl) were placed in 1.5 ml tubes to which equal volumes of OT (50 µg) either in water or in milk (spiked with OT and boiled for 20 min to simulate household boiling) was added and incubated in a shaking water bath at 37 °C for a period of 2 h. At the beginning (0 h) and at the end of incubation (2 h), 100 µl aliquots of the samples were analyzed by HPLC. To study the effect of disulphide bond integrity on pepsin digestion, OT peptide was first reduced with DTT and alkylated with iodoacetamide as described previously[21] and then subjected to peptic digestion exactly as above.

Digestion with simulated intestinal fluid: Simulated intestinal fluid (SIF) was prepared as described by Astwood *et al*[19], consisting of 10 mg/ml pancreatin in 0.05 M NaH_2PO_4 buffer *p*H 7.5 (PB). Aliquots (65 µl) of SIF were diluted to 200 µl with PB and mixed with equal volumes of OT (50 µg) for 2 h. All the incubations were carried out in triplicates. At 0, 60 and 120 min time points, 100 µl aliquots were analyzed by HPLC. Digestion reactions were also carried out similarly with pancreatin solutions pretreated with 10 mM phenylmethylsulphonyl fluoride (PMSF), a specific inhibitor of serine proteases.

Statistical analysis: Each analysis was done in triplicates and the experiments were repeated at least twice to generate six observations. The per cent OT was computed assuming the peak area of OT in control (without heat, pepsin or pancreatin treatment) sample as 100 per cent. Means between treatments were compared by one-way analysis of variance (ANOVA) followed by post-hoc Least significant differences (LSD) test (SPSS software, version 11.0, USA).

Results

The OT content of milk samples varied from 0.015 to 0.17 ng/ml with a mean OT content of 0.06±0.03 ng/ml (n=241). The mean OT content of milk samples obtained without OT injections (0.06±0.031 ng/ml, n=120) and with OT injections (0.06±0.028 ng/ml, n=121) remained similar.

In silico digestion analysis revealed that pepsin (gastric protease), chymotrypsin and elastase (intestinal proteases) possessed specific proteotolytic cleavage sites in OT amino acid sequence while trypsin (intestinal protease) had no such sites (Table).

HPLC analysis of oxytocin and characterization of its identity by MALDI-MS: HPLC analysis of OT either in its pure form (Fig. 1A) or when it is spiked in to milk (Fig. 1B) revealed several minor peaks at about 4 to 6 min and a major peak at 10.6 min, which could be OT. To confirm, this peak fraction was analysed using MALDI-TOF MS spectroscopy, which demonstrated peaks at 1007 atomic mass unit (amu), representing molecular weight of OT with internal disulphide bond, and two other peaks at 1029 and 1045 (amu), representing the Na (22 amu) and K (38 amu) adducts of OT (Fig. 2A). Similarly, reduced OT showed three peaks similar to that of native OT, except that all the peaks were shifted to a 2 amu higher mass, consistent with the addition of two hydrogen atoms during reduction (Fig. 2B). The reduced and alkylated OT also produced a similar chromatographic profile, except that it was eluted 0.9 min later compared to native OT (data not shown).

Stability of oxytocin: The mean per cent OT remained similar during milk boiling over a period of 0 (100 ± 4.7%) to 60 min (96.4 ± 7.4%). The mean per cent OT remained similar after 0 h (100 ± 5.2% and 100 ± 9%) or 2 h (97.8 ± 10.2% and 101 ± 10.1%) digestion with gastric pepsin at *p*H 2 either in the absence (Fig. 3A) or presence of milk (Fig. 3B), respectively. In contrast, incubation of reduced OT with gastric pepsin for a period of 2 h led to 99.6 per cent ± 0.2 and 99.3 ± 0.4 per cent reduction in the initial peptide concentration (100%) either in the absence and presence of milk, respectively (Fig. 3).

The mean per cent OT content significantly reduced upon incubation with pancreatin for a period of 1 h (0.37 ± 0.2%) compared to 0 h (100 ± 7.2%) time point in the absence (Fig. 3C) or presence of milk (Fig. 3D), respectively. Further, time course studies revealed

Table. *In silico* digestion of oxytocin with gastric and intestinal proteases

Enzyme	*Amino acid sequence of generated peptides	Predicted molecular weight (Da)	Cutting site after amino acid (no. in the sequence)
-	C-Y-I-Q-N-C-P-L-G	1010.44	No cutting
Pepsin	Q-N-C-P-L-G	574.26	3rd amino acid
	Q-N-C-P-L	631.28	3rd and 8th amino acid
	I-Q-N-C-P-L	687.34	2nd and 8th amino acid
Chymotrypsin	I-Q-N-C-P-L	687.34	2nd and 8th amino acid
	I-Q-N-C-P-L-G	744.37	2nd amino acid
	C-Y-I-Q-N-C-P-L	953.42	8th amino acid
Trypsin	C-Y-I-Q-N-C-P-L-G	1010.44	No cutting
Elastase	I-Q-N-C-P-L	687.34	2nd and 8th amino acid
	I-Q-N-C-P-L-G	744.37	2nd amino acid
	C-Y-I-Q-N-C-P-L	953.42	8th amino acid

*Amino acid sequence is given in FASTA format. The amino acids are Cysteine (C), Tyrosine (Y), Isoleucine (I), Glutamine (Q), Asparagine (N), Proline (P), Luecine (L) and Glycine (G)

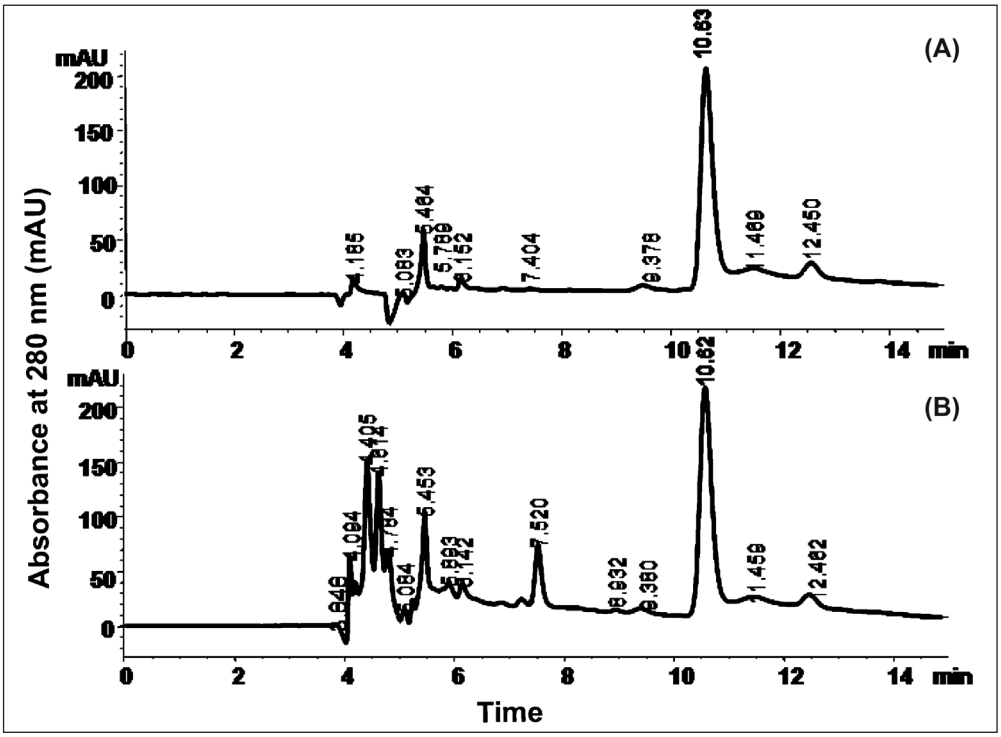

Fig. 1. Representative HPLC chromatograms of oxytocin in the absence **(A)** and presence **(B)** of milk: One hundred µl aliquots of purified oxytocin (50 µg/ml in 50mM phosphate buffer, *p*H 7.5) or in milk (spiked with oxytocin, 50 µg/ml) were subjected to reverse phase HPLC analysis. The peak eluting at 10.6 min represents oxidized oxytocin. mAU, mili Absorption Unit.

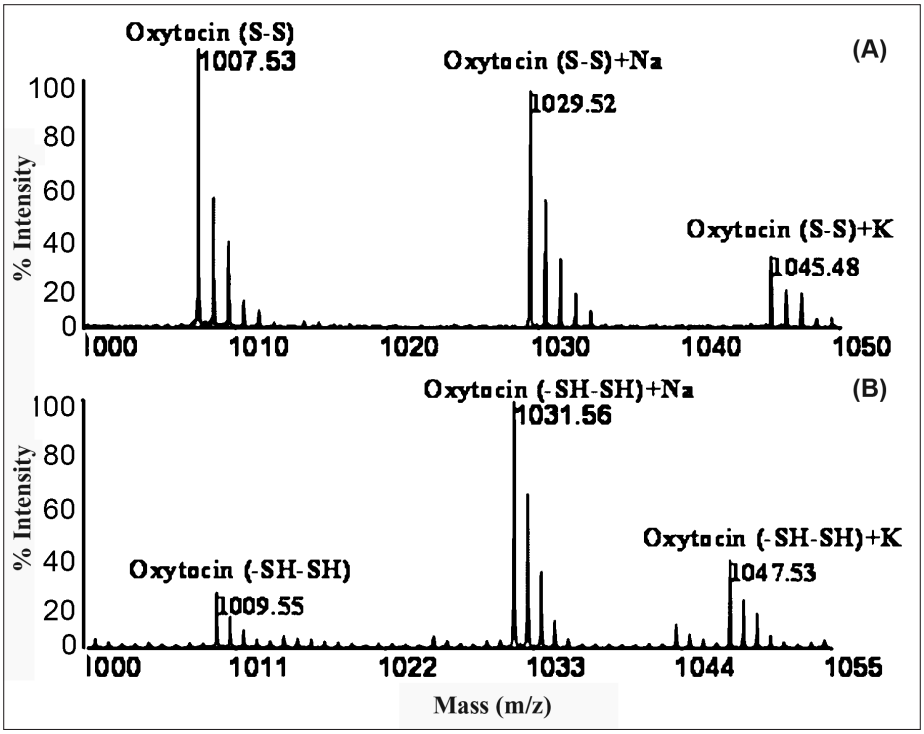

Fig. 2. MALDI-TOF analysis of oxytocin disulphide bond integrity: The oxytocin peak fraction eluting at 10.6 min was subjected to MALDI-TOF mass analysis before **(A)** and after **(B)** reduction with dithiothreitol (DTT). The shift of 2 amu mass, in oxytocin and its Na and K adducts after reduction with DTT indicates addition of 2-H atoms.

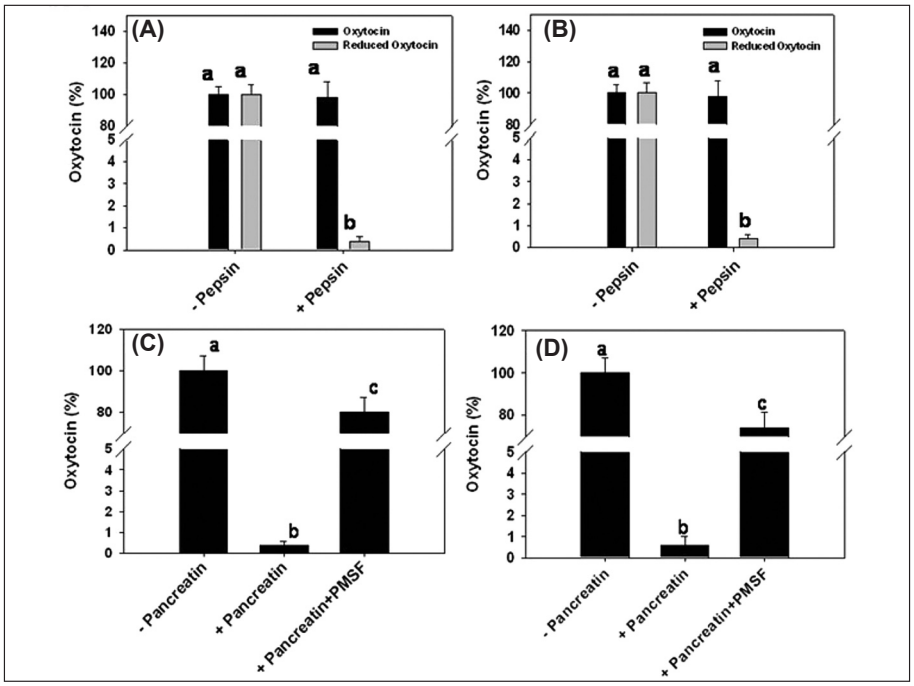

Fig. 3. Stability of oxytocin during gastric and intestinal phase of digestion: Native or reduced oxytocin in the absence **(A)** or presence of milk **(B)** was incubated 0 to 2 h with gastric fluid at 37 °C. Oxytocin in the absence **(C)** or presence of milk **(D)** was incubated with intestinal fluid with and without 10 mM phenylmethyl sulphonyl fluoride (PMSF) for 0 to 1h. Aliquots (100 µl) of these samples were analyzed by HPLC. The % oxytocin was calculated assuming the peak area of oxytocin at 0 h as 100%. The bars represent mean±SD (n=6) and bars that do not share common superscript differ significantly (P<0.05).

that as little as 5 min incubation with pancreatin was sufficient to digest more than 90 per cent OT (data not shown). Pretreatment of pancreatin with PMSF abrogated the pancreatic digestion of OT as evidenced by higher mean per cent OT remained after the digestion (Fig. 3). Similarly, pancreatin also digested the reduced OT completely (data not shown).

Discussion

It is known that OT is naturally present in milk and is derived from the mother. Further, transfer of small amount of labelled OT from the maternal blood to milk has been demonstrated in humans[22]. Therefore, it is possible that part of the injected OT is excreted through milk. However, in the current study OT content of milk samples remained similar regardless of the OT injections used. It has been reported that the half-life of injected OT is around 5-7 min in the plasma[14, 23], which might hinder its excretion in the milk. Non significant effect of OT injections on actual milk OT levels has been reported in Brown Swiss cows[24]. It is noteworthy that mean OT content of milk samples was (0.06±0.02 ng/ml) very close to the minimum detection limit (0.07 ng/ml) of the EIA assay method used in our study. Therefore more sensitive methods need to be used for analysis of oxytocin in milk.

In silico enzymatic digestion has revealed that OT is digested by gastric pepsin and intestinal proteases to smaller peptides with the exemption of trypsin, consistent with the lack of positively charged amino acid residue in the primary structure of OT. Initial studies to estimate the OT content of milk after the digestion reactions using EIA method failed due to severe interference of gastric, intestinal proteases and other buffer salts used during digestion (data not shown). Therefore, we used a RP-HPLC method for analysis of OT stability in milk. Purified OT was eluted as a symmetric peak at 10.6 min on reverse phase column. The greater retention of OT on RP column was consistent with the presence of hydrophobic amino acids in the peptide back-bone. However, HPLC analysis of milk samples or milk peptides obtained after the 3kDa cut-off filtration did not show peak with retention time close to that of OT standard (data not shown). Since OT content of milk is in sub-nano gram range, UV absorption based methods may not be sensitive enough to detect such concentrations. To overcome this technical limitation, we spiked milk samples with exogenous OT. The HPLC analysis of OT spiked milk samples produced peak with similar retention to that of pure OT standard at 10.6 min, except that several other minor contaminating peaks also appeared between 4-8 min. The oxidized form of OT (with internal disulphide bond intact) is biologically active, but it is also reported to exist in reduced form[8]. MALDI-MS analysis of HPLC peak fraction (eluted at 10.6 min) gave a molecular mass indicative of oxidized OT (1007 Da) and its sodium (1007+22 Da) and potassium (1007+39 Da) adducts. As expected, reduction of OT with DTT, led to an expected increase in the molecular mass by 2 amu (due to addition of 2 hydrogen atoms), implying that under the experimental conditions the integrity of OT disulphide bond was not affected. Therefore, this method was ideally suited to study the thermal and digestive stability of OT either in the absence or presence of milk.

Boling of milk samples for 0 to 60 min did not decrease the actual OT content as assessed by HPLC method, suggesting that OT was stable to heat denaturation. Therefore, milk processing methods such as pasteurization (70-80 ^{o}C) should not have influence on milk OT levels. Similarly, peptic digestion of OT at gastric *p*H for 2 h has failed to hydrolyze the OT in the absence or presence of milk. Further, increasing the peptic digestion time to 12 h did not result in OT hydrolysis (data not shown). OT or arginine-vasopressin are reported to be stable during digestion with human gastric juice[25]. These results were in contrast to the *in silico* studies where specific proteolytic sites for pepsin were detected at the 3rd and 8th amino acid of OT. Interestingly, reduced OT was completely digested by pepsin, indicating that partial cyclic nature of peptide due to disulphide bond could resist the peptic digestion. The fact that the OT spiked milk samples were boiled before the digestion, and yet did not show peptic digestion implied no effect of milk boiling on the redox-status of OT. These results suggested that OT was stable to heat and gastric pepsin digestion, which could be attributable to its internal disulphide bond.

In contrast to pepsin, incubation of OT with pancreatin led to complete digestion either in the absence or presence of milk, and similar results were observed with reduced OT as well (data not shown). These results were in agreement with *in silico* studies with pancreatic serine proteases chymotrypsin and elastase. Pre-incubation of pancreatin with PMSF abrogated the pancreatin induced digestion of OT, confirming the role of serine proteases in pancreatin mediated digestion. Further, incubation of OT with purified chymotrypsin led to its hydrolysis (data not shown). Corroborating these observations, the bioavailability of OT or its analogues was reported to

be very poor when given sublingually in humans or orally to pigs[26,27]. The bioavailability of OT analogues has been reported to be increased significantly when the pancreatic juice was diverted via a pancreatic duct catheter[27]. Fjellestad-Paulsen *et al*[25], have reported that OT degradation activity is exclusively resided in the intestinal juice rather than mucosal preparations. These observations together with *in silico* and *in vitro* digestion studies reported in the present study confirm the role of intestinal proteases in digesting the OT.

In conclusion, the results of the present study demonstrate that OT is a natural constituent of buffalo milk and injection of exogenous OT for milk letdown has no effect on its milk content. Further, despite its stability to heat and gastric pepsin digestion, OT present in milk is rapidly degraded during intestinal phase of digestion, thus ruling out the possibility of its intestinal absorption and speculated adverse health consequences, if any.

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