

## The effect of chronic oxytocin-treatment on the bovine mammary gland immune system

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**ABSTRACT:** The aim of this study was to evaluate the effect of chronic oxytocin (OT) treatment on the mammary gland immune system. In *Experiment I* fourteen healthy cows were used to study the effect of chronic intramuscular (im) OT administration on concentration of milk somatic cells and white blood cells (WBC). Cows in the OT-group (6) were im injected with 50 IU OT (5 ml) whereas animals of the C-group (6) were im injected with 5 ml of saline (9 g/l) for eight days (Day 1–8) before each milking. Milk samples were taken during normal milking time on Day 0–3, 5, 7, 9–11 and 18. Blood samples were taken immediately after each milking and analysed for WBC count, polymorphonuclear neutrophils, potassium (K), sodium (Na) and chloride (Cl) ions, and blood lactose. All milk samples were analysed for somatic cell counts (SCC), lactose, Na, Cl and electrical conductivity (EC). Furthermore mRNA expression of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, IL-8 and cyclooxygenase-2 (COX2) in milk cells were measured via real-time RT-PCR. None of the investigated milk and blood parameters changed significantly in response to the OT treatment. The mRNA-expression of TNF $\alpha$  decreased ( $P < 0.05$ ) to a minimum on Day 3 in response to OT administration. IL-1 $\beta$  and IL-6-mRNA expression decreased ( $P < 0.05$ ) to a minimum within three day. IL-8 and COX2 expression did not change in response to OT treatment. In *Experiment II* twelve cows, randomly divided into two groups of six, were used to investigate the effect of chronic im OT administration on mammary tissue. Cows were im administered 50 IU OT (OT-group) or 5 ml saline (9 g/l; C-group) before each milking during eight days. Biopsy samples were taken after every morning milking. The mRNA expression of various inflammatory factors and the tight junction (TJ) proteins occludin (OCLN) and zonula occludens (ZO)-1, ZO-2 and ZO-3 were measured via real-time RT-PCR. TNF $\alpha$ -mRNA expression decreased (Day 2 with  $P < 0.05$ ) within the first four days of OT administration and increased ( $P < 0.05$ ) in the C-group on Day 2. IL-1 $\beta$  expression levels of the OT-group increased transiently and decreased on Day 3 and in the C-group values increased significantly on Day 3 as compared to Day 0. IL-6 expression in the OT-group decreased ( $P < 0.05$ ) to a minimum on Day 1 and increased ( $P < 0.05$ ) as compared to Day 0 on Day 7 and increased significantly on Day 1 and Day 5 compared to Day 0 in group C. IL-8 and COX2 expression did not change in response to OT administration. The mRNA-expression of OCLN and ZO-3 decreased ( $P < 0.05$ ) as compared to Day 0 with a minimum on Day 7. ZO-1 and ZO-2 expression did not change due to OT administration. ZO-2-mRNA expression in C-group decreased significantly on Day 2 compared to Day 0. In conclusion, chronic OT administration induced increasing SCC and EC levels in milk as well as K and lactose in blood while nearly all investigated cytokines in milk cells and mammary tissue were down regulated. The mRNA expressions of the TJ proteins OCLN and ZO-3 were down-regulated in response to the OT treatment what indicates an increasing TJ permeability. Besides the effect on TJ proteins there was no obvious change of the immunological competence of the mammary gland in response to OT. However, a more complete milk ejection should help to remove pathogens during milking.

**Keywords:** mastitis; oxytocin; cattle

For decades oxytocin (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 either the sucking calf, hand or machine milking. OT causes the contrac-

tion of myoepithelial cells surrounding the alveoli forcing the milk into the milk ducts and cisternal cavities (Lefcourt and Akers, 1983; Nostrand et al., 1991; Bruckmaier et al., 1994; Knight et al., 1994; Pfeilsticker et al., 1996). OT also enhances the differentiation and proliferation of the mammary myoepithelial cells (Sapino et al., 1993). Many dairies use synthetically manufactured OT to facilitate milk ejection, and also as a treatment for mastitis (Guterbock et al., 1993; Knight et al., 2000; Macuhova et al., 2004).

It is thought that high doses of OT relax the mammary gland tight junctions (TJ) (Stelwagen et al., 1997; Nguyen and Neville, 1998), thus potentially causing increased milk somatic cells counts (SCC) and electrolyte levels, and decreased lactose levels in milk, while increasing lactose levels in blood (Linzell, 1967; Linzell et al., 1975; Allen, 1990; Stelwagen et al., 1997; Nguyen and Neville, 1998). However, TJ also become leaky during intramammary infection (IMI) (Stelwagen et al., 1997; Nguyen and Neville, 1998). TJ are the most apically situated part of the junctional complex and surround each adjacent epithelial cell like gaskets. In the mammary gland TJ are dynamic structures. Around parturition and onset of lactation their state changes from a permeable condition during pregnancy to an impermeable condition during lactation (Nguyen and Neville, 1998; Schneeberger and Lynch, 2004). TJ are formed by transmembrane proteins such as occludin (OCLN) and the cytoplasmatic plaque proteins zonula occludens 1 to 3 (ZO-1, ZO-2, ZO-3) (Haskins et al., 1998; Nguyen and Neville, 1998).

The aim of this study was to evaluate the effect of chronic intramuscular (im) OT treatment on mammary immune status and integrity of TJ. We hypothesized that OT treatment influences the immune system of the mammary gland, and that high doses of OT induce TJ permeability to allow migration of immune cells from blood into milk that would result in a more efficient immune response to infection.

## MATERIAL AND METHODS

### Animals and husbandry

For both experiments animal care and use protocols were submitted to and approved by the responsible Animal Care and Use Committee (Regierung von Oberbayern, Germany).

In *Experiment I* fourteen German Braunvieh cows were used to investigate the effect of chronic im OT administration on milk somatic cells and white blood cells (WBC). In particular, the milk somatic cell mRNA expression of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukins (IL, -1 $\beta$ , -6, -8) as well as the key enzyme of prostaglandin biosynthesis cyclooxygenase-2 (COX2) was investigated. Four cows were in their first lactation, six in their second, three in their third, two in their fourth and one in its sixth. All animals were free of clinical udder health problems. The animals were kept in a loose-housing barn and milked twice daily at 500 and 1 600 in a milking parlour. Feeding consisted of maize and grass silage, hay and concentrate according to their individual production levels. Water was available *ad libitum*. Cows were randomly assigned to two groups of seven cows each, OT- and control (C)-group. All quarters were bacteriologically negative immediately before the investigation.

In *Experiment II* twelve German Braunvieh cows were used to investigate the effect of chronic im OT administration on mammary tissue. Besides the cytokine- and COX2-mRNA expression, mRNA expression of the tight junction proteins OCLN, ZO-1, ZO-2 and ZO-3 were also measured. The animals were kept in a tethered barn and milked twice daily at 500 and 1 700. They were fed with maize silage, hay and concentrate. Water was available *ad libitum*. Five cows were in their first lactation, six in their second and one in its fifth. Cows were randomly selected into two groups of six cows each, OT- and C-group. All cows were bacteriologically negative before the investigation.

## Experimental procedure

### Experiment I

The experiment lasted for 19 days. From Day 1 to Day 8 one group was administered an im injection of 50 IU oxytocin (Oxytocin-S, Intervet GmbH, Tonisvorst, Germany) while the C group was injected im with 0.9% sterile saline before each milking. Throughout the experiment milk from one selected quarter of every cow was collected at the morning milking in a quarter milker (GEMA – Bruno Gelle GmbH, Wangen) on Day 0, 1, 2, 3, 5, 7, 9, 10, 11, 18. Immediately after milking, a blood sample was collected into vacutainer tubes containing EDTA for haematological studies.

### Measurement of milk constituents

Lactose concentration and somatic cell counts (SCC) were analysed in the laboratory of the Milchprüfing Bayern e.V. (Wolnzach, Germany), by using a MilkoScan 4500 analyser (FOSS Elektrik, Hillerød, Denmark). Whole milk aliquots of each sample were frozen at  $-20^{\circ}\text{C}$  immediately after sampling for the determination of electrical conductivity (EC), sodium (Na) and chloride (Cl) ion concentrations.

Na and Cl in milk were measured by potentiometer determination with ion selective electrodes model 9811 and model 9617BN (pH; Ise Meter, Modell 720 Aplus, Orion Research, Beverly MA, USA). EC was measured at  $25^{\circ}\text{C}$  using the LDM electrode from WTW (LDM 130, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany).

### Determination of blood parameters

Total and differential WBC were measured with a Advia 120 (Bayer Diagnostics, Fernwald, Germany). Blood Na and Cl were measured with a Modular E170 (Roche Diagnostics GmbH, Mannheim, Germany) at Vet Med Labor GmbH (Ludwigsburg, Germany).

Blood lactose was measured by an enzymatic method (R-Biopharm AG, Darmstadt, Germany).

### Milk cell isolation

Quarter milk samples (200 g) were centrifuged to obtain the cell pellet according to Sarikaya et al. (2004). After washing twice with PBS the pellet was resuspended in 500  $\mu\text{l}$  peqGold TriFast™ (Peqlab Biotechnologie GmbH, Erlangen, Germany) for total RNA isolation.

### Experiment II

Cows were milked at 05:00 and 17:00 hours for the duration of the experiment. Beginning at evening milking time on Day 0, cows were administered 50 IU OT im before every milking for eight consecutive days.

### Biopsy procedure

On Day 0 after the morning milking cows were sedated with an intravenous injection of 0.8 ml xyla-

zine (Xylazin 2%, CP-Pharma, Burgdorf, Germany). The selected glands were clipped and cleaned. The biopsy site, which was carefully selected to avoid the cisternal region and larger subcutaneous blood vessels, was washed and sterilized with 70% ethanol. The glands were anaesthetized by a subcutaneous injection of 3.5 ml lidocain (Lidocain 2%, Chassot, Ravensburg, Germany). A puncture incision was made through the skin with a sterile single use scalpel. One or two biopsies of one single quarter was carried out through this incision using a human Bard® Magnum™ Biopsy Instrument with a Bard® Magnum® Core Tissue Biopsy Needle (12 g  $\times$  10 cm) (Bard Inc., Covington, USA), a core of mammary tissue (20 mg) was extracted. Until the next morning the incision was dressed with a swab and an adhesive Fixomull® stretch (Beiersdorf AG, Hamburg, Germany) plaster to avoid wound infection. More mammary biopsies were taken in the manner described for the next eight days (1–8). Neither during the experiment nor after it did any cow need anti-inflammatory or antibiotic therapy. The wound dressing was removed two days after the last biopsy. During this period cows were milked twice daily.

### Mammary tissue preparation

Tissue homogenisation was implemented by using a FastPrep 120 after transferring the tissue samples in Green-Caps (MP Biomedicals, Solon, OH) with Matrix Green (by Obiogene, Morgan Irvine, CA). Total RNA of mammary biopsy samples was isolated using peqGold TriFast™ (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturers instruction.

### Total RNA quantification and complementary DNA (cDNA) synthesis

The optical density (OD) was determined at three different dilutions of the final RNA preparations at 260 nm in order to quantify the extracted RNA. RNA integrity was verified by the  $\text{OD}_{260}/\text{OD}_{280}$  absorption ratio 1.6–1.8. Synthesis of first strand complementary DNA (cDNA) was performed with reverse transcriptase (MMLV-RT, Promega, Madison, WI, USA) and random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturers instructions. The con-

centration of reverse transcribed total RNA was 25 ng/μl.

### Oligonucleotide primers

All the primers for each housekeeping or target gene were synthesized commercially (MWG Biotech, Ebersberg, Germany) using published bovine specific primer sequences (Wittmann et al., 2002; Schmitz et al., 2004). All primer information is listed in Table 1.

### Quantification by real-time PCR

The quantitative analysis of the different RT-products was performed on the RotorGene 3000

(Corbett Life Science, Sydney, Australia) via one-step qRT-PCR. 3.8 μl of extracted mRNA solutions (15 ng/μl) were used. According to the manufacturers instruction 6.2 μl Master Mix (Super Script™ III Platinum SYBR® Green One-Step qRT-PCR Kit, Invitrogen GmbH, Karlsruhe, Germany) was prepared including 5 μl 2 × SYBR® Green Reaction Mix, 0.2 μl SYBR® Green One-Step Enzyme Mix, 0.5 μl (10 pM) forward primer and 0.5 μl (10 pM) reverse primer.

Crossing points (CP) were achieved by the RotorGene software 5.0. A normalisation of the target genes was performed with an endogenous standard. Therefore, the expression levels of two reference genes glyceraldehyde-3-phosphatedehydrogenase (GAPDH) and Ubiquitin (UbQ) were measured. The relative mRNA levels were calculated by normalisation of the CP of the target gene

Table 1. Sequences of PCR primers (for = forward; rev = reverse), PCR product length and EMBL accession number of the used nucleic acid sequences as well as product specific Rotor-Gene-PCR conditions

Primer	Sequence (5' → 3')	Length (bp)	EMBL (accession No.)	Annealing (°C)
UbQ for	AGA TCC AGG ATA AGG AAG GCA T	198	Z18245	60
UbQ rev	GCT CCA CCT CCA GGG TGA T			
GAPDH for	GTCTTCACTACCATGGAGAAGG	197	U85042	58
GAPDH rev	TCATGGATGACCTTGCCAG			
TNF-α for	TAA CAA GCC GGT AGC CCA CG	277	AF011926	64
TNF-α rev	GCA AGG GCT CGG GAT GGC AGA			
COX2 for	TCT TCC TCC TGT GCC TGA T	358	AF031698	64
COX2 rev	CTG AGT ATC TTT GAC TGT GG			
IL-1β for	TTC TCT CCA GCC AAC CTT CAT T	198	M37211	60
IL-1β rev	ATC TGC AGC TGG ATG TTT CCA T			
IL-6 for	GCT GAA TCT TCC AAA AAT GGA GG	200	NM173923	60
IL-6 rev	GCT TCA GGA TCT GGA TCA GTG			
IL-8 for	ATG ACT TCC AAG CTG GCT GTT G	149	AF232704	60
IL-8 rev	TTG ATA AAT TTG GGG TGG AAA G			
OCN for	CTG GAT CAG GGA ATA TCC ACC	176	AJ313191	64
OCN rev	ACT CTT CAC TTT CTT CTC TAT AGT		AJ313182	
ZO-1 for	AGA AGA TAG CCC TGC AGC CAA	272	AJ313188	64
ZO-1 rev	CCT CTC CTT TGT TAA AAC TAA GTC		AJ313183	
ZO-2 for	GAC CAG ATT CTG AAG GTG AACACA	197	AJ313187	64
ZO-2 rev	TCC TTC TCA CAT TCA AAG TGG CT		AJ313184	
ZO-3 for	AAC GAC GTG GGC ATC TTC GT	251	AJ313186	64
ZO-3 rev	GTG CGG ATG TAG AAG GAG TC		AJ313185	

UbQ = ubiquitin; GAPDH = glyceraldehydes-3-phosphatedehydrogenase; TNFα = tumor necrose factor alpha; COX2 = cyclooxygenase-2; IL = interleukin; OCLN = occludin; ZO = zonula occludens

to the mean CP of the two reference genes UbQ and GAPDH.

## Statistical analyses

Data are presented as means  $\pm$  SEM. Differences between OT treated and C quarters were tested for significance ( $P < 0.05$ ) by analysis of variance, using the MIXED procedure of the SAS program. The MIXED model included the animal and the sample as class variables. Within each sample the treatment was the repeated term. Because SCC values could not be assumed to be normally distributed they were logarithmised ( $\log_{10}$ ) for statistical calculations.

## RESULTS

### Experiment I

#### Milk parameters

SCC values showed a numerical but non-significant increase at Day 2 and Day 3 in the OT-group whereas SCC levels in C-group remained unchanged (Figure1).

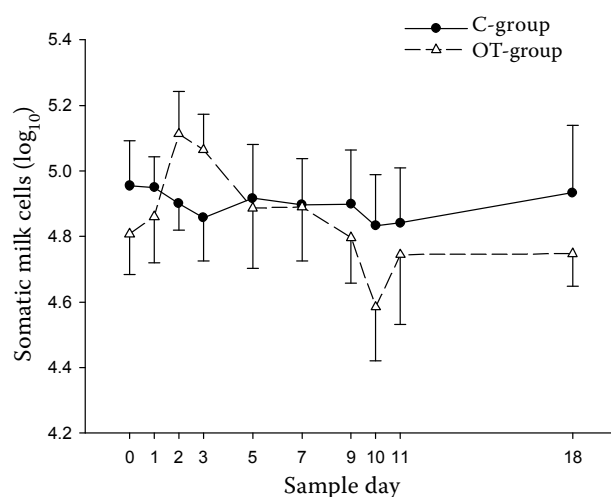


Figure 1. Somatic cell counts before (Day 0), during (Day 1–7) and after (Day 9–18) a daily *i.m.* treatment with 50 IU oxytocin

C-group: means of cows treated with 5 ml of saline as controls

OT-group: means of cows treated with 50 IU oxytocin (5 ml)

As documented in Table 2 none of the investigated milk parameters changed significantly in response to chronic OT treatment. A numerical decrease was observed for lactose levels from Day 2 to Day 7, whereas Na and Cl levels increased numerically within the first 3 days in response to the OT treatment. A numerical increase was observed for EC levels between Day 2 and Day 9.

## Blood parameters

All investigated blood parameters documented in Table 3 did not change significantly in response to chronic OT treatment. Potassium (K) and blood lactose levels of the OT-group showed a slight non-significant increase on Day 2.

## Cytokine expression in milk cells

TNF $\alpha$ -mRNA expression levels in the OT-group decreased ( $P < 0.05$ ) on Day 2 and reached a minimum on Day 3 and increased thereafter with variations to baseline whereas in C levels increased slightly up to Day 3 and decreased thereafter to baseline values.

IL-1 $\beta$  and IL-6-mRNA expression values in the OT-group decreased until Day 3 ( $P < 0.05$ ) and increased thereafter to baseline levels. C-group values for both parameters undulated during the course of investigation.

IL-8 and COX2-mRNA expression did not change significantly in response to the OT treatment.

### Experiment II

#### Cytokine expression in mammary tissue

TNF $\alpha$ -mRNA expression levels in the OT-group decreased until Day 4, significantly on Day 2 ( $P < 0.05$ ) and then tended to increase. For the C-group, values increased significantly on Day 2 compared to Day 0, then decreased to baseline levels.

IL-1 $\beta$ -mRNA expression values in the OT-group increased within the first two days then decreased to its minimum ( $P < 0.05$ ) on Day 3, and then increased again and remained elevated until the end of the experiment. In the C-group, IL-1 $\beta$ -mRNA expression values increased slightly but significantly reaching a maximum on Day 3 and decreased thereafter to baseline levels.

Table 2. Investigated milk parameter levels before (Day 0), during (Day 1–7) and after (Day 9–18) long term intramuscular (im) infusion of 50 IU oxytocin

Parameter	Sample day									
	0	1	2	3	5	7	9	10	11	18
Lactose (g/l)	OT	4.7 ± 0.1	4.7 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.7 ± 0.2	4.7 ± 0.1
	C	4.7 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	4.3 ± 0.4
EC (mS/cm)	OT	4.3 ± 0.3	4.3 ± 0.3	4.5 ± 0.3	4.5 ± 0.4	4.4 ± 0.4	4.5 ± 0.4	4.3 ± 0.4	4.3 ± 0.4	4.3 ± 0.3
	C	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.2 ± 0.2	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3
Na (mmol/l)	OT	15.9 ± 1.5	16.7 ± 1.7	17.0 ± 1.6	18.0 ± 1.8	17.3 ± 1.6	13.7 ± 2.9	17.7 ± 2.2	18.0 ± 2.4	15.6 ± 1.2
	C	17.6 ± 1.5	16.5 ± 1.4	16.5 ± 1.5	16.2 ± 1.5	16.8 ± 1.5	16.2 ± 1.3	16.1 ± 1.4	15.9 ± 1.2	15.7 ± 1.1
Cl (mmol/l)	OT	40.2 ± 3.1	40.8 ± 3.6	44.0 ± 3.9	45.0 ± 3.9	43.4 ± 4.2	42.0 ± 5.2	42.0 ± 5.1	41.2 ± 5.6	39.2 ± 4.2
	C	39.7 ± 2.4	39.4 ± 2.2	40.2 ± 2.7	40.0 ± 3.3	40.5 ± 2.9	40.6 ± 2.4	39.0 ± 2.9	40.7 ± 2.4	40.5 ± 2.6

Lactose = milk lactose; EC = electrical conductivity; Na = sodium; Cl = chloride; OT = means of all cows treated with 50 IE oxytocin im (5 ml); C = means of control cows treated with 5 ml saline im (9 g/l)

Table 3. Investigated blood parameter levels before (Day 0), during (Day 1–7) and after (Day 9–18) long term intramuscular (im) infusion of 50 IU oxytocin

Parameter	Sample day									
	0	1	2	3	5	7	9	10	11	18
WBC (G/l)	OT	6.1 ± 0.3	5.7 ± 0.3	6.2 ± 0.2	6.3 ± 0.3	5.9 ± 0.2	5.8 ± 0.3	5.3 ± 0.3	5.3 ± 0.2*	6.9 ± 0.5
	C	6.2 ± 0.6	6.5 ± 0.5	6.5 ± 0.7	6.8 ± 0.8	6.4 ± 0.6	6.4 ± 0.6	6.2 ± 0.5	6.2 ± 0.5	6.7 ± 0.7
PMN (%)	OT	48.2 ± 4.9	46.5 ± 5.9	43.1 ± 2.7	46.7 ± 4.4	45.5 ± 2.6	39.3 ± 3.7	52.8 ± 5.4	40.3 ± 1.7	45.8 ± 5.1
	C	43.0 ± 4.4	51.6 ± 4.7	45.3 ± 3.1	42.4 ± 2.2	40.9 ± 1.3	40.9 ± 2.8	46.4 ± 5.3	46.6 ± 6.5	38.2 ± 2.1
bNa (mmol/l)	OT	143.1 ± 0.6	143.7 ± 0.5	141.7 ± 2.7	143.4 ± 0.7	142.8 ± 1.4	143.0 ± 0.4	144.2 ± 1.1	144.7 ± 0.6	144.3 ± 1.2
	C	143.3 ± 0.6	140.4 ± 2.9	143.6 ± 1.6	143.4 ± 0.4	142.6 ± 1.0	144.0 ± 0.4	143.1 ± 0.9	144.7 ± 0.8	144.3 ± 0.6
bCl (mmol/l)	OT	98.4 ± 0.5	100.6 ± 1.0	95.0 ± 1.7	97.0 ± 1.5	100.4 ± 2.7	95.7 ± 0.6	97.7 ± 1.2	99.3 ± 1.0	99.2 ± 2.2
	C	98.3 ± 1.2	98.0 ± 2.4	96.9 ± 1.2	97.7 ± 0.7	99.9 ± 1.7	97.0 ± 1.1	96.9 ± 1.6	99.9 ± 0.9	98.7 ± 1.5
K (mmol/l)	OT	4.2 ± 0.1	4.4 ± 0.1	4.6 ± 0.2	4.4 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.3 ± 0.1
	C	4.3 ± 0.1	4.1 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.3 ± 0.1
β-lactose (g/l)	OT	38.7 ± 13.0	51.7 ± 18.0	73.6 ± 22.4	46.7 ± 10.9	59.9 ± 19.5	54.3 ± 12.6	55.0 ± 17.8	49.8 ± 19.9	51.2 ± 19.1
	C	43.7 ± 12.5	41.4 ± 12.1	36.6 ± 10.5	48.5 ± 17.4	41.4 ± 13.7	46.4 ± 14.3	31.6 ± 7.6	33.3 ± 9.8	62.9 ± 38.1

WBC = leukocytes; PMN = polymorphonuclear neutrophils; bNa = blood sodium; bCl = blood chloride; K = potassium; b-lactose = lactose measured in blood; OT = means of all cows treated with 50 IE oxytocin im (5 ml); C = means of control cows treated with 5 ml saline im (9 g/l); \* means within day are significantly different ( $P < 0.05$ ) between OT- and C-group

Table 4. Changes of mRNA expression of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and COX2 in milk cells relative to sample day = 0 (log<sub>2</sub>), during (Day 1–7) and after (Day 9–18) a long term intramuscularly (*i.m.*) injection of 50 IU oxytocin

Factor	Group	Sample day								
		1	2	3	5	7	9	10	11	18
TNF $\alpha$	OT	-0.9 $\pm$ 0.3	-2.3 $\pm$ 0.8 <sup>*a</sup>	-2.6 $\pm$ 0.4 <sup>**a</sup>	-1.4 $\pm$ 0.6	-0.9 $\pm$ 0.4	-1.3 $\pm$ 1.4	0.2 $\pm$ 0.5	0.4 $\pm$ 0.7	-0.8 $\pm$ 0.7
	C	-0.8 $\pm$ 0.5	-0.1 $\pm$ 0.3	0.3 $\pm$ 0.4	-0.2 $\pm$ 0.3	-0.8 $\pm$ 0.6	-0.6 $\pm$ 0.6	0.2 $\pm$ 0.4	-0.3 $\pm$ 0.4	-0.5 $\pm$ 0.5
IL-1 $\beta$	OT	-0.3 $\pm$ 0.3	-0.9 $\pm$ 0.6	-1.5 $\pm$ 0.4 <sup>*a</sup>	-0.5 $\pm$ 0.4	0.0 $\pm$ 0.6	-0.8 $\pm$ 0.9	0.3 $\pm$ 0.6	0.7 $\pm$ 1.0	0.3 $\pm$ 0.6
	C	-1.1 $\pm$ 0.7	-0.7 $\pm$ 0.5	-0.4 $\pm$ 0.4	-0.7 $\pm$ 0.3	-1.1 $\pm$ 0.5	-1.2 $\pm$ 0.5	-0.8 $\pm$ 0.5	-0.7 $\pm$ 0.6	-1.1 $\pm$ 0.6
IL-6	OT	0.3 $\pm$ 0.8	-1.2 $\pm$ 0.8	-1.1 $\pm$ 0.8 <sup>*</sup>	-0.5 $\pm$ 0.5	-0.4 $\pm$ 0.7	-1.5 $\pm$ 1.6	0.3 $\pm$ 0.3	0.4 $\pm$ 0.5	-0.1 $\pm$ 0.6
	C	-1.4 $\pm$ 0.9	-0.3 $\pm$ 0.5	-0.1 $\pm$ 0.5	-0.7 $\pm$ 0.4	-1.3 $\pm$ 0.6	-1.8 $\pm$ 0.4	-0.9 $\pm$ 0.4	-0.3 $\pm$ 0.6	-1.6 $\pm$ 0.6
IL-8	OT	0.3 $\pm$ 0.6	-0.2 $\pm$ 0.5	-0.4 $\pm$ 0.5	-0.2 $\pm$ 0.5	0.3 $\pm$ 0.5	-0.8 $\pm$ 0.9	0.4 $\pm$ 0.4	0.6 $\pm$ 0.9	0.3 $\pm$ 0.5
	C	-0.7 $\pm$ 0.6	-0.8 $\pm$ 0.6	-0.4 $\pm$ 0.5	-0.7 $\pm$ 0.3	-0.8 $\pm$ 0.6	-1.1 $\pm$ 0.6	-0.5 $\pm$ 0.5	-0.6 $\pm$ 0.6	-0.9 $\pm$ 0.7
COX2	OT	-0.3 $\pm$ 0.5	-0.4 $\pm$ 0.9	-0.9 $\pm$ 0.5	-0.1 $\pm$ 0.6	0.1 $\pm$ 0.9	-0.2 $\pm$ 1.2	0.6 $\pm$ 0.6	0.1 $\pm$ 0.8	0.5 $\pm$ 0.7
	C	-1.2 $\pm$ 0.6	-0.7 $\pm$ 0.6	-0.2 $\pm$ 0.5	-0.9 $\pm$ 0.3	-1.1 $\pm$ 0.6	-1.8 $\pm$ 0.6	-1.3 $\pm$ 0.9	-1.1 $\pm$ 0.8	-1.7 $\pm$ 0.7

TNF $\alpha$  = tumor necrosis factor- $\alpha$ ; IL-1 $\beta$  = interleukin-1 $\beta$ ; IL-6 = interleukin-6; IL-8 = interleukin-8; COX2 = cyclooxygenase-2; OT = means of all cows treated with 50 IE oxytocin im (5 ml); C = means of control cows treated with 5 ml saline im (9 g/l)

\*means of OT and C quarters are significantly different ( $P < 0.05$ )

\*\*means of OT and C quarters are significantly different ( $P < 0.0001$ )

<sup>a</sup>means within quarter differ significantly ( $P < 0.05$ ) from time Day 0

IL-6-mRNA expression in the OT-group decreased to a minimum ( $P < 0.05$ ) within the first day of the experiment, then increased on Day 2, then decreased again on Day 3 and 4. Another increase occurred thereafter with a maximum on Day 7 ( $P < 0.05$ ) compared to Day 0, then decreased on Day 8. In the C-group, values increased significant compared to Day 0 on Day 1 and 5, all other values tended to the higher when compared to Day 0.

IL-8-mRNA expression levels did not differ significantly in response to the OT treatment but there were significantly elevated levels compared to Day 0 on Days 2, 5, 7 and 8 in the OT-group as well as IL-8-mRNA levels on Day 1 to 5, 7 and 8 in the C-group.

COX2-mRNA expression values did not differ significantly in either the OT- or C-group.

### Tight junction protein expression in mammary tissue

OCLN-mRNA expression values in both groups did not show significant differences within the first

6 days of the OT treatment. However, values in the OT-group diminished to minimal values ( $P < 0.05$ ) on Day 7. The value on Day 7 was significantly lower than that on Day 0.

ZO-1-mRNA expression levels did not show any significance in response to the OT treatment.

ZO-2-mRNA expression values did not differ significantly in response to the long term OT administration. However, values in for the C-group on Day 2 diminished significantly to levels observed on Day 0.

ZO-3-mRNA expression levels did not differ significantly within the first six days of the OT administration. But values in the OT-group decreased ( $P < 0.05$ ) on Day 7 which were also significant less when compared to levels on Day 0.

### DISCUSSION

In most dairy practices OT is a widely used drug. It has become popular as a treatment for mastitis caused by environmental pathogens such as coliforms or streptococci. Although it is reported that

Table 5. Changes of mRNA expression of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, COX-2, OCLN, ZO-1, ZO-2 and ZO-3 in mammary tissue relative to day = 0 ( $\log_2$ ), during (Day 1–7) and after a long term intramuscularly (*i.m.*) injection of 50 IU oxytocin (OT) or 5ml 0.9% NaCl solution (C)

Factor	Group	Sample day							
		1	2	3	4	5	6	7	8
TNF $\alpha$	OT	-0.2 $\pm$ 0.7	-0.3 $\pm$ 0.8*	-0.5 $\pm$ 0.4	-0.7 $\pm$ 0.2	0.3 $\pm$ 0.2	-0.1 $\pm$ 0.2	0.7 $\pm$ 0.7	0.0 $\pm$ 0.4
	C	1.0 $\pm$ 0.8	2.0 $\pm$ 1.2 <sup>a</sup>	1.2 $\pm$ 0.8	1.4 $\pm$ 1.1	1.2 $\pm$ 0.7	0.9 $\pm$ 1.3	0.9 $\pm$ 0.9	0.4 $\pm$ 1.3
IL-1 $\beta$	OT	0.1 $\pm$ 0.8	1.5 $\pm$ 1.2	-0.3 $\pm$ 0.8*	0.5 $\pm$ 0.5	1.6 $\pm$ 0.8	0.9 $\pm$ 0.5	1.0 $\pm$ 1.1	1.7 $\pm$ 0.6
	C	1.4 $\pm$ 0.6	1.1 $\pm$ 0.6	1.9 $\pm$ 1.6 <sup>a</sup>	0.8 $\pm$ 0.7	1.6 $\pm$ 0.8	0.6 $\pm$ 0.5	0.7 $\pm$ 0.5	1.7 $\pm$ 0.4
IL-6	OT	-0.6 $\pm$ 0.6*	0.2 $\pm$ 0.8	-0.3 $\pm$ 0.1	-0.4 $\pm$ 0.8	0.1 $\pm$ 0.5	0.5 $\pm$ 0.5	3.5 $\pm$ 2.0 <sup>a</sup>	0.9 $\pm$ 1.1
	C	2.5 $\pm$ 1.0 <sup>a</sup>	1.6 $\pm$ 1.2	1.9 $\pm$ 1.1	1.9 $\pm$ 1.1	2.5 $\pm$ 0.7 <sup>a</sup>	1.9 $\pm$ 1.1	1.2 $\pm$ 1.2	2.1 $\pm$ 0.4
IL-8	OT	0.7 $\pm$ 0.4	3.4 $\pm$ 1.6 <sup>a</sup>	0.4 $\pm$ 0.6	1.2 $\pm$ 1.1	3.5 $\pm$ 1.0 <sup>a</sup>	1.7 $\pm$ 0.3	3.4 $\pm$ 1.0 <sup>a</sup>	2.0 $\pm$ 0.6 <sup>a</sup>
	C	2.5 $\pm$ 0.8 <sup>a</sup>	2.2 $\pm$ 1.1 <sup>a</sup>	3.0 $\pm$ 1.1 <sup>a</sup>	2.8 $\pm$ 1.0 <sup>a</sup>	3.2 $\pm$ 0.8 <sup>a</sup>	2.5 $\pm$ 0.3	2.3 $\pm$ 0.9 <sup>a</sup>	3.6 $\pm$ 0.5 <sup>a</sup>
COX2	OT	-0.1 $\pm$ 1.0	-0.4 $\pm$ 0.8	-0.7 $\pm$ 1.3	-1.0 $\pm$ 1.1	0.1 $\pm$ 1.7	-0.4 $\pm$ 0.7	-0.2 $\pm$ 1.0	-0.5 $\pm$ 0.7
	C	0.4 $\pm$ 1.7	0.3 $\pm$ 1.6	1.7 $\pm$ 1.8	1.0 $\pm$ 1.4	0.6 $\pm$ 1.0	1.1 $\pm$ 1.4	-0.6 $\pm$ 1.2	0.0 $\pm$ 1.9
OCLN	OT	-0.7 $\pm$ 0.2	-0.3 $\pm$ 0.3	-0.7 $\pm$ 0.4	-0.4 $\pm$ 0.2	-0.6 $\pm$ 0.3	-0.3 $\pm$ 0.3	-1.7 $\pm$ 0.8 <sup>a</sup>	-0.3 $\pm$ 0.3
	C	0.2 $\pm$ 0.6	0.3 $\pm$ 0.7	0.4 $\pm$ 0.3	0.2 $\pm$ 0.7	-0.6 $\pm$ 0.2	-0.4 $\pm$ 0.4	-0.1 $\pm$ 0.5	0.0 $\pm$ 0.9
ZO-1	OT	-0.9 $\pm$ 0.4	-0.2 $\pm$ 0.5	-0.5 $\pm$ 0.4	-0.2 $\pm$ 0.2	-0.7 $\pm$ 0.4	-0.1 $\pm$ 0.2	-0.5 $\pm$ 0.3	0.0 $\pm$ 0.3
	C	-0.4 $\pm$ 0.7	-0.4 $\pm$ 1.0	-0.2 $\pm$ 0.5	-0.4 $\pm$ 0.6	-0.7 $\pm$ 0.2	-0.9 $\pm$ 0.3	-0.5 $\pm$ 0.6	0.0 $\pm$ 1.2
ZO-2	OT	-0.1 $\pm$ 0.6	-0.1 $\pm$ 0.4	-0.7 $\pm$ 0.4	-0.5 $\pm$ 0.1	-1.4 $\pm$ 1.0	-0.4 $\pm$ 0.1	-0.9 $\pm$ 0.5	-0.2 $\pm$ 0.4
	C	-0.9 $\pm$ 0.9	-1.2 $\pm$ 1.0 <sup>a</sup>	0.5 $\pm$ 1.0	-1.1 $\pm$ 0.3	-1.3 $\pm$ 0.3	-0.9 $\pm$ 0.2	-0.3 $\pm$ 0.6	-0.1 $\pm$ 1.0
ZO-3	OT	-0.1 $\pm$ 0.8	-0.3 $\pm$ 0.2	-1.3 $\pm$ 0.6	-0.2 $\pm$ 0.2	-1.0 $\pm$ 0.3	-0.6 $\pm$ 0.3	-2.2 $\pm$ 1.7 <sup>a</sup>	-0.4 $\pm$ 0.4
	C	-1.0 $\pm$ 1.0	-0.6 $\pm$ 0.8	0.8 $\pm$ 0.8	-1.1 $\pm$ 0.4	-0.7 $\pm$ 0.8	-0.4 $\pm$ 0.5	0.1 $\pm$ 0.6	0.2 $\pm$ 0.8

TNF $\alpha$  = tumour necrosis factor  $\alpha$ ; IL-1 $\beta$  = interleukin-1 $\beta$ ; IL-6 = interleukin-6; IL-8 = interleukin-8; COX2 = cyclooxygenase-2; OCLN = occludin-3; ZO-1 = zonula occludens-1; ZO-2 = zonula occludens-2; ZO-3 = zonula occludens-3; OT = means of all cows treated with 50 IE oxytocin im (5 ml); C = means of control cows treated with 5 ml saline im (9 g/l)

\*means of OT and C quarters are significantly different ( $P < 0.05$ )

<sup>a</sup>means within quarter differ significantly ( $P < 0.05$ ) from time Day 0



OT can be as effective as antibiotics for the treatment of IMI (Guterbock et al., 1993; Knight et al., 2000) there is no information on the mode of action of OT treatment (Hillerton and Semmens, 1999). It has been reported that OT is not effective in treating mastitis caused by *Streptococcus uberis* (Hillerton and Kliem, 2002) and *Staphylococcus aureus* (Knight et al., 2000). Basically, OT can act on an immunological level, or, more likely through a more complete udder evacuation during milking because the dosage of OT used is beyond physiological concentrations (Bruckmaier, 2003). This investigation was performed in order to determine the functional role of OT during mastitis therapy.

Although there were only a few significant results in this study, our findings of numerically increasing numbers of somatic cells, Na, Cl and EC as well as decreasing concentrations of lactose in milk, and increasing blood lactose and K levels after OT administration agree with earlier investigations (Linzell and Peaker, 1971; Linzell et al., 1975; Allen, 1990). These findings might emphasize our hypothesis that high dosages of OT causes increased permeability of TJ. It was earlier suggested that open TJ are associated with this pattern of changes (Stelwagen et al., 1997; Nguyen and Neville, 1998; Bruckmaier et al., 2004). In contrast to these results we did not observe decreases in circulating WBC and PMN (this is the first time that you use PMN, need to define PMN in your introduction) in response to the OT treatment. Similar to IMI activated udders (Persson et al., 1993; Schmitz et al., 2004) we assumed that if OT was able to initiate TJ permeability then there should be migration of WBC and PMN from blood into milk and consequently an enhanced mammary gland immune status.

Macrophages, the predominant cell type in milk from healthy udders (Burvenich et al., 1994; Paape et al., 2002; Sarikaya et al., 2004), release the acute phase cytokines TNF $\alpha$ , IL-1 $\beta$  and IL-6 that are primarily responsible for the first host response to invading pathogens (Blum et al., 2000; Sordillo and Streicher, 2002; Prgomet et al., 2005). The recruitment of PMN is triggered by IL-8 a potent chemo-attractant for the accumulation of PMN at sites of infection (Riollet et al., 2000; Sordillo and Streicher, 2002). Further, COX1, COX2 in the synthesis of prostanoids. COX2 synthesis is cytokine induced and therefore the predominant enzyme at sites of inflammation (Mitchell et al., 1995; Wittmann et al., 2002; Pfaffl et al., 2003). Milk somatic cells are

described to be the major source of TNF $\alpha$  (Pfaffl et al., 2003). TNF $\alpha$  is elevated in udders with IMI (Blum et al., 2000) and increasing SCC (Sarikaya et al., 2006). Therefore increasing amounts of TNF $\alpha$  could be expected after OT administration because we found slightly increased SCC. In contrast our results showed decreasing TNF $\alpha$ -mRNA expression levels in the OT-group suggesting that the increased of SCC caused by OT administration is not related to an increased number of potent immune cells. IL-1 $\beta$ -mRNA expression levels in our study showed the same pattern as TNF $\alpha$  values, which agrees with results of others (Sarikaya et al., 2006) and confirmed our suggestion that the somatic cells increase after OT infusion is not equivalent to an increase in mammary gland immunity. IL-6-mRNA expression levels in our study showed the same pattern as IL-1 $\beta$  values, which agrees with results of Prgomet et al. (2005) and confirmed our suggestion that the somatic cells increase after OT infusion is not equivalent to an increase in mammary gland immunity. The expression levels of IL-8 and COX2 did not show remarkable differences in response to OT administration which could be due to a wide range of variation among cows or because OT treatment did not cause inflammation.

In *Experiment II* the influence of chronic (8 days) im treatment of 50 IU OT on immunological important factors and TJ status in mammary tissue were studied over a period of nine days. Therefore mRNA-expression of some cytokines and well-known TJ associated proteins were measured by real-time RT-PCR.

To confirm our earlier findings with milk somatic cells we measured the same cytokines in mammary tissue. Similar to what we observed for milk somatic cells, expression of TNF $\alpha$  and IL-1 $\beta$  decreased within the first two to three days of OT infusion, which confirmed our hypothesis that OT administration did not enhance the immune response. In mammary tissue there were also decreasing IL-6-mRNA expression levels within the first day of OT treatment. Although the decrease did not follow that of IL-1 $\beta$  this result agrees with findings by Prgomet et al. (2005) who described that IL-6 mRNA expression showed the same pattern as IL-1 $\beta$ . IL-8 expression levels did not show any response to OT treatment. COX2-mRNA expression showed no response to either OT administration or to repeated mammary biopsies, and is not in agreement with results of Schmitz et al. (2004) who found increasing COX2 levels also in the C-group.

This lack of agreement is most likely due to a much lower biopsy sampling frequency as compared with the previous study.

Over the years many investigators (Allen, 1990; Stelwagen et al., 1994, 1995, 1997; Nguyen and Neville, 1998; Sloth et al., 2003; Bruckmaier and Meyer, 2005) assumed that elevated SCC and EC levels in milk and the occurrence of lactose in blood might be signs of changes in TJ protein structure. Although these changes are usually related to IMI or intramammary challenge with lipopolysaccharides and were therefore always related to decreased milk yield, we hypothesised that these changes could also occur after OT administration. Consequently we measured the TJ associated proteins OCLN, ZO-1, ZO-2 and ZO-3-mRNA expression in mammary tissue samples before, during and after chronic OT infusion.

OCLN that is embedded in intramembranous strands of the TJ was the first discovered TJ associated protein (Furuse et al., 1993; Fujimoto, 1995; Nguyen and Neville, 1998). A synthetic peptide of OCLN might be able to increase TJ permeability but its principal function has not yet been determined. However, it does not appear to be involved in the configuration of ion-selective pores of the TJ (Wong and Gumbiner, 1997; Schneeberger and Lynch, 2004). In our study OCLN did not change within the first six days of OT administration but its decrease ( $P < 0.05$ ) on Day 7 might indicate that OT influenced this component of the TJ complex.

The cytoplasmatic plaque protein ZO-1, ZO-2 and ZO-3 are members of the MAGUK (membrane-associated guanylate kinase homologue) family and in contrast to other members of this group each consists of an acidic domain, a basic arginine-rich region and a proline-rich domain (Haskins et al., 1998; Wittchen et al., 1999; Itoh et al., 1999; Schneeberger and Lynch, 2004). All ZO proteins are able to bind directly to OCLN, whereas ZO-2 and ZO-3 interact with ZO-1 but not with each other (Haskins et al., 1998; Wittchen et al., 1999; Schneeberger and Lynch, 2004). Wittchen et al. (1999) suggested that the complexity of these protein-protein interactions at the site of TJ has functional as well as regulatory effects. In our study ZO-1-mRNA expression did not show any differences in response to OT treatment. ZO-2-mRNA expression in the OT-group did not differ in response to OT treatment. The significant (compared to Day 0) decrease in the C-group may be due to variation among cows and not a response to the

saline administration. In contrast, the expression levels of ZO-3-mRNA showed the same pattern as OCLN did; the decrease ( $P < 0.05$ ) on Day 7 does suggest that OT influenced this part of the TJ, but only after a long-term treatment.

## CONCLUSION

This study confirmed that chronic OT administration induced increases in SCC and EC levels in milk as well as increasing K and lactose levels in blood, nearly all investigated cytokines in milk cells and mammary tissue were down regulated after OT treatment. Furthermore, the expression of the TJ proteins OCLN and ZO-3 appeared to be down-regulated. It can be assumed that this resulted in increased permeability of TJ in response to the chronic OT treatment. While OT stimulation of the mammary gland immune system was not apparent, chronic OT treatment will help in the elimination of pathogens due to a more complete milk ejection.

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