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Effects of oxytocin and PGF2α on uterine contractility in cows with and without metritis—An *in-vitro* study

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ABSTRACT

The aim of this study was to investigate the effects of $PGF_{2\alpha}$ and oxytocin *in vitro* on myometrial contractility in puerperal uteri. Thirteen puerperal uteri were removed and perfused after euthanasia of cows with ($n = 7$) and without metritis ($n = 6$). Measurement of uterine contractility was done using four piezoelectric crystals, which were implanted into the myometrium along the greater curvature of the uterine horn where fetal implantation occurred during the previous pregnancy. After 30 min of equilibration, oxytocin (5 IU) or $PGF_{2\alpha}$ (2.5 mg Dinoprost) was administered randomly into both uterine arteries, and 30 min later, the second administration of either oxytocin or $PGF_{2\alpha}$ occurred. Treatment with oxytocin induced contractions in uteri with metritis and uteri without metritis ($P < 0.05$). In uteri with metritis, greater uterine contractions occurred after stimulation with oxytocin than in uteri without metritis ($P < 0.05$). Treatment with $PGF_{2\alpha}$ did not ($P > 0.05$) result in increased contractions in the uteri without metrtitis, however, induced an initial decrease in contractions followed by an increase ($P < 0.05$) in contractions in uteri with metritis. Myometrial and endometrial gene expression of $PGF_{2\alpha}$ (FPR) and oxytocin receptor (OTR) was greater ($P < 0.05$) in uteri with metritis than in uteri without metritis. The results suggest that oxytocin, but not $PGF_{2\alpha}$, is an effective uterotonic drug in puerperal cows. Uteri in which metritis was diagnosed contracted more strongly after treatment with oxytocin than uteri in which metritis was not diagnosed. This effect was paralleled by greater gene expression of OTR as well as FPR in uteri with metritis compared with uteri in which metritis was not diagnosed.

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1. Introduction

Puerperal uterine diseases, including retained fetal membranes and metritis, are important in dairy cows (Esslemont and Peeler, 1993; Drillich et al., 2001;) for various reasons. Substantial economic loss stems from decreased milk yield, reduced fertility, increased treatment costs, and greater culling rates (Bartlett et al., 1986; Esslemont and Peeler, 1993; Lewis, 1997). Metritis is characterized by uterine enlargement and fetid lochia with or without systemic illness. Metritis usually occurs in the first 10 days after parturition and is associated with delayed uterine involution (Sheldon et al., 2006).

In addition to local and systemic antibiotics (Smith et al., 1998; Drillich et al., 2001), treatment of puerperal uterine diseases often includes uterotonic agents such as $PGF_{2\alpha}$ and oxytocin. Theoretically, these drugs could be expected to increase uterine contractility thereby promoting drainage of uterine contents and enhancing uterine involution and, eventually, fertility (Bajcsy et al., 2006). Reports on the effects of uterotonic agents on uterine contractility and subsequent fertility are ambiguous (Frazer, 2005). Oxytocin has consistently been reported to have a positive effect on uterine contractility (Eiler et al., 1984; Kündig et al., 1990a; Gajewski et al., 1999; Bajcsy et al., 2006), but its use in the early puerperal period had no effect on reproductive performance in healthy cows and cows with uterine diseases (Tian and Noakes, 1991; Barrett et al., 2009; Jeremejeva et al., 2010). Prostaglandin $F_{2\alpha}$ treatment of healthy postpartum cows had positive (Kündig et al., 1990a; Gajewski et al., 1999) or no effects (Eiler et al., 1981; Kündig et al., 1990a) on myometrial contractility. Fertility of dairy cows was positively affected (Young et al., 1984; Nakao et al., 1997; Melendez et al., 2004) or not affected (Tian and Noakes, 1991; Jeremejeva et al., 2010; Jeremejeva et al., 2012) by $PGF_{2\alpha}$ treatment in the early postpartum period. Of note, the cited studies did not include cows with puerperal uterine disease when assessing the effect of uterotonics on uterine contractility.

In addition to oxytocin and $PGF_{2\alpha}$, other hormones are involved in uterine contractility in cows. Estrogen and progesterone (P4) have indirect as well as direct effects on myometrial contractility (Hixon and Flint, 1987; Fomin et al., 1999). For a hormone to have an effect on a target cell, it must first bind to its receptor. The efficacy of a hormone, therefore, depends not only on the concentration of the hormone but also on the numbers of receptors in the target organ. To our knowledge, uterine expression of receptor genes of ecbolic hormones has not been previously investigated in cows in the early puerperal period.

Uterine contractility can be investigated *in vitro* using isolated perfused uteri or myometrial strips mounted in an organ bath (Hirsbrunner et al., 2002; Gorriz-Martin et al., 2017). Compared with other *in-vitro* techniques, extracorporeal perfusion of organs allows estimation of the electric and mechanic activity of the whole organ and preserves the structure and function of the organ (Dittrich et al., 2003). Several studies reported that the model of isolated perfused uteri is suitable for measuring uterine contractility as well as investigating the effects of uterotonics and tocolytics in humans (Bulletti et al., 2004; Richter et al., 2006), swine (Dittrich et al., 2003; Künzel et al., 2014), and cattle (Bock, 2004). Comparison of the effects of oxytocin on uterine contractility obtained using extracorporeally perfused uteri and an *in-vivo* model in cattle showed basic agreement between the two methods (Bock, 2004).

Sonomicrometry is a novel technique which has been used successfully for *in-vivo* assessment of uterine involution (Heppelmann et al., 2015a; Heppelmann et al., 2015b) and for measuring the cervical diameter in the periparturient period in dairy cows (Breeveld-Dwarkasin et al., 2002; Van Engelen et al., 2007). Sonomicrometry allows the objective measurement of the distance between individual piezoelectric crystals based on the time an ultrasonic signal requires to travel between a transmitter and a receiver (Adelson and Million, 2004). This technique has also been used for investigation of cardiac and skeletal muscle contractility in different species (Horiuchi et al., 2012; Robertson and Biewener, 2012; Askov et al., 2013).

The primary goal of this study was to investigate the effects of $PGF_{2\alpha}$ and oxytocin on myometrial contractility using sonomicrometry in isolated perfused uteri from euthanized puerperal cows with and without metritis. In the present study, it was hypothesized that $PGF_{2\alpha}$ and oxytocin would have no effect on uterine contractility in the early puerperal period, particularly in uterine specimens collected from cows with metritis. It was speculated that findings associated with acceptance of this hypothesis would contribute to a reduction in the use of hormones in dairy cows. A secondary goal was to examine the gene expression for hormonal receptors (estrogen receptor α [ERα], progesterone receptor [PR], $PGF_{2\alpha}$ receptor [FPR], and oxytocin receptor [OTR]) in these uteri.

2. Materials and methods

2.1. Uteri

The uteri were taken from 13 Holstein Friesian cows that were euthanized 5–21 days postpartum. Reasons for euthanasia were traumatic diseases of the locomotor system ($n = 3$), lameness ($n = 2$), peritonitis ($n = 2$), endocarditis ($n = 1$), pericarditis ($n = 1$), amyloid nephrosis ($n = 1$), bronchopneumonia ($n = 1$), sepsis ($n = 1$), and posterior functional stenosis ($n = 1$). All cows were patients of the Clinic for Cattle of the University of Veterinary Medicine, Hannover, Germany.

2.2. Study design

Blood samples were collected from a jugular vein into EDTA and serum tubes (Sarstedt AG & Co, Nürnbrecht, Germany) for hormone analysis. The cows were euthanized by intravenous injection of 30 g of pentobarbital sodium (Release®, WDT eG, Garbsen, Germany). A transverse incision was then made in the ventral abdomen of the dead cows and the uterus including the cervix and ovaries was immediately removed. The uterine blood vessels were immediately flushed with heparinized (Heparin-Natrium Braun, B. Braun Melsungen AG, Melsungen, Germany; 150 IU/mL medium) Tyrodés solution containing 136.8 mmol/L NaCl, 5.5 mmol/L glucose*H_2O, 11.9 mmol/L $NaHCO_3$, 2.7 mmol/L KCl, 0.416 mmol/L NaH_2PO_4, 1.05 mmol/L $MgCl_2$*$6H_2O$, and 1.8 mmol/L

$CaCl_2$*$2H_2O$ to avoid clotting of the blood. Lochia were drained from the uteri via the cervix, and the uteri were weighed. The lochia were evaluated according to Sheldon et al. (Sheldon et al., 2009), and the uteri were divided into those with (M+) and without metritis (M−). Ovaries were evaluated for presence and size of corpora lutea and follicles. For histologic examination and measurement of gene expression, an intercaruncular tissue sample (size 1.0 × 0.5 cm) including all uterine layers was taken from the dorsal part of the formerly gravid uterine horn, and the defect thus created was closed using a continuous Lembert suture pattern. The uterine arteries and ovarian veins, which are the main afferent and efferent blood vessels, were cannulated with silicone tubes. Four sonometric crystals (Sonometrics, London, Ontario, Canada) were then implanted into the myometrium of the greater curvature of the formerly gravid uterine horn. The uteri were placed on a fixation mattress (CP-Pharma, Burgdorf, Germany) in a heated incubator (Incubator S.I. 60, Stuart Scientific, United Kingdom) at 39 °C. The perfusion medium was a carbogen-gassed (95% oxygen, 5% carbon dioxide; Carbogen LAB, Linde Gas Therapeutics GmbH, Oberschleißheim, Germany) isotonic Tyrodés solution. After 30 min of equilibration, sonometric measurements of spontaneous contractility were made for 30 min. Oxytocin (CP-Pharma, Burgdorf, Germany; 5 IU per uterine horn) or $PGF_{2\alpha}$ (Dinoprost, Zoetis, Berlin, Germany; 2.5 mg per uterine horn) was then administered into the cannulated uterine arteries. The order of treatment with the two drugs was random, and 30 min later, the second drug was given. After each stimulation, sonometric measurements were made for 30 min. This protocol amounted to a total perfusion time of 2 h. At the completion of the perfusion trial, a second tissue sample was collected from the uterine wall for histologic evaluation. The uteri were then weighed again to estimate the amount of perfusion medium in the tissue.

2.3. Perfusion system

The perfusion system consisted of a Tyrodés reservoir (5 L), a waterbath (Grant OLS 200, Grant Instruments, Cambridge, United Kingdom), and two peristaltic pumps (Reglo digital, Ismatec, Wertheim-Mondfeld, Germany). In the reservoir, the Tyrodés solution was saturated with carbogen ($95\%O_2/5\%CO_2$). From the reservoir, the perfusion solution was pumped through a glass coil submerged in the water bath at 39 °C, which served to warm the solution. From the glass coil, the solution ran through a silicon tube that was split by a Y-tube connector so that both uterine arteries had a separate influx of perfusion solution. A separate pump was used for each uterine horn so that the perfusion rate could be adjusted to uterine horn size. Perfusion flow rate ranged from 8 to 35 mL/min/uterine horn depending on the size of the horn.

2.4. Tissue viability parameters

The viability of the uterine tissue during perfusion was monitored by measuring glucose consumption and lactate generation as described by Bäumer et al. (Bäumer et al., 2002). To this end, perfusate samples were collected from the cannulated ovarian veins at the beginning of perfusion and then every half hour (at 30, 60, 90, and 120 min) and stored at −20 °C until analysis. Glucose und L-lactate concentrations were measured using the ABX Pentra 400® (Horiba, Medical, Kyoto, Japan). Glucose consumption per uterine horn and hour (g/h) was calculated by the difference between glucose content of the Tyrodés solution (g/l) and the perfusate sample, multiplied by the perfusion flow per hour. Lactate generation per uterine horn and hour (g/h) was calculated by multiplying the lactate content of the perfusate sample with the perfusion flow per hour.

2.5. Sonometric system, measurements and analysis

The sonomicrometry system (Sonometrics Corp., London, ON, Canada) was recently described in detail (Heppelmann et al., 2015b). In brief, the system consists of four piezoelectric crystals connected to a sonomicrometer (TRX8), a channel selector box and a Sonometrics data acquisition computer. The sonomicrometer generates ultrasonic signals in the piezoelectric crystals and converts the received analogue signals into digital signals for further analysis. The four piezoelectric crystals were implanted in a sagittal plane along the greater curvature of the formerly gravid uterine horn. The caudalmost crystal (no. 4) was placed near the bifurcation of the uterus and the other 3 (nos. 3, 2, and 1) cranially at regular intervals depending on the size of the uterine horn. For fixation of the crystals, an incision was made through the serosa and outermost layer of the myometrium, and the crystal head was buried using no 2 silk in a single seromuscular cruciate suture pattern. The SonoSOFT software (Version 3.4.30 RC1, Sonometrics Corp., London, Canada) was used for data acquisition and analysis. Only the data from adjacent crystals were used. From each possible crystal transmission pair (e.g., 1 to 2 and 2 to 1), the direction with the best signal quality was selected. This resulted in three distances between crystals that remained for final analysis, and these measurements were presented graphically using the software SonoVIEW (Sonometrics Corp., London, Canada). The distances were filtered with a SonoSOFT filter, during which single point outliers were assigned to the actual distance between crystals. The Shapiro-Wilk test showed that most distributions of the residuals of the sonometrically determined distances were not normal and, therefore, medians were calculated. In addition, for each distance and for each measuring unit (each 3 min) medians were calculated; one unit before stimulation and ten units after stimulation. The crystals were not entirely equidistant within and between uteri and, therefore, not directly comparable. Relative changes (%) in the distances between neighboring crystals were, therefore, used to characterize uterine contractility based on a reference value obtained before stimulation (100%).

2.6. Blood samples

The samples were immediately placed on ice after collection until centrifugation (2000*g*, 20 min at 4 °C). Serum and plasma were

harvested and stored at −20 °C until analysis.

The serum P4 concentration was measured using a commercial coat-a-count radioimmunoassay according to the manufacturer's instructions (P4 Coat-a-Count, TKPG1, Siemens Medical Diagnostics, CA, USA). The analytical sensitivity was 0.02 ng/mL, the intra-assay coefficient of variation (CV) was 6.5%, and the inter-assay CV was 7.8%. Total estrogen concentration was determined after ether extraction with a direct enzyme-immunoassay on microtiter plates using a secondary-antibody coating technique and horseradish peroxidase as the enzyme label. The antiserum used was raised against 17β-estradiol hemisuccinate (cross reactivity 17β-estradiol 100%, estrone 100%, 17α-estradiol 70%). The 17β-estradiol hemisuccinate horseradish peroxidase was used as steroid-enzyme conjugate. The minimal detectable concentration was 8 pg/mL. Recovery ranged from 84.6% to 96.2%, the intra-assay CV was 13.2%, and the inter-assay CV was 19.7%.

The total serum calcium concentration was measured using a Cobas Mira biochemistry analyzer (intra-assay coefficient of variation, 2.53%; Hoffmann-La Roche & Co AG Diagnostika, Basel, Switzerland).

2.7. Histological examination

For histological examination, samples of uterine wall were fixed in 4% neutral buffered formalin for 24–48 h and then rinsed in tap water for 20 h. The samples were dehydrated in a graded series of alcohol and embedded in paraffin (Leica Surgi Path Paraplast, Leica Mycrosystems, Wetzlar, Germany). The paraffin-embedded tissue was sectioned at 3–4 μm using a rotation microtome (Leitz 1512®, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). The sections were mounted on glass slides coated with alum gelatin (Engelbrecht, Edermünde, Germany) and dried overnight at 60 °C in a heating chamber. The sections were stained with hematoxylin and eosin (H&E). All slides were examined using the same light microscope (Zeiss, Oberkochen, Germany), and assessed at x 40, x 100 and x 400 magnification. For estimation of histologic changes attributable to perfusion *per se*, the vacuolization of cells of the luminal and glandular epithelium and signs of edema were assessed.

2.8. Gene expression

For gene expression analysis, endometrium and myometrium were dissected, frozen in liquid nitrogen, and stored at −80 °C in a sterile DNase- and RNase-free cryotube (Fa. Brand, Wertheim, Germany) until analysis. The relative abundance of endometrial and myometrial mRNA of estrogen receptor α (ERα), progesterone receptor (PR), $PGF_{2\alpha}$ (FPR), and oxytocin receptor (OTR) was quantified using a quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Total RNA was extracted from the biopsies with peqGOLD TriFast™ (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. Final RNA quantity and purity was determined by absorbance at 260 nm and 280 nm using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Germany). Subsequently, 1 μg of total RNA was reverse transcribed with Moleney Murine Leukemia Virus Reverse Transcriptase RNase H minus, Point Mutant (MMLV-RT, Promega, Madison, WI, USA) using random hexamer primers (Invitrogen, Leek, The Netherlands). Amplification and quantification of the target cDNA was performed by qPCR in a Rotor-Gene 6000 (Corbett Research, Sydney, Australia). Threshold values (cycle threshold; Ct) were achieved by Rotor Gene software version 1.7.75. The Ct values of target genes were normalized to the abundance of four housekeeping genes (GAPDH, β-actin, ubiquitin, and YWHAZ) according to the following equation: ΔCt = Ct (target gene) – Ct (arithmetic mean of housekeeping genes). The four housekeeping genes were relatively consistent in amounts in all samples with variations within < 1 cycle. Primers for the housekeeping and target genes were synthesized commercially (Microsynth, Balgach, Switzerland) using the sequences shown in

Table 1
Sequences and accession numbers of the PCR primers for mRNA of estrogen receptor α (ERα), progesterone receptor (PR), $PGF_{2\alpha}$ receptor (FPR) and oxytocin receptor (OTR).

Primer*		Sequence (5′ → 3′)	Accession no.*	Product length (bp)
GAPDH	for	GTC TTC ACT ACC ATG GAG AAG G	NM 01034034	110
	rev	TCA TGG ATG ACC TTG GCC AG		
β-actin	for	CAT CAT GAA GTG TGA CGT CGA C	NM_173979.3	231
	rev	CTG ATC CAC ATC TGC TGG A		
Ubiquitin	for	AGA TCC AGG ATA AGG AAG GCA T	NM174133	198
	rev	GCT CCA CCT CCA GGG TGA T		
YWHAZ	for	CAGGCTGAGCGATATGATGAC	NM174814	141
	rev	GACCCTCCAAGATGACCTAC		
ERα	for	GAA GTG GGC ATG ATG AAA GG	AY_538775	135
	rev	AAG GTT GGC AGC TCT CAT GT		
PR	for	TCG AGC TCA CAG CGT TTC TA	NM_001205356	106
	rev	CCC GGG ACT GGA TAA ATG T		
FPR	for	AGCCTTGCCATTGCTATCC	NM 181025	127
	rev	TAGTTCCATTGATGAGGTGCC		
OTR	for	ACGGTGTCTTCGACTGCTG	NM 174134	110
	rev	GGTGGCAAGGACGATGAC		

* Sequences are available in the Genbank database under the given accession numbers (Kenngott et al., 2011; Lüttgenau et al., 2011; Zbinden et al., 2014; Lüttgenau et al., 2016).

Table 1.

2.9. Statistical analysis

Statistical analyses were conducted using the Statistical Analysis System (SAS Institute, Cary, North Carolina). The Shapiro-Wilk test was used to test for normality of the distribution of all variables. For relative changes in sonometric measurements, blood, and viability variables, the median and the median absolute deviation (MAD) values were calculated. The effect of group and day postpartum on sonometric measurements and blood values were calculated using the Kruskal-Wallis-test (PROC NPAR1WAY). Differences between the two groups were analyzed using the non-parametric Wilcoxońs rank sum test (PROC NPAR1WAY). The effect of time after stimulation on repeated measurements was determined using the Friedman two-way ANOVA (PROC FREQ). Differences between duration of perfusion and measuring units within groups were calculated using the Wilcoxońs signed rank test (PROC UNIVARIATE). All other variables were normally distributed and were given as mean ± SD. Differences between the two groups were analyzed using the *t*-test (PROC TTest). Comparison of uterine weight before and after perfusion was made using the paired *t*-test (PROC MEANS). The effect of day postpartum on gene expression was analyzed using the one-way ANOVA (PROC GLM). Categorical data of histologic examinations were compared using the chi-square test of homogeneity or Fisher's exact test when sample size was five or less (PROC FREQ). Differences were considered significant at $P < 0.05$.

3. Results

Of the 13 uteri, six were normal (M−) and seven had gross signs of metritis (M+). The cows of Group M− were 5.3 ± 1.4 years old and the cows of group M+ were 5.0 ± 2.0 years old. Days postpartum were 12.7 ± 5.4 days for Group M− and 12.9 ± 4.2 days for Group M+. These variables did not differ ($P > 0.05$) between groups.

3.1. Viability variables

Glucose consumption was similar ($P > 0.05$) during perfusion in the uterine horn in which the implantation of the previous pregnancy occurred (gravid) was not different from the contralateral uterine horn (non-gravid). Lactate generation in the gravid uterine horn decreased ($P < 0.05$) from 30 to 60 and from 60 to 90 min of perfusion. In the non-gravid uterine horn, lactate generation decreased ($P < 0.05$) after 30 min of perfusion. There were no differences ($P > 0.05$) between Groups M− and M+ regarding both viability variables. The values for the two groups were, therefore, not listed separately in Table 2.

3.2. Sonometric measurements

The order of stimulation and day after calving did not affect ($P > 0.05$) sonometric measurements. After stimulation with oxytocin, all three distances (1–2, 2–3, 3–4) were affected by group ($P < 0.05$; Fig. 1). Contraction of distance 12 at 18 min after initiation of stimulation was greater in Group M+ than M− ($P < 0.05$). After stimulation with oxytocin, contraction was also affected by time ($P < 0.05$) in Group M+ at distance 1–2. In Group M+, distance 1–2 was decreased from 3 to 30 min and distance 3–4 was decreased from 18 to 30 min ($P < 0.05$). In Group M−, distance 3–4 was shorter at 9, 15, and 18 min ($P < 0.05$) compared with values before stimulation.

There was no effect of group ($P > 0.05$) after stimulation with $PGF_{2\alpha}$ (Fig. 2). In Group M+, distance 3–4 was affected by time ($P < 0.05$); there was a decrease (27 min) and an increase (30 min) of distance 3–4 ($P < 0.05$).

3.3. Uterine weight and ovarian functionr

Before perfusion, the uteri weighed 3,947.0 ± 1,997.0 g in Group M- and 3,420.0 ± 1,016.0 g in Group M+. After infusion, the weights were 4,217.0 ± 2,102.0 g and 3,712.0 ± 1,147.0 g, respectively. Perfusion caused an increase ($P < 0.05$) in weight of 270.0 ± 154.0 g (7%) in Group M− and of 293.0 ± 215.0 g (9%) in Group M+. This difference was not significant ($P > 0.05$).

Table 2
Glucose consumption (g/h) and lactate generation (g/h) of puerperal uteri ($n = 13$) during 2 h of isolated perfusion of the formerly gravid (gh) and non-gravid uterine horn (ngh).

Duration of perfusion (min)	Glucose consumption		Lactate generation	
	gh	ngh	gh	Ngh
30	0.12 ± 0.13	0.07 ± 0.06	0.34 ± 0.13	0.22 ± 0.06
60	0.09 ± 0.13	0.05 ± 0.03	0.30 ± 0.13*	0.14 ± 0.03*
90	0.09 ± 0.11	0.07 ± 0.02	0.25 ± 0.11*	0.15 ± 0.04
120	0.13 ± 0.09	0.09 ± 0.03	0.25 ± 0.11	0.13 ± 0.03

Values are median ± median absolute deviation.
* Values differed from those of the chronologic previous measurement ($P < 0.05$).

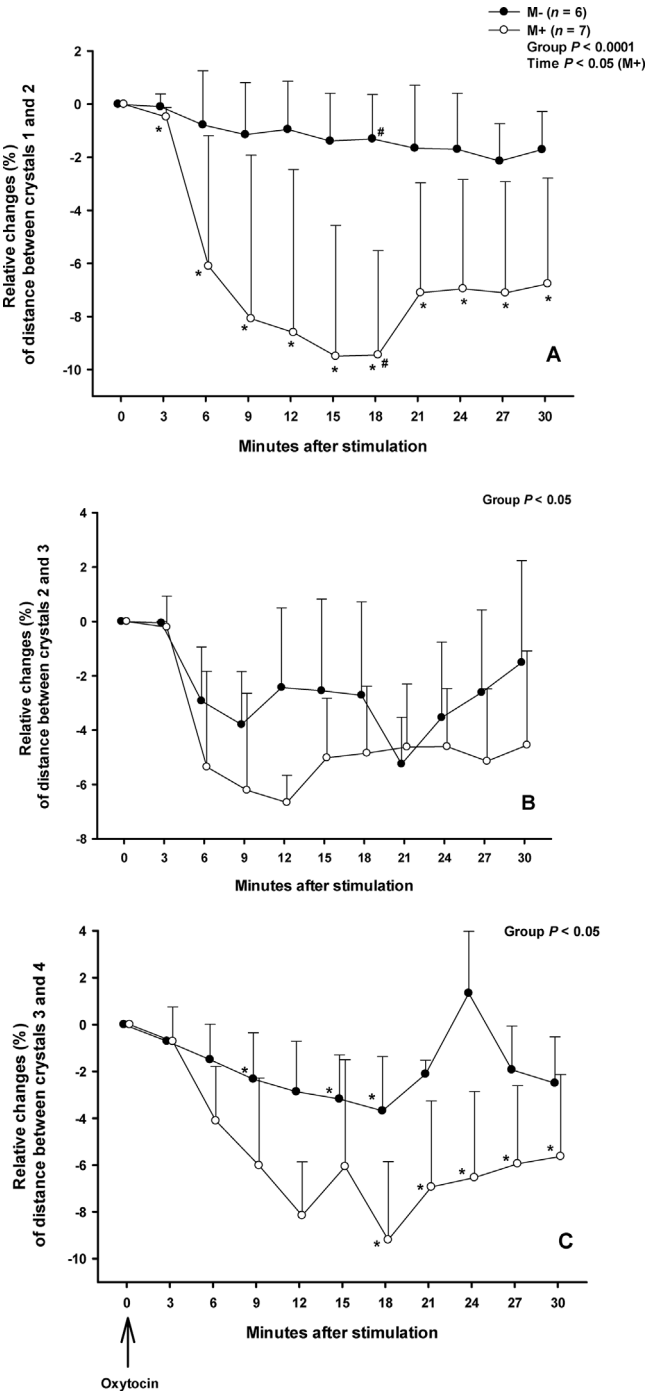

Fig. 1. Relative changes (median ± median absolute deviation) of distances between sonometric crystals 1 and 2 (A), 2 and 3 (B), and 3 and 4 (C) in 13 isolated perfused uteri (n = 6 without metritis [M−]; n = 7 with metritis (M+]) 30 min after stimulation with oxytocin (values before stimulation = 0%); *Values differed from those before stimulation ($P < 0.05$); # Values differed between groups ($P < 0.05$).

The ovaries of both groups had no functional corpora lutea and no follicles > 10 mm.

3.4. Histologic findings

One tissue sample of Group M+ could not be evaluated because of poor quality, and in four others, the luminal epithelium was absent. All post-perfusion samples of Group M− and two (29%) of Group M+ had mild edema of the lamina propria, and four samples (57%) of the Group M+ had moderate edema. Mild vacuolization of the luminal epithelium was seen in one sample (17%) of

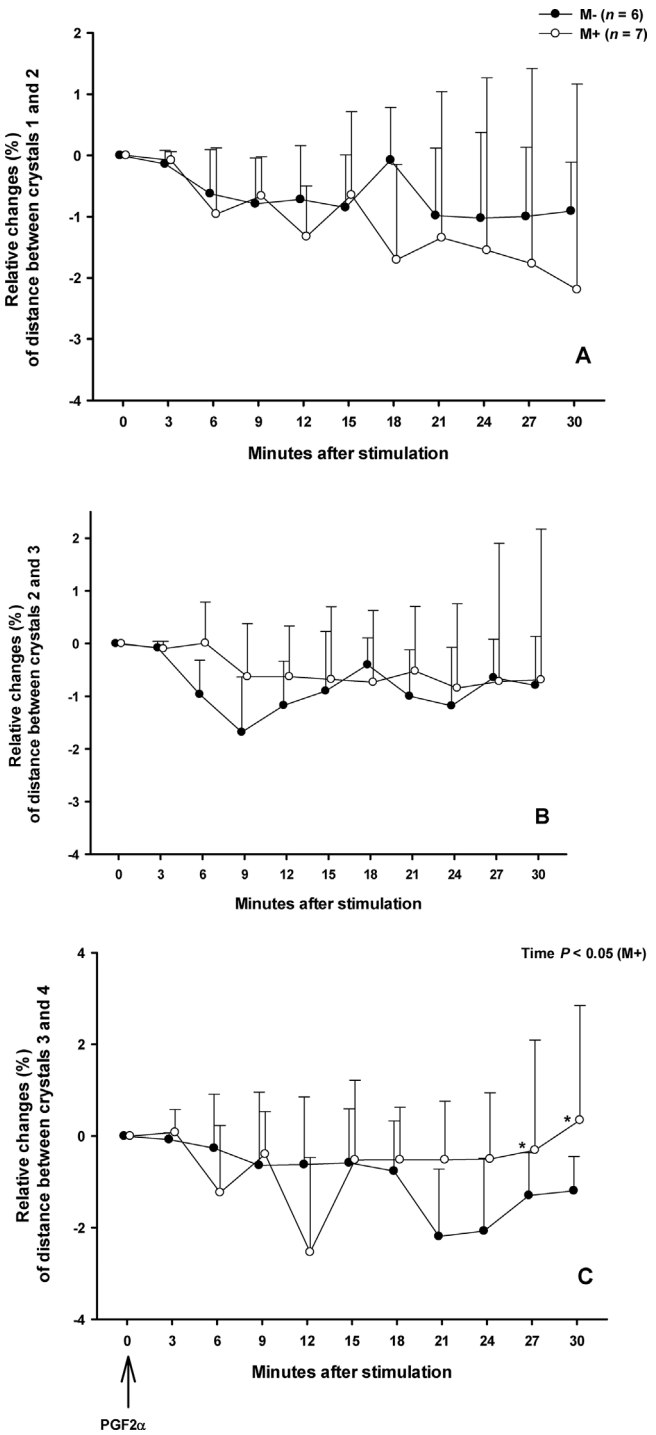

Fig. 2. Relative changes (median ± median absolute deviation) of distances between sonometric crystals 1 and 2 (A), 2 and 3 (B), and 3 and 4 (C) in 13 isolated perfused uteri ($n = 6$ without metritis [M−]; $n = 7$ with metritis (M+]) 30 min after stimulation with $PGF_{2\alpha}$ (values before stimulation = 0%); *Values differed from those before stimulation ($P < 0.05$).

Table 3
Endometrial and myometrial relative abundance of mRNA (ΔCt) of estrogen receptor α (ERα), progesterone receptor (PR), $PGF_{2\alpha}$ receptor (FPR) and oxytocin receptor (OTR) of uteri from cows with (M+; $n = 7$) and without metritis (M–; $n = 6$) diagnosed between 5 and 21 days postpartum.

	Type of receptor	M−	M+
Endometrium	ERα	13.5 ± 0.6	10.3 ± 2.8
	PR	13.1 ± 0.8*	11.2 ± 1.3*
	FPR	7.3 ± 3.4*	14.8 ± 2.0*
	OTR	12.5 ± 0.5*	15.7 ± 1.4*
Myometrium	ERα	13.7 ± 1.4	12.1 ± 1.3
	PR	13.1 ± 0.8	12.2 ± 1.1
	FPR	8.6 ± 1.1*	14.1 ± 1.0*
	OTR	12.0 ± 1.1*	15.1 ± 1.0*

Values are mean ± SD.
* Values differed between groups M– and M+ ($P < 0.05$).

Group M− and in two (67%) samples of Group M+. One sample (17%) of Group M+ had mild vacuolization of the glandular epithelium. Histologic changes attributable to perfusion did not differ ($P > 0.05$) between groups.

3.5. Blood variables

The concentrations of P4, total estrogens, and ionized calcium did not differ ($P > 0.05$) between groups. The P4 concentrations for Groups M+ and M− were 0.2 ± 0.2 and 0.7 ± 0.6 ng/mL, respectively. Total estrogen concentration for Groups M+ and M− were 19.8 ± 10.8 and 75.2 ± 46.1 pg/mL, respectively. Serum calcium concentrations for Groups M+ and M− were 2.1 ± 0.1and 1.9 ± 0.2 mmol/L, respectively. Day postpartum had no effect ($P > 0.05$) on blood variables.

3.6. Gene expression of hormonal receptors

Endometrial and myometrial expression of FPR and OTR genes was greater ($P < 0.05$) in Group M+ than Group M−. Endometrial expression of the PR gene was greater ($P < 0.05$) in Group M− than M+. Gene expression for other receptors did not differ ($P > 0.05$) between groups (Table 3), and days post-calving had no effect ($P > 0.05$) on gene expressions for hormonal receptors.

4. Discussion

In the present *in-vitro* study, effects were investigated of oxytocin and $PGF_{2\alpha}$ on contractility of extracorporeally perfused uteri from euthanized puerperal cows with and without metritis. Contractility measurement was achieved with a sonomicrometry technique, which was used previously to monitor *in vivo* uterine involution of cows (Heppelmann et al., 2015b). Gene expression of hormonal receptors (ERα, PR, FPR, and OTR) was also determined in the uteri because the effects of uterotonic drugs depend significantly on the respective receptor concentrations (Hoffmann, 1994).

It is important that the viability and function of the organ under investigation be maintained during a perfusion experiment (Richter et al., 2000). In the present study, the viability of the perfused uteri was monitored by measuring glucose consumption and lactate generation, by determining any increase in uterine weight during perfusion, and by assessing histologic changes attributable to perfusion *per se* (Bäumer et al., 2002). Of note, the occurrence of metritis did not affect viability variables. Inflammation, therefore, did not impair the viability of the uterus during perfusion. Glucose consumption remained constant during the entire perfusion experiment, which was in agreement with the results of other studies (Bäumer et al., 2002; Bock, 2004). Likewise, the decrease in lactate production documented in the present experiment confirmed earlier observations (Bäumer et al., 2002; Bock, 2004). Lactate production was greatest at the start of the perfusion experiment, and it is suspected that this was because of transient anaerobic metabolism during transport of the organs to the laboratory. Comparison of the total glucose consumption and lactate production in the present study with values of other uterine perfusion models for cattle (Bäumer et al., 2002; Bock, 2004) is complicated by the fact that the uteri varied in size and with respect to inflammation, and originated from cows at different stages of the estrous cycle. The increase in uterine weight by 7% (M−) to 9% (M+) by the end of the 2-h perfusion period was caused by edema. In other studies (Bäumer et al., 2002; Braun, 2002) a much greater weight increases of 20% to 22% were observed n similar experiments, however, in these studies a perfusion time of 5 h was used. Histologic changes attributable to perfusion *per se* were minimal to moderate edema in the lamina propria, which has been reported previously (Bäumer et al., 2002).

Even though the cows used in the present study were recruited from cases at Hanover Foundation clinic and, therefore, were difficult to select for age and days postpartum, the two groups were homogeneous with respect to these variables. This was particularly important with regard to a similar number of days postpartum at initiation of the study because it was shown that the spontaneous contractility pattern in the early postpartum period may be irregular (Zerobin, 1970; Kündig et al., 1990b). Furthermore, one study described a decrease of the uterotonic efficacy of dinoprost and oxytocin after day 5 postpartum (Kündig et al., 1990a).

The effect of uterotonic agents on uterine contractility depends greatly on the hormonal status (P4 and estrogen) of the cow (Kündig et al., 1990a; Gajewski et al., 1999). Progesterone may be excluded as a factor causing a difference in the contractility pattern between the two groups because its concentration was < 1 ng/mL in all cows, consistent with the lack of functional corpora lutea. Likewise, total estrogen concentrations did not differ significantly between groups even though cows of the M+ group had numerically greater concentrations (75.2 compared with 19.8 pg/mL). The causal relationship between the latter finding and the occurrence of metritis is not clear. Administration of estradiol into the uterus of cows during early puerperium resulted in an increase in pathogenic anaerobic bacteria in the uterus (Sheldon et al., 2004). Treatment with estradiol cypionate on the first day postpartum suppressed the coordinated myometrial activity for up to 5 days (Burton et al., 1990). The ovarian follicles in the present study were all smaller than 10 mm and, therefore, are not a likely source of estrogens that were detected in the blood samples. Furthermore, a study that used the same estrogen assay provided evidence that during the estrous cycle of cows, estrogen concentrations did not exceed 30 pg/mL (Meyerholz et al., 2015). Cows with metritis had total estrogen concentrations of 75.2 pg/mL, which may have reflected a decreased hepatic elimination rate of free estrogens that remained in the circulation from gestation; the assay used detected free as well as sulfated estrogens and estrogens with glucoronate attached. One could, therefore, speculate that greater estrogen concentrations are implicated in the pathogenesis of metritis. Further studies are required to clarify the relationship between estrogen concentrations and metritis in cattle.

Calcium is important in mediating smooth muscle contractions. Cows with hypocalcemia have less myometrial contractility in the puerperal period (Al-Eknah and Noakes, 1989) and delayed uterine involution (Heppelmann et al., 2015a). Total serum calcium concentrations did not differ between the two groups and, therefore, differences in myometrial contractility cannot be attributed to differences in calcium homeostasis of the cows before they were euthanized.

It is possible that pentobarbital affected *in-vitro* uterine contractility, although to the best of our knowledge this has not been studied. Reports of the effect of pentobarbital on esophageal and gastric smooth musculature in humans have been ambiguous and a ranged from inhibitory to stimulatory (Sehhati-Chafai, 1979). Likewise, it is conceivable that uterine changes associated with the disease that led to euthanasia of the cow affected uterine contractility. Increased concentrations of systemic $PGF_{2\alpha}$ associated with inflammatory disease may have enhanced myometrial tone.

In the present study, a uterine contraction was defined as significant shortening of the distance between two sonomicrometric crystals in response to stimulation with an uterotonic drug. Oxytocin caused significant shortening of one distance in Group M− and of two distances in Group M+. This confirms that oxytocin enhances uterine contractility in the puerperal period in cows and is consistent with results of *in-vivo* studies in postpartum cows without metritis, in which oxytocin had a positive effect on uterine contractility (Kündig et al., 1990a; Gajewski et al., 1999; Bajcsy et al., 2006). A lesser effect of oxytocin after day 4–5 postpartum described previously in cows in *in vivo* studies (Kündig et al., 1990a) did not occur in the present study, and days postpartum had no effect on contractility pattern after stimulation with oxytocin. Surprisingly, oxytocin treatment resulted in greater contractions in uteri of cows with metritis than in uteri without metritis, which was consistent with greater expression of the myometrial and endometrial OTR gene in uteri of Group M+. It is suspected that inflammatory cytokines are capable of inducing transcriptional activation of OTR in the uterus of mice and women (Gimpl and Fahrenholz, 2001). It is conceivable that the same happens in cows with metritis and that increased concentrations of inflammatory cytokines in the endometrium (Chapwanya et al., 2009) enhance OTR gene expression. Another possible reason for the greater uterine contractility in cows with metritis is increased prostaglandin synthesis associated with this disease (Heppelmann et al., 2014). Prostaglandins enhance myometrial sensitivity to oxytocin (Chan, 1983) and contribute to formation of gap junctions in the myometrial syncytium (Garfield et al., 1980). Whether $PGF_{2\alpha}$ concentrations differed between the cows with and without metritis was not addressed in the present study.

After stimulation with $PGF_{2\alpha}$, one inter-crystal distance decreased after 27 min and then increased 3 min later in Group M+. It can, thus, be concluded that $PGF_{2\alpha}$ is not suitable for enhancing myometrial contractility in puerperal cows. This is in agreement with results of an *in–vivo* investigation in a previous study (Eiler et al., 1984). It has been reported that only dinoprost given intravenously had an uterotonic effect *in vivo*, wheras cloprostenol given intravenously and dinoprost and cloprostenol given intramuscularly had no effect on myometrial contractility (Kündig et al., 1990a). The reason $PGF_{2\alpha}$ has no effect on uterine contractility *in vivo* has been explained by the short half-life of $PGF_{2\alpha}$ after systemic administration with enzymatic elimination in the lungs (Davis et al., 1980; Frazer, 2005). In the present study, $PGF_{2\alpha}$ was injected via the uterine artery directly into the uterus and, therefore, insufficient concentrations or pulmonary enzymatic elimination are not likely causes of its lack of efficacy. Even though cows of Group M+ had greater myometrial and endometrial expression of the FPR gene, unlike oxytocin, this was not paralleled by increased uterine contractility in response to stimulation with $PGF_{2\alpha}$. Greater concentrations of endogenous prostaglandin of cows with metritis (Delvecchio et al., 1994; Heppelmann et al., 2014) is a possible explanation for the lack of uterotonic effect of parenterally administered $PGF_{2\alpha}$ in the present study.

Analogous to the greater expression of the OTR gene in Group M+ as a result of receptor induction by inflammatory cytokines (Gimpl and Fahrenholz, 2001), increased expression of OTR and FPR genes in cows of the M+ group could be attributable to the slightly greater serum estrogen concentrations in these cows; estrogen increases the number of OTR (Bale and Dorsa, 1997) and FPR (Dong and Yallampalli, 2000) receptor. It appears, therefore, that postpartum uterine disease stimulates an expression pattern of uterine hormonal receptor genes that facilitate enhanced myometrial contractility.

To summarize, based on the present *in-vitro* study, the hypothesis that $PGF_{2\alpha}$ has no effect on uterine contractility in puerperal cows was accepted and, therefore, $PGF_{2\alpha}$ cannot be recommended for the ecbolic treatment of cows with metritis. In contrast, oxytocin had a positive effect on myometrial contractility and the hypothesis with respect to this hormone was rejected. Surprisingly, oxytocin induced greater contractions in uteri with metritis than those without metritis. This effect was paralleled by a greater myometrial and endometrial expression of the OTR gene in uteri where metritis was present compared with uteri where there was no

metritis diagnosed. This suggests that the administration of oxytocin can be an effective treatment for enhancement of uterine contractility in cows with metritis. Further study is, however, needed to corroborate these findings using *in vivo* investigations.

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