Effects of *Escherichia coli*- and *Staphylococcus aureus*-induced mastitis in lactating cows on oocyte developmental competence

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Abstract

Mastitis is associated with decreased fertility in dairy cows. In the current study, we created an experimental model to simulate short-term mastitis by a single intramammary administration of Gram-negative endotoxin of *Escherichia coli* origin (G−), or Gram-positive toxin of *Staphylococcus aureus* origin (G+), to examine the effect of mastitis on oocyte developmental competence. Healthy Holstein cows were synchronized, and follicular fluid (FF) of cows treated with G+ or G− and of uninfected cows (controls) was aspirated from the preovulatory follicles by transvaginal ultrasound procedure. The aspirated FF was used as maturation medium for in vitro embryo production. The distribution of matured oocytes into different cortical granule classes and meiotic stages was affected by G− administration (P<0.05) but not by G+ administration. The proportion of oocytes that cleaved to two- and four-cell stage embryos (44 h postfertilization) was lower in both G+ and G− groups than in controls (P<0.05). Blastocyst formation rate (7–8 days postfertilization) was lower in the G− group (P<0.05) and numerically lower in the G+ group compared with their uninfected counterparts. The total cell number in blastocysts did not differ among groups; however, the apoptotic index was higher in the G+ group (P<0.05), but not in the G− group, relative to controls. Examining mRNA relative abundance in oocytes and early embryos revealed mastitis-induced alterations in PTGS2 (COX2), POU5F1, and HSF1 but not in SLC2A1 (GLUT1) or GDF9. Results indicate a differential disruptive effect of mastitis induced by G− and G+ on oocyte developmental competence in association with alterations in maternal gene expression.

Reproduction (2014) 147 33–43

Introduction

Mastitis is one of the major diseases affecting dairy cattle worldwide. It causes large economic losses to the dairy industry, due to both loss of milk production and low milk quality. Mastitis also has deleterious effects on reproductive performance. For instance, the time from parturition to first insemination is longer and the number of services for conception is larger in mastitic cows (Schrick et al. 2001, Maizón et al. 2004, Lavon et al. 2011a). Other epidemiological studies have demonstrated that conception rates (Loeffler et al. 1999, Santos et al. 2004) and pregnancy rates (Harman et al. 1996) are lower in mastitic vs healthy cows.

Oocyte developmental competence is acquired in a progressive manner throughout follicular development and includes a variety of molecular and cellular modifications that are required for the oocyte to complete meiosis, successful fertilization, maternal zygote transition, and further pre- and postimplantation development (Coticchio et al. 2004). The ovarian follicular fluid (FF) in which the oocyte is enclosed and developed is of plasma origin (Edwards 1974). Thus, it is reasonable to assume that elevation of biomolecules in the plasma upon physiological and/or pathological impairments, such as mastitis, might affect FF composition and the follicle-enclosed oocyte. In support of this, Nakajima et al. (1997) reported the presence of tumor necrosis factor α (TNFα) and interleukin 6 (IL6) in the milk and serum of cows with naturally occurring coliform mastitis. Blum et al. (2000) reported an increase in TNFα and NOx (nitrite and nitrate) in the milk and plasma upon *Escherichia coli*-induced mastitis. Hisaeda et al. (2001) detected interferon-γ and TNFα in serum and whey samples from naturally occurring coliform mastitis. Supplementation of lipopolysaccharide (LPS), prostaglandin F₂α (PGF₂α), nitric oxide (NO) generator, or sodium nitroprusside to the maturation or culture medium deleteriously affected the developmental competence of bovine oocytes (Soto et al. 2003a). Exposing bovine embryos to TNFα increased the proportion of apoptotic blastomeres in the developed blastocyst (Soto et al. 2003b). In addition, TNFα decreases the number of cells in the inner cell mass in mouse embryos and lowers embryonic survival (Pampfer et al. 1994, Wuu et al. 1999). Nevertheless, the concentration of inflammatory mediators in the FF of the preovulatory follicle has never been examined. Here, we hypothesized that
mastitis-induced alterations in the FF content have a deleterious effect on both maturation and developmental competence of the follicle-enclosed oocyte.

We established an experimental model based on: i) a single intramammary injection of Gram-negative endotoxin of *E. coli* origin (G−) or Gram-positive toxin of *Staphylococcus aureus* origin (G+); ii) in vivo aspiration of the preovulatary FF; and iii) *in vitro* maturation (IVM) of bovine oocytes using the aspirated FF (i.e., physiologically relevant conditions) as maturation medium. Several analyses were performed to examine the oocytes’ competence to undergo nuclear and cytoplasmic maturation (maturation competence), be fertilized, cleave, and develop to the blastocyst stage (developmental competence). Blastocyst quality was estimated by total cell count and apoptotic index in the developing blastocyst. Expression of stress-related genes such as *PTGS2* (COX2) and *HSF1* (Dubois et al. 1998, Santoro 2000) and those involved in folliculogenesis and early embryonic development such as *GDF9* and *POU5F1* (Niwa et al. 2000, Gui & Joyce 2005) and cell metabolism such as *SLC2A1* (Leppens-Luisier et al. 2001) was determined by real-time PCR in metaphase II (MII) stage oocytes and four-cell stage embryos, before activation of the embryonic genome.

Materials and methods

Animals and milk samples

The study was carried out on 19 healthy, cyclic Holstein cows in their first to fifth lactation. The average DIM was 125 days. Cows enrolled in the study were with somatic cell count (SCC) <150 000 cell/ml milk and with no bacteriological findings as determined by 3-monthly routine milk test prior to the experiment (by Fossomatic 360 instrument, Foss Electric, Hillerød, Denmark). In addition, SCC was determined (by Z1 Coulter counter, Coulter Electronics, Luton, UK) in three sequential aseptic milk samples taken from each of the four mammary quarters once a week before the experiment was begun, as described previously (Younis et al. 2003, Leitner et al. 2006). Bacterial examination was performed according to the International Dairy Federation procedures using accepted standards for bacteriological typing (Oliver et al. 2004).

Induction of mastitis

Mastitis was induced as described previously by Lavon et al. (2011b). The experiment was approved by the local ethics committee of the Hebrew University. Briefly, healthy cows were randomly separated into three groups and received a single intramammary injection into one quarter as follows: i) control cows (n=6) received 10 ml of sterile nonpyrogenic saline; ii) the G− group (n=6) received 10 μg LPS (*E. coli* O55:B5, Sigma Chemical Co.) dissolved in 10 ml nonpyrogenic saline; and iii) the G+ group (n=7) received 40 μg *S. aureus* FR2449/1 extract dissolved in 10 ml nonpyrogenic saline, as described previously (Leitner et al. 2002, Younis et al. 2003).

Intramammary injections were performed following careful cleaning of the teat, using a sterile syringe mounted with a sterile adapter via the teat canal into the gland, as described previously (Lavon et al. 2008). Clinical symptoms such as swelling, rigidity, and high sensitivity of the udder were checked frequently throughout the day of administration. Body temperatures were recorded hourly for 12 h, and SCC was determined 24 h following toxin injection.

Synchronization and follicular aspiration

Cows were synchronized by the OvSynch protocol (Fig. 1). GNRH analog (200 μg Gonadorelin, GonaVex, Parnell Laboratories, Alexandria, NSW, Australia) was injected (i.m., day −8) followed by injection of PGF<sub>2α</sub> analog (500 μg Cloprostenol, Estroplan, Parnell Laboratories; day −2) and a second GNRH administration (day 0). Ultrasonographic scanning (7.5 MHz linear probe with SSD Aloka 900, Tokyo, Japan) was performed through days 0–6 of the cycle to confirm ovulation and corpus luteum formation and to characterize the pattern of growth of the first-wave dominant follicle. On days 6 and 7 of the synchronized cycle, cows received an injection of PGF<sub>2α</sub> to induce regression of the corpus luteum and development of preovulatory follicle.

FF of the developing preovulatory follicle was aspirated 42 h after the second injection of PGF<sub>2α</sub> (Fig. 1A). Briefly, cows were sedated with xylazine hydrochloride (Sedaxylan; Eurovet Animal Health BV, Bladel, Holland), and caudal epidural anesthesia was induced with lidocaine. Aspiration was performed with an
ultrasound scanner (Pie Medical, Maastricht, The Netherlands) equipped with a 7.5 MHz vaginal transducer and 19 G needle connected to a sterile syringe. For each experimental group, the aspirated FFs were pooled and kept at −20°C.

**Hormone concentrations**

Estradiol concentrations in the FF were determined by solid-phase RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA) as described previously (Lavon et al. 2011b). The assay sensitivity was 8 pg/ml, and the intra-assay coefficient of variation was 3%. Progesterone concentration in the FF was analyzed with the solid-phase RIA kit against a standard curve prepared from ovariec-tomized cow plasma (Lavon et al. 2011b). The minimum detected amount was 0.2 ng/ml and the intra- and inter-assay coefficients of variation were 8.6 and 9.9% respectively.

**Chemicals and media for in vitro procedures**

All chemicals, unless otherwise stated, were purchased from Sigma. Follicle-stimulating hormone (FSH) isolated from ovine pituitary extract (Ovagen) was from ICP Bio (Auckland, New Zealand). Double-distilled water (DDW) was from Merck. Dulbecco’s PBS, FCS, and RQ1 RNase-free DNase I were from Promega. Diethylpyrocarbonate (DEPC)-treated water was from Biological Industries (Beit Haemek, Israel). Paraformaldehyde (16%) was from Electron Microscopy Sciences (Hatfield, PA, USA). Superscript II reverse transcriptase, Dynabeads mRNA DIRECT Kit, non-essential amino acids, and essential amino acids were from Invitrogen. DyNAmo ColorFlash SYBR Green qPCR Kit was from Finnzymes (Espoo, Finland). In situ cell death detection kit was from Roche. Fluoromount was from Diagnostic Biosystems (Pleasanton, CA, USA). The culture media HEPES–Tyrode’s lactate (TL), SP-TL, and IVF-TL were prepared in our laboratory: HEPES–TL was supplemented with 0.3% (w/v) BSA, 0.2 mM sodium pyruvate, and 0.75 mg/ml gentamicin (HEPES–TALP); SP–TL was supplemented with 0.6% BSA, 1 mM sodium pyruvate, and 0.2 mg/ml gentamicin (SP–TALP); IVF–TL was supplemented with 0.6% essential fatty acid–free BSA, 0.2 mM sodium pyruvate, 0.05 mg/ml gentamicin, and 0.01 mg/ml heparin (IVF–TALP) as described by Parrish et al. (1986). Oocyte maturation medium (OMM) was made up of TCM-199 and Earle’s salts supplemented with 10% (v/v) heat-inactivated FCS, 0.2 mM sodium pyruvate, 50 μg/μl gentamicin, 2.2 g/l sodium bicarbonate, 2 μg/ml 17β-estradiol, and 1.32 μg/ml FSH. Potassium simplex optimized medium (KSOM) contained 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH₂PO₄, 0.2 mM MgSO₄·7H₂O, 0.8% (v/v) sodium lactate, 0.2 mM sodium pyruvate, 0.2 mM d(+)-glucose, 25 mM NaHCO₃, 0.01 mM phenol red, 1 mM l-glutamine, and 0.01 mM EDTA supplemented with 1.7 mM CaCl₂·2H₂O, 0.1 mg/ml polyvinyl alcohol, 10 μl/ml essential amino acids and 5 μl/ml non-essential amino acids, 100 U/ml penicillin-G, and 0.1 mg/ml streptomycin.

**In vitro production of embryos**

In vitro production (IVP) of embryos was performed as described previously by Gendelman & Roth (2012a, 2012b). Briefly, Holstein cow ovaries were obtained from a local abattoir and transported to the laboratory within 60–90 min in physiological saline solution at 37°C with 50 μg/ml penicillin–streptomycin. Ovaries were placed over a trans-illuminator (Arav 2001) and cumulus–oocyte complexes (COCs) were aspirated from 3 to 8 mm follicles. Groups of ten COCs were transferred to 50 μl droplets of OMM or FF overlaid with mineral oil and incubated in humidified air with 5% CO₂ for 22 h at 38.5°C. Groups of 30 matured oocytes were transferred to four-well plates containing, per well, 600 μl IVF–TALP and 25 μl PHE (0.5 mM penicillamine, 0.25 mM haptotaurine, and 25 μM epinephrine in 0.9% NaCl) and IVF with Percoll-purified spermatozoa (~1×10⁷) from the same bull (i.d. Pazil 3421 ‘Sion’ Hafetz-Haim, Israel) for 18 h at 38.5°C, 5% CO₂. After fertilization, putative zygotes were denuded of cumulus cells by gentle vortexing in HEPES–TALP containing 1000 U/ml hyaluronidase and randomly placed in groups of ten in 25 μl droplets of KSOM. All embryo droplets were overlaid with mineral oil and cultured for 8 days (38.5°C, 5% CO₂, and 5% O₂). The experiments included six IVP runs with 57–68 oocytes per experimental group per run.

**Nuclear and cortical granule staining**

At the end of maturation, oocytes were denuded of cumulus cells and placed in permeabilization solution containing PBS with 1 mg/ml polyvinylpyrrolidone (PVP) and 0.1% (v/v) Triton X-100 for 5 min at 39°C in the oven. Then oocytes were fixed in 4% (v/v) paraformaldehyde in PBS for 15 min at room temperature and stored in PBS with 1 mg/ml PVP (PBS–PVP) at 4°C. Oocytes were washed three times in PBS–PVP and placed in blocking solution containing PBS with 1 mg/ml 0.1% (v/v) Triton X-100, 2% (v/v) normal goat serum, 0.1 M glycine, 1% (w/v) powdered skim milk, and 0.5% (w/v) BSA (pH 7.4) for 1 h at 39°C in the oven.

Cortical granules were stained with 100 μg/ml FITC–peanut agglutinin (PNA) in PBS–PVP for 30 min at 39°C in the oven. Nuclear staining was performed with 10 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS–PVP for 15 min at room temperature. Stained oocytes were washed and placed in drops of Fluoromount and examined under inverted fluorescence microscope (Nikon, Tokyo, Japan) using Nis Elements Software (Nikon).

The oocytes were classified into three types according to the observed distributional pattern of the cortical granules as defined by Izadyar et al. (1998): class I, large aggregates of cortical granules distributed over the entire cytoplasm; class II, cortical granules localized in the cortical cytoplasm and distributed as individual particles as well as small aggregates; and class III, cortical granules more or less evenly dispersed in the cortical cytoplasm aligning the oolemma (Fig. 2A, B, and C). The same oocytes were also classified into four meiotic nuclear stages as described previously by de los Reyes et al. (2005): germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), and MI (Fig. 2D, E, F, and G). The examination included 50 oocytes per group from five different IVM runs.
A

B

C

D

E

F

G

H

I

J

Figure 2 Cellular features in matured oocytes and blastocysts. Cortical granules were stained with FITC–PNA (A, B, and C). Oocytes were classified into class I (A), class II (B), and class III (C). Nuclei of the same oocytes were stained with DAPI (D, E, F, and G) and classified into four meiotic stages: germinal vesicle (D), germinal vesicle breakdown (E), metaphase I (F), and metaphase II (G). Representative images of 8-day blastocyst stained with Hoechst 33342 (H, blue nuclei), staining positive in TUNEL assay (I, green nuclei), and merged pictures (J).

Detection of DNA fragmentation by TUNEL assay

TUNEL assay (Roche) was used to detect DNA fragmentation as previously performed in our laboratory (Kalo & Roth 2011). Briefly, 8-day embryos were washed three times in PBS–PVP, fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, and stored in PBS–PVP at 4 °C. On assay, samples were placed in permeabilization solution containing PBS with 1 mg/ml PVP, 0.3% Triton X-100, and 0.1% (w/v) sodium citrate for 20 min at room temperature in an humidified box. For positive and controls, samples were incubated in 50 μl drops of 50 U/ml RNase-free DNase at 37 °C for 1 h in the dark. After RNase–free DNase treatment, samples were incubated in 50 μl droplets of TUNEL reaction mixture (containing FITC-conjugated dUTP and TdT) for 1 h at 37 °C in the dark. The negative control was incubated under the same conditions, but without TdT. Finally, samples were stained with 1 μg/ml Hoechst 33342 in PBS–PVP for 15 min at room temperature, washed three times in PBS–PVP, and placed in drops of Fluoromount. Labeling was examined under an inverted fluorescence microscope using Nis Elements Software (Fig. 2H, I, and J). The apoptotic cell ratio for each blastocyst was determined by calculating the number of TUNEL-positive blastomeres out of the total number of blastomeres. The examination included 7-day blastocysts, 20 per experimental group, from three different runs.

Gene quantification

Oocytes were collected after 22 h of IVM and denuded of cumulus cells as described above. The examination included 100 oocytes (five samples of 20 oocytes each) taken from five different IVP runs. In addition, four-cell stage embryos were collected at 42–44 h postfertilization. The examination included 50 embryos (five samples of ten embryos each) taken from five different IVP runs. All collected samples were washed in PBS, snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Poly(A) RNA was isolated using Dynabeads mRNA DIRECT Kit according to the manufacturer’s instructions (Invitrogen) as described previously by Gendelman & Roth (2012a, 2012b). In brief, oocytes and embryos were lysed by adding 100 μl lysis-binding buffer to each sample. Prewashed oligo(dT)25 Dynabeads (20 μl) were added to each tube and mixed for 5 min at room temperature to allow binding of poly(A) to the beads. The samples were put into a magnetic separator to remove the lysis buffer while retaining the Dynabeads. The Dynabeads were washed twice with 100 μl washing buffer A (100 mM Tris–HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, pH 8, and 5 mM dithiothreitol), twice with 100 μl washing buffer B (10 mM Tris–HCl, pH 7.5, 0.15 M LiCl, and 1 mM EDTA), and once with 100 μl 10 mM Tris–HCl. After removal of HCl, 8 μl sterile DEPC water was added and the samples were immediately subjected to RT.

RT was performed in a total volume of 20 μl. The first step was incubation at 70 °C with 8 μl RNA sample, 1 μl oligo(dT)12–18 (500 μg/ml), 1 μl RNaseout, 1 μl dNTPs (10 mM each), and 1 μl (50 ng) random primer, followed by 50 min incubation at 42 °C and 5 min at 70 °C with RT mix containing 4 μl 5X reverse transcriptase buffer, 200 U SuperScript II reverse transcriptase, 2 μl 0.1 M dithiothreitol, and DEPC water. The samples were transferred to −20 °C until use.

Quantitative RT-PCR was carried out with primers for PTGS2 (also known as COX2), HSFl, GDF9, POU5F1, and SLCL2A1 (also known as GLUT1), using YWHAZ as a reference gene (Gendelman & Roth 2012a, 2012b). The primers were derived from bovine sequences found in GenBank and designed using Primer Express Software (Life Technologies, Carlsbad, CA, USA; Table 1). Briefly, real-time PCR was conducted on an Mx3000p cycler (Stratagene, La Jolla, CA, USA) using SYBR Green in a final volume of 20 μl containing ultrapure water, 500 nM of each primer, and 3 μl diluted cDNA. The reaction efficiency ranged between 90 and 110% with R2 >0.995. The amplification program included preincubation at 95 °C for 7 min to activate taq polymerase, followed by 40 amplification cycles of denaturation at 95 °C for 10 s and annealing–elongation at 60 °C for 15 s. All samples were run in duplicate in 96-well plates. A melting curve analysis was performed at the end of the amplification to confirm single-gene specificity. Fluorescence was recorded to determine the threshold cycle during the log-linear phase of the reaction at which fluorescence rises above background. Gene expression was quantified and analyzed by MxPRO QPCR Software for
Table 1 Primers used in this study for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Accession number</th>
<th>Size (bp)</th>
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</table>
| YWHAZ | Forward: GCATCCACAGCAATTCC  
Reverse: GCAAGACACATTGAGACCA | NM_174814        | 124       |
| GDF9  | Forward: TGGTCTTGCTGAAGCATCTAGA  
Reverse: ACAGTTGTGAGGTTGCTCTTCT | NM_174681        | 202       |
| POU5F1| Forward: ATATACCCAGGCCGATGTTGG  
Reverse: TGCA-CAAGGGTGTCTCTGCTTCT | M60448          | 167       |
| HSF1  | Forward: AAAGATTCCGCCAGGACAGTG  
Reverse: CATGCACACCTTGGGCTTA | NM_001076809     | 151       |
| PTGS2 | Forward: GAAATGATC-TACCCCGCTCA  
Reverse: TCTGGAA-CAACTGCTCATCG | NM 174445       | 161       |

Mx3000p and Mx3005p QPCR ver. 3, and the ΔΔCt method was used to calculate the relative expression of each gene.

**Experimental design**

The experimental model included two stages. In the first (Fig. 1A), mastitis was induced *in vivo* using doses of G− or G+ toxins as described above and aspirated prevulatory FF. Briefly, lactating Holstein cows were synchronized, and on days 6 and 7 of the cycle, PGF$_2\alpha$ was injected to induce luteolysis and development of a preovulatory follicle. Mastitis was induced 36 h post-PGF$_2\alpha$ injection and the prevulatory follicles were aspirated 6 h later. For each experimental group, the aspirated FFs were pooled and kept at −20°C.

In the second stage (Fig. 1B), oocytes aspirated from Holstein cow ovaries obtained from a local abattoir were matured *in vitro* in the undiluted FF aspirated in the first stage. The rationale for using this model was to mature oocytes in a physiologically relevant follicular environment. At the end of maturation, from each experimental group, subgroups of oocytes were examined for cortical granule distribution (FITC–PNA) and meiotic status (DAPI). In addition, matured oocytes were fertilized, cultured for 8 days, and the proportion of oocytes that cleaved and developed to blastocysts was recorded 44 h and 7 and 8 days postfertilization respectively. Blastocyst quality was estimated by total cell count and apoptotic index (TUNEL assay). Gene expression was examined in both matured oocytes and four-cell stage embryos (real-time PCR).

**Statistical analysis**

Differences between treatments were subjected to one-way ANOVA (JMP-7; SAS Institute, Cary, NC, USA) followed by Tukey–Kramer test. To examine the model’s reliability, oocyte maturation in a standard OMM was compared with that in undiluted FF aspirated from control uninfected cows. To examine the effect of maturation in FF aspirated from mastitic cows, G− and G+ groups were compared with controls. The variables were as follows: SCC in milk samples, estradiol and progesterone concentrations in the FF, proportion of oocytes that cleaved to the two- and four-cell stages and developed to blastocysts (data were arcsine-transformed before analysis), relative mRNA abundance in matured oocytes and four-cell stage embryos, total cell count, and proportion of TUNEL-positive blastomeres in 8-day blastocysts. Data are presented as mean ± S.E.M. and $P<0.05$ was considered significant. Analysis of likelihood ratio was performed to examine differences in oocyte distribution to cortical granule classes (I–III) and nuclear meiotic stages (GV, GVBD, MI, and MII). $P<0.05$ was considered significant.

**Results**

**Mastitis effects on body temperature, milk yield, and SCC**

Induction of G− mastitis increased body temperature by 2°C ($P<0.05$), which lasted for 4 h above the body temperature of the control group. In the cows treated with G+, body temperature was only slightly higher (+0.2°C, NS) than that of controls. Induction of mastitis did not affect milk yield for 3 days after toxin administration. In the G− induced mastitis group, SCC increased to 5×10$^6$ cells/ml 24 h after LPS administration, and in the G+ group, SCC increased to 2.5×10$^6$ cells/ml. Local clinical symptoms in the udder, such as swelling, rigidity, and high sensitivity, were detected in the G− quarters but not in the G+ quarters (data not shown).

**Validation of the experimental model**

To examine the model’s reliability, oocyte maturation in standard OMM was compared with that in undiluted FF aspirated from control uninfected cows. The experiment included three to five runs with 50 oocytes per run per experimental group. Cellular and nuclear maturation characteristics, total cell count, and apoptotic index in blastocysts did not differ between groups (Fig. 3A and A’). Cleavage into two- to four-cell stage embryos and blastocyst formation rates (72 vs 76% and 12.3 vs 13% respectively) were similar in both groups (Fig. 3B and B’). In light of these findings, FF aspirated from control cows was used as a control medium for the IVM procedure. For each experimental group, FFs were pooled. The average progesterone concentration did not differ between groups and were 96.1, 135.5, and 236.2 ng/ml for G−, G+, and control groups respectively. The average estradiol concentration did not differ between groups and were 1067.4, 1109.3, and 1060.0 ng/ml for G−, G+, and control groups respectively.

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Reproduction (2014) 147 33–43
postfertilization, and (B) the percentage of oocytes cleaved to two- to four-cell stage embryos, 42–44 h postfertilization and analyzed for gene expression. The expression of PTGS2 and POU5F1 in oocytes matured in G– FF was lower than that of the control, whereas the expression of these two genes in oocytes matured in G+ FF did not differ from those in the control (P<0.05; Fig. 7A). The expression of HSF1 was lower in both the G– and G+ groups than in the controls (P<0.05; Fig. 7A).

Increased PTGS2 expression was detected in four-cell stage embryos of both G– and G+ groups relative to controls (P<0.05; Fig. 7B). The expression of POU5F1 was lower than in G– and G+ groups relative to controls (P<0.05; Fig. 7A).

**Total cell count and apoptotic rate in blastocysts**

Eighty-day blastocysts were subjected to TUNEL procedure. The range of total cell numbers was 88–95 cells per blastocyst and did not differ among groups (Fig. 6A). However, the proportion of TUNEL-positive (apoptotic) cells per embryo was higher in the G+ embryos than in the controls (P<0.05; Fig. 6B) and that in the G– embryos did not differ from controls.

**Gene expression**

 Matured oocytes were collected at the end of 22-h maturation and embryos at the four-cell stage were collected 42–44 h postfertilization and analyzed for gene expression. The expression of PTGS2 and POU5F1 in oocytes matured in G– FF was lower than that of the control, whereas the expression of these two genes in oocytes matured in G+ FF did not differ from those in the control (P<0.05; Fig. 7A). The expression of HSF1 was lower in both the G– and G+ groups than in the controls (P<0.05; Fig. 7A).

Increased PTGS2 expression was detected in four-cell stage embryos of both G– and G+ groups relative to controls (P<0.05; Fig. 7B). The expression of POU5F1 was lower than in G– and G+ groups relative to controls (P<0.05; Fig. 7A).
was lower in G\textsubscript{C} and higher in G\textsubscript{K} groups relative to controls (P \!<\! 0.05; Fig. 7B). The expression of HSF1 was lower in the G\textsubscript{K} group than in the control group (P \!<\! 0.05; Fig. 7B).

**Discussion**

Mastitis is one of the risk factors for disrupted reproductive performance in dairy cows, but the mechanism by which it impairs the ovarian pool of oocytes is not entirely clear. Here, we provide evidence for the differential impairment of maturation and developmental competence of oocytes in association with alterations in both cellular and molecular features by LPS of *E. coli* (G\textsubscript{K}) vs *S. aureus* extract (G\textsubscript{C}) mastitis. The experimental approach was based on induced mastitis (*in vivo*) followed by IVM of oocytes in the preovulatory FF aspirated from the treated cows. Given the limited ability to identify clinical mastitis in a timely fashion, this was found to be a good simulation approach. One of the main advantages of this model was that mastitis events were well controlled: cows were synchronized and toxin administration was performed at the same stage of the estrous cycle. Another advantage was that IVM in the FF enabled the use of a large number of oocytes rather than a single oocyte per cow aspirated from a single preovulatory follicle. Moreover, maturation of oocytes in the FF did not have any deleterious effects on oocyte developmental competence, as the proportion of oocytes that cleaved and developed to the blastocyst stage did not differ from that of oocytes matured in the standard maturation medium. Similarly, previous studies have reported that oocyte maturation in FF or addition of a certain concentration of FF can even improve embryonic development (Ali *et al*. 2004, Coleman *et al*. 2007). By contrast, another study showed that maturation of oocytes in FF reduces developmental competence (Avery *et al*. 2003). Differences between studies might be due to the use of FF aspirated at different stages of follicular growth or the estrous cycle. It should be noted, however, that maturation of oocytes aspirated from 3 to 8 mm follicles (i.e. immature oocytes), used in the current study, differ from that of preovulatory follicle-enclosed oocytes.

Oocyte developmental competence is acquired in a progressive manner throughout follicular development. The findings of the current study suggest that mastitis not only impairs follicular function (Lavon *et al*. 2011\textsuperscript{b}) but can also affect the follicle-enclosed oocyte. Mastitis induced by G\textsubscript{K} affected both nuclear and cytoplasmic maturation. The proportion of oocytes that resumed
meiosis and reached the MII stage (i.e. nuclear maturation) was lower in the G− group. Similarly, the proportion of oocytes defined according to their cortical granule distribution as class I (i.e. cytoplasmic maturation) was also affected in the G− group compared with the control group. On the other hand, G+ mastitis did not affect either nuclear or cytoplasmic maturation but lowered the cleavage rate. These findings suggest that different mechanisms for each type of toxin, resulting in differential effects on oocyte maturation, could be related to the degree of the inflammatory response, i.e. larger in the mastitic group induced by G− toxin than in that induced by G+ toxin.

With respect to oocyte developmental competence, maturation in FF obtained from both mastitic groups (induced by G− and G+) reduced the proportion of oocytes that fertilized and cleaved to two- and four-cell stage embryos. Moreover, both types of bacteria deleteriously affected the proportion of embryos that developed to the blastocyst stage, with a prominent effect in the G− mastitic group. Because in the current study oocytes were matured in FF aspirated from mastitic cows, the deleterious effect on oocyte developmental competence should be considered a direct effect on the prevulatory enclosed oocyte that is carried over to the blastocyst stage. Such alterations might explain, in part, the reduced fertility in mastitic cows (Lavon et al. 2011b). An epidemiological study (Lavon et al. 2011b) showed a long-term effect of E. coli-induced mastitis on conception rate. In particular, a past clinical event occurring up to 10 days prior to AI significantly reduced the probability of conception. Furthermore, we have recently shown that E. coli mastitis occurring during a 90-day period prior to performing IVM/IVF disrupts the ovarian pool of GV stage oocytes, resulting in a decrease in blastocyst formation rate (Roth et al. 2013). Taken together, the findings support the view that not only the developing embryo but also the oocyte is highly susceptible to pathogenic (mastitis) stress.

Given the prominent effect on oocyte maturation and the long-lasting effect on embryonic development, one might expect that blastocysts developed from oocytes matured in FF aspirated from mastitic cows will be of inferior quality. However, the findings are complex and not entirely clear. While the total cell count for the blastocysts did not differ between groups, the apoptotic cell count was higher in the G+ but not in the G− group relative to controls. Modest apoptosis is essential for embryonic survival (Paula-Lopes & Hansen 2002), whereas a high level of apoptotic blastomeres might decrease the inner cell mass, reduce embryonic survival, and increase embryonic death (Pampier et al. 1994, Wu et al. 1999). This phenomenon has been well documented for preimplantation embryos exposed to thermal stress (Paula-Lopes & Hansen 2002). Nevertheless, in the current study, neither oocytes nor embryos were exposed to hyperthermia and the transient hyperthermia developed in vivo in the G− treated cows has nothing to do with oocytes matured in G− FF. On the other hand, as the oocytes were matured in FF, it is possible that immune-activated factors pass via the circulation into the FF and in turn impair oocyte developmental competence. Supporting this assumption are reports that mastitis impairs the concentrations of inflammation mediators such as IL6, TNFα, and NO in milk and blood (Nakajima et al. 1997, Blum et al. 2000, Hisaeda et al. 2001). Although not examined in the current study, an increase in inflammation mediators might also be expressed in the FF. Previous studies have reported an increase in the inflammatory component in the plasma within 6–12 h of mastitis induction (Mehrzad et al. 2007). In the current study, maturation in FF aspirated 6 h after induction of mastitis impaired oocyte developmental competence, which might support this assumption. It is also possible that mastitis induced changes in the hypothalamus–pituitary–ovarian axis might affect steroid concentration in the preovulatory follicle (Lavon et al. 2011b), which in turn might affect the oocyte microenvironment.

Cortical granule distribution has been shown to be affected by environmental culture conditions (Liu et al. 2005). For instance, supplementation of antioxidant and growth factors to the maturation medium increases the proportions of class-I oocytes and developing blastocysts (Izadyar et al. 1998, Cordova et al. 2010). Exposing oocytes to elevated temperature during maturation impairs cortical granule distribution (Payton et al. 2004). Therefore, passage of immune-activated factors into the FF, as suggested above, might affect oocyte development.

**Figure 7** Effect of induced mastitis on gene expression. MII stage oocytes were collected at the end of 22-h maturation (A) and four-cell stage embryos were collected 42 h postfertilization (B). Presented are relative transcript levels of PTGS2, HSF1, POU5F1, GDF9 and SLC2A1. Quantification relative to YWHAZ is presented as means ± S.E.M.; different letters indicate treatment effect within a specific gene, \( P<0.05 \). The examination included 100 oocytes and 50 embryos per group from five different runs.
cytoplasmic maturation upon induction of mastitis by G−. Increased inflammatory factors in the FF might also disrupt the maternal mRNA transcripts stored in the oocyte. Oocyte maturation in FF aspirated from mastitic cows was associated with altered gene expression in a stage-dependent (MI stage oocytes and four-cell stage embryos) and bacterial-type-dependent (G− and G+) manner. Moreover, the alterations documented in the oocyte through the four-cell-stage embryos (i.e. before embryonic genome activation) indicate a long-lasting effect on the maternal transcriptional level. In particular, maturation in G− FF reduced the expression of PTGS2 in matured oocytes, whereas maturation in G+ FF further reduced POU5F1 expression in four-cell stage embryos. This is an important finding because POU5F1 is a member of the POU family of transcriptional activators (Ryan & Rosenfeld 1997) and essential for maintenance of totipotency/pluripotency of embryonic stem cells and primordial germ cells. The level of POU5F1 governs embryo fate, and a critical level of POU5F1 is required to maintain embryonic stem cell renewal. Up- or downregulation of POU5F1 has been found to induce changes in developmental programs (Niwa et al. 2000). Therefore, the impaired (i.e. increased or decreased) POU5F1 expression noted in the current study might explain, in part, the reduced oocyte developmental competence in mastitic groups. Whether these alterations are further expressed in advanced stages of embryonic development is not clear.

COX2 is highly inducible by diverse stimuli, including cytokines, growth factors, and mitogen and tumor promoters (Hla & Neilson 1992, Smith et al. 1994). In mice, Ptgs2-deficient females are infertile due to abnormal processes of ovulation, fertilization, implantation, and decidualization (Dinchuk et al. 1995, Langenbach et al. 1995, Lim et al. 1997). It is therefore possible that the altered PTGS2 expression noted here resulted from exposure to mediators secreted into the circulation upon mastitis induction and eventually ending up in the FF. In particular, the reduced PTGS2 expression in oocytes matured in G− FF and the increased PTGS2 expression in four-cell stage embryos in both G− and G+ groups are suggested to underlie the reduced developmental competence of oocytes in mastitic cows. COX is an initiator enzyme in the cascade of prostaglandin (PG) formation and plays an important role in early embryonic development (Lewis 1989). In vivo studies have reported that mastitis and/or endotoxin administration are associated with increased PGF2α in the circulation (Giri et al. 1991, Huszenicza et al. 2005). Short-term in vitro exposure of oocytes and early-stage embryos to PGs, mimicking the acute phase of clinical intramammary injection, disrupts both oocyte maturation and embryonic development (Soto et al. 2003a, 2003b). In addition, COX2-derived PGII plays an important role in blastocyst development (Pakrasi & Jain 2007) and has a part through embryo hatching (Huang et al. 2003, 2004). Although not examined here, involvement of PG via COX activation in disruption of oocyte and embryo development cannot be ruled out.

HSF1 is a member of the heat-shock transcription factor family responsible for stress-induced expression of heat-shock proteins (HSPs). HSP level rapidly increases in response to stress (Santoro 2000) or under some physiological and pathogenic conditions such as fever, inflammation, cell or tissue trauma, aging, and infection (Feige & van Eden 1996, Morimoto & Santoro 1998). In turn, HSPs have protective properties including protein synthesis, refolding of denatured proteins, protection of folded proteins and targeting irreversibly denatured proteins for removal (Knowlton 2006). Moreover, HSPs have direct anti-inflammatory and protective effects on membrane integrity and mitochondrial function, as well as an inhibitory effect on apoptosis (Beere et al. 2005, Voss et al. 2005). However, in the current study, the expression of HSF1 mRNA isolated from both oocytes and four-cell stage embryos was generally lower in the mastitic groups than in the control, with a prominent reduction in the G− group. Reduced expression of HSF1 mRNA might lead to reduced levels of HSP, which in turn might impair oocyte competence to cope with stress. In addition, HSF1 is essential for oocyte meiosis (Metchat et al. 2009) and plays a role in reproductive success (Christians et al. 2000). It is therefore suggested to be involved in the mechanism underlying decreased embryonic development in the mastitic groups. Supporting this assumption is the fact that reduced HSF1 gene expression in cultured cells decreases their response to stress in association with delayed HSP activation (Westerheide et al. 2009).

Unlike the impaired gene expression discussed above, GDF9 transcription level did not change in oocytes or embryos upon induction of mastitis. GDF9 is a germ cell marker and a member of the large transforming growth factor β (TGFβ) superfamily (McPherron & Lee 1993), which plays a pivotal role in folliculogenesis. Homozygous knockout female mice are sterile due to blockage of follicles at the primary stage (Dong et al. 1996). It also stimulates proliferation of theca cells derived from bovine small follicles and is therefore defined as a mitogenic factor (Spicer et al. 2008). GDF9 is involved in oocyte maturation via regulation of cumulus cell function in the preovulatory follicle (Gui & Joyce 2005). A recent study reported lower GDF9 expression in primordial, primary, and secondary follicles in cows with subclinical mastitis (Rahman et al. 2012). It is therefore possible that the effect on GDF9 expression is associated with subclinical mastitis, rather than the acute mastitis examined here.

In summary, the model used in the current study enabled examining the effect of follicular microenvironment (i.e. FF aspirated from mastitic cows) on oocyte competence. Findings indicate that mastitis impairs both
nuclear and cytoplasmic maturation, as well as oocyte developmental competence; this effect seems to be dependent on bacterial type. Moreover, observed alterations in transcriptional levels in the oocyte carried over to the four-cell stage embryo. These findings might explain, in part, the reduced fertility in mastic cows.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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