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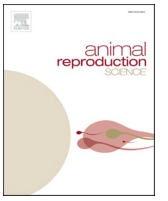

Bovine salpingitis: Histopathology, bacteriology, cytology and transcriptomic approaches and its impact on the oocyte competence

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ABSTRACT

The present study was performed to examine the histopathology, cytology, bacteriology and expression pattern of a targeted set of genes of cytokines in the oviduct of cows with inflammation (Experiment 1). In addition, the effects of oviductal fluid from cows with salpingitis on the oocyte maturation and fertilization in vitro were examined (Experiment 2). The most frequent bacterial co-infection was *Escherichia coli* and *Fusobacterium necrophorum*, which was always associated with severe histopathologic salpingitis. Out of 15 cows with histologically healthy uterus, only one cow (6.7%) displayed the histologic signs of mild salpingitis, whereas from 50 cows with endometritis, 48 cows (96%) showed histologically different grades of salpingitis. The mRNA expression of *IL1β, CD14, IL8* and *CASP3* was significantly different among all groups of salpingitis ($P < 0.05$) with the highest level of mRNA expression in the sever grade of salpingitis. Results of experiment 2 showed a significant decline in the oocytes with peripheral free mitochondria and fertilization rate in the salpingitis group than the no- salpingitis group ($P < 0.05$). In conclusion, our results showed that histologically detected salpingitis is in most cases associated with histologic and cytologic endometritis. The pattern of the gene expression of chemokines and cytokines was altered in association with different grades of salpingitis. Further, we observed a decline in the peripherally located mitochondria and lower fertilization rate in oocytes following addition of oviductal fluid collected from the cows with sapingitis to the maturation media.

1. Introduction

Bacterial contamination of the bovine uterus is an inevitable post-calving event that occurs in a large percentage of high producing cows reared in intensive farms (Bromfield et al., 2015; Lima, 2018; Sheldon et al., 2019). Uterine contaminations may result in the development of uterine diseases, including acute septic metritis, clinical and subclinical forms of endometritis, pyometra, and salpingitis (Sheldon et al., 2019). Even after the treatment and the disappearance of clinical signs of uterine infections, fertility may

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still be less than optimum in dairy cows (Noakes et al., 2018). The lower fertility in these cows is presumed to be associated with undetectable endometritis before breeding which is a carryover consequence of uterine infections in the early postpartum period (Ribeiro et al., 2016). Recently, it was shown that the transcriptome of the endometrium, oviduct, and even granulosa cells is altered even after three months of the induction of intrauterine infusion of pathogenic bacteria in virgin Holstein heifers (Horlock et al., 2020). These findings may explain the low fertility in cattle with uterine infections even after the voluntary waiting period and before the first artificial insemination. Uterine inflammatory diseases, before breeding, reduce oocyte fertilization rate and development to the blastocyst stage which in turn result in disruptions in embryo quality and conceptus development (Bromfield et al., 2015).

The oviduct has various crucial tasks, including carrying sperm and oocyte and in addition provides a suitable microenvironment for final gamete maturation, fertilization, and early embryo development (Marey et al., 2014). Any undesirable changes, therefore, such as salpingitis, an infection and inflammation in the oviduct, could detrimentally impact normal oviductal functions. Salpingitis is regarded as a major cause of subfertility in humans and animals (Shivhare et al., 2012; Schindlbeck et al., 2014) and among reproductive tract disorders, endometritis and salpingitis have been reported as the most common causes of infertility in bovine. Most cows with pyometra also have salpingitis with no significant bacterial invasion of the oviductal wall (Karstrup et al., 2017). In a previous study, Owhor et al. (2019) revealed that endometritis is always correlated with salpingitis in the cow. Furthermore, it was observed that various species of microorganisms, such as *Trueperella pyogenes, Fusobacterium necrophorum, Streptococcus*, and *Porphyromonas levii,* could potentially invade bovine oviducts as ascending infection from the uterus (Karstrup et al., 2017; Owhor et al., 2019). Although many reports deal with the histopathology, cytology, and bacteriology of endometritis-affected cows, not much information on the relationship among the histopathology, cytology, bacteriology, and the inflammatory related gene expression of cows with salpingitis is available. In addition, to the best of our knowledge, no study has examined the effects of the oviductal fluid of inflamed oviducts on the maturation and fertilization of oocytes in dairy cows.

The immunity of bovine uterus and oviduct at the molecular level has been a very important topic of research among bovine reproduction researchers. Cows with uterine disease experience rise in gene expression of several chemokines and cytokines. Downregulation of oviductal transcriptome related to immunological functions is a vital prerequisite for the establishment of pregnancy (Maillo et al., 2015). The cytokine interleukin-1β *(IL1β)* is a very important mediator of the inflammatory reactions and has an essential role in the host resistance to pathogens. IL1β is a key mediator to generate rapid and vast proinflammatory activity in endometrial cells (Schaefer et al., 2005; Gärtner et al., 2016). Interleukin-8 (*IL-8*) is a chemoattractant cytokine produced by various tissue and blood cells. IL8 has target specificity to attract and activates neutrophils in inflamed tissues. CD14 functions as a co-receptor with the Toll-like receptor (*TLR4*) to detect bacterial LPS (Kitchens, 2000; Tapping and Tobias, 2000) as well as other pathogen-associated molecular patterns such as lipoteichoic acid (Ranoa et al., 2013). CD14 is mainly expressed by macrophages, neutrophils and monocytes (Funda et al., 2001). CASP3 is a biological indicator in the signaling pathways of apoptosis, necrosis, and inflammation. The increased expression level of the mRNA of the *CASP3* in bovine oviduct epithelial cells has been observed in in vitro and ex vivo studies following exposure to LPS (Ibrahim et al., 2015; O'Doherty et al., 2016).

With this background in mind, the current study was designed to examine (1) the histopathology, cytology, bacteriology, and expression pattern of a targeted set of genes of cytokines in the oviduct of cows with salpingitis with different severities (Experiment 1), and (2) the effects of oviductal fluid from cows with salpingitis on the oocyte maturation and fertilization in vitro (Experiment 2).

2. Materials and methods

2.1. Ethics

This study was approved by the Research and Animal Welfare Committee of Shiraz University (99GCB2M1251).

2.2. Experiment 1: histopathology, cytology, bacteriology, and gene expression of bovine salpingitis with different severities

2.2.1. Collection of reproductive tracts

Reproductive tracts of 300 Holstein dairy cattle, including uterus, oviducts, and ovaries, were collected from the slaughterhouse. The tracts were free from perimetritis, uterine and ovarian adhesions and segmental aplasia in the oviducts, uterine horns and ovarian cysts. The cranial end of each uterine horn, the cervix, and the end of each ampulla were closed with nylon ties to prevent subsequent contamination of the lumen of different parts of the tract. The tracts were then placed in individual plastic bags and immediately transported to the pathology laboratory at 4 °C in an insulated container. Subsequently, all the tracts with gross signs of uterine infections (Studer and Morrow, 1978; Sheldon and Noakes, 1998; Knutti et al., 2000; LeBlanc et al., 2002; Williams et al., 2005; De Boer et al., 2014) and at least a mature corpus luteum on their ovary were subjected to histopathology and cytology (n = 70); a subset of samples was used for bacteriology (n = 24). In addition, a total of 20 tracts with a grossly involuted uterus without signs of uterine infections were used as the healthy control group. The reason we used the reproductive tracts with at least a corpus luteum was to rule out any confounding factors in histological features associated to other stages of estrus cycle. Genital tracts with accumulated urine in the uterus were excluded from the study.

2.2.2. Preparation for samplings

In the laboratory, the outer surface of the whole tracts was rinsed with sterile phosphate-buffered saline (PBS); the oviducts from designated tracts were then exscinded from ampulla as well as utero-tubal junction. The oviducts were trimmed free of corresponding connective tissues and fats to open up its coils and convoluted parts. Moreover, both ends of the oviduct were closed using hemostats

and rinsed twice externally with sterile PBS to clear out external blood and contaminations (Abe and Hoshi, 1997; Way, 2006; Alfradique et al., 2019; Kumaresan et al., 2019). All samplings were performed within 2 h after slaughtering the cows.

2.2.3. Histopathological examination of the uterus and oviduct

A 2 × 1.5 cm full-thickness piece of the uterine wall was excised by scissors from the dorsal aspect of the uterine body. In addition, two pieces of 1 cm in length, one from proximal parts of the ampulla and the isthmus, were separated from both oviducts (four samples from each tract); uterine and oviductal samples were subsequently separately placed in 10% buffered formaldehyde solution (Karstrup et al., 2017; Owhor et al., 2019). Formalin-fixed samples were then processed by routine methods, sectioned at 5 μm thicknesses, and stained with hematoxylin and eosin. The samples were then evaluated under a light microscope for bovine endometritis (Bonnett et al., 1991; Chapwanya et al., 2009; Meira et al., 2012; Fuentes et al., 2018) and salpingitis (Karstrup et al., 2017; Owhor et al., 2019) (Table 1). Both uterine and oviductal samples were graded from healthy to mild, moderate, or severe inflammation.

2.2.4. Cytological examination of the oviduct

After sampling for histopathology, a 3 cm of ampulla ipsilateral to the ovary bearing corpus luteum was incised longitudinally to expose their lumens, and then a cytobrush (Heinz Herenz, Hamburg, Germany) was rolled against the luminal surface and rotated on a microscope slide. Afterward, oviductal smears were fixed and stained using Diff Quick staining kit (Hooshmand Fanavar, Tehran® Co, Iran). The percentage of polymorphonuclear neutrophils (PMN) was determined by counting 300 cells at 400 × magnification (Melcher et al., 2014). Oviductal smears showing more than 3% of PMNs were diagnosed as salpingitis (Marey et al., 2016).

2.2.5. Bacteriological analysis of the oviduct

Samples for bacterial culture were collected under sterile conditions by flushing the oviducts with 1 mL sterile PBS using 22-gauge needles. The flushing of the oviducts was performed from the ampullary end directly into the Stuart transporting medium (Zistroyesh®, Shahrekord, Iran). Bacterial samples were immediately transported to the microbiology laboratory in an insulated container at 4 °C (Kumaresan et al., 2019; Owhor et al., 2019).

2.2.6. Bacterial culture

Sheep blood agar was used for aerobic gram-positive bacteria isolation, and aerobic gram-negative bacteria were isolated using Eosin Methylene Blue and MacConkey agars; all were subsequently incubated aerobically at 37 °C for 48 h. A supplemented sheep blood agar was also utilized for the identification of *Trueperella pyogenes*, by employing a microaerophilic incubator. For microaerophilic culture, sheep blood agar was supplemented with 0.5% yeast extract, vitamin K (10 μg/mL, V3501–1G, Sigma, St. Louis, USA), and Haemin (5 μg/mL, Sigma, St. Louis, USA) (Markey et al., 2013). Moreover, for culturing non-sporing anaerobes, an anaerobic atmosphere was prepared using an anaerobic jar and a gas pack A type (Merck KGaA, Darmstadt, Germany). The anaerobic jar was then incubated at 37 °C for seven days. Finally, a cooked meat broth medium with 0.4% glucose was utilized for the cultivation of the rest of the anaerobic bacteria. Bacterial identification was performed considering colony characteristics, gram staining, bacterial cell morphology, hemolytic capability, and biochemical profile (Markey et al., 2013). Afterward, according to histopathology, the isolates from 32 oviduct flushes consisting of healthy (n = 8), mild (n = 8), moderate (n = 7), and severe (n = 9) salpingitis were recorded as the bacteriology of the oviduct.

Table 1
Criteria used for the diagnosis and grading of the bovine salpingitis; * : Hpf indicate 640 × magnification (equal to 0.1 mm^2).

Variable	Category		Assessment
Lumen			
Secretions and cell detritusNormal	Absent		Normal
	Present		Abnormal
Epithelium and lamina properia			
Bulging of secretory cells	Absent		Normal
	Present		Abnormal
Mucus covering	Absent		Normal
	Present		Abnormal
Mucus covering	Absent		Normal
	Present		Abnormal
Cilliary clumping	Absent		Normal
	Present		Abnormal
Edema	Absent		Normal
	Present		Abnormal
Congestion	Absent		Normal
	Present		Abnormal
Lymphocyte infiltration intensity		0–5 cells/ hpf*	No inflammation
		5–10 cells/hpf	Mild
		10–15 cells/hpf	Moderate
		> 15 cells/hpf	Severe

2.2.7. Evaluation of the gene expression

Real-time RT-PCR test was conducted on the endometrium and oviduct samples for the relative quantification of gene expression. To compare the transcriptome of the inflamed oviducts with a control group, the longitudinally opened oviducts were placed on a microscope slide and gently scraped with a scalpel blade to recover epithelial cells. The recovered cells (~250 mg) were immediately placed in a 1.5 mL tube full of RNA preservatives (RNAlater, Denazist Asia, Mashhad, Iran) and put in a − 70 °C for subsequent gene expression surveys (Maillo et al., 2015). A total of six samples from each severity group of cows with salpingitis were taken (a total of 18 samples from cows with salpingitis and six samples from control no salpingitis cows) based on the results of the histopathology of the oviduct. The Hybrid-R™ kit (GeneAll®, Seoul, South Korea) was employed to extract total RNA from 150 mg of fragmented frozen oviduct tissue based on the manufacturer's protocol. Moreover, total RNA was eluted in 50 μL, and RNA quality and quantity were assessed by a Nanodrop spectrophotometer (Epoch, BioTek®, Santa Clara, USA). Total RNA yield was between 400 and 2000 ng/μL, and the 260/280 absorbance ratio was between 1.8 and 2.18. The reverse transcription of 1 μg RNA to cDNA was accomplished using the ExcelRT™ Reverse Transcription kit (SMOBIO®, Hsinchu City, Taiwan) by random hexamer primers and according to the manufacturer's protocol. No-template and no-RT controls were included in the reverse transcription and real-time PCR experiments. In addition, primer pairs were designed using Beacon Designer software (PREMIER Biosoft®, San Francisco, USA). Then their specificity was checked using BLAST server which was supplied by Bioneer®, Seoul, South Korea. As the primer pairs were intron spanning, or placed on exon- exon junctions, there was no need for DNase treatment of RNA samples before cDNA synthesis. Primer sequences and types with more details are shown in Table 2. Real-time qPCR was performed on cDNA samples in triplicate using a LightCycler® 96 instrument (Roche®, Penzberg, Germany). A Master Mix was prepared for each assay containing 2 × SYBR green PCR mix (Ampliqon®, Odense M, Denmark), forward and reverse primers, and nuclease-free water. For each sample and gene transcript, a total volume of 14 μL was prepared by mixing of 7 μL Master Mix, 0.28 μL forward primer (10 μM), 0.28 μL reverse primer (10 μM), 4.94 μL distilled water, and 1.5 μL cDNA. In addition, real-time qPCR was conducted with initial denaturation for 15 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 59 °C for 20 s, and 72 °C for 10 s. Subsequently, the melting curve analysis program was performed from 60 ºC to 95 ºC. In order to qualify real-time qPCR tests, standard curves were depicted for each gene, separately. *GAPDH* was used as the reference gene, and fold change (expression ratio) of genes was calculated using the delta delta-Ct method.

2.3. Experiment 2: The effects of oviductal fluid from cows with salpingitis on the oocyte maturation and fertilization in vitro

2.3.1. In vitro maturation of oocytes

The oviductal fluid samples from cows with histopathologically healthy oviducts (n = 5) and salpingitis (n = 5) were collected and then pooled to be substituted as serum supplement in the oocyte maturation media in each experimental group. Fetal calf serum (FCS) was also used as a supplement in the control group of in vitro oocyte maturation. This technical control group was employed to compare the results of routine standard in vitro oocyte maturation with the results of the oviductal fluid groups. For oocyte collection, the ovaries of slaughtered cows were collected and transferred to the laboratory within 2 h at 32–35 °C in PBS. The cumulus oocyte complexes (COCs) with more than three layers of cumulus cells and a finely granulated homogenous ooplasm were selected and transferred to a place containing in vitro maturation culture medium (Lorenzo et al., 1994). In total, 557 good-quality COCs were assigned to three groups. In the technical control group, COCs (n = 176) were cultured in TCM-199 medium (M4530, Sigma, St, Louis, USA) supplemented with 10% FCS, 5 IU/mL hCG (Karma-HCG, Bioscience GmbH, Baesweiler, Germany), 10 ng/mL EGF (Sigma, St. Louis, USA), and 0.1 IU/mL human FSH (Follitrope, Geonbuk-do, South Korea). On the other hand, in the non-salpingitis group, COCs (n = 182) were cultured in TCM-199 medium supplemented with 10% filtered oviductal fluid. In addition, in the salpingitis group, COCs (n = 199) were cultured in TCM-199 medium supplemented with 10% filtered oviductal fluid. All cultures were carried out in four well culture dishes (NuncTM, Roskilde, Denmark). In all maturation media, 50 μg/mL of Gentamicin (Sigma, St. Louis, USA) was also added, and the groups of 30–50 COCs were cultured for 24 h in 500 μL culture medium at 38.5 °C and 5% CO_2.

2.3.2. Evaluation of the nuclear maturation of oocytes

After 24 h, cumulus expansion was scored using a stereomicroscope from 0 (no expansion) and 1 (partial expansion) to 2 (complete expansion). In addition, COCs were assessed for nuclear maturation using the aceto-orcein staining method as previously described (Azari et al., 2017). Oocytes with no polar body and the chromosomes arranged in metaphase plate were classified as metaphase I (MI);

Table 2
Nucleotide sequence details of primers for RT-PCR analysis.

Gene	Primer sequences (`5-`3)	Primer pair types	Amplicon length (bp)	Accession number
IL8	For: GCTGGCTGTTGCTCTCTT Rev: GGGTGGAAAGGTGTGGAA	Intron spanning	126	NM_173925.2
IL1B	For: TATTCTCTCCAGCCAACCTTCA Rev: GTCATCAGCCTCAAATAACAGC	Intron spanning	126	NM_174093.1
CD14	For: ATCTACTGACTATGGTGTGCGT Rev: GAAATCGTCGTCGTCCAGC	Exon- exon junction	110	NM_174008.1
CASP3	For: CGGAAGCAGACCAGGAGT Rev: GCCAGTATTTTCGTGGAAGTTC	Intron spanning	249	NM_001077840.1
GAPDH	For: GCTCTCTGCTCCTGCC Rev: CCGTTCTCTGCCTTGACTG	Intron spanning	259	NM_001034034.2

moreover, oocytes with a polar body and the metaphase II plate were classified as mature. In addition, oocytes that did not reach the metaphase or showed chromosomal abnormality were classified as immature. The experiment was performed in five replicates. The status of nuclear maturation was not possible to determine in sixty one COCs in different groups. These COCs were not included in the statistical analysis.

2.3.3. Evaluation of the mitochondrial distribution of oocytes

The mitochondrial distribution pattern was evaluated in a subset of each replicate of the in vitro matured oocytes in the technical control (n = 103), non-salpingitis (n = 117), and salpingitis groups (n = 95) (Uppangala et al., 2015). In summary, oocytes were incubated in TCM-199 containing 10 μg/mL of Rhodamine 123 (Cat No. R8004, Sigma Aldrich, St. Louis, USA) for 20 min at 37 °C and in 5% CO_2. Afterward, oocytes were washed and transferred onto a microscopic slide containing approximately 20 μL of fluorescent mounting medium (Cat. No. S3023, Dako, USA). The slides were then carefully observed under UV light (405–435 nm) using a fluorescent microscope (Primo Star, Zeiss, Germany) (Uppangala et al., 2015). The following patterns of mitochondrial distribution were distinguished: i) uniform distribution: mitochondria spread uniformly throughout the cytoplasm; ii) aggregated distribution: mitochondria spread in irregular clumps in different areas of the cytoplasm; iii) peripheral distribution: mitochondria distributed mostly in the peripheral areas of the cytoplasm; iv) central distribution: localized mostly in the central area of the cytoplasm (Liu et al., 2010). Evaluation of the mitochondrial distribution was performed in five replicates.

2.3.4. In vitro fertilization

In vitro maturation of the oocytes was performed in three groups as mentioned above. After in vitro maturation of the oocytes, the groups containing 35–50 COCs were transferred into four-well culture dishes with 500 μL Tyrode's medium as fertilization medium. Frozen-thawed sperm of a high fertility bull, previously tested in the laboratory, was used for in vitro fertilization. Motile spermatozoa from frozen/thawed semen were recovered using the swim-up method, and were then added to the wells containing the oocytes at a final concentration of 10^6 spermatozoa/mL (Azari et al., 2017). In addition, oocytes and spermatozoa were co-incubated at 38.5 °C for 18 h in 5% CO_2.

2.3.5. Assessment of the fertilization rate

After the fertilization period, the surrounding cumulus cells of presumptive zygotes were removed by repeated pipetting; next, denuded oocytes were mounted on glass slides under coverslips. The fertilization rate in the oocytes was determined using the aceto-orcein staining method as previously described (Azari et al., 2017). Presumptive zygotes with two pronuclei were classified as fertilized. Moreover, presumptive zygotes with more than two pronuclei were classified as polyspermy. Presumptive zygotes with no pronuclei or second polar body were classified as non-fertilized. The experiment was performed in six replicates.

2.3.6. Statistical analyses

Statistical analysis was performed using SPSS 24.0 software (version 16). The normality assumption of studied parameters was assessed using a Shapiro–Wilk test, and Levene's test was also used to evaluate the equality of variances before we proceed with other analyses. The difference in the mean percentage of PMN among different grades of histopathologic endometritis groups was assessed using the one-way ANOVA and Tukey's post hoc tests. Pearson's correlation coefficient (r) was used to assess the correlations between the cytological and histopathological findings. The linear by linear association between severities of endometritis and salpingitis was evaluated using a chi-squared test. The agreement between cytology and histopathology of the results from paired oviducts was

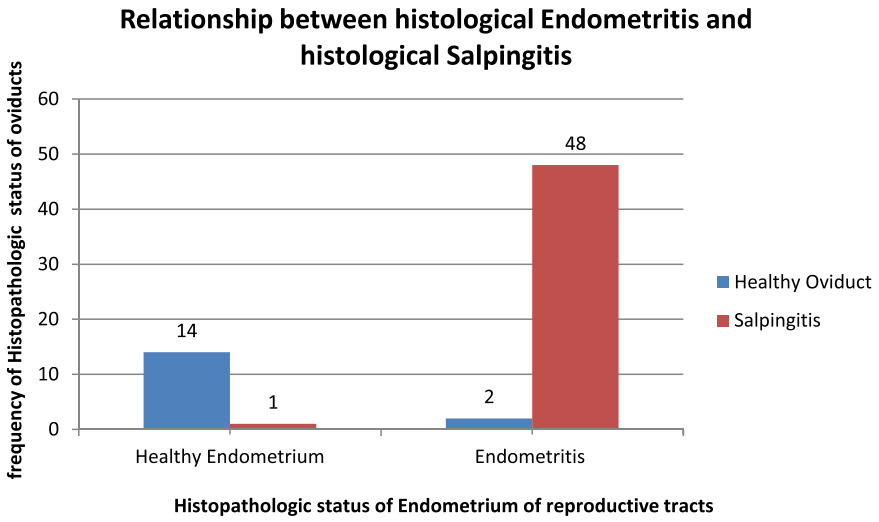

Fig. 1. Relationship between the histological endometritis and histological salpingitis.

compared using Cohen's Kappa test. Moreover, the Shapiro-Wilk and Levene's tests were employed to examine the normality of the distribution of data sets and the equality of variances, respectively. Then, one-way ANOVA was applied for the statistical analysis of Real-time RT-PCR results. The differences in the cumulus expansion, maturation, and fertilization rates among groups were statistically analyzed using an ANOVA test (Tukey's post hoc). In all statistical tests, probability values less than 0.05 and 0.01 were considered statistically significant and highly significant, respectively.

3. Results

3.1. Experiment 1

3.1.1. Uterus and oviduct histopathology

Among 70 tracts with gross signs of uterine infections, excluding those with urovagina, 50 uteri were histopathologically diagnosed with endometritis (mild inflammation n = 15; moderate inflammation n = 17; severe inflammation n = 18) (Fig. 1). Moreover, out of 50 cows with endometritis, a total of 48 oviducts (96%) showed different histological grades of inflammation (Fig. 2), including mild (n = 18), moderate (n = 7), or severe (n = 23) grades of inflammation (Fig. 3). Of 20 control cows with the grossly involuted uterus and no signs of uterine infections, 15 (75%) uteri were histopathologically diagnosed as negative for endometritis, of which only one oviduct displayed histological signs of mild inflammation (6.7%). The mean (± SD) number of lymphocytes was 3.12 ± 1.15, 7.45 ± 0.96, 12.68 ± 1.30 and 35.74 ± 23.14/0.1 mm^2 in the healthy oviduct, mild, moderate and sever salpingitis, respectively. The relationship between histologically diagnosed endometritis and salpingitis was significant (R = 0.87; $P = 0.01$). Furthermore, there was a linear relationship between the degree of inflammation in the endometrium and the oviduct, in a way that as the severity of endometritis increased, the grade of salpingitis also increased simultaneously ($P = 0.01$). In addition, 79.2% of the endometritic cows had bilateral salpingitis (38 out of 48), and 20.8% of the cows had unilateral salpingitis (10 out of 48).

3.1.2. Relationship between the uterus and oviduct histopathology and oviduct cytology

Of 50 cows with histological endometritis, 13 (26%) had cytologic salpingitis. Furthermore, it was revealed that 53.8% of the cows with cytological salpingitis belonged to the cows with severe grade of histological endometritis. Moreover, there was a linear relationship between the degree of histological endometritis and cytological salpingitis. As the severity of endometritis increased, the incidence of cytological salpingitis was also increased (R = 0.39; P = 0.01). A significant relationship (P = 0.01) was found between the results of histological evaluation of the oviduct and the cytology of the oviduct. The degree of agreement between the histology and cytology of the oviduct, however, was low (kappa = 0.15) for the presence of inflammation. Most cases of cytologic salpingitis (84.6%, or 11 out of 13) belonged to the severe grade of histologically inflamed oviducts.

3.1.3. Bacteriology of the oviduct

The isolated bacteria from the inflamed oviducts are shown in Table 3. Bacteria were isolated from 3 out of 8 (37.5%) healthy oviducts, whereas cultures from all cases of salpingitis (100%) resulted in bacterial isolation. In most cases (62.5%), no bacterium was isolated from the healthy oviducts. *Escherichia coli, Trueperella pyogenes* and *Fusobacterium necrophorum* were the most common bacterial species isolated from the salpingitis groups. The most frequent bacterial co-infections diagnosed in severe salpingitis cases were *Escherichia coli* and *Fusobacterium necrophorum*, which were always associated with severe histopathological salpingitis. *Escherichia coli* was isolated only from two samples of the healthy oviducts.

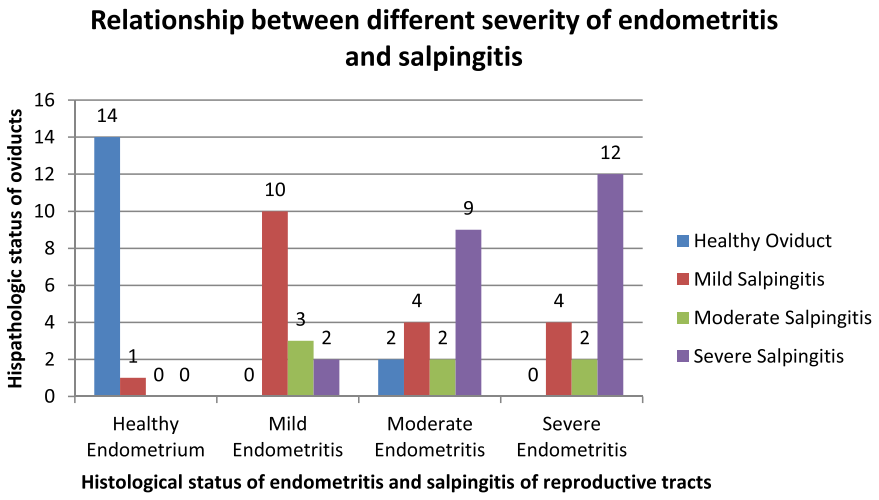

Fig. 2. The relationship between severity of histological endometritis and histological salpingitis. As the severity of endometritis increased, the incidence of cytological salpingitis was also increased (R = 0.39; P = 0.01).

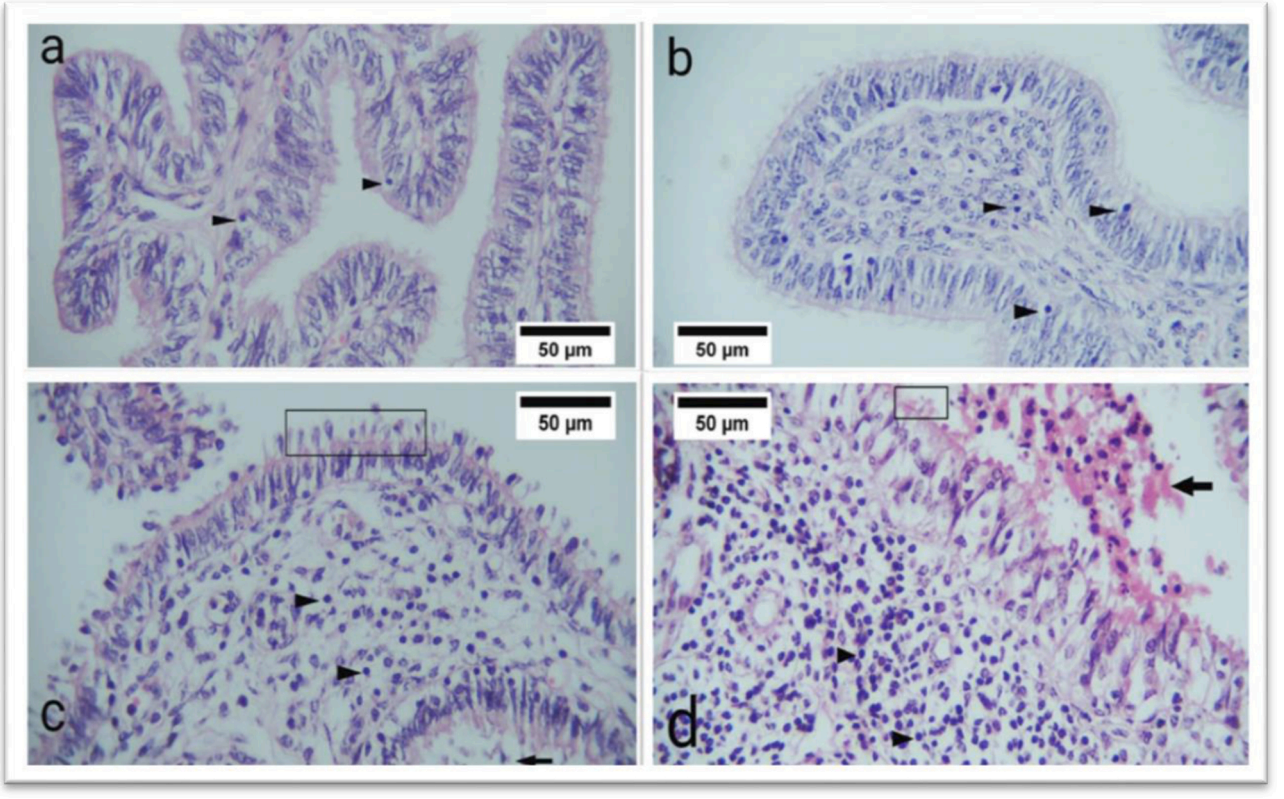

Fig. 3. Different grades of histological salpingitis in the ampullary region, a: healthy oviduct with clear lumen and normal epithelial cells, lymphocyte infiltration intensity is only 3 per Hpf, b: mild salpingitis, c: moderate salpingitis and, d: severe salpingitis, (arrow) is pointing to the accumulation cellular debris and secretions in the lumen. (Arrow heads) are demonstrating lymphocytes and (quadrates) are exhibiting cilliary clumping, bulging of secretory cells and mucus covering of epithelium. The mean ($\pm$ SD) number of lymphocytes was 3.12 ± 1.15, 7.45 ± 0.96, 12.68 ± 1.30 and $35.74 \pm 23.14/0.1$ mm^2 in the healthy oviduct, mild, moderate and sever salpingitis, respectively.

Table 3
Incidence of bacteria* isolated from 32 oviductal flushes including no-salpingitis (healthy), mild, moderate and severe salpingitis.

Bacterial Species	Salpingitis			
	Healthy n = 8	Mild n = 8	Moderate n = 7	Severe n = 9
Coagulase negative Staphylococcus	0	1	0	0
Staphylococcus aureus	1	1	1	1
Escherichia coli	2	2	2	6
Fusobacterium necrophorum	0	1	0	3
Trueperella pyogenes	0	0	0	4
Dichelobacter nodosus	0	1	0	0
Clostridium perfringens	1	2	0	1
Streptococcus uberis	0	2	0	0
Bacillus subtilis	1	1	0	1
Klebsiella pneumonia	0	0	1	0
Peptostreptococcus	0	0	1	0
Pseudomonas aeruginosa	1	1	3	1
Proteus mirabilis	0	0	2	0
Salmonella dublin	0	1	0	0
Rhodococcus equi	1	0	0	0
Campylobacter fetus	0	1	0	0

*Different bacteria were isolated from the same samples. From three healthy oviducts, seven species of bacteria were isolated.

3.1.4. Gene expression assay

The results are presented in detail in Figs. 4 and 5. In the healthy control group, transcript abundances in most samples were not measurable (Without Ct or Ct > 40). The mRNA expression of cytokines interlekin-1β (*IL1β), CD14,* and *IL8* was significantly higher in the moderate and sever salpingitis group than the healthy control group ($P = 0.01$). Furthermore, the severe salpingitis group exhibited the highest expression of mRNA of *IL1β, CD14* and *IL8*. The mRNA expression of *CASP3* was significantly higher in salpingitis groups compared to the healthy control group ($P < 0.05$). Mild salpingitis groups revealed higher mRNA expression of *CASP3* compared to those of moderate and severe groups of salpingitis ($P < 0.05$).

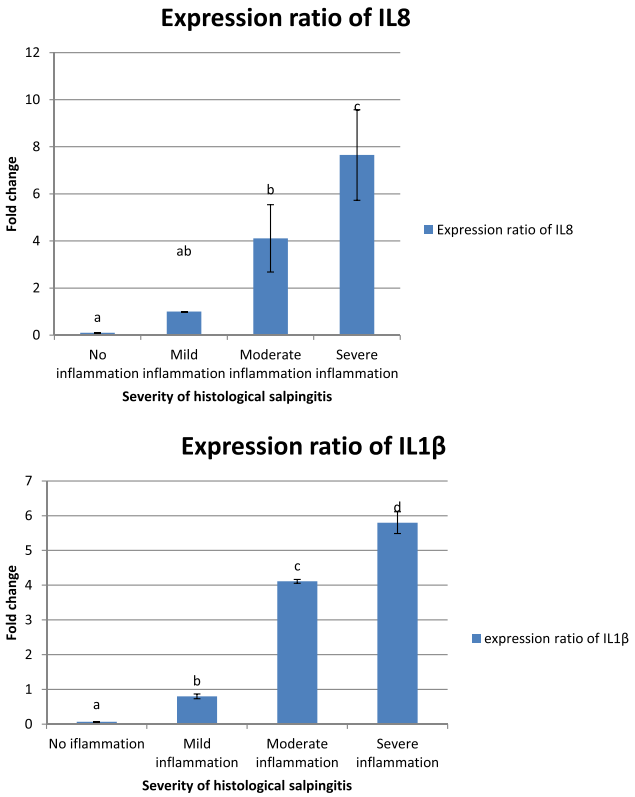

Fig. 4. The expression ratio (mean ± SD) of *IL1β, CD14, IL8* and *CASP3* in different severity groups of histological salpingitis.

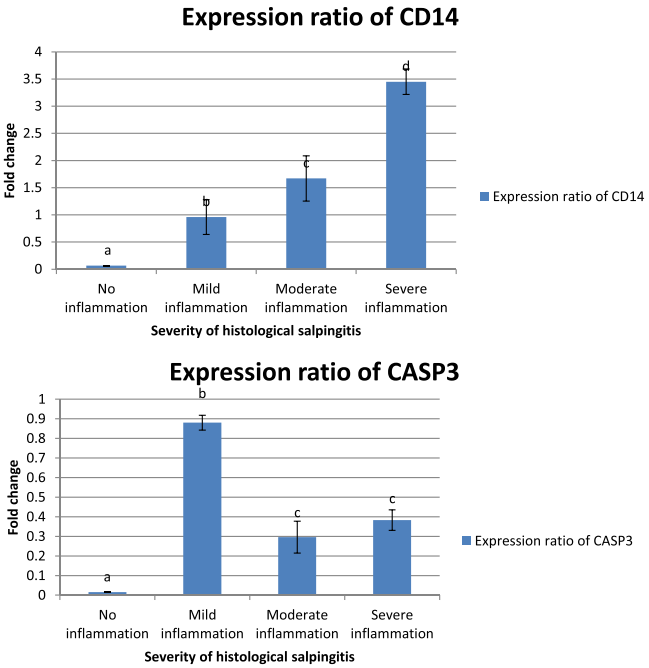

Fig. 5. The expression ratio of *IL1β, CD14, IL8* and *CASP3* in different severity groups of 775 histological salpingitis.

3.2. Experiment 2

3.2.1. In vitro maturation and mitochondrial distribution of oocyte

The mean (± SD) percentage of COCs showing fully expanded cumulus cells in the technical control group (78.8 ± 4.9) did not significantly differ from those in the non-salpingitis and salpingitis groups (77.8 ± 7.2 and 69.3 ± 6.8, respectively; $P > 0.05$). Similarly, the percentage of fully expanded cumulus cells between non-salpingitis and salpingitis groups was not significantly different ($P > 0.05$). The percentages of oocyte nuclear maturation in the technical control group were not significantly different from those of the other groups ($P > 0.05$; Table 4). Moreover, the mitochondrial distribution pattern in the matured oocytes were not different when the technical control group and the non-salpingitis group were compared. In contrast, a significant decline in the percentage of the oocytes with peripheral free mitochondria was observed in the salpingitis group as compared to the non-salpingitis group (29.5 vs 56.3%; $P < 0.01$), respectively.

3.2.2. In vitro fertilization

The mean (± SD) percentage of normal oocyte fertilization was significantly higher in technical control and non-salpingitis groups than in the salpingitis group (69.7 ± 5.0 and 64.9 ± 9.0 vs 36.9 ± 8.6, $P < 0.05$, respectively). The results are shown in detail in Table 5.

4. Discussion

The present study aimed to examine the histopathology, bacteriology, cytology and the expression pattern of relevant inflammation-related genes in bovine salpingitis. We observed that histologically detected salpingitis was, in most of the cases, associated with histological endometritis. Furthermore, the severity grade of salpingitis was associated with the level of the mRNA expression of inflammation-related genes, including *IL1β, CD14,* and *IL8.* In addition*, Escherichia coli, Fusobacterium necrophorum* and

Table 4
Mean (± SD) percentages of nuclear maturation following addition of oviductal fluid collected from cows with no salpingitis and with sapingitis to the maturation media.

Groups	No.	MII oocytes, n (%)	Immature oocytes, n (%)	MI oocytes, n (%)
Control	173	147 (85.6 ± 3.4)	20 (11.6 ± 1.8)	6 (3.5 ± 2.3)
No saplingitis	158	129 (82.5 ± 2.8)	22 (13.9 ± 8.7)	7 (4.4 ± 3.0)
Salpingitis	165	125 (76.9 ± 6.9)	33 (20.1 ± 6.8)	7 (4.2 ± 3.1)

In control group, oocytes were cultured with FCS.
MII (Metaphase II), MI (Metaphase I).

Table 5
Mean (± SD) percentages of normally fertilized oocytes following addition of oviductal fluid collected from cows with no salpingitis and with sapingitis to the maturation media.

Groups	No.	Fertilization, n (%)	Non fertilized, n (%)	Polyspermy,n (%)
Control	192	134 (69.7 ± 5.0)[a]	43 (22.4 ± 2.0)[a]	15 (7.8 ± 5.5)
No saplingitis	220	138 (64.9 ± 9.0)[a]	70 (31.8 ± 2.6)[a]	12 (5.5 ± 2.0)
Saplingitis	199	74 (36.9 ± 8.6)[b]	107 (53.7 ± 4.6)[b]	18 (9.1 ± 5.1)

Different letters within a column differs significantly ($P < 0.05$).

Trueperella pyogenes were isolated from the majority of cows with severe salpingitis. Our results also indicated a reduction in the peripherally located mitochondria and a lower fertilization rate in the oocytes following the addition of oviductal fluid, collected from cows with salpingitis, to the oocyte maturation media.

The delicate microenvironment of the oviduct is responsible for the final capacitation of the male gamete, fertilization, and early embryo development (Avilés et al., 2015; Maillo et al., 2015). The present study is the first to show the effects of oviductal fluid collected from cows with salpingitis on the oocyte nuclear maturation, mitochondrial distribution and fertilization rates using an in vitro model. Our results showed no difference in the rate of nuclear maturation in the oocytes following the addition of oviductal fluid, collected from cows with salpingitis to the maturation medium compared to non-salpingitis and technical control groups. The distribution pattern of mitochondria, however, was different in the oocytes matured using the oviductal fluid of the cows with salpingitis when compared to the other experimental groups. A significant decrease in the oocytes with peripheral free mitochondria was observed in the salpingitis group compared to the distribution pattern of mitochondria in oocytes cultured in maturation medium supplemented with either FCS or the oviductal fluid from the cows with no salpingitis. Normal nuclear and cytoplasmic maturation in the oocyte is the vital prerequisite events for the proper fertilization of the oocyte (Dey et al., 2012; Lonergan and Fair, 2016). Previous studies on bovine oocytes and embryos showed a correlation between the reorganization of the mitochondria in the oocytes after IVM, the ATP levels and the total number of cells in the blastocysts (Ferreira et al., 2009). Moreover, the occurrence of all these events requires a normal oviductal microenvironment. Any dysfunction in the epithelial cells of the oviduct can cause abnormal maturation and fertilization capability of the oocytes or sperm cells. Owhor et al. (2019), using an *ex-vivo* model, showed that salpingitis impaired sperm survival and motility. Furthermore, they observed that inflamed oviducts secreted high amounts of mucus which contained glycoproteins and acidic mucopolysaccharides compared to healthy oviducts (Owhor et al., 2019). These secretions caused impairment in the transport and nourishment of the sperm cells and oocytes. Similarly, our results clearly showed that salpingitis caused morphological and functional alterations in the epithelial cells of the oviduct, including ciliary clumping, the bulging of secretory cells, and the accumulation of mucus and cell debris in the oviduct lumen. The functional alterations in the epithelial cells of the oviduct in the present study could have been the consequence of the alteration in the mRNA expression of chemokines and cytokines, including *IL1B*, *CD14,* and *IL8* in Experiment 1 of the present study. These findings imply that inflammatory products in the inflamed oviducts that we used in the maturation media could have created an abnormal microenvironment for mitochondrial distribution in the cytoplasm of the oocytes leading to a low fertilization rate. Downregulation of the oviductal transcriptome related to immunological functions was previously shown as a vital prerequisite for the establishment of pregnancy in heifers (Maillo et al., 2015).

We found a strong association between the simultaneous presence of endometritis and salpinigitis. Our finding is similar to that reported by Owhor et al. (2019). Previous studies showed that cows with endometritis had an elevated mRNA expression of several chemokines and cytokines, including *IL1β, CD14,* and *IL8* in the endometrial cells (Chapwanya et al., 2009; Galvão et al., 2011; Sheldon et al., 2019). Similarly, the results of the present study showed a significant rise in the level of mRNA expression of *IL1B, CD14,* and *IL8* in the oviductal epithelium in cows with salpingitis. It was previously showed that downregulation of oviductal transcriptome related to immunological functions was a vital prerequisite for the establishment of pregnancy in heifers (Maillo et al., 2015). The cytokine interleukin-1β *(IL1β)* is a key mediator of the inflammatory response and has an essential role in the host response and resistance to pathogens. *IL1β* is pivotal for generating rapid and extensive proinflammatory activity in the epithelial cells of the uterus (Schaefer et al., 2005; Gärtner et al., 2016). Using an in vitro model, a similar role for the *IL1β* was observed when the oviductal epithelial cells were exposed to pathogenic bacteria such as *Trueperella pyogenes* (Mesgaran et al., 2018). In addition, Ibrahim et al. (2015) showed that the expression level of *IL1β* increased when bovine oviductal epithelial cells were exposed to LPS. In the present study, gram-negative bacteria such as *Escherichia coli* and *Fusobacterium necrophorum* were frequently isolated from the majority of cows with moderate and severe salpingitis. Taking the results of these studies and our findings together, it can be concluded that the increase in the level of mRNA expression of *IL1β* in the oviductal cells could have been associated with salpingitis due to the pathogenic gram-negative bacteria, and mainly LPS secretion. Although the LPS concentration was not measured in the oviductal fluid of cows with salpingitis in the present study, our previous studies revealed that the LPS concentration was high in the follicular fluid of cows with subclinical (Heidari et al., 2019) and clinical endometritis (Salary et al., 2020). *CD14* acts as a co-receptor for the Toll-like receptor (*TLR4*) and *MD-2* to detect bacterial LPS (Kitchens, 2000; Tapping and Tobias, 2000) as well as other pathogen-associated molecular patterns such as lipoteichoic acid (Ranoa et al., 2013). *CD14* is mainly expressed by macrophages, neutrophils and monocytes (Funda et al., 2001). Additionally, the expression of the *CD14* gene by endometrial and oviductal epithelial cells has been reported in previous studies (Herath et al., 2006, 2009; Chapwanya et al., 2009; Ibrahim et al., 2015; Marey et al., 2016). The present study similarly showed an increasing pattern in the mRNA expression of the *CD14* with a rising pattern along with the severity of histological salpingitis. *IL-8*, also known as the neutrophil chemotactic factor, has two important functions; induction of chemotaxis in target cells, primarily neutrophils and other granulocytes, as well as stimulation of the phagocytosis (Pekalski et al., 2017). The results

of the present study showed a significant severity-dependent rise in the mRNA expression level of *IL-8* in the inflamed oviducts. *CASP3* is a biological indicator in the signaling pathways of apoptosis, necrosis, and inflammation. The increased expression level of the mRNA of the *CASP3* in bovine oviduct epithelial cells has been reported in in vitro and *ex vivo* studies following exposure to LPS (Ibrahim et al., 2015; O'Doherty et al., 2016). Our results showed a significant increase in the expression level of the mRNA of the *CASP3* in the inflamed oviducts compared to the oviducts of healthy cows. The significant higher level of mRNA expression of *CASP3* in the mild salpingitis compared with moderate and sever salpingitis cannot be explained in the preset study. This requires further research before a solid conclusion can be reached.

No information is available regarding the relationship between histological and cytological salpingitis. We only used the diestrus reproductive tracts to rule out the possible effects of the stage of the estrous cycle on the cytology of the oviduct. The results of the present study showed that 26.5% (13 out of 49) of the histologically diagnosed cows with salpingitis had cytological salpingitis. A significant relationship between the results of histological evaluation of the oviduct and the cytology of the oviduct was observed; however, the degree of agreement between the histology and cytology of the oviduct was low for the presence of inflammation in the present study. Similarly, Fuentes et al. (2018) reported a significant correlation between the endometrial inflammatory infiltrate and the uterine cytology results, while there was a low degree of agreement between the uterine histology and cytology for the detection of inflammation. This was attributed to the fact that cytology fails to detect most of the oviducts with low degree of inflammation. In addition, the cytology examines the superficial layer of the oviductal epithelium and then it may not detect the inflammatory cells in deeper layers (Madoz et al., 2014; Fuentes et al., 2018).

5. Conclusions

In summary, our study demonstrated that in most cases, the histologically diagnosed endometritis is associated with salpingitis in bovine. Moreover, the pattern of the gene expression of cytokines was altered in association with different grades of salpingitis. These alterations in the microenvironment of the oviduct resulted in the abnormal mitochondrial distribution and low fertilization rates in bovine oocytes.

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CRediT authorship contribution statement

Mohammad Sadeghi: Investigation. **Mehdi Azari:** Investigation. **Mojtaba Kafi:** Conceptualization, Investigation, Supervision. **Hossein Nourani:** Investigation, Supervision. **Mehran Ghaemi:** Investigation. **Davoud Eshghi:** Investigation.

Conflict of interest

The authors declare no conflict of interest.

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