Aus dem Institut für Veterinär-Biochemie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

# Evaluating cultured primary bovine oviductal epithelial cell responses under physiological and pathophysiological conditions

**Inaugural-Dissertation**
zur Erlangung des Grades eines
Doctor of Philosophy (PhD) in Biomedical Sciences
im Dahlem Research School (DRS) Programm
an der
Freien Universität Berlin

vorgelegt von
**Sadjad Danesh Mesgaran**
Agrarwissenschaftler (Animal Physiology)
aus Mashhad, Iran

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***To my Ensieh,***

***For your advice, your patience, and your faith,***

***Because you always understood.***

***To my loving sweet parents,***

***Your affection, love, encouragement and prays of day and night make me able to get such success and honor.***

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## List of abbreviations

| | |
|---|---|
| 15-PGDH | 15-hydroxyprostaglandin dehydrogenase |
| AA | Arachidonic acid |
| *B. pumilus* | *Bacillus pumilus* |
| BDH2 | 3-hydroxybutyrate dehydrogenase |
| BOEC | Bovine oviductal epithelial cells |
| C5a | Complement component 5a |
| CL | Corpus luteum |
| COC | Cumulus-occyte complex |
| CXCL | Chemokine (C-X-C motif) ligand |
| CXCR | Chemokine (C-X-C motif) ligand receptor |
| DNA | Deoxyribonucleic acid |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| G6P | Glucose 6-phosphate |
| h | Hour |
| HK | Hexokinase |
| IFN | Interferon |
| IL | Interleukin |
| MAPK | Mitogen-activated protein kinase |
| MCP-1 | Monocyte chemoattractant protein 1 |
| MIP3α/CCL20 | Macrophage inflammatory protein 3 alpha |
| mM | Millimolar |
| mRNA | Messenger ribonucleic acid |
| MOI | Multiplicity of infection |
| MUC | Mucin |

| | |
|---|---|
| MYD88 | Myeloid differentiation primary response gene 88 |
| NK | Natural killer cells |
| NFκB | Nuclear factor-kappa B |
| OVGP1 | Oviduct-Specific Glycoprotein 1 |
| OXCT2 | 3-oxoacid CoA transferase |
| P0 | Passage 0 |
| P3 | Passage 3 |
| PI3K | Phosphatidyl-inositol 3-kinases |
| PG | Prostaglandin |
| $PGE_2$ | Prostaglandin $E_2$ |
| $PLA_2$ | Phospholipase $A_2$ |
| PLO | Pyolysin |
| PRR | Pattern recognition receptors |
| PTGES | Prostaglandin $E_2$ synthase |
| PTGS1/2 | Prostaglandin-endoperoxide synthase1/2 |
| qPCR | Quantitative polymerase chain reaction |
| ROS | Reactive oxygen species |
| SDHA | Succinate dehydrogenase complex, subunit A |
| SEA | Statistical energy analysis |
| SLO | Cholesterol-binding cytotoxin streptolysin O |
| *T. pyogenes* | *Trueperella pyogenes* |
| TLR | Toll-like receptor |
| TNFA | Tumor necrosis factor alpha |
| VNTR | Variable number tandem repeat |
| WGA | Wheat germ agglutinin |

# 1. Introduction

The oviduct provides an optimal environment for gamete maturation, fertilization and early development of the embryo (Hunter 1998, Coy *et al.* 2012). Oviduct functions are mediated mainly through the epithelial cells. Oviductal epithelial cells do not only promote normal physiological events, but also serve as part of the mucosal immune system, protecting the oviduct from various potential pathogens (Wira *et al.* 2005b).

Cell culture models of bovine oviductal epithelial cells (BOEC) are used widely to study the cells' role in reproductive physiology and to elucidate the different interactions of the oviduct with the spermatozoa or the zygote (Ulbrich *et al.* 2010). These models allow researchers to study cells without any systemic variation that might occur during an *in vivo* trial. Nevertheless, various culturing conditions require standardization to produce results that more closely resemble *in vivo* conditions. A significant amount of research has outlined the impact of underestimating culturing conditions, e.g. cell passaging on different aspects of cellular responses (Arkell & Jackson 2003, Neumann *et al.* 2010, Aldarmahi *et al.* 2014).

Noticeable advances in dairy cow management conditions have led to tremendously increased yields in the modern cattle milk (Barkema *et al.* 2015). Intensified management as well as deliberate genetic alteration for milk production traits have resulted in current high-producing dairy cows (Oltenacu & Broom 2010). However, this constant striving for milk production has been associated with poor fertility and lower pregnancy rates in the animals (Wathes *et al.* 2014). Dairy cattle fertility rates and pregnancy outcomes are influenced by nutrition, environment and disease, ultimately affecting the profitability and sustainability of the dairy production industry (Chebel *et al.* 2004). Sub-optimal fertility in modern dairy cattle requires a multidisciplinary strategy involving veterinarians, animal physiologists and molecular biologists in order to fully reveal different aspects of this phenomenon (Leroy *et al.* 2008).

The impact of pathogenic bacteria and their pathogenesis mechanisms on the bovine reproductive tract is of high importance, as infection often results in post-parturient diseases and impairment of dairy cattle health (Sheldon *et al.* 2006). A significant number of studies have focused on elucidating the immune interactions of the bovine uterus, as it is the first organ of the upper reproductive tract that is exposed to pathogens, in general, and the endometrium more specifically, to detrimental bacteria (Healy *et al.* 2015, Ibrahim *et al.* 2016). Nevertheless, recent work has indicated that up to 40% of the dairy cows still show clinical signs of uterine infection at 60 days postpartum, the period in which breeding should start (Yasui *et al.* 2014). A recent study reported the presence of different pathogenic bacterial species on the oviductal epithelial surface in early postpartum dairy cows with pelvic infection (Karstrup *et al.* 2017). Therefore, it would be interesting to understand whether the upper reproductive tract organs, e.g. the oviduct might also be vulnerable to the invaded bacteria. So far, very little is known about the interaction of different pathogens with bovine oviductal cells. Expanding on this knowledge is very important for providing better understanding on how pathogens impact the bovine reproductive system.

The present project aims to describe a standardized monolayer cultured model of primary BOEC by observing the responses of BOEC under the influence of various culturing conditions. These cell

responses are revealed by determining the transcription and proteomic levels of molecules involved in oviduct function. The established BOEC model is further used to reveal the immune interactions of BOEC, e.g. cellular inflammatory responses against pathogenic microorganisms.

# 2. Overview of the literature

## 2.1. Oviduct contribution in successful reproduction

### 2.1.1. Structure of the oviduct

The first anatomical observation of the mammalian oviduct was reported in the 16$^{th}$ century (Menezo & Guerin 1997). This organ in bovine is roughly 21-28 cm long and encompasses mucosa of ciliated and secretory columnar epithelial cells, i.e. endosalpinx. The endosalpinx is surrounded by smooth muscle layers namely myosalpinx and the perisalpinx, which consists of the blood vessels and connective tissues (Ellington *et al.* 1991). Generally, the oviduct contains three anatomically distinct sections. These sections have dissimilar histomorphometric structures as well as mucosal topography particularly during phases of the estrous cycle (Mokhtar 2015).

The infundibulum, located close to the ovarian surface, opens by an ostium and is coated with highly vascularized finger-like projections called fimbriae forming the oviductory funnel (Eddy & Pauerstein 1980). The mucosa of this part is highly folded with tall primary and further secondary folds. The ampulla has a large diameter with apparent extended mucosal folds adjacent to a thin layer of smooth muscle cells (Crisman *et al.* 1980). Ampulla mucosa is in shape of different branched leaf-like folds, which are interconnected with each other (Mokhtar 2015). The isthmus connects the ampulla to the utero-tubal junction covering the remaining part of the tube. The thick wall and well narrowed lumen of this segment allows it to function as a spermatozoa reservoir along with the entrance point for the conceptus to the uterus (Hung & Suarez 2010). Isthmus' mucosa comprise of longitudinal lines of low and thick primary folds with less divergence compared with the other sections (Yaniz *et al.* 2000). Secondary folds of isthmus mucosa are formed in apical areas and progress toward the basement section of the folds in a direction from the uterus to the ovaries.

### 2.1.2. Gamete maturation, fertilization and embryo development

Several studies have focused on revealing the gamete and oviduct interactions. Oviductal secretion contains molecules such as organ-specific glycoproteins (further described in Section 2.1.5.2.) or other proteins that contribute to the regulation of gamete interactions and to the early stages of embryo development.

Gamete transport within the oviductal lumen is a highly controlled process (Suarez 2008, Kolle *et al.* 2009). In cattle, various molecules such as sperm binder proteins are involved in the interaction between spermatozoa and epithelial cells in the formation of these reservoirs (Leblond *et al.* 1993, Calvete *et al.* 1994). Heparin in oviductal fluid induces the synchronous release of sperm adhering to the bovine oviduct epithelium (Gualtieri *et al.* 2005). A study on bovine spermatozoa oviduct interaction revealed that uncapacitated sperm adhered to the oviduct, and that their release was associated with capacitation (Talevi *et al.* 2007). The authors suggested that thiol-reducing agents in the oviductal fluid could cause spermatozoa release from oviduct epithelium.

Oviductal epithelial cells are constantly in contact with the cumulus-oocyte complex (COC) facilitating its movement through the organ to the site of fertilization. *In vitro* studies revealed that the oviduct via its cadherin expression is involved in oocyte-sperm adhesion (Caballero *et al.* 2014). A study on bovines showed that the presence of oviductal fluid collected during the follicular phase of the estrous cycle increased the number of binding sperm to the oocyte zona than the fluid collected during the luteal stage (Way *et al.* 1997).

During the preimplantation phase, the fertilized zygote undergoes initial cell divisions, and then the intracellular contact increases, leading to blastocyst (around day 5 in cattle) formation and cell differentiation. The cellular interaction of the oviduct through various bioactive compounds, e.g. glutamate and bicarbonate, forms an optimal milieu for these pivotal events, leading to an effective embryo-maternal dialogue (Leese *et al.* 2008). Co-culture of bovine embryos with oviduct epithelial cells or oviductal fluid conferred significant benefits on the rate of embryo development (Lopera-Vasquez *et al.* 2015). Via their metabolic capacity, oviductal cells can reduce the atmospheric oxygen pressure and the levels of embryonic development inhibitors. It has been suggested that growth factors secreted in oviductal fluid are involved in preimplantation embryo development (Swangchan-Uthai *et al.* 2011).

### 2.1.3. Bovine reproductive cycle

Reproductive (estrous) cycle in bovine takes around 21 days (17-24 days). Duration of estrus is around 15 hours with a range of 6 to 24 hours (Senger 2003). The estrous cycle is divided into two endocrine phases i.e. the follicular and luteal phases, predominantly regulated by hormones. The follicular phase in cows lasts for about 4 days (including proestrus and estrus stages), while the length of the luteal phase is about 17 days (Senger 2003). The follicular phase is associated with high levels of estrogen produced by the ovarian follicles, which will be released into the blood stream affecting the anterior pituitary with positive feedback on cows' nervous system causing the animal to show signs of standing estrus. The luteal phase is characterized by high levels of progesterone produced by corpus luteum (CL) that releases into the blood and has negative feedback on anterior pituitary preparing the uterus for the fertilized ovum reception. The luteal phase (including metestrus and diestrus) starts from the time of ovulation and ends after regression of the CL (luteolysis). The duration of metestrus is about 5 days. This phase is characterized by CL formation (luteinization). Diestrus corresponds to the mid luteal phase, with mature CL producing high levels of progesterone. During the last 2-3 days of the luteal phase, luteolysis occurs. This results in decreasing amounts of progesterone in the serum leading to the removal of the negative feedback of this hormone on the hypothalamus (Senger 2003).

### 2.1.4. Oviductal epithelial cells

The oviduct epithelium is a columnar layer of ciliated and secretory cells (Senger 2003). Secretory cells produce various molecules promoting embryonic development, while ciliated cells facilitate the transport of oocytes and zygotes by ciliary beating. These cells are tightly bound together into a sheet of epithelia. Moreover, they are recognized as a polarized type of cell, where the plasma membrane is elaborately compartmentalized into two distinct apical and basolateral poles (Alberts *et al.* 2008). The

apical part lies in the direction of the oviductal lumen, comprising distinct attributes such as ciliated cells, while the basolateral pole encompasses the other cell types. The proportion of bovine oviduct's ciliated cells is abundant at the follicular phase, whereas the proportion of secretory cells gradually increases with the formation of the CL (Abe *et al.* 1995). The factors involved in these changes are relatively unknown.

Oviductal epithelial cells express estrogen and progesterone receptors and undergo cyclical changes related to the estrous cycle (Saruhan *et al.* 2011). In the presence of progesterone, the decrease in estradiol levels is followed by the loss of ciliated epithelium, and the secretory cells tend to lose their biosynthetic structure. On the other hand, estrogens stimulate oviductal epithelium secretion, which is highest in the proliferative phase (Killian 2011).

#### 2.1.4.1 Mucins

Most epithelia require strict defense mechanisms to maintain the integrity of the epithelial barrier. Mucins (MUC) at the apical surface of epithelial cells are highly pivotal parts of that defense system. They are further evolved as more complex transmembrane structures that participate in the protection, repair and survival of epithelia in mammals, including bovine (Kufe 2009). The expression and localization of different MUC in female reproductive organs, particularly the oviduct, has been demonstrated (Gipson *et al.* 1997). Through sub-cellular localization MUC can be grouped as either cell-surface (MUC1, -4 and -16) or gel-forming (MUC6) (Senapati *et al.* 2010). Eleven known genes encode cell-surface MUC expressed by a wide diversity of mucosal tissues, with substantial redundancy in many tissues. Cell-surface MUC contain large extracellular variable number tandem repeat (VNTR) domains, which confer the ability to form rigid elongated structures. Their unique structure and high expression rate prove that MUC are likely to be a prominent component of the glycocalyx, which may form a barrier that limits access of other cells and large molecules to the cell surface. During synthesis, it appears that most cell-surface MUC are cleaved into two subunits in a region known as the statistical energy analysis (SEA) module, which is often flanked by epidermal growth factor-like domains (Wreschner *et al.* 2002). Gel-forming MUC, which are major constituents of mucus and confer its viscoelastic properties, are encoded by a pool of four highly related genes on chromosome 11 (Desseyn *et al.* 1998, Desseyn *et al.* 2000) and a similar gene on chromosome 12 (Chen *et al.* 2004). Gel-forming MUC comprise N- and C-terminal cysteine-rich domains that are both involved in homo-oligomerization mediated by inter-molecular disulfide bonds (Gum *et al.* 1994).

Table 1 highlights the studies investigating the expression and localization of various MUC in different female reproductive organs. Steroid hormones impact the glycoprotein production of the female reproductive tract (Horne *et al.* 2006). Transcription levels of MUC differs based on the reproductive cycle status, which leads to changes in the overall properties of mucus (Vigil *et al.* 2009). Higher blood levels of estradiol released from ovarian follicles during the proliferative phase result in mucus becoming thin in vagina, cervix and uterus with lower viscosity to facilitate sperm movement towards the fertilization site. However, higher blood progesterone levels released from CL during the luteal phase induce thick and viscous mucus, especially in the cervicovaginal section, that prevents the movement of material towards the upper reproductive tract (Wira *et al.* 2015).

Zona pellucida MUC are produced in the ovary and surround mature ova (Wassarman & Litscher 1995). MUC1 expression in normal ovary tissues has been detected in different studies, although the specificities of the detecting monoclonal antibodies in those studies are conflicting (Wang *et al.* 2007a, Van Elssen *et al.* 2010). Moreover, MUC1 and MUC-associated carbohydrates motifs are markedly elevated in ovarian tumors (Tashiro *et al.* 1994).

The oviduct typically expresses MUC genes in a non-transformed state. Among the typical mucin genes, the oviduct expresses MUC1 in its epithelium (Gipson *et al.* 1997). Nevertheless, a secreted protein from oviductal epithelial cells that has MUC-type properties has been proposed and designated MUC9 (Lapensee *et al.* 1997). This oviduct-specific protein is further described in section 2.1.5.2.

**Table 1. MUC expression in mammalian female reproductive tract tissues**

| Tissue | Detected MUC | References |
|---|---|---|
| Ovary | MUC1 | (Tashiro *et al.* 1994, Van Elssen *et al.* 2010) |
| Ovum | MUC1 | (Wassarman & Litscher 1995) |
| Oviduct | MUC1, MUC9 | (Lapensee *et al.* 1997, Danesh Mesgaran *et al.* 2016) |
| Uterus | MUC1, MUC6, MUC8, MUC12, MUC16 | (Gipson *et al.* 1997, Kasimanickam *et al.* 2014, Wagener *et al.* 2017) |
| Cervix | MUC1, MUC4, MUC5AC, MUC5B, MUC6, MUC8 | (Gipson *et al.* 1997, Gipson *et al.* 2001, Brunelli *et al.* 2007) |
| Vagina | MUC1, MUC4 | (Gipson *et al.* 1997) |

MUC1 and other mucins are present at the apical surface of luminal and glandular uterine epithelia (Hey *et al.* 1994, Surveyor *et al.* 1995). Different studies on various species have focused extensively on revealing the functions of MUC, particularly MUC1, within the uterus. MUC1 knock-down mice showed chronic infection and inflammation of the uterus as a result of increased infection by reproductive tract pathogens (DeSouza *et al.* 1999). Other work has indicated that cells expressing high levels of MUC1 at the cell surface resist blastocyst attachment, while removing MUC1 from the cell surface allows attachment (Chervenak & Illsley 2000). Therefore, blastocysts appear to produce factors that lead to local MUC1 loss on uterine epithelial cells (Meseguer *et al.* 2001). Moreover, it has been shown that bovine endometrial epithelial cells express MUC1, which was upregulated when the cells were treated with bacterial endotoxins (Davies *et al.* 2008). Experiments on dairy cows showed higher expression of the studied MUC1,-4 and -12 mRNA in diseased and/or subfertile animals compared with the controls (Kasimanickam *et al.* 2014, Wagener *et al.* 2017). Nevertheless, the role of these MUC in bovine fertility has to be further studied.

### 2.1.4.2 Mucin glycosylation

Mucosal surface carbohydrate formation completely differs based upon cell lineage, tissue location and developmental stage. Mucin glycosylation can alter in response to mucosal infection and inflammation, and this may be an important mechanism for unfavorably changing the niche occupied by mucosal pathogens. Studies were able to show fluorescently labelled lectins can detect glycosylation changes (Bird-Lieberman *et al.* 2012). Wheat germ agglutinin (WGA) is a lectin, which binds to N-acetyl-D-glucosamine and sialic acid residues (Lis & Sharon 1986). These monosaccharides are part of MUC that makes them suitable as an indirect indicator for the presence of MUC on the surface of epithelial cells (Valdizan *et al.* 1992).

*O*-glycosylation occurs in MUC at extensive rate in order to protect them from proteolytic enzymes and prompt a relatively extended conformation (Hanisch 2001). The oligosaccharides on secreted mucins are clustered into heavily glycosylated domains (typically 600 – 1,200 amino acids long) separated by shorter non-glycosylated regions (Hanisch 2001). The *O*-linked glycans contain 1 – 20 residues, which occur both as linear and branched structures. The carbohydrate chain is initiated with an *N*-acetylgalactosamine linked to serine or threonine and is elongated by the formation of the so-called core structures followed by the backbone region (type-1 and type-2 chains). Moreover, sulfation of galactose and *N*-acetylglucosamine residues causes further diversification. MUC additionally contain a smaller number of *N*-linked oligosaccharides, which have been implicated in folding, oligomerization, or surface localization (McCool *et al.* 1999, Kui Wong *et al.* 2003). Specific glycosyl transferases expression highly indicates the carbohydrate structure on mucin surface. Therefore, glycosylation of mucins is controlled by enzyme's polymorphisms, tissue-specific enzyme expression as well as host and environmental factors influencing transferase expression (Hanisch 2001).

## 2.1.5. Epithelial cell secretions

Interactions with gametes and the early conceptus require an active oviductal environment, which is mainly established by epithelial cells secretions. The oviductal fluid comprises amino acids, proteins, simple and complex carbohydrates, ions, lipids, and phospholipids. Some of these components are metabolic substrates, such as lactate, pyruvic acid, amino acids, and glucose, whose levels differ from those present in the uterine fluid and the serum (Aviles *et al.* 2010, Hugentobler *et al.* 2010). The complex mixture of proteins present in the oviductal fluid partially comes from serum transudate, but the oviduct epithelium also synthesizes and secretes specific proteins. The volume and some protein components of the oviductal fluid change throughout the estrous cycle. Oviductal secretion is generally greater under estrogen than progesterone domination. There is significantly greater secretion activity in ovine and bovine oviducts at estrus than on other days of the cycle (Malayer *et al.* 1988, Buhi *et al.* 1991). In addition, oviductal fluid components are altered in the presence of gametes and the conceptus (Caballero *et al.* 2014, Garcia *et al.* 2017).

#### 2.1.5.1 Prostaglandins

Prostaglandins (PG) are pivotal compounds released into the oviductal lumen, facilitating fertilization via contraction and relaxation of the oviduct muscle layers (Wijayagunawardane *et al.* 2001). Particularly prostaglandin $E_2$ ($PGE_2$) release by epithelial cells is stimulated by the presence of sperm and contributes to immunosuppressive effects by the female reproductive tract towards the existing sperm (Marey *et al.* 2014). Others have pointed out that synthesized PG might also be able to interact with the early embryo as part of the embryo-maternal dialogue in the oviduct (Odau *et al.* 2006). During an immune response, epithelia, fibroblasts and infiltrating inflammatory cells are major sources of $PGE_2$. $PGE_2$ synthesis involves phospholipase $A_2$ ($PLA_2$) family members that mobilize arachidonic acid (AA) from the cellular membranes (Lambeau & Lazdunski 1999); PG-endoperoxide synthase 1 and 2 (PTGS1 and -2), which convert AA into $PGH_2$, and microsomal and cytosolic prostaglandin $E_2$ synthase (PTGES2 and -3, respectively), which are required for $PGE_2$ synthesis (Park *et al.* 2006). While the rate of $PGE_2$ synthesis and the resulting inflammatory process can be affected by additional factors, such as the local availability of AA, the rate of $PGE_2$ synthesis in most physiologic conditions, is controlled by the local expression and activity of PTGS2 (Ricciotti & FitzGerald 2011).

The $PGE_2$ turnover rate is high and $PGE_2$ is eliminated from tissues and circulation at a great rate (Forstermann & Neufang 1983). $PGE_2$ degradation in individual tissues is regulated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Tai *et al.* 2002). Apoptotic cancer cells apparently modulate prostanoid production by enhancing macrophage expression of PTGS2 and PTGES2, while suppressing 15-PGDH (Brecht *et al.* 2011). These observations indicate that the $PGE_2$ decay rate may also contribute to immunopathology and constitute a potential target for immunomodulation (Hirsch & Lippman 2005).

$PGE_2$ regulates various facets of inflammation and the functions of different immune cells (Phipps *et al.* 1991). This lipid generally induces active inflammation via local vasodilatation and local attraction and activation of neutrophils, macrophages and mast cells at the early stages of inflammation (Yu & Chadee 1998, Wang & Lau 2006). Nevertheless, the ability of $PGE_2$ to suppress the production of multiple pro-inflammatory cytokines allows it to limit nonspecific inflammation, promoting the immune suppression associated with chronic inflammation and cancer (Wang *et al.* 2007b). It has been revealed that this mediator suppresses both innate and antigen-specific immunity at multiple molecular and cellular levels (Harris *et al.* 2002). Based on these investigations, it is believed that $PGE_2$ holds the paradoxical status of a pro-inflammatory factor with immunosuppressive activity.

#### 2.1.5.2 Oviduct-specific glycoprotein

The secretion of oviduct-specific glycoprotein 1 (OVGP1), an organ-specific glycoprotein, is important. Different studies have indicated that it modifies gamete physiology and fertilization (Coy *et al.* 2008).

OVGP1, also termed oviductin or MUC9, is a major secretory glycoprotein that has been identified as being secreted in particular by the oviduct of several species including bovine (Buhi 2002). The carboxyl-terminal region of OVGP1 contains MUC-type VNTR sequences of different amino acids,

hence its inclusion as a member of the MUC family (Paquette *et al.* 1995, Buhi 2002). These VNTR regions are heavily O-glycosylated in different species, but the structures of these glycans have not been determined (Yang *et al.* 2012). Differences in tandem repeats and the distribution of the N- and O-linked carbohydrate chains may confer species specificity and regulate the biological activity of OVGP1 (Malette *et al.* 1995, Pradeep *et al.* 2011).

Specific OVGP1 biological characteristics could partially be due to post-translational modifications. These modifications may influence protein–protein or protein–cell interactions with sperm membranes, zona pellucidae, the vitelline membrane of oocytes, or early embryos (Satoh *et al.* 1995). Studies using lectin binding and chemical deglycosylation determined that both N- and O-linked glycosylation occur. Such examples of glycosylated carbohydrates include terminal N-acetylneuraminic acid (sialic acid), galactose, N-acetylgalactosamine, a-fucose, galactosyl-β(1,3)-N-acetylgalactosamine, N-acetylglucosamine and mannose (DeSouza & Murray 1995, Satoh *et al.* 1995).

In mammalian species, OVGP1 is involved in different roles during fertilization and embryonic development. King *et al.* (1994) treated bovine spermatozoa with enriched or semi-purified OVGP1 and reported significantly increased capacitation and ability to fertilize bovine oocytes. Moreover, a dose-dependent increase in motility and viability of bovine spermatozoa was observed following the addition of OVGP1 (Abe *et al.* 1995). Lower concentration of semi-purified OVGP1 increased the viability of pig spermatozoa (McCauley *et al.* 2003). The addition of increasing concentrations of OVGP1 to pig spermatozoa during homologous *in vitro* fertilization maintained high penetration rates at low concentrations, with a significant decrease in polyspermy (Kouba *et al.* 2000).

In most domestic animals, including bovine and porcine, treatment with OVGP1 alone prior to fertilization influenced the oocytes positively. In pigs, high penetration and fertilization rates were maintained and polyspermy was significantly reduced when oocytes were treated with low OVGP1 concentrations (McCauley *et al.* 2003). Pre-incubating bovine oocytes with OVGP1 resulted in significant increases in fertilization rates (Martus *et al.* 1998).

Direct embryotrophic effects of OVGP1 in bovine zygotes have not yet been detected. Contrarily, when this protein was added to porcine zygotes fertilized *in vivo*, a significant increase in incorporation of radiolabelled amino acid into proteins at the four-cell stage was observed (Kouba *et al.* 2000). These data support a possible role for OVGP1 during embryonic development *in vitro* and pinpoint its importance during fertilization and first cleavage events.

#### 2.1.5.3 Energy substrates

Embryo development and survival occur in cattle when the embryos are still dependent on the nutrients provided by the oviductal fluid (Diskin *et al.* 2011). The *in vitro* development of bovine embryos requires a significant amount of metabolic activity (Tiffin *et al.* 1991, Thompson *et al.* 1996). Various energy substrates from different metabolic pathways are utilized by the embryo during its early development (Rieger, 1992; Edwards et al., 1997). For example, lactate and pyruvate have been identified as key energy sources for cattle embryos up to the blastocyst stage (Takahashi & First 1992, Kim *et al.* 1993). The embryo consumes glucose at a relatively low rate during early preimplantation development, yet glucose consumption increases dramatically at blastocyst formation. It has been

demonstrated that the absence of glucose up to the morula stage stimulates blastulation (Kim *et al.* 1993).

Oviductal cells obtain their energy for secreting and synthesizing components to support early embryo development through different metabolic pathways. Glycolysis, citric acid cycle and respiratory chain reaction are among such pathways (Graziano *et al.* 2017). Hexokinase (HK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex, subunit A (SDHA) are enzymes with crucial roles in energy production within the abovementioned pathways.

Glycolysis is the metabolic pathway that converts glucose into pyruvate. The first step in glycolysis is glucose phosphorylation, conducted by HK to form glucose 6-phosphate (G6P). A recent study on oviduct metabolites profiling indicated an intense glycolytic metabolism based on HK activity and low pyruvate levels that could be exported to compose the lumenal fluid, particularly in mid-portions of the organ where fertilization and early embryo development take place (Gomes *et al.* 2010). Hugentobler *et al.* (2008) observed similar glucose concentrations in oviductal fluid compared with plasma on days 0-8 postpartum, while lactate was around eight-fold higher in the oviductal lumen.

It appears that oviductal cells can utilize ketone bodies, particularly during energy deficiency situations (Wathes *et al.* 2007). Acetoacetate and 3-hydroxybutyrate are water-soluble ketone bodies synthesized from acetyl-CoA that results from the beta-oxidation of fatty acids (Laffel 1999). Energy generation from ketone bodies takes place via the citric acid cycle and oxidative phosphorylation and therefore requires proper mitochondrial function. These substrates can directly enter the citric acid cycle as acetyl-CoA. Furthermore, 3-hydroxybutyrate dehydrogenase (BDH2) and 3-oxoacid CoA transferase (OXCT2) are vital enzymes in the degradation of these ketone bodies (Newman & Verdin 2014).

## 2.2. Bovine oviductal epithelial cell culture

Cell culture models of bovine oviductal epithelium facilitate research regarding this organ and avoid time-consuming and expensive *in vivo* studies. Organ cultures of oviducts in bovine and other species have been conducted to reveal the pathogenesis of sexually transmitted diseases by determining the transcription/protein levels of different immune factors (Stalheim *et al.* 1976, Maisey *et al.* 2003). Although *ex vivo* culturing can bridge the gap between cultures of isolated cells and *in vivo* conditions, there are pitfalls associated with this method. The cultured explants or tissues begin to deteriorate after a few days or weeks in culture (Van Soom *et al.* 2010).

A short-term suspension culture was introduced in order to avoid cell dedifferentiation by maintaining cilia and secretory granules, which could be beneficial for studying embryo-maternal communication in the oviduct (Rottmayer *et al.* 2006). Comprehensive light and electron microscopy confirmed the functional morphology and ultrastructural integrity of the cultured cells. However, there is an obvious, major time-limitation with this model.

Embryo-maternal cross-talk studies have used monolayer culture of BOEC successfully as a source of feeder cells for the development of the early embryo *in vitro* (Ulbrich et al. 2010). Others have shown the application of this culturing approach in understanding the interaction of oviductal cells with both

spermatozoa and COC (Kodithuwakku *et al.* 2007, Gabler *et al.* 2008). Moreover, this conventional epithelial cell culture appears to be an inevitable tool for understanding the wide range of molecular mechanisms that underlie the strategies of microbial virulence such as host receptor ligand binding and may possibly afford critical insight in what drives host defense strategies (Duell *et al.* 2011). Almost all cells in the *in vivo* environment are surrounded by other cells and extracellular matrix in a three-dimensional fashion, therefore conventional monolayer cell culture might not adequately take into account the natural environment of cells (Birgersdotter *et al.* 2005).

Polarized cell culture systems allowing bidirectional cell exposure via hanging inserts of BOEC have been used by a few research groups (Gualtieri *et al.* 2012, Jordaens *et al.* 2015, Jordaens *et al.* 2017). Recent studies using polarized BOEC culture systems have revealed physiological cellular responses that resembles the *in vivo* conditions more closely (Jordaens *et al.* 2017).

Very recently, a research group in the Netherlands was able to use 3D printing technology along with microfluidics creating a 3D oviduct-on-a-chip model with a U-shaped porous membrane allowing BOEC polarization that could be maintained in long-term culture (> 2 months) (Ferraz *et al.* 2017).

## 2.3. Oviduct immune response

The bovine reproductive tract is constantly in contact with invading microorganisms and pathogens, which can ascend from the lower to the upper parts, particularly the oviduct. The inflammatory reaction within the oviduct can be considered as a normal and necessary physiological occurrence against pathogen invasion. The epithelial cells, pivotal participants of the first-line defense mechanism, are included in the immediate, early, and nonspecific immune responses against pathogens (Wira *et al.* 2005b).

Microbial pathogens recognition is facilitated by germ-line encoded pattern recognition receptors (PRR) (Akira & Takeda 2004). Wide ranges of Toll-like receptors (TLR), major families of PRR, recognize distinct pathogen structures within the reproductive tract epithelium (Akira & Takeda 2004). Most recently, TLR2 and -4 expression was reported in cultured BOEC (Kowsar *et al.* 2013). Most TLR share a common adaptor molecule, i.e., myeloid differentiation primary-response protein 88 (MyD88), in generating their downstream pathways. Through different complexes, MyD88 triggers the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor-kappa B (NFκB) pathway, which are both equally vital for generating inflammatory responses. Transcription of most common genes associated with these two pathways comprises interleukin (IL) 1A, -1B, -6, tumor necrosis factor alpha (TNFA) and chemokines such as IL8, also known as CXCL8, and CXCL1-5 (Kopp & Medzhitov 2003, Kawai & Akira 2007). Messenger ribonucleic acid (mRNA) regulation of $PGE_2$ synthesis mediators and enzymes genes are also induced through the MAPK and NFκB pathways. Various studies have observed the pro- and anti-inflammatory hemostatic function of $PGE_2$ (Kalinski 2012). This physiologically active compound is shown to shift from pro-inflammatory responses to other forms of immunity through antagonistic activity and the suppression of different cytokines (Wu *et al.* 1998).

The proposed dual role for immunomodulation in the oviduct (acceptance of allogeneic tissue, e.g. early embryo and immune protection against pathogens) suggests a fine balance between the presence

of suppressive and pro-inflammatory cytokines, possibly contrasting in different oviductal parts. IL1 and TNFA are major pro-inflammatory cytokines that promote inflammation and stimulate an acute-phase response (Parsey *et al.* 1998). IL10 on the other hand is considered to be a potent suppressor of the effector functions of macrophages, T cells and natural killers (NK) cells (Moore *et al.* 2001). IL10 acts to terminate the inflammatory response and limits inflammation-induced tissue changes by de-activating macrophages and inhibiting their synthesis of pro-inflammatory cytokines (Fiorentino *et al.* 1991).

### 2.3.1 Cytokines

Cytokines are small pleiotropic glycoprotein mediators whose biological actions are locally mediated by specific receptors (Balu *et al.* 2002). Chemokines are small chemotactic cytokines, locally acting, which are well known for their function in recruitment of leukocytes to inflamed sites and their activation (Salamonsen *et al.* 2007). Chemokines attract immune cells to the tissue, while IL along with TNFA differentiate and activate these cells (Wira *et al.* 2005a). Various number of investigations have observed secretion of numerous cytokines, including granulocyte-macrophage colony-stimulating factor, TNFA, IL1, IL6 and of chemokines such as monocyte chemoattractant protein 1 (MCP1) and IL8 by polarized epithelial cells of different female reproductive tract tissues (Kaushic *et al.* 2000, Kayisli *et al.* 2002, Duerst & Morrison 2003, Healy *et al.* 2015). The majority of these mediators was secreted into the apical/luminal compartment resulting in a gradient for stimulation and attracting immune cells to the epithelial surface (Fahey *et al.* 2005).

IL8 secreted by uterine epithelial cells has been shown to result in migration of neutrophils along the epithelium (Carolan *et al.* 1997). Cytokines secreted into the basal compartment of endometrial epithelial cells have been shown to impact function and development of immune cells (Eriksson *et al.* 2006, Ochiel *et al.* 2010). Moreover, TNFA and IL6 facilitate differentiation of leukocytes to more active pro-inflammatory cells (Ochiel *et al.* 2008). Other important cytokines like interferon (IFN) beta induce expression of anti-viral molecules such as macrophage inflammatory protein 3 alpha (MIP3α/CCL20) indicating their important protective roles during viral infections (Hickey *et al.* 2011). Immunoregulatory action of cytokines in the female reproductive tract is partially done through immune cells, including monocytes, macrophages, NK and dendritic cells (Cooper *et al.* 2001, Eriksson *et al.* 2004).

Steroid hormones have been shown to influence the secretion of these mediators in a direct or indirect manner (Ochiel *et al.* 2008, Hickey *et al.* 2011). Lower concentrations of progesterone lead to the up-regulation of IL8, MCP1, and PTGS2, eventually activating monocytes and neutrophils (Critchley *et al.* 2001). Indirect influence of steroid hormones was noticed when treatment with specific dosage of estradiol induced higher hepatocyte growth factor secretion that in turn regulated TNFA and MIP3α/CCL20 production by uterine epithelial cells (Grant-Tschudy & Wira 2005, Coleman *et al.* 2009).

Expression of cytokines is also critical in preparation for fertilization, implantation, and successful pregnancy, as well as uterine involution during each reproductive cycle (Tabibzadeh 1996, Dominguez *et al.* 2002). Studies indicated that particular cytokines trigger the generation of reactive oxygen species

by spermatozoa (Rajasekaran *et al.* 1995). Increased levels of oxidative stress induce deoxyribonucleic acid (DNA) fragmentation in sperms causing morphological abnormal spermatozoa and reduced rates of fertilization (Alvarez *et al.* 2002).

Successful pregnancy is often associated with anti-inflammatory mediators, while the pro-inflammatory reactivity is detrimental to a pregnancy (Raghupathy *et al.* 2000). Evidences revealed members of IL6 family are involved during endometrial transformation into receptive stage and embryo-endometrial interaction, which are vital for implantation (Aghajanova 2004). Spontaneous abortions in human have been correlated with increased levels of IL2 and interferon (IFN) gamma and lower IL10 concentration compared with normal pregnancy (Marzi *et al.* 1996). Investigation in dairy cattle indicated a differential mRNA expression of candidate cytokine factors in animals that repeatedly fail to conceive (Wagener *et al.* 2017).

## 2.4. Bacterial pathogenic strains associated with bovine reproductive tract diseases

After parturition, the reproductive tract in most dairy cattle is rapidly challenged with various microorganisms, most of them categorized based on their pathogenesis in the bovine uterus (Williams *et al.* 2007). These bacterial strains are recognized as pathogenic, potentially pathogenic or opportunistic contaminant bacteria (Williams *et al.* 2005). Previous studies have supported the theory that pathogens in the lower reproductive tract can routinely reach the oviducts (Kunz *et al.* 1996). An investigation in cattle reported oviduct infection with *Chlamydia spp.* indicating the presence of these pathogens in 37.5 and 18.1 percent of studied animals with healthy and pathological reproductive tract, respectively (Appino *et al.* 2015). Signs of prevalent pathogenic species have been proven in humans showing higher rates of *Chlamydia trachomatis* and *Mycoplasma genitalium* in oviductal specimens that eventually could lead to 'tubal infertility' in women (Ashshi *et al.* 2015). Therefore it is vital to study the impact of these microorganisms on the viability and cellular response of oviductal cells.

### 2.4.1. *Trueperella pyogenes*

*Trueperella pyogenes* is one of the commonly isolated pathogenic bacteria highly associated with abnormal vaginal discharge in infected animals (Werner *et al.* 2012). It has been postulated that it is not solely the trigger factor in the development of postpartum disease, yet the synergism between *T. pyogenes* and other bacteria may be more important (Silva *et al.* 2008). Formerly known as *Arcanobacterium pyogenes*, *T. pyogenes* is generally characterized as a Gram-positive, non-motile, non-sporeforming bacterium that is a common inhabitant of the mucous membranes of the upper respiratory, urogenital and gastrointestinal tracts of various livestock species including bovine (Jost & Billington 2005). Several diseases are caused by *T. pyogenes* including abortion, abscesses, arthritis, endocarditis, mastitis, pneumonia, osteomyelitis, uterine infections leading to infertility and vesiculitis (Hagan *et al.* 1988, Lewis 1997). Management control strategies for preventing *T. pyogenes* infections in domestic animals appear relatively difficult. Intravaginal vaccination with culture filtrates or inactivated *T. pyogenes* has been ineffective in protecting these animals (Machado *et al.* 2014). Despite its opportunistic behavior and its presence in the environment and normal microflora of the mucous

membranes in livestock, control of fly vector populations, reduced contact of udders with water or humid environments, and isolation and/or culling of animals with severe draining abscesses may limit animal-to-animal spread of *T. pyogenes* infection (Ribeiro *et al.* 2015).

*T. pyogenes* encompasses several putative virulence factors, which are necessary for adherence, with subsequent colonization further causing host tissue damage associated with infection by the bacteria. A crucial virulence factor for this bacterial species is the potent extracellular toxin, pyolysin (PLO) (Ding & Lammler 1996, Jost *et al.* 1999). The endotoxin induces hemolysis of red blood cells in a wide range of animal species, and is responsible for the characteristic beta hemolysis exhibited by *T. pyogenes* grown on blood-containing medium (Ramos *et al.* 1997). All *T. pyogenes* strains produce PLO (Jost *et al.* 1999), and it is expressed primarily in stationary phase cultures, where it is detected in culture supernatant as a 55 kD protein (Billington *et al.* 1997). PLO belongs to the cholesterol-dependent cytolysin family of toxins, which exert their cytolytic effects through the formation of large pores in eukaryotic cell membranes (Billington *et al.* 2000). Moreover, these toxins can manipulate host cell signaling events, such as the expression of cytokines (Ruiz *et al.* 1998) and other inflammatory substances, including $PGE_2$ (Mitchell & Andrew 1997). It has been determined that *T. pyogenes* can survive within epithelial cells for at least 72 h, although bacterial numbers decline over this period, suggesting that the bacteria do not replicate in these cells. One reason might be that *T. pyogenes* moves along the epithelial cells (Jost & Billington 2005).

### 2.4.2. *Bacillus pumilus*

Scientists have observed other bacterial groups thus far uncultured or unidentified that could play key roles in the establishment of detrimental diseases (Santos & Bicalho 2012). Wagener *et al.* (2015) reported that *Bacillus pumilus* comprised almost 5 percent of postpartum dairy cattle uterine microflora and suggested the need for further investigation of the role of this bacteria in bovine health. These bacterial species are characterized as potential pathogens where they have been successfully isolated from uterine lumen of endometritis cases, but are not predominantly linked with uterine lesions (Williams *et al.* 2007). Gärtner *et al.* (2016) recently showed that *B. pumilus* induced an evident pro-inflammatory response from cultured endometrial cells.

*B. pumilus* belongs to the genus Bacillus, and is a Gram-positive, aerobic, rod-shaped endospore-forming bacteria widely represented in the soil (Priest 1993). *Bacillus* spp. are closely linked with food poisoning via their heat-labile enterotoxins, namely, hemolysin BL (B is a binding component; L is a lytic component) and the nonhemolytic enterotoxin protein complexes such as enterotoxic protein-T (McKillip 2000, Phelps & McKillip 2002). The relevance of other *Bacillus* species as food poisoning organisms and as etiological agents in nongastrointestinal infections, including local, deep-tissue, and systemic infections, is being increasingly recognized. Raw milk contamination through infection of bovine udder, and serious herd damages of bovine mastitis have been attributed to members of this genus (Kirkbride 1993). *Bacillus* spp. have also been implicated as causative agents of mastitis and abortions in other animals, including cows (Kirkbride 1993), pigs (Kirkbride *et al.* 1986), water buffalo (Galiero & De Carlo 1998), and dromedary camels (Rowan *et al.* 2003). This bacterium is highly resistant to extreme environmental conditions such as low or no nutrient availability, desiccation,

irradiation, and hydrogen peroxide and chemical disinfections (Nicholson *et al.* 2000). Two isolates of these bacteria have been shown to be the main cause of severe sepsis in neonatal infants (Kimouli *et al.* 2012). The presence of *Bacillus* spp. has been detected in different organs of the bovine reproductive tract (Panangala *et al.* 1978, Dolezel *et al.* 2010).

# 3. Aims of the study

The current literature provides an overview about bovine oviducts' important roles in gamete maturation and fertilization as well as supporting the development of the early embryo. *In vitro* monolayer culturing of oviducts' epithelial cells has enabled the researchers to unveil most of the mentioned roles of this organ. However, an evident gap has been observed on fine-tuning and standardizing this method of *in vitro* studies. A standardized culture model of oviductal cells could result in cellular responses more closely to what happens *in vivo*. Moreover, the bovine oviduct is known to provide a nearly bacteria-free environment to support important reproductive events. However, there is a need to observe the interactions of this organ with pathogenic bacteria, which invade the reproductive tract of dairy cows during early days after calving in detail. These microorganisms might disrupt the oviducts' integrity, its functions and potentially terminate the pregnancy in cattle at very early stages.

Therefore, the present work was set-up based on two main hypotheses. The initial hypothesis is that sub-optimal culturing conditions alter bovine oviductal cell responses, particularly their immune and metabolic system. The second is that BOEC early inflammatory response to the presence of pathogenic bacteria with distinct pathogenic characteristics is different.

**Therefore, the specific aims were to investigate:**

Part 1:

- the mRNA expression pattern of various candidate genes in bovine oviductal cells whose encoded proteins are crucial for the cellular defense system and metabolism as well as the animals' reproductive success. The chosen candidate genes were as follows: a) mucins and IL8, which are part of oviducts' innate immune system, b) enzymes that are particularly involved in glucose metabolism and synthesis of ketone bodies and c) key enzymes in $PGE_2$ synthesis. The mRNA expression of the studied genes were determined: 1) between cells obtained directly from tissue, resembling the *''in vivo''* state, and the cultured BOEC to understand oviducts' epithelial cell responses as they introduce to the new "*in vitro*" environment; 2) within cultured BOEC during three consecutive cell passages, in order to observe the effect of *in vitro* conditioning; 3) between BOEC cultured with HIGH- or LOW-glucose concentrations of the cell culture medium, in order to notice the possibility of culturing cells with glucose content in the medium closer to that of dairy cows' body and 4) under influence of the estrous cycle phase during *in vivo* and *in vitro*. Moreover, the concentration of $PGE_2$, OVGP1 and IL8 in the cultured BOEC media was determined to observe oviductal cells ability to release some of the important bioactive compounds in particular stages of cell culture. This would further compliment the observed mRNA expression data of the selected genes.

Part 2:

- the impact of distinct bacterial species, *Trueperella pyogenes* as a classic pathogen of bovine reproductive tract and *Bacillus pumilus* with potentially pathogenic characteristic, on bovine oviducts' cellular pro-inflammatory responses. *Trueperella pyogenes* or *B. pumilus* incubation with BOEC was done in a standardized *in vitro* culture model imitating bacterial infection in order to determine: 1) the influence of the studied bacterial species on viability of BOEC during

co-culture; 2) the mRNA expression of different pro-inflammatory factors comprising IL, chemokines and PG synthesis enzymes as an indicator of early cellular responses to bacterial infection and 3) the release of IL8 and $PGE_2$ from BOEC after 24 h incubation with bacteria, which helps to understand the cellular inflammatory responses at a functional level.

## 4. Subsuming the published work

Publication 1: **mRNA expression pattern of selected candidate genes differs in bovine oviductal epithelial cells *in vitro* compared with the *in vivo* state and during cell culture passages.**

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**mRNA expression pattern of selected candidate genes differs in bovine oviductal epithelial cells in vitro compared with the in vivo state and during cell culture passages**

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**Abstract**

**Background**

The mammalian oviduct provides the optimal environment for gamete maturation including sperm capacitation, fertilization, and development of the early embryo. Various cell culture models for primary bovine oviductal epithelial cells (BOEC) were established to reveal such physiological events. The aim of this study was to evaluate 17 candidate mRNA expression patterns in oviductal epithelial cells 1) in transition from in vivo cells to in vitro cells; 2) during three consecutive cell culture passages; 3) affected by the impact of LOW or HIGH glucose content media, and 4) influenced by different phases of the estrous cycle in vivo and in vitro. In addition, the release of a metabolite and proteins from BOEC at two distinct cell culture passage numbers was estimated to monitor the functionality.

**Methods**

BOEC from 8 animals were isolated and cultured for three consecutive passages. Total RNA was extracted from in vivo and in vitro samples and subjected to reverse transcription quantitative polymerase chain reaction to reveal mRNA expression of selected candidate genes. The release of prostaglandin $E_2$ ($PGE_2$), oviduct-specific glycoprotein 1 (OVGP1) and interleukin 8 (IL8) by BOEC was measured by EIA or ELISA after 24 h.

**Results**

Almost all candidate genes (prostaglandin synthases, enzymes of cellular metabolism and mucins) mRNA expression pattern differed compared in vivo with in vitro state. In addition, transcription of most candidate genes was influenced by the number of cell culture passages. Different glucose medium content did not affect mRNA expression of most candidate genes. The phase of the estrous cycle altered some candidate mRNA expression in BOEC in vitro at later passages. The release of $PGE_2$ and OVGP1 between passages did not differ. However, BOEC in passage 3 released significantly higher amount of IL8 compared with cells in passage 0.

**Conclusion**

This study supports the hypothesis that candidate mRNA expression in BOEC was influenced from the in vivo situation to the new in vitro environment and during consecutive passages. The consequence of cell culture passaging on BOEC ability to release bioactive compounds should be considered.

**Keywords:**

Cell culture passage, culture medium, enzymes of cellular metabolism, mRNA expression, mucin, oviduct, prostaglandin synthase

**Background**

The mammalian oviduct is the site of gamete maturation including sperm capacitation, fertilization, and development of the early embryo [1, 2]. Oviductal epithelial cells play an important role in these reproductive events. In addition, the epithelium layer of the oviduct, which is covered at its apical site with a mucosal surface containing mucins (MUC), provides a physical barrier against invading pathogens [3, 4]. These MUC are grouped as either cell-surface (MUC1, -4, -16) or gel-forming (MUC6) ones [5]. The epithelial cells as the first defense line are also involved in the innate immune response against pathogens. After pathogen recognition through Toll-like receptors [6], epithelial cells release several chemokines, e.g. interleukin 8 (IL8), which are able to attract immune cells to the infected site generating an inflammatory response against microbes [7]. Additionally, oviduct-specific glycoprotein 1 (OVGP1), also named MUC9, is secreted into the lumen of the oviduct and interacts with the gametes assuring the success of fertilization in different species [8].

To fulfill their physiological functions, oviductal epithelial cells can utilize different metabolic pathways as energy source: glycolysis, citric acid cycle, and finally the respiratory chain. Key enzymes of the glycolysis are hexokinase (HK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Especially higher HK activity was observed in the region of the presumptuous fertilization site compared with other regions [9]. An important enzyme as part of the citric acid cycle as well as of the respiratory chain is the succinate dehydrogenase complex, subunit A (SDHA). Ketone bodies are used as a source of energy especially during an energy deficit situation in the cow [10]. Oviductal cells are also supposed to use ketone bodies utilizing specific enzymes as 3-hydroxybutyrate dehydrogenase (BDH2) and 3-oxoacid CoA transferase (OXCT2).

The generated energy is used e.g. for the synthesis of several prostaglandins (PG), which are important for oviduct muscle layers contraction and relaxation supporting the transportation of gametes [11]. Particularly, $PGE_2$ release by epithelial cells was stimulated by the presence of sperm and contributes to female's immunosuppressive effects towards the existing sperm [12]. The $PGE_2$ synthesis is conducted starting from the arachidonic acid by the key enzymes PG-endoperoxide synthase 1 and 2 (PTGS1 and -2) to generate $PGH_2$, which is converted by specific synthases (PTGES1-3) into $PGE_2$ [13].

In order to better understand the abovementioned physiological events, various cell culture models for primary bovine oviductal epithelial cells (BOEC) were established in the last decades [14-16]. Cell monolayer culture models are widely used to elucidate the interactions of the oviduct with spermatozoa [17] or cumulus-oocyte complexes [18] and as feeder cells for embryo development in vitro [19].

Primary cultures resemble cells, which are closer to the tissue origin present in vivo than permanent cell lines. Transcriptome data indicated significant divergence of cell lines from their initial primary cells [20, 21]. In addition, number of passaging strongly effects the mRNA expression of porcine oviductal cells [22] and of human synovial fibroblasts [23]. In this context, cell culture passaging also influences cellular responses by significantly reducing the expression rate of different proteins in synovial and gingival fibroblast cells [24, 25].

Therefore, in vitro conditions should be standardized to achieve the in vivo state of cells as closely as possible. Culture medium plays a crucial part providing proper source of energy and compounds

supporting cell growth in vitro. Glucose content should be applied similar to the plasma concentrations of the animal restoring cells in normal condition, because conventional high-glucose medium may be induce undesirable metabolic stress as observed in primary cultured rat myocardial cells [26].

This leads to the important question, how mRNA expression of cultured primary BOEC is perturbed during different numbers of in vitro culturing passages. The groups of investigated candidate genes are (a) mucins and IL8 as parts of the defense system against pathogens, (b) enzymes of cellular metabolism, and (c) key enzymes of the $PGE_2$ synthesis. Therefore, the aim of the present study was to evaluate the mRNA expression pattern of these candidate genes in bovine oviductal cells (1) in transition from in vivo state to the in vitro situation in the same animals; (2) monitoring during three consecutive passages; (3) determining the effect of two different glucose concentrations in the cell culture medium; and (4) elucidating the effect of the estrous cycle phase in vivo and in vitro. In addition, the release of a metabolite ($PGE_2$) and proteins (OVGP1 and IL8) was determined from BOEC at two distinct cell culture passage numbers. This was primarily done to reflect changes on the mRNA level also on the protein level.

## Methods

### Collection and classification of bovine oviducts

The procedure of oviduct collection and oviductal epithelial cell isolation was performed as previously described [27]. Briefly, both oviducts from non-pregnant Holstein cows (n=8) were collected at the abattoir, transported on ice to the laboratory and processed within 3 h after animal death. Samples from reproductive tracts with signs of inflammation or anatomic defects were not considered. The stage of the estrous cycle was determined based on the assessment of the ovaries (presence of follicle or corpus luteum) along with the evaluation of the uterus and cervix appearance [28, 29]. The oviducts were classified into the following phases of the estrous cycle (two animals per each phase): post-ovulatory (Day 1 to 5), early-to-mid luteal (Day 6 to 12), late luteal (Day 13 to 18), and pre-ovulatory (Day 19 to 21). Oviductal epithelial cells were collected by gently scraping the luminal surface of the opened oviducts with a rubber policeman. Scraped cells from each oviduct were flushed with 1 ml Dulbecco's phosphate-buffered saline without $Ca^{2+}$/$Mg^{2+}$ (PBS; PAA, Cölbe, Germany) into a 1.5 ml reaction tube, centrifuged at 570 g for 5 min, and the supernatant was removed. Then, cells from both oviducts of each animal were combined by suspending them with 1 ml PBS. A small aliquot of this cell suspension (200 µl) was removed, centrifuged at 570 g for 5 min, and the resulting pellet immediately stored at -80 °C for further analysis representing the in vivo samples. The remaining suspended cells were used for cell culturing.

### Cell culture and passaging

The combined oviductal epithelial cells of one cow were washed twice and suspended with Dulbecco's Modified Eagle Medium (DMEM) containing 10% dialyzed fetal calf serum (FCS) (Sigma, Seelze, Germany), 1 g/l (5.5 mM) glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 1 µg/ml amphotericin B (all Biochrom, Berlin, Germany) along with 10 µg/ml reduced glutathione

and 10 µg/ml ascorbic acid as antioxidants (both Sigma). Subsequently, 5 ml of the obtained cell suspension (approximately 1-2 × $10^5$ cells/ml) were seeded in six-well plates at 37 °C in a humidified atmosphere of 5% $CO_2$. The abovementioned culturing medium represents the LOW glucose medium. Dialyzed FCS was supplemented to the LOW glucose medium in order to avoid an external additional source of glucose in the medium for cultured cells.

Additionally, HIGH glucose culturing medium was also applied within this study, which contained 4.5 g/l (25 mM) glucose and was supplemented with superior FCS (Biochrom). The other supplements of the medium contents were the same as for the LOW glucose medium. Cultured cells were considered as primary bovine oviductal epithelial cells (BOEC) at passage 0 cultured in LOW or HIGH glucose culture medium, named in the following P0-LOW or P0-HIGH, respectively. Monolayer formation (>80% confluence) was reached 5 days after cell seeding and cells were detached using accutase (Sigma) until single cells appeared. Detached cells were then subjected to successive subculturing and seeded at a density of 5 × $10^5$ cells/well in 6-well plates in 5 ml of the abovementioned culture medium with LOW or HIGH glucose concentration for the next three passages (P1-LOW or P1-HIGH; P2-LOW or P2-HIGH; P3-LOW or P3-HIGH). Monolayer of >80 % confluence was achieved 72 h after subculturing in the passages 1, 2 and 3.

**Cytokeratin immunofluorescence staining**

The purity and homogeneity of each passage were evaluated by staining oviductal cells with a monoclonal mouse anti-human cytokeratin antibody as a marker for epithelial cells (DakoCytomation, Glostrup, Denmark) as previously described [30] with some modifications. Briefly, cells at each passage were cultured with the density of 1.25 × $10^5$ cells/well in multi-well chamber slides (Sarstedt, Nümbrecht, Germany). After reaching confluence, cells were rinsed with Dulbecco's PBS (PAA) and fixed using ice cold acetone (Merck, Darmstadt, Germany) for 10 min. After treatment with PBS/1% bovine serum albumin (Sigma) (blocking medium) for 1 h to block non-specific binding-sites, cells were incubated with the anti-cytokeratin antibody (1.2 µg/ml in blocking medium) for 1 h at room temperature. Cells were washed once for 30 s and two times each for 10 min with PBS. Afterwards, samples were incubated with the goat anti-mouse-IgG secondary antibody conjugated with DyLight 488 (Bio-Rad AbD Serotec, Puchheim, Germany; 1.2 µg/ml in blocking medium) for 1 h at room temperature in the dark. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Roche, Mannheim, Germany; 200 ng/ml in PBS) followed by three washing steps with PBS. Finally, slides were mounted with mounting medium (50% glycerol in PBS) and viewed under a Leica epifluorescence microscope (Leica DMI 6000 B, Wetzlar, Germany). The exposure length and gain remained constant for all samples by using the excitation and emission wavelengths at 494 nm and 519 nm, respectively. Negative controls were performed by substituting the primary antibody with the secondary antibody.

**Wheat germ agglutinin staining**

The lectin wheat germ agglutinin (WGA) binds to N-acetyl-D-glucosamine and sialic acid residues [31]. These monosaccharides are part of mucins [32], which makes WGA suitable as an indirect indicator for the presence of MUC on the surface of epithelial cells [33].

The procedure of WGA staining for paraffin embedded oviduct sections and cultured BOEC was carried out as described by manufacturer's instructions (Invitrogen, Darmstadt, Germany).

In brief, oviductal cross-sections of 3-5 µm from each estrous cycle stage were adhered to SuperFrost Plus microscope slides, fixed overnight at 56 °C, and stored until use at room temperature [27]. In order to liquefy the paraffin, slides were heated for 60 min at 60 °C. Afterwards, sections were immediately deparaffinized in xylene, rehydrated through graded ethanol, rinsed with deionized water, and washed with PBS for 10 min. Subsequently, slides were incubated with WGA Alexa Fluor 594 conjugate [Invitrogen, 5 µg/ml in Hank's balanced salt solution (HBSS; Biochrom)] for 15 min at room temperature in the dark. Slides were washed twice with HBSS and nuclei were stained with DAPI (200 ng/ml in PBS) for 10 min. Finally, tissues were rinsed, washed once for 10 min with PBS, and mounted with mounting medium (50% glycerol in PBS). Pictures were taken with a Leica epifluorescence microscope (Leica DMI 6000 B). The exposure length and gain remained constant for all samples by using the excitation and emission wavelengths at 590 nm and 617 nm, respectively.

Oviductal cells of each passage with the density of $1.25 \times 10^5$ cells/well were cultured in multi-well chamber slides (Sarstedt) until confluence. Afterwards, cells were fixed with 4% formaldehyde for 15 min. Following three times washing with HBSS, cells were incubated with WGA Alexa Fluor 594 conjugate (5 µg/ml in HBSS) for 10 min at room temperature in the dark. Cells were washed once for 30 s and two times each for 10 min with PBS and nuclei were stained with DAPI (200 ng/ml in PBS) for 10 min. Finally, slides were rinsed, washed twice with PBS, and mounted with mounting medium (50% glycerol in PBS) to view under a Leica epifluorescence microscope with the same conditions as described above.

**Total RNA extraction and cDNA synthesis**

Total RNA from oviductal cell pellets representing the in vivo samples was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Cultured oviductal cells after reaching >80% confluence at each passage were subjected to total RNA isolation performed with the InviMag Universal RNA Mini Kit (Stratec Molecular, Berlin, Germany) using the KingFisherFlex (Thermo Scientific, Langenselbold, Germany) according to the manufacturer's instructions with a slight modification. Cells were lysed with buffer RLT (Qiagen, Hilden Germany) supplemented with 1% (v/v) 2-mercaptoethanol (Sigma) immediately after medium removal within the culture plates. Quantification of the extracted total RNA was done photometrically at 260 nm using NanoDrop ND-1000 UV-VIS spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany). The integrity of total RNA was determined by using RNA 6000 Nano Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Reverse transcription (RT) of 0.25 µg total RNA was carried out in a total volume of 60 µl as previously described [27]. Complementary DNA was synthesized using 200 U RevertAid Moloney-Murine Leukaemia Virus Reverse Transcriptase, 2.5 µM random hexamer primers, 0.66 mM dNTPs, and 1x of the supplied RT buffer (all Fermentas, St. Leon-Rot, Germany). DNA digestion was performed before RT in order to remove genomic DNA contaminations [34]. Samples without the

reverse transcriptase were included to confirm the absence of any genomic DNA or other contaminations. The synthesized cDNA was stored in aliquots at -20 °C until use.

**Quantitative PCR**

Quantification of mRNA expression was performed as previously described [27] according to minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [35]. Each reaction with a total volume of 10 µl contained 1 µl cDNA, 0.4 µM of forward and reverse primer each (details are given in Tables 1 and 2; synthesized by Eurofins MWG, Ebersberg, Germany), and 1x SensiMix SYBR HI-ROX (Bioline, Luckenwalde, Germany). The amplification was done by a StepOne Plus device (Applied Biosystems, Darmstadt, Germany) and performed with an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at temperature indicated in Tables 1 and 2 for 20 s, and extension at 72 °C for 30 s. After amplification, samples were heated at the rate of 0.5 °C/15 s from 50 to 99 °C with continuous reading of fluorescence to obtain a melting curve followed by a final step of cooling to 40 °C. Negative controls by substituting template DNA with water and samples without RT enzyme were also included in each run.

Serial dilutions of the appropriate purified amplicons were used as a standard curve for gene quantification. Standards were generated as previously described [27]. Besides the melting curve, the obtained amplicons were checked for specificity by sequencing (GATC Biotech, Konstanz, Germany) and showed a 100% homology to the published bovine sequences.

**ELISA and EIA**

Medium from oviductal cells of P0-HIGH and P3-HIGH cultured in 24-well plates was removed after reaching confluence. Cells were incubated with 0.5 ml DMEM HIGH glucose culture media at 37 °C in a humidified atmosphere of 5% $CO_2$. After 24 h, the supernatant was collected and stored at -80 °C for further analysis. Prior to each assay, samples were centrifuged at 13,000 g for 5 min to pellet any dead/floating cells remaining in the supernatants. Duplicates of each sample (100 µl) were used to estimate in a 96 well microtiter plate the release of $PGE_2$ (Parameters kit; R&D Systems, Wiesbaden, Germany), OVGP1 (MyBioSource, San Diego, USA) and IL8 (Human CXCL8/IL8 antibody DuoSet; R&D Systems) from BOEC using commercially available kits. Every assay was conducted based on the manufacturer's instructions. Samples were three-fold diluted for the $PGE_2$ assay with the same BOEC culture medium. Previous work confirmed the cross-reactivity of human CXCL8/IL8 antibodies with bovine IL8 [36]. All assays were carried out using BOEC culture supernatants from different cows (IL8: n=4; $PGE_2$: n=3; OVGP1: n=3). Amounts of the mentioned factors in supernatants were evaluated with a microtiter reader (iMark Bio Rad, Bio-Rad Laboratories, Munich, Germany) after optical density measurements at 450 nm based on the generated standard curve. The range of standard curve for $PGE_2$, IL8 and OVGP1 were 15.6-2000 pg/ml, 39-2500 pg/ml and 0-1000 ng/ml, respectively.

**Table 1** Primer pairs for amplification of genes of interest

| Gene | Sequence of nucleotide | Accession No. | Product size (bp) | Tm (°C) |
|---|---|---|---|---|
| **PTGS1** | **for** 5' -CAG ATG CGG AGT TTC TGA GTC G- 3'<br>**rev** 5' -GGG TAG TGC ATC AGC ACG G- 3' | [27] | 313 | 60 |
| **PTGS2** | **for** 5' -CTC TTC CTC CTG TGC CTG AT- 3'<br>**rev** 5' -CTG AGT ATC TTT GAC TGT GGG AG- 3' | [27] | 359 | 60 |
| **PTGES2** | **for** 5′ -CCT CCT ACA GAA AGG TGC C- 3′<br>**rev** 5′ -GTG ATG ATG TCT GCC AGG G- 3′ | [56] | 133 | 56 |
| **PTGES3** | **for** 5′ -TGC AAA GTG GTA CGA TCG G- 3′<br>**rev** 5′ -TAA CCT TGG CCA TGA CTG G- 3′ | [56] | 253 | 61 |
| **MUC1** | **for** 5′ -ATG ACC ACC CGC TCT ATG TC- 3′<br>**rev** 5′ -GGA GGT GGA AAG TGC TAT GC- 3′ | NM_174115.2 | 189 | 60 |
| **MUC4** | **for** 5′ -ACG TCA CTG TGC ATC TTT GG- 3′<br>**rev** 5′ -AAG CTC TTG ATG GAC GGT TG- 3′ | XM_002684819.3 | 199 | 60 |
| **MUC6** | **for** 5′ -CAG CAG TCC CAC TTC CTC TG- 3′<br>**rev** 5′ -CAG TGA TGG AGC TGG CTA GG- 3′ | XM_005197398.1 | 206 | 65 |
| **MUC16** | **for** 5′ -CAG GTC TCA AAA TCC CAT CC- 3′<br>**rev** 5′ -TGC TGG AGG TGT TGA TAT GG- 3′ | XM_002688785.3 | 256 | 62 |
| **OVGP1** | **for** 5′ -GGG AAA GGT TCG TCA GTT CA- 3′<br>**rev** 5′ -CAT ACG CTT TCT GGA CGA CA- 3′ | NM_001080216.1 | 240 | 60 |
| **BDH2** | **for** 5′ -ATG TCC TCT GTG GCT TCC AG- 3′<br>**rev** 5′ -CAC AAA CTC CAG CCT CCA TC- 3′ | NM_001034488.2 | 347 | 59 |
| **OXCT2** | **for** 5′ -CAC AGT GAG AAC GGG ATC TTG- 3′<br>**rev** 5′ -GTG CAC TTC TCC ACG ATC TTG- 3′ | XM_002704022.2 | 347 | 55 |
| **GAPDH** | **for** 5′ -CCC AGA AGA CTG TGG ATG G- 3′<br>**rev** 5′ -AGT CGC AGG AGA CAA CCT G- 3′ | [57] | 306 | 62 |
| **SDHA** | **for** 5′ -GGG AGG ACT TCA AGG AGA GG- 3′<br>**rev** 5′ -CTC CTC AGT AGG AGC GGA TG- 3′ | [57] | 219 | 60 |
| **HK1** | **for** 5′ -GCG TTT CCA CAA GAC TCT GC- 3′<br>**rev** 5′ -AGA TCC AGG GCC AAG AAG TC- 3′ | NM_0010112668.1 | 324 | 61 |
| **IL8** | **for** 5′ -CGA TGC CAA TGC ATA AAA AC- 3′<br>**rev** 5′ -CTT TTC CTT GGG GTT TAG GC- 3′ | [58] | 153 | 56 |

**Selected gene transcripts, primer sequences and annealing temperatures (Tm) used for quantitative PCR with resulting amplicon length**

**Table 2** Primer pairs for amplification of references genes

| Gene | Sequence of nucleotide | Accession No. | Product size (bp) | Tm (°C) |
|---|---|---|---|---|
| **HDAC1** | **for** 5' –CCA GTG CAG TT GTCT TGC AG- 3'<br>**rev** 5' –TTA GGG ATC TCC GTG TCC AG- 3' | NM_001037444.2 | 217 | 60 |
| **UXT** | **for** 5' –CGC TAC GAG GCT TTC ATC TC- 3'<br>**rev** 5' –TGA AGT GTC TGG GAC CAC TG- 3' | NM_001037471.2 | 207 | 61 |
| **PPIA** | **for** 5' – CTG AGC ACT GGA GA GAAA GG- 3'<br>**rev** 5' – TGC CAT CCA ACC ACT CAG TC- 3' | NM_178320.2 | 259 | 60 |
| **RPL19** | **for** 5′ -GGC AGG CAT ATG GGA ATA GG- 3′<br>**rev** 5′ -CCT TGT CTG CCT TCA GCT TG- 3′ | NM_001040516.1 | 232 | 60 |
| **SUZ12** | **for** 5′ -TTC GTT GGA CAG GAG AGA CC- 3′<br>**rev** 5′ -GTG CAC CAA GGG CAA TGT AG- 3′ | [59] | 286 | 60 |

**Selected gene transcripts, primer sequences and annealing temperatures (Tm) used for normalization of quantitative PCR with resulting amplicon length**

## Statistical analysis

GeNorm tool [37], which calculates a normalization factor based on the geometric mean of expression levels of reference genes, was assessed to normalize the sample to sample variation of the mRNA expression data. Initially, real-time PCR data of histone deacetylase 1 (HDAC1), ubiquitously-expressed transcript (UXT), peptidylprolyl isomerase A (cyclophilin A) [PPIA], suppressor of zeste 12 homolog (SUZ12) and 60S ribosomal protein L19 (RPL19) were tested with geNorm. Due to their higher expression stability among samples, HDAC1 and UXT were chosen for normalizing mRNA expression of the genes of interest in this study. Normalized data were used for generation of box and whiskers plots with GraphPad Prism 6 (GraphPad Software, La Jolla, USA) displaying median values with 50% of data within the box. Outliers are shown as circles.

Shapiro-Wilk test was performed to determine the normality of data distribution. Based on the normality test output, either one-factor analysis of variance (ANOVA) or Kruskal-Wallis-Test was performed followed by *post hoc* Tukey or Mann-Whitney *U* test to analyze the effect of passaging. Either *t*-test or Mann-Whitney *U* test was conducted to test the effect of glucose medium content at each cell culture passage. Mann-Whitney *U* test was used to test the effect of estrous cycle phases (luteal phase versus non-luteal phase; n=4 each) on in vivo cells or on cultured BOEC at each number of cell culture passage. Fold changes were calculated as the approximate ratio of the mean of normalized mRNA expression to compare different groups.

Bar charts generated from ELISA data are presented as the mean ± SEM (n=3 cows for OVGP1; n=4 for IL8; n=3 for $PGE_2$). Mann-Whitney *U* test was conducted to compare rate of abovementioned contents between P0-HIGH and P3-HIGH cell culture supernatants.

All statistical analyses were performed with SPSS Statistics for Windows Version 20 (SPSS, Chicago, USA) and the level of significance was set at $P \leq 0.05$.

## Results

### Cytokeratin staining and morphology

A purity >99% of epithelial cells at each passage was determined with cytokeratin immunofluorescence staining as a specific marker for epithelial cells (Fig. 1a-d). Oviductal stromal cell contamination in all in vitro cell culture passages was below 1%. Furthermore, there was no difference in the purity cultured with either LOW or HIGH glucose medium content at each passage. In the negative control no specific staining for cytokeratin was observed (Fig.1e).

Cells through all passages within this study retained their epithelial cell heterogeneity. Oviductal cells in P0, P1 and P2 (Fig. 1a, b and c, respectively) had a polygonal structure. However, it was observed that cultured BOEC in P3 showed a tendency towards morphology change to lose this type of structure to appear more elongated (Fig. 1d). There was no evident difference of BOEC morphology with either LOW or HIGH glucose medium at each passage.

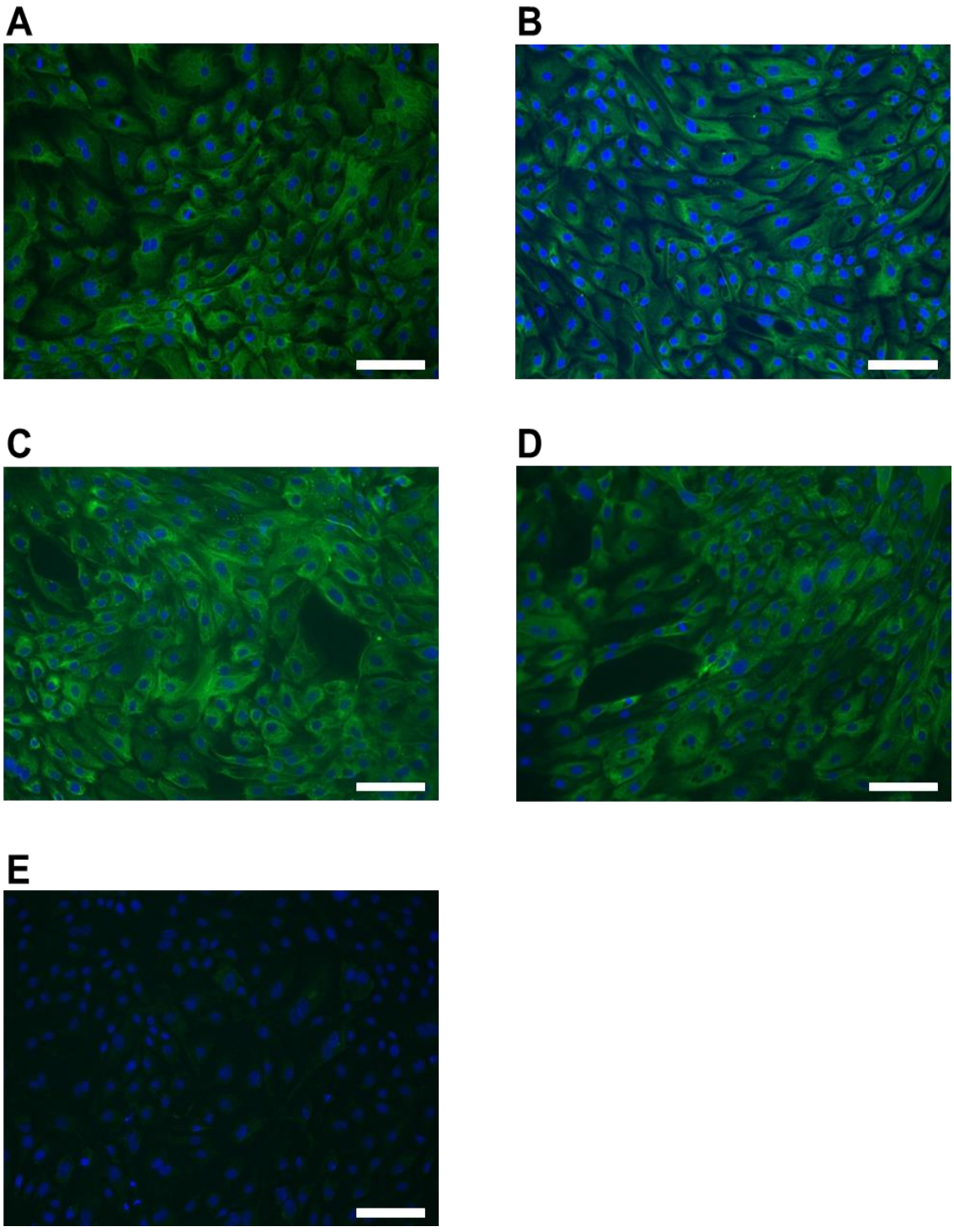


**Fig. 1** Cytokeratin immunostaining of cultured BOEC in different number of cell culture passages. Immunostaining with anti-cytokeratin antibody of cultured BOEC in: **a** passage 0; **b** passage 1; **c** passage 2; **d** passage 3; and **e** negative control. Goat anti-mouse-IgG DyLight 488 conjugate (green) was used for staining cytokeratin as a secondary antibody. DAPI (blue) was used to visualize nuclei. Magnification was set at 200 X. Bar in each figure represents 100 µm. Representative pictures of BOEC cultured in LOW glucose medium are shown.

**Wheat germ agglutinin staining**

The presence of N-acetyl-D-glucosamine and sialic acid residues as parts of mucins was detected on oviductal cells both in vivo and in vitro by WGA staining (Fig. 2).

In detail, oviductal cells in vivo were positively stained with WGA in samples obtained from the luteal as well as from the non-luteal phase without any obvious differences. A representative picture from the stained section obtained during the luteal phase is presented (Fig. 2a). The WGA staining was observed only at the apical surface of the epithelium lining. The basal surface of the epithelial cells was not stained. In addition, staining could neither be detected on stromal nor on endothelial cells.

Concerning oviductal cells in vitro, WGA staining was observed on the entire surface of cultured cells in all passages. However, the WGA staining of the minimum and maximum number of cell passages (P0 and P3, respectively) showed differences. The staining appeared on more cells in P0 compared with P3 (Fig. 2b and c). In addition, the staining was noted to be stronger on more positive stained cells in P0 than in P3.

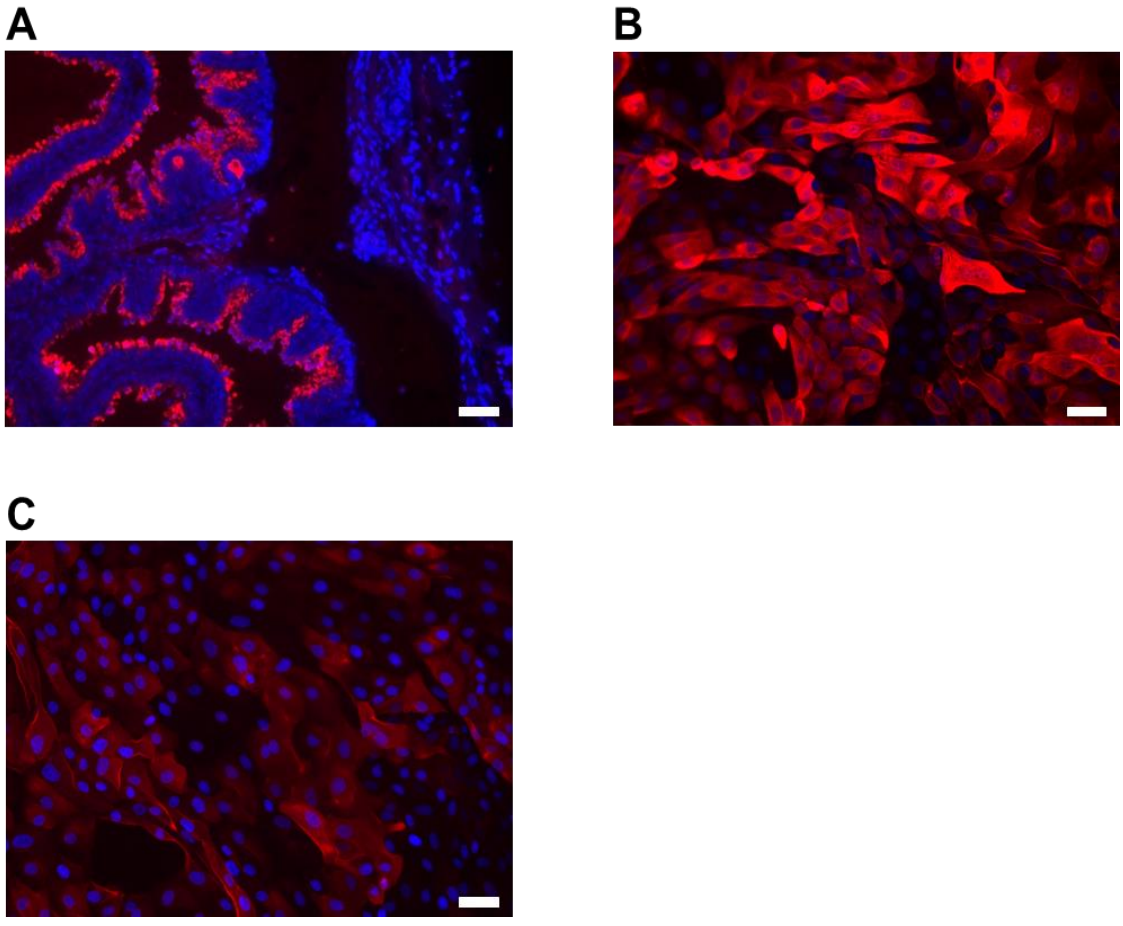


**Fig. 2** WGA staining in sections of oviduct tissue and cultured BOEC. WGA-Alexa Fluor 594 (red) staining of: **a** oviductal cross-section of the ampulla collected obtained during the luteal phase represents in vivo sample; **b** primary cultured BOEC in passage 0; **c** primary cultured BOEC in passage 3. DAPI (blue) was used to visualize nuclei. Magnification was set at 200 X. Bar in each figure represents 50 µm. Representative pictures of BOEC cultured in HIGH glucose medium are shown.

**mRNA expression of selected PG synthesis enzymes**

The mRNA for PTGS1 was about 20-fold more highly expressed in cultured oviductal cells compared with cells obtained from the in vivo state (Fig. 3a). However, PTGS1 mRNA expression was neither affected by the number of cell culture passages nor by the different medium glucose content. In addition, there was no difference of PTGS1 mRNA expression in BOEC obtained at different phases of the estrous cycle cultured with either LOW or HIGH glucose content medium at all passages (data not shown).

Similar as observed for PTGS1, PTGS2 mRNA expression in oviductal cells differed tremendously in the in vitro state with at least tenfold higher contents compared with the *in vivo* state (Fig. 3b). The glucose content of the culturing medium impacted the mRNA expression pattern of PTGS2. Throughout the passages P1, P2 and P3, BOEC cultured in HIGH glucose medium showed more than threefold higher PTGS2 mRNA expression than their cultured counterparts with LOW glucose medium in the same cell culture passage ($P < 0.05$). Additionally, oviductal cells in P0-HIGH tended to show higher transcript amount for PTGS2 than in P0-LOW cells ($P = 0.07$). In contrast, mRNA expression of PTGS2 was not affected by the number of cell culture passages. The stage of estrous cycle did not affect PTGS2 transcription in BOEC cultured with LOW glucose medium (Fig. 3c), whereas oviductal cells collected around ovulation cultured with HIGH glucose medium showed in P3 a significant sevenfold higher PTGS2 mRNA expression compared with cells harvested during the luteal phase (Fig. 3d).

There was an alteration of PTGES2 transcription in BOEC from the in vivo state to in vitro cells with a higher PTGES2 mRNA content at least after the first passage (Fig. 3e). The expression of PTGES2 mRNA was also influenced by the cell culture passage in the presence of HIGH glucose medium but not with LOW glucose containing medium. The mRNA amount of PTGES2 in P2-HIGH BOEC was significantly lower ($P < 0.05$) compared with cells in P0-HIGH. In contrast, the different content of glucose in the medium did not influence significantly the mRNA expression pattern of PTGES2 ($P > 0.05$). Regardless to the glucose content in media, BOEC obtained around ovulation had significant higher ($P < 0.05$) PTGES2 mRNA abundance in P3 than cells harvested during the luteal phase (Fig. 3f and 3g).

In opposite to the other PG synthases, oviductal cells in vitro showed approximately twofold lower PTGES3 mRNA expression than in the in vivo state (Fig. 3h). On the other hand, mRNA expression pattern of PTGES3 was neither influenced by the number of cell culture passages nor by the different glucose content of the medium. Furthermore, the phase of estrous cycle did not show any significant effect on the transcription rate of PTGES3 in cultured BOEC with either LOW or HIGH glucose medium (data not shown).

The mRNA expression of all investigated PG synthases in oviductal cells in vivo was not influenced by the phase of estrous cycle grouping them into the non-luteal (around ovulation; n=4) or luteal (n=4) phase (data not shown).

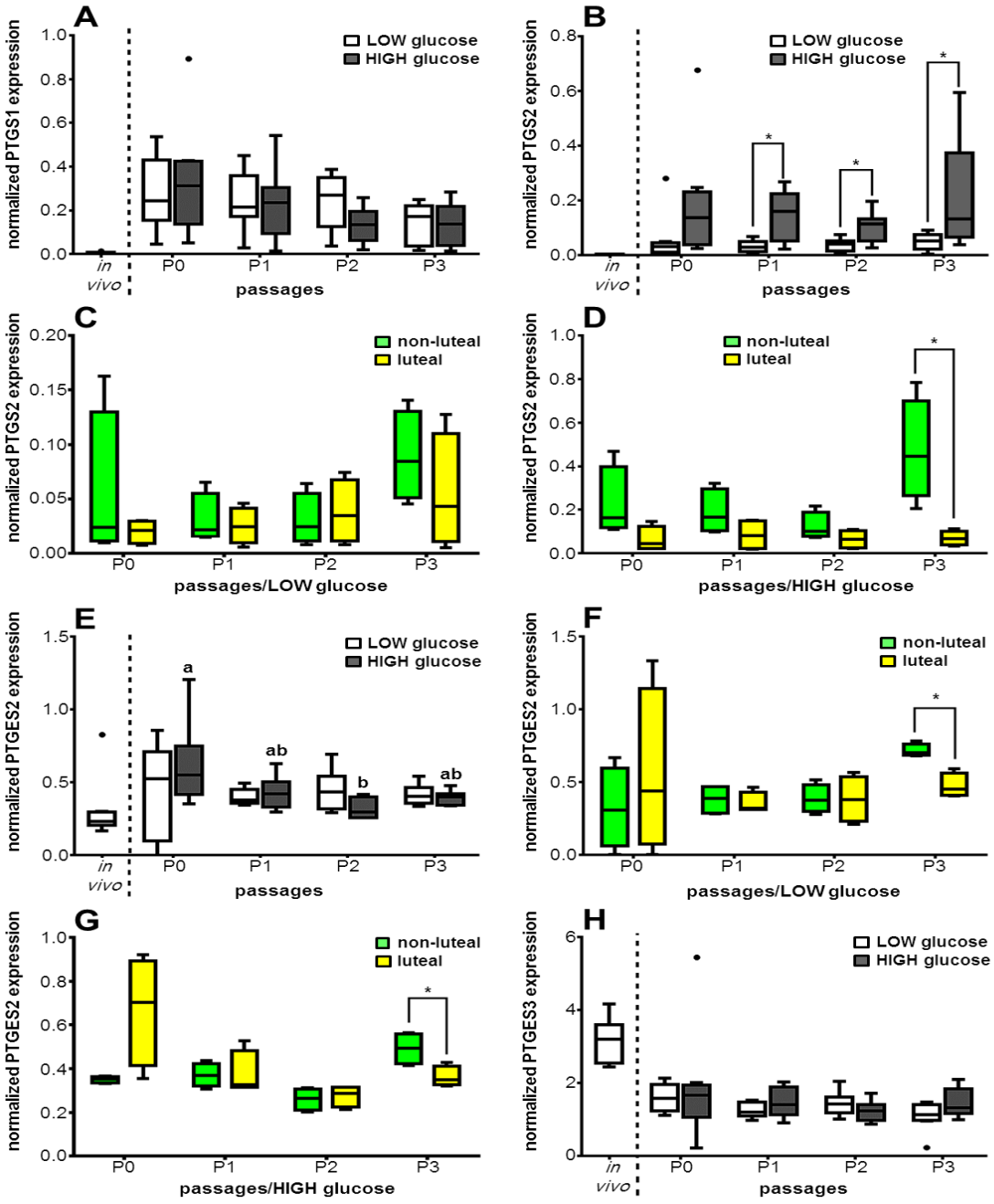


**Fig. 3** Effect of passage number or estrous cycle stage on PG synthesis enzyme mRNA expression pattern. Normalized mRNA expression of **a** PTGS1; **b** PTGS2; **e** PTGES2; and **h** PTGES3 in BOEC of in vivo samples and of cell culture passages P0, P1, P2 and P3 with LOW and HIGH glucose medium content (n= 8) as well as normalized mRNA expression depending of the phase of the estrous cycle on day of collecting cells of **c** PTGS2 in LOW glucose medium; **d** PTGS2 in HIGH glucose medium; **f** PTGES2 in LOW glucose medium; and **g** PTGES2 in HIGH glucose medium (n= 4). Different letters indicate significant difference ($P < 0.05$) between cell culture passages of the same glucose medium content. Asterisk on top of the line represents significant difference between different glucose content of medium at each cell culture passage or between the different estrous cycle phases on day of collection ($P < 0.05$).

**mRNA expression of selected mucins and IL8**

The mRNA expression of MUC1 was about twofold higher in oviductal cells in vitro compared with cells from the in vivo state (Fig. 4a). There was no significant effect of different glucose concentration in the culturing medium on the MUC1 mRNA expression. However, cell culture passage influenced the mRNA expression pattern of MUC1 ($P < 0.05$) in cells cultured with LOW glucose medium but not with a HIGH glucose medium. Oviductal cells in P2-LOW showed a twofold higher MUC1 mRNA expression than in P3-LOW ($P < 0.05$). The phase of estrous cycle did not impact MUC1 mRNA expression in BOEC cultured with LOW glucose medium (data not shown). However, there was a significant higher ($P < 0.05$) MUC1 mRNA expression in P0-HIGH in oviductal cells harvested during the non-luteal phase compared with oviductal cells collected during the luteal phase (Fig. 4b).

Although oviductal cells in P0 showed similar MUC4 mRNA expression pattern to cells in the in vivo state, an apparent decrease was detected in BOEC at the other passages compared with in vivo cells (Fig. 4c). The transcription rate of MUC4 was influenced by the number of cell culture passages. Cultured cells in P0-LOW and P0-HIGH displayed an approximately tenfold higher MUC4 mRNA expression compared with cultured BOEC in P3-LOW and P3-HIGH, respectively ($P < 0.05$). Different glucose concentration in the medium did not influence MUC4 mRNA expression ($P > 0.05$). The MUC4 transcript amount in BOEC collected around ovulation was significantly fivefold higher ($P < 0.05$) in LOW glucose medium in P3 compared with oviductal cells harvested during the luteal phase (Fig. 4d). In contrast, cells cultured with HIGH glucose medium were not affected by the stage of the estrous cycle in their MUC4 mRNA expression (data not shown).

In general, mRNA expression of MUC6 was the lowest one of all investigated mucins. Bovine oviductal cells showed an approximately tenfold decrease regarding the MUC6 mRNA expression from the in vivo situation to the in vitro state (Fig. 4e). Number of cell culture passages as well as different glucose contents of the medium did not affect MUC6 mRNA expression ($P > 0.05$). The stage of estrous cycle did not impact the LOW glucose cultured oviductal cells (data not shown). However, in oviductal cells collected during the luteal phase cultured with HIGH glucose medium MUC6 mRNA was 40-fold more highly expressed in P1 ($P < 0.05$) than in BOEC harvested during the non-luteal phase (Fig. 4f).

Cultured BOEC displayed a twofold decrease of MUC16 mRNA expression, especially in cells cultured with LOW glucose medium, compared with oviductal cells in vivo (Fig. 5a). The number of cell culture passages concerning LOW glucose containing medium had a significant effect ($P < 0.05$) on the MUC16 mRNA expression with significant fourfold higher contents in cells in P2-LOW than in P3-LOW. In contrast, HIGH glucose medium did not affect the MUC16 mRNA expression. Moreover, different glucose content in the medium influenced the MUC16 mRNA expression revealing in P2-HIGH and P3-HIGH about threefold higher transcription rate of MUC16 than in P2-LOW and P3-LOW cells, respectively ($P < 0.05$). The phase of estrous cycle had a significant impact in P3 on MUC16 mRNA expression in BOEC cultured with a LOW glucose medium (Fig. 5b) but not with a HIGH glucose medium (data not shown). Cultured BOEC obtained during the non-luteal phase had a significant twofold higher ($P < 0.05$) MUC16 mRNA amount in comparison with cells harvested during the luteal phase.

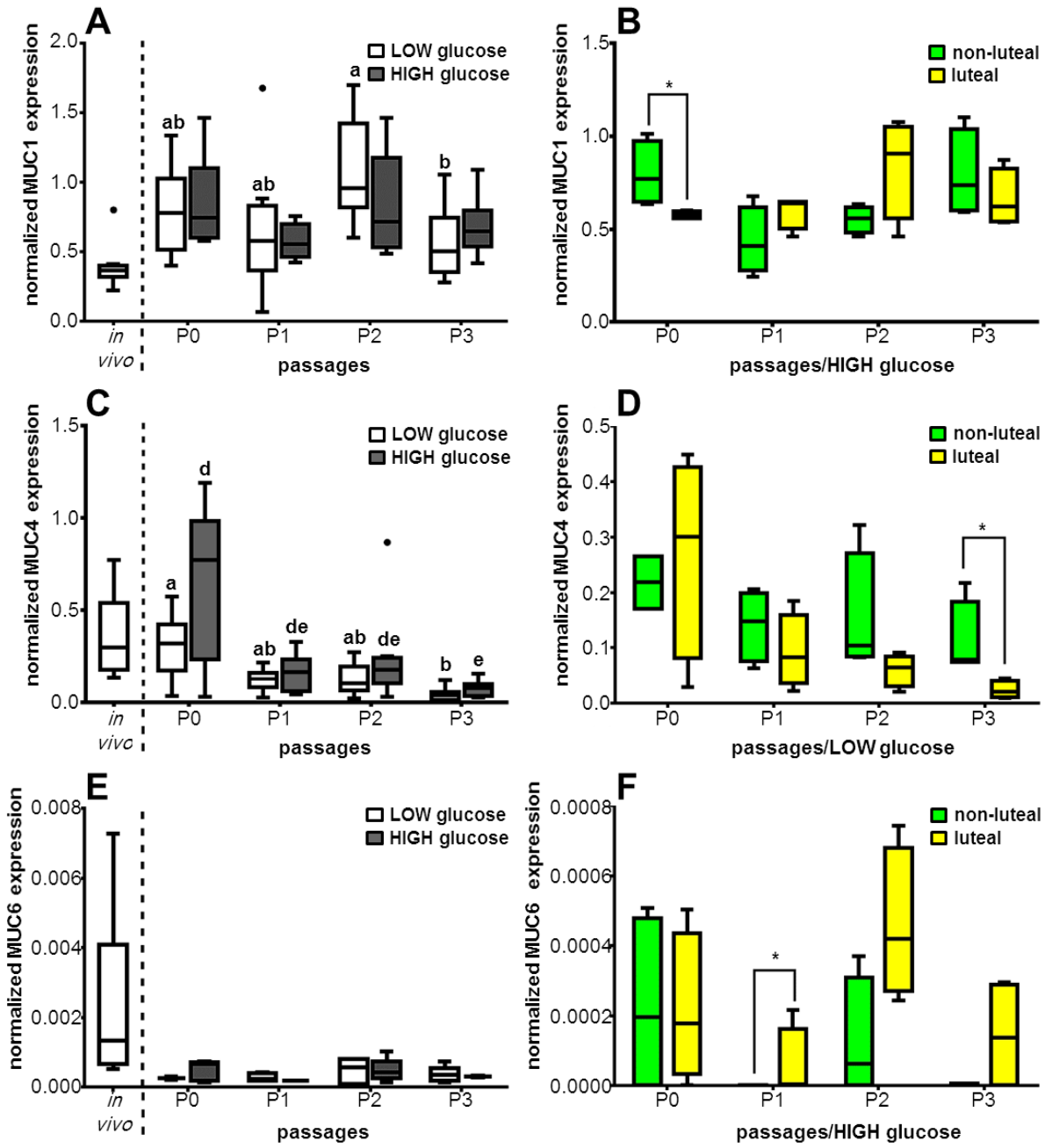


**Fig. 4** Effect of passage number or estrous cycle stage on selected mucin mRNA expression pattern. Normalized mRNA expression of **a** MUC1; **c** MUC4; and **e** MUC6 in BOEC of in vivo samples and of cell culture passages P0, P1, P2 and P3 with LOW and HIGH glucose medium content (n= 8) as well as normalized mRNA expression depending of the phase of the estrous cycle on day of collecting cells of **b** MUC1 in HIGH glucose medium; **d** MUC4 in LOW glucose medium; and **f** MUC6 in HIGH glucose medium (n= 4). Different letters indicate significant difference ($P < 0.05$) between cell culture passages of the same glucose medium content (ab and de for LOW and HIGH glucose medium content, respectively). Asterisk on top of the line represents significant difference between the different estrous cycle phases on day of collection ($P < 0.05$).

When BOEC were cultured, the OVGP1 mRNA expression decreased tremendously to more than 400-fold lower levels compared with the in vivo state (Fig. 5c). The cell culture passage had also a significant effect ($P < 0.05$) on the OVGP1 mRNA expression pattern in oviductal cells, which in both P0-LOW and P0-HIGH was more than 20-fold higher than in the other passages (Fig. 5d). The different glucose concentration of the medium did not influence significantly OVGP1 mRNA expression ($P > 0.05$). The stage of estrous cycle did not affect OVGP1 transcription in oviductal cells in vitro regardless to the glucose content of media (data not shown).

In addition to the presented candidate mucins, mRNA expression of MUC5AC, -12, and -15 was investigated. Oviductal cells in vivo displayed mRNA expression of these mentioned mucins. However, this was in most samples in vitro near or below the detection limits (data not shown).

Transcript amount of each investigated mucin in oviductal cells in vivo showed no influence by the phase of estrous cycle grouping them into the non-luteal (around ovulation; n=4) or luteal (n=4) phase (data not shown).

Cultured oviductal cells displayed a minimum 40-fold higher expression of IL8 mRNA than in vivo (Fig. 5e). Number of cell culture passage did not affect IL8 mRNA expression pattern. The effect of glucose concentration in the culture medium was apparent in P0 and P3 where cells cultured with HIGH glucose medium in both passages showed higher IL8 mRNA expression ($P < 0.05$) compared with LOW glucose medium cultured cells. The stage of estrus cycle did not influence the IL8 transcription level of in vivo collected oviductal cells (data not shown). BOEC cultured in P1 with HIGH glucose medium obtained in the non-luteal phase tended ($P= 0.08$) to show higher IL8 mRNA expression than cells in the luteal phase (Fig. 5f). The estrous cycle stage didn't show any influence on IL8 mRNA abundance in cultured BOEC with LOW glucose content medium (data not shown).

**mRNA expression of selected enzymes of cellular metabolism**

Cultured BOEC in P1, P2 and P3-HIGH had similar BDH2 transcript amount compared with oviductal cells in the in vivo state, except in P0 independently from the glucose medium content as well as in P3-LOW, when BDH2 mRNA expression was lower (Fig. 6a). In addition, mRNA expression of BDH2 was not influenced by the different glucose medium content ($P > 0.05$). However, BDH2 transcription rate in BOEC differed through the cell culture passages with a relatively similar pattern in LOW and HIGH glucose medium cultured cells. Oviductal cells in P2-LOW showed almost three-fold higher BDH2 mRNA expression compared with BOEC in P0-LOW and P3-LOW, respectively ($P < 0.05$). In addition, BDH2 mRNA was significantly threefold more highly expressed in cells in P2-HIGH than in cells in P0-HIGH ($P < 0.05$). Oviductal cells collected during the luteal phase showed in P3 cultured with LOW and HIGH glucose medium significant twofold higher ($P < 0.05$) BDH2 transcription than cells harvested during the non-luteal phase, respectively (Fig. 6b and 6c). Furthermore, oviductal cells collected around ovulation cultured with HIGH glucose medium showed in P0-HIGH a significant higher BDH2 mRNA expression than cells harvested during the luteal phase (Fig. 6c).

A ten- to 20-fold lower OXCT2 mRNA expression was observed in BOEC in vitro in all cell culturing passages compared with the in vivo situation (Fig. 6d). However, oviductal cells showed similar OXCT2 mRNA expression pattern ($P > 0.05$) in different number of cell culture passages (Fig. 6e).

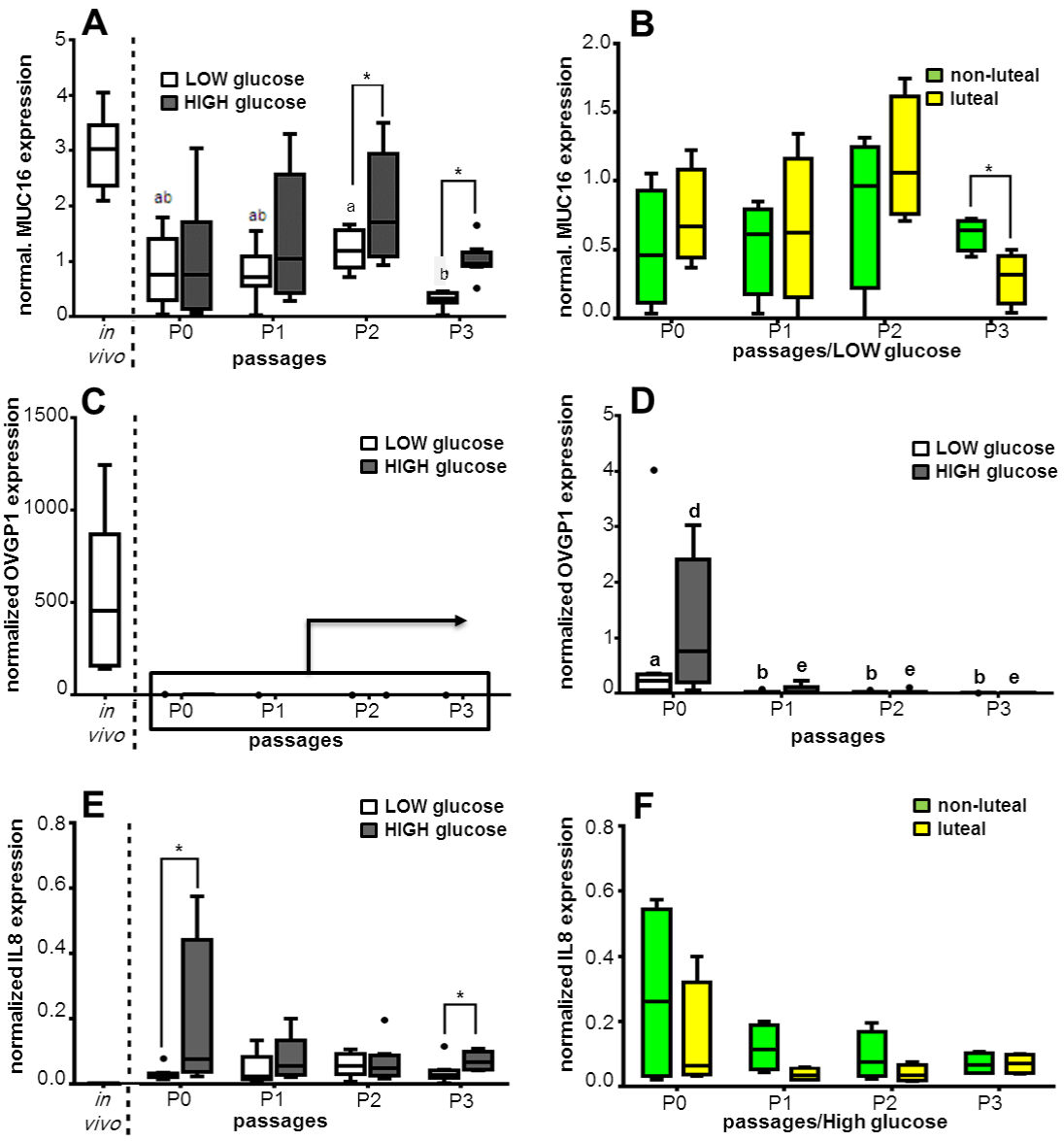


**Fig. 5** Effect of passage number or estrous cycle stage on MUC16, OVGP1 and IL8 mRNA expression pattern. Normalized mRNA expression of **a** MUC16; **c** OVGP1; and **e** IL8 in BOEC of in vivo samples and of cell culture passages P0, P1, P2 and P3 with LOW and HIGH glucose medium content (n= 8); and **d** magnified inset of OVGP1 in BOEC in cell culture passages P0, P1, P2 and P3 with LOW and HIGH glucose medium content as well as normalized mRNA expression depending of the phase of the estrous cycle on day of collecting cells of **b** MUC16 in LOW glucose medium; and **f** IL8 in HIGH glucose medium (n= 4). Different letters indicate significant difference ($P < 0.05$) between cell culture passages of the same glucose medium content (ab and de for LOW and HIGH glucose medium content, respectively). Asterisk on top of the line represents significant difference between different glucose content of medium at each cell culture passage or between the different estrous cycle phases on day of collection ($P < 0.05$).

Additionally, different glucose medium content did not influence OXCT2 mRNA expression. The OXTC2 transcription rate was in vitro altered by the estrous cycle phase in cultured BOEC in LOW and HIGH glucose medium. In detail, oviductal cells obtained during the luteal phase cultured with LOW glucose medium in P1 displayed an about 20-fold significant higher ($P < 0.05$) OXCT2 mRNA expression than cells harvested during the non-luteal phase (Fig. 6f). In contrast, mRNA expression of OXCT2 in BOEC harvested during the non-luteal phase was significantly higher ($P < 0.05$) in P3-LOW compared with cells collected during the luteal phase. In addition, there was in P2 in presence of HIGH glucose medium an about fivefold higher OXCT2 mRNA expression in BOEC obtained during the luteal phase compared with cells collected during the non-luteal phase (Fig. 6g).

The GAPDH mRNA expression was approximately twofold higher in BOEC in the in vitro situation compared with the in vivo state (Fig. 7a). The number of cell culture passages influenced the GAPDH mRNA amount in the presence of LOW glucose medium but not with HIGH glucose medium. GAPDH mRNA was significantly twofold more highly expressed in BOEC in P0-LOW than in cells in P3-LOW ($P < 0.05$). Furthermore, different glucose medium content altered GAPDH mRNA expression in BOEC showing in P3-HIGH higher contents than in P3-LOW ($P < 0.05$). An approximate twofold increase of GAPDH mRNA expression was observed in P3 in BOEC obtained during the luteal phase cultured with LOW and HIGH glucose medium compared with cells harvested around ovulation, respectively (Fig. 7b and 7c).

The mRNA expression of SDHA in BOEC in all cell culture passages, except in P3, was about twofold higher compared with the in vivo state (Fig. 7d). The number of cell culture passages had an impact on the transcription rate of SDHA of BOEC cultured in LOW glucose medium but not of cells incubated in HIGH glucose medium. The SDHA mRNA expression in P3-LOW cultured BOEC was significantly lower than in P0-LOW and P2-LOW cells, respectively ($P < 0.05$). Different glucose concentration of the culturing medium did not affect SDHA mRNA expression ($P > 0.05$). The mRNA content of SDHA in P0-LOW cultured BOEC harvested around ovulation was significantly higher ($P < 0.05$) compared with cells obtained during the luteal phase (Fig. 7e). Furthermore, the SDHA mRNA expression in P2 and P3 cultured with HIGH glucose medium in BOEC collected during the luteal phase was significantly higher ($P < 0.05$) than in cells obtained during the non-luteal phase, respectively (Fig. 7f).

The mRNA of HK1 was twofold more highly expressed in oviductal cells in P0 than in BOEC in the in vivo situation (Fig. 7g). However, BOEC in other cell culture passages showed similar HK1 mRNA expression compared with cells in the in vivo state. The cell culture passages altered HK1 transcription in LOW glucose medium cultured cells but not in HIGH glucose medium cultured BOEC. Oviductal cells in P0-LOW and P2-LOW had an about twofold higher HK1 mRNA amount than in P3-LOW cells ($P < 0.05$). Different glucose content of the medium had no impact on HK1 mRNA expression ($P > 0.05$). There was no significant differential regulation of HK1 transcription depending on different phases of the estrous cycle in cultured oviductal cells (data not shown).

The different estrous cycle phase did not affected the mRNA expression of the investigated cell metabolism enzymes in the in vivo state in oviductal cells, when comparing samples obtained during non-luteal (pre- and post-ovulatory phase; n=4) with samples from the luteal (n=4) phase (data not shown).

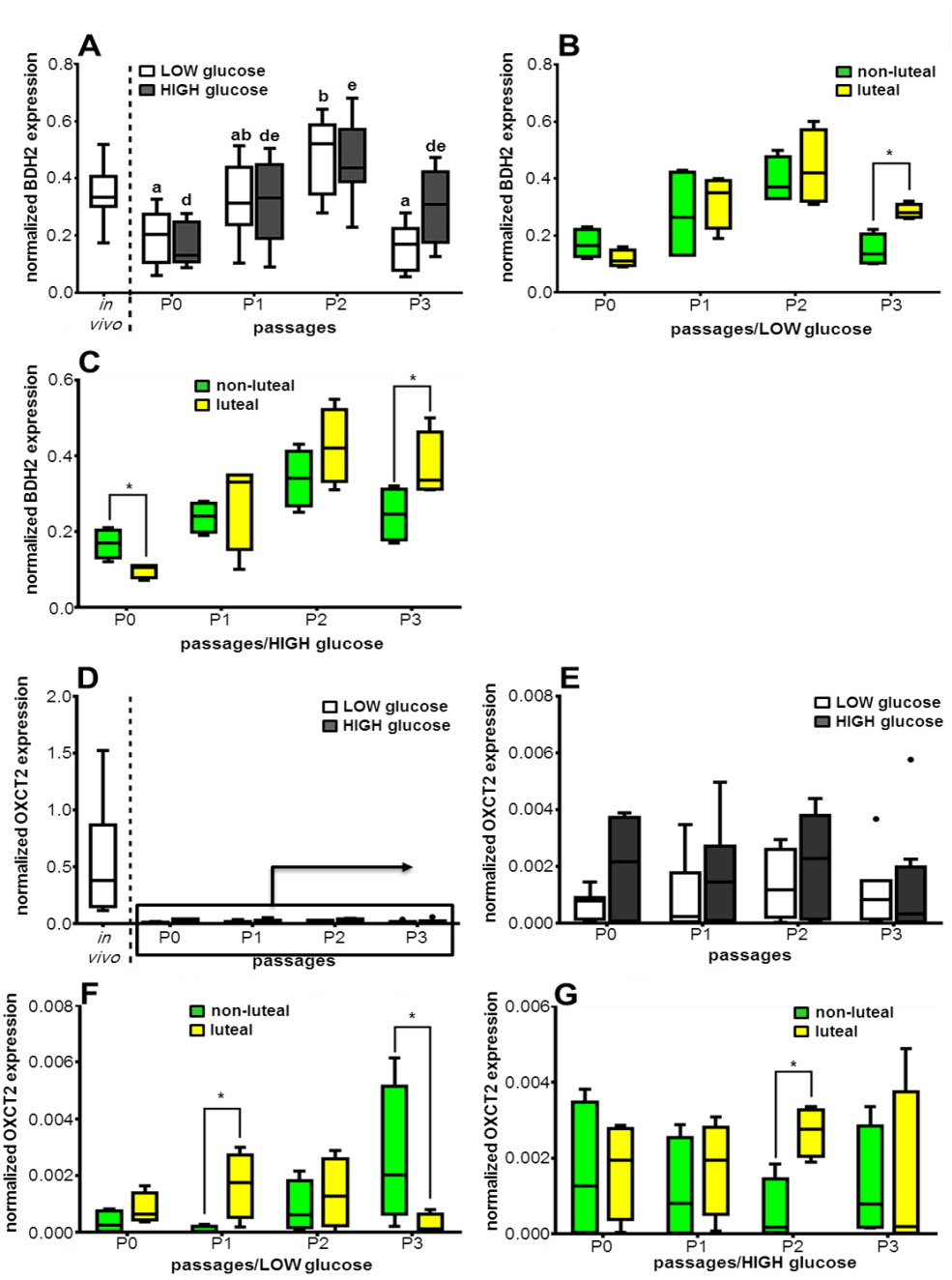


**Fig. 6** Effect of passage number or estrous cycle stage on enzymes of cellular metabolism mRNA expression. Normalized mRNA expression of **a** BDH2; and **d** OXCT2 in BOEC of in vivo samples and of cell culture passages P0, P1, P2 and P3 with LOW and HIGH glucose medium content (n= 8); and **e** magnified inset of OXCT2 in BOEC in cell culture passages P0, P1, P2 and P3 with LOW and HIGH glucose medium content as well as normalized mRNA expression depending of the phase of the estrous cycle on day of collecting cells of **b** BDH2 in LOW glucose medium; **c** BDH2 in HIGH glucose medium; **f** OXCT2 in LOW glucose medium; and **g** OXCT2 in HIGH glucose medium (n= 4). Different letters indicate significant difference ($P < 0.05$) between cell culture passages of the same glucose medium content (ab and de for LOW and HIGH glucose medium content, respectively). Asterisk on top of the line represents significant difference between the different estrous cycle phases on day of collection ($P < 0.05$)

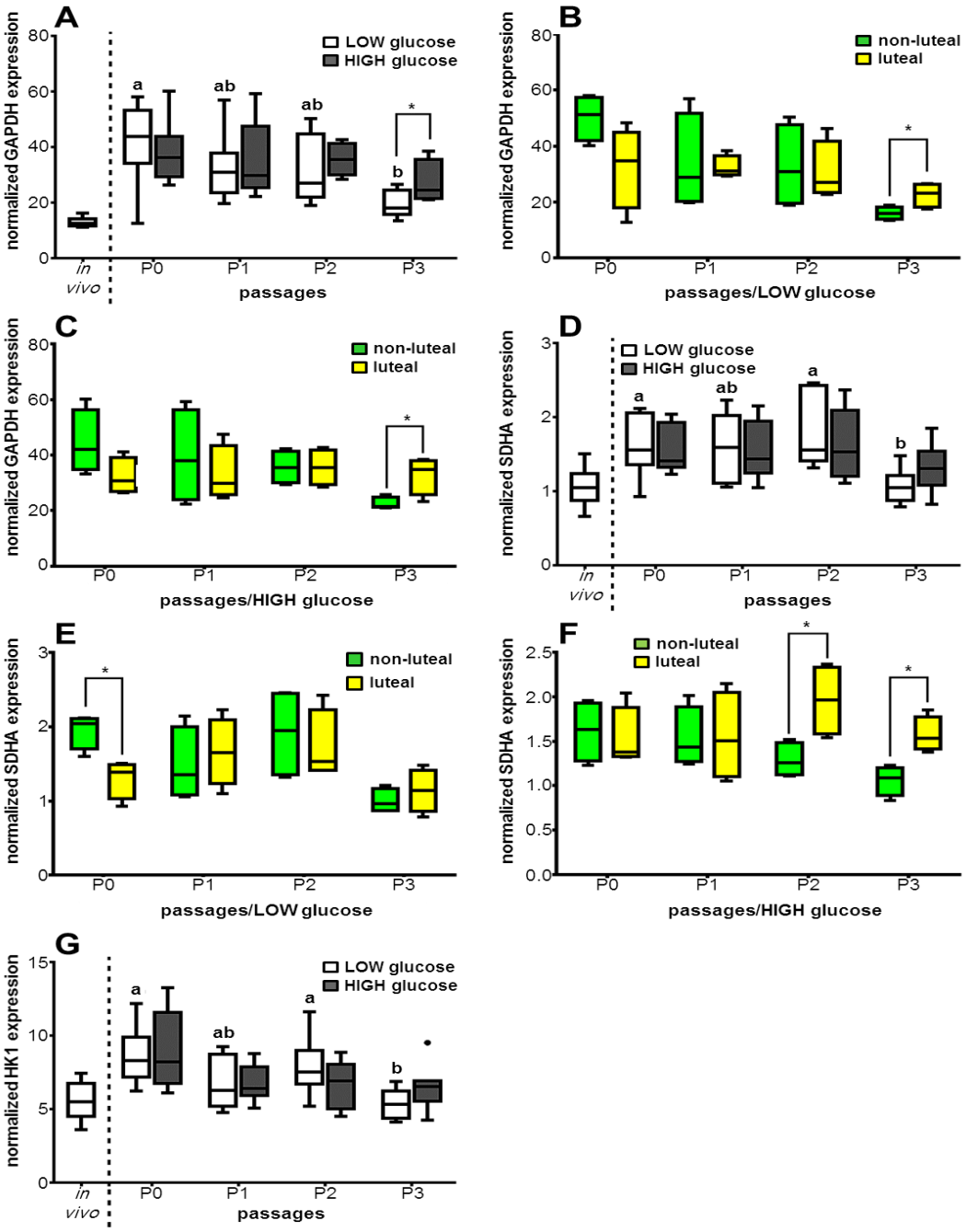


**Fig. 7** Effect of passage number or estrous cycle stage on GAPDH, SDHA and HK1 mRNA expression. Normalized mRNA expression of **a** GAPDH; **d** SDHA; and **g** HK1 in BOEC of in vivo samples and of cell culture passages P0, P1, P2 and P3 with LOW and HIGH glucose medium content (n= 8) as well as normalized mRNA expression depending of the phase of the estrous cycle on day of collecting cells of **b** GAPDH in LOW glucose medium; **c** GAPDH in HIGH glucose medium; **e** SDHA in LOW glucose medium; and **f** SDHA in HIGH glucose medium (n= 4). Different letters indicate significant difference ($P < 0.05$) between cell culture passages of the same glucose medium content. Asterisk on top of the line represents significant difference between different glucose content of medium at each cell culture passage or between the different estrous cycle phases on day of collection ($P < 0.05$).

### $PGE_2$, OVGP1 and IL8 release from BOEC

The amount of $PGE_2$, OVGP1 and IL8 were estimated from supernatants of BOEC in P0-HIGH and P3-HIGH. These particular passage numbers and the type of cell culturing medium were chosen based on the mRNA expression results, which showed the most evident differences in the transcription levels of the candidate genes.

There was no significant change ($P > 0.05$) in $PGE_2$ release between P0 and P3 BOEC (Fig. 8a).

Oviductal cells in P0 released higher amounts of OVGP1 (1.5-fold) compared with their cultured counterparts in P3 (Fig 8b); although this higher release did not reach the significant level.

Cell culture passaging clearly influenced the rate of IL8 release (Fig. 8c). BOEC in P3 released higher amounts of this cytokine (~ three-fold; $P < 0.05$) compared with their counterparts in P0.

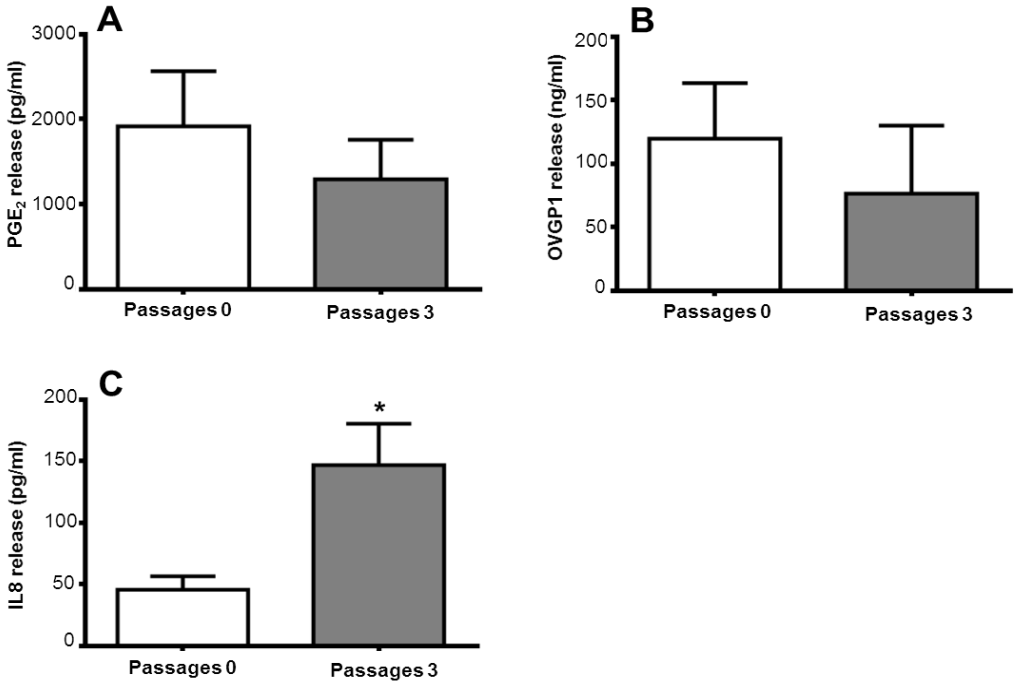


**Fig. 8** Effect of passage number on $PGE_2$, OVGP1 and IL8 release from BOEC. Release estimated in P0-HIGH and P3-HIGH BOEC of **a** $PGE_2$ (n=3); **b** OVGP1 (n=3); and **c** IL8 (n=4). Each bar chart illustrates data as means ± SEM. Asterisk depicts significant difference ($P < 0.05$) between P0 and P3.

## Discussion

Cell culture models are useful tools for revealing specific effects and mechanisms of pure cell populations. However, to obtain valid and reliable information of the treated cells, cell populations should be as close as possible in the in vitro state to the in vivo situation as occur in the organ environment. The present study indicated that each candidate gene differed in its mRNA expression

pattern between the in vivo state and the situation in the cultured oviductal cells in vitro until the third passage.

The immunofluorescence staining with cytokeratin as a specific epithelial cell marker revealed that the monolayer cell culturing model within this study contained a pure epithelial cell population throughout all passages as also observed in many other studies [14, 38]. However, it seems very likely that the cell composition of the so called “in vivo” preparation contained as contamination other cell types, e.g. immune cells or fibroblasts. This difference could alter the mRNA expression pattern of cells from the in vivo state to the in vitro situation but does not explain such dramatic change for some genes in their transcription rate.

A possible explanation for the altered mRNA expression rate is the phenomenon described with the term ‘culture shock’ to point out the tremendous stress encountering the primary cells, which leads to higher production of reactive oxygen species (ROS) inducing oxidative stress to the cultured cells [39, 40]. These findings were accompanied by the up-regulation of especially PTGS2 mRNA expression in presence of ROS via the NFκ-pathway, which is also likely in the oviduct [18, 41]. One further reason could be due to the stress prompted by hyperglycemia. It has been stated that hyperglycemia strongly induces stress responses specifically in postinjury and postsurgical situations and in systemic inflammatory response syndrome [42], which is similar after tissue collection with disruption of the cells out of their tissue resulting in a higher mRNA expression of PTGS2 and IL8 in this study.

The present study provided a series of data indicating that the mRNA expression of some of the selected genes in BOEC is different starting from P0 throughout the in vitro passages of culturing until P3. More importantly, the release of some of their encoded proteins such as IL8 differed between distinct cell culture passage numbers. This was in accordance with previous investigations, which have shown the consequence of cell culture passages on growth rate, protein level and mRNA expression profile alteration in primary cultured endothelial cells [43, 44]. Especially cells in the LOW glucose medium showed a decrease of mRNA expression of metabolic enzymes. This could be due to the fact that the cell metabolism is reduced and the cells are less proliferative, which is likely in the tissue in most situations when no stimulus is present. Such findings were observed in the untreated controls in vitro [18, 27].

In contrast, specific enzymes for the synthesis of PG were expressed un-regulated throughout all cell culture passages. Constant transcription level of these enzymes along with relatively unchanged release of $PGE_2$ indicates that BOEC in this study are functional cells. Prostaglandin $E_2$ has been stated the most abundant of the PG in the bovine oviduct conducting a wide range of physiological and immunological functions within this organ [45]. This indicates that the expression of $PGE_2$ synthesizing enzymes in cultured BOEC is a functional requirement, which is increased under different physiological treatments, e.g. progesterone, estradiol, cumulus-oocyte-complexes or sperm [17, 18, 27].

Another group of functional proteins are MUC in the oviduct, especially the importance of OVGP1 as the main constitute of oviductal fluid in early embryonic enhancement in different species was described [8, 46, 47]. The WGA staining indicated the presence of mucins on the apical surface of BOEC in vivo, which indicates MUC as an additional functional epithelial cell marker for cell culture approaches, because other cell types were not stained. In addition, a staining was also observed in vitro but the intensity decreased with higher number of cell culture passages, which was accompanied by the

decrease of mRNA expression of most MUC in this study. This may be the consequence of oviductal cell dedifferentiation as they go through consecutive subculturing. The anti-adhesive nature of mucins provides the epithelial cells with the first layer of defense mechanism towards pathogens [48]. This alteration of mucins mRNA expression patterns within the in vitro stages of culturing during this study may affect the immune response of cultured BOEC. Additionally, lower expression of OVGP1 at both transcriptome and proteomic level in BOEC cultured in higher cell culture passages could implicate the decrease of secretory granules. The loss of secretory granules in cultured BOEC has been stated [38].

The switch of MUC presence observed in the present investigation is in accordance with studies indicating the effect of in vitro cell culture passage number on metabolic and biochemical activity of primary cells or cell lines from other tissues [49-51]. Halliwell et al. [39] stated that cells, which survive from the 'culture shock' induce multiple changes in their metabolic activity and relative metabolic enzyme levels in order to adapt to the new environment. In the present study was observed a higher GAPDH mRNA expression in BOEC incubated with higher glucose medium content. This may result in cells in vitro having metabolic profile far different from their in vivo situation. GAPDH and SDHA mRNA expression decreased by higher number of cell culture passages in the presence of LOW glucose medium content, which within this study supports the statement that these genes are not overall well suited endogenous control genes due to their regulation in different metabolic states [52].

The defined composition of cell culture medium provides good experimental reproducibility in mammalian cell culture. Typical glucose concentration in the culturing medium is 4.5 g/l (25 mM) in the majority of the studies regarding BOEC, which is higher than the blood glucose concentration in bovine of about 0.7 g/l (3.8 mM) [53]. Additionally, this is also higher than the glucose concentration observed in oviductal fluid at different days of the estrous cycle [54]. The actual environment in the cows was simulated for the BOEC in vitro by using cell culture medium containing 1 g/l (5.5 mM) glucose in this study. The mRNA expression data showed that most studied genes, except PTGS2, MUC16, IL8 and GAPDH, had similar pattern in LOW and HIGH glucose medium groups indicating no influence on BOEC in all passages. In this study could be shown for the first time that oviductal cells expressed enzymes for the use of ketone bodies, which would minimize the situation of a negative energy balance when higher ketone bodies were present in the blood [10]. However, the mRNA expression was unexpected independently from the glucose medium content.

Beside the glucose content in the medium, the long term effect of the estrous cycle phase with high progesterone (luteal phase) or high estradiol (around ovulation) contents on BOEC mRNA expression was observed for many investigated factors. Short-time effects of up to 6 h treatment with progesterone or estradiol were observed with up-regulation of PTGS2 mRNA expression in BOEC [27]. However, most of the alterations in mRNA expression pattern occurred in cells cultured at later passages. One explanation might be that subsequent subculturing of BOEC would modify the micro RNA profile regulating present candidate genes mRNA expression. It has been previously mentioned that these noncoding RNA can complement target mRNA, thereby causing translational repression or mRNA degradation [55].

## Conclusion

This study supports the hypothesis that candidate gene mRNA expression patterns in oviductal cells were influenced when they were incubated in the new in vitro environment. Findings from this study might be a good indication of how much in vitro cell culturing alone could alter the mRNA expression of BOEC and to some extent the functionality level of these cells. The obtained results indicate that in vitro situation in general and more importantly the in vitro cell culture passage of the oviductal cells should be carefully considered in studies involving this organ. On the other hand, the application of two different concentrations of glucose in the medium showed that there may be a possibility to culture BOEC with the environment as closely as possible to in vivo. Therefore, researchers conducting experiments with oviductal cells are encouraged considering the impact of cell culture passage number on BOEC response as well as functionality with the advice to use BOEC of P0 when possible.

## Abbreviations

BDHA, 3-hydroxybutyrate dehydrogenase; BOEC, Bovine oviductal epithelial cells; cDNA, complemantary DNA; DAPI, 4' 6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDAC1, histone deacetylase1; HBSS, Hank's balanced salt solution; HK1, hexokinase 1; IL8, interleukin 8, mRNA, messenger RNA; MUC, mucin; OVGP1, oviduct-specific glycoprotein; OXCT2, 3-oxoacid coA transferase; P, passage; PBS, Dulbecco's phosphate-buffered saline without $Ca^{2+}$/$Mg^{2+}$; PG, prostaglandin; PPIA, peptidylpropyl isomerase A (cyclophilin A); PTGES2 and -3, prostaglandin $E_2$ synthase 2 and -3; PTGS1 and 2, prostaglandin-endoperoxide synthase 1 and 2; RPL19, 60S ribosomal proteinL19; SDHA, succinate dehydrogenase complex, subunit A; SUZ12, suppressor of zeste 12 homolog; UXT, ubiquitously-expressed transcript; WGA, wheat germ agglutinin.

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## Availability of data and material

The datasets supporting the conclusions of this article are included within the article.

## Authors' contributions

SDM performed all the experiments, analysis and wrote the manuscript; JS help with immunostaining and manuscript editing; RE gave over all guidance and help with manuscript editing; CG conceived the study, assisted with data analysis and interpretation and manuscript preparation. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

## Consent for publication

Not applicable

**Ethics approval and consent to participate**

As only abattoir waste was used for sample collection, institutional or national research council guidelines did not have to be adhered to.

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Publication 2: **Different inflammatory responses of bovine oviductal epithelial cells *in vitro* to bacterial species with distinct pathogenicity characteristics and passage number**

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**Different inflammatory responses of bovine oviductal epithelial cells *in vitro* to bacterial species with distinct pathogenicity characteristics and passage number**

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**Abstract**

The bovine oviduct provides the site for fertilization and early embryonic development. Modifications to this physiological environment, for instance the presence of pathogenic bacterial species, could diminish reproductive success at early stages of pregnancy. The aim of this study was to elucidate the inflammatory responses of bovine oviductal epithelial cells (BOEC) to a pathogenic bacterial species (*Trueperella pyogenes*) and a potentially pathogenic bacterium (*Bacillus pumilus*). BOEC from four healthy animals were isolated, cultured in passage 0 (P0) and passaged until P3. Trypan blue staining determined BOEC viability during 24 h co-culture with different multiplicities of infection (MOI) of *T. pyogenes* (MOI 0.01, 0.05, 0.1 and 1) or *B. pumilus* (MOI 1 and 10). BOEC remained viable when co-cultured with *T. pyogenes* at MOI 0.01 and with *B. pumilus* at MOI 1 and 10. Extracted total RNA from control and bacteria co-cultured samples was subjected to reverse transcription-quantitative polymerase chain reaction (RTq-PCR) to determine mRNA expression of various studied genes. The rate of release of interleukin 8 (IL8) and prostaglandin $E_2$ ($PGE_2$) from BOEC was measured by ELISA after 24 h co-culture with bacteria. RT-qPCR of various selected pro-inflammatory factors revealed similar mRNA expression of pro-inflammatory factors in BOEC co-cultured with *T. pyogenes* and in the controls.

Higher mRNA expression of IL 1A, -1B, tumor necrosis factor alpha and CXC ligand (CXCL) 1/2, -3, -5 and IL8 and PG synthesis enzymes in BOEC co-cultured with *B. pumilus* was observed. In the presence of *B. pumilus* a higher amount of IL8 and $PGE_2$ was released from BOEC than from controls. The viability and pro-inflammatory response of P3 BOEC incubated with bacteria was lower than in P0 BOEC. These findings illustrate the pathogenicity of *T. pyogenes* towards BOEC in detail and the potential role of *B. pumilus* in generating inflammation in oviductal cells. Culturing conditions influenced the pro-inflammatory responses of BOEC towards bacteria. Therefore, researchers conducting epithelial-bacterial *in vitro* co-culture should not underestimate the effects of these parameters.

**Keywords:**

Bovine oviductal epithelial cells, postpartum diseases, Gram-positive bacteria, dairy cattle fertility, *in vitro* cell passaging

## 1. Introduction

The uteri of most dairy cows postpartum are invaded by a variety of pathogens, leading to clinical diseases that persists beyond 21 d postpartum in 50% of affected animals [1]. Among these pathogens, *Trueperella pyogenes*, a Gram-positive pathogen, provokes endometrial inflammation resulting in reproductive disorders [1]. However, other bacterial groups may also play a key role in the establishment of these diseases [2]. *Bacillus* spp. are also Gram-positive bacteria that have been detected in the bovine uterus [3]. These bacterial species are characterized as potential pathogens that have been isolated from the uterine lumen of endometritis cases, yet they have not been specifically linked with uterine lesions [4]. *Bacillus* spp. were isolated from healthy and diseased bovine uteri with a frequency of 10% and half of the strains belonged to the species *B. pumilus* [5]. *B. pumilus* was also reported to promote pro-inflammatory reactions in bovine endometrial epithelial cells *in vitro* [6].

Several studies have investigated the immune responses of bovine ovaries or uteri to pathogenic bacteria and their endotoxins [7-9]. In this context, *Chlamydia* spp. were found in the oviducts of 37% and 18% of cows with a healthy and a pathological reproductive tract, respectively [10]. In addition, *T. pyogenes* was detected on the oviductal epithelial surface in cows with pelvic infection early postpartum [11]. Any disruption to the oviduct could affect the reproductive rate because this organ provides the environment for gamete maturation, fertilization, and early embryo development [12].

The columnar epithelial cells of the oviduct are the host's first line of defense and have evolved to modulate the innate immune system in the presence of pathogens [13]. This includes activation of pathways leading to the transcription of several genes including interleukin (IL) 1A, -1B, -6, -8, tumor necrosis factor alpha (TNFA) and CXC ligand (CXCL) 1–5 as seen in the uterus [14]. These factors are crucial for evoking the migration of immune cells to the infected site to generate the inflammatory response [15]. Prostaglandins (PG) also play a major role during inflammation. Prostaglandin-endoperoxide synthase 2 (PTGS2) and microsomal $PGE_2$ synthase 1 (PTGES) are key enzymes for PG, particularly $PGE_2$, synthesis [16].

*In vitro* culturing enabled most of the abovementioned insights into the physiology of epithelial cell interactions with pathogens. Primary bovine oviductal epithelial cell (BOEC) cultures have been widely used to understand various functions of this organ [17,18]. Clear differences in BOEC transcription between particular cell culture passage numbers indicated the influence of *in vitro* passage number, which should be carefully considered to prevent ambiguous results [19].

The objective of this study was to elucidate the influence of two bacterial species with different pathogenicity attributes, *T. pyogenes* as a pathogenic strain and *B. pumilus* as a potential pathogen strain, on pro-inflammatory responses in BOEC. The effects of two particular numbers of cell culture passage 1) on viability, 2) on the mRNA expression of selected pro-inflammatory factors, and 3) on the release of IL8 and $PGE_2$ were investigated.

## 2. Materials and methods

### *2.1. Primary bovine oviductal epithelial cell culture*

All procedures for oviduct collection and the isolation and cell culture of BOEC were carried out as described previously [20]. In brief, oviducts from healthy non-pregnant Holstein cows (n=4) were collected at a local abattoir within 15–20 min of death. In the laboratory, the luminal surface of the opened oviducts was scraped using a rubber policeman and the isolated cells from one oviduct were suspended with 1 ml phosphate buffered saline solution without $Ca^{2+}$/$Mg^{2+}$ (PBS; Sigma, Seelze, Germany). Isolated cells from both oviducts of each animal were combined, washed again with PBS, and suspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS; Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, 1 µg/ml amphotericin B (all Biochrom, Berlin, Germany) and, as antioxidants, 10 µg/ml reduced glutathione and 10 µg/ml ascorbic acid (both Sigma). Epithelial cell suspensions of 500 µl (~1–2 × $10^5$ cells/ml) were incubated in 24-well plates at 37 °C in a humidified atmosphere of 5% $CO_2$. The cultured cells were considered as primary BOEC in passage 0 (P0). Adhered monolayers of BOEC reached confluence (>80%) after 5 days and cells were passaged with the density of 3 × $10^5$ cells/well in 24-well plates in 0.5 ml of the abovementioned BOEC culture medium. The passaging was repeated until confluent primary BOEC in cell passage number 3 (P3) were obtained. The purity of epithelial cells in P0 and P3 was confirmed by immunofluorescence staining against cytokeratin as described previously [19]. The contamination of oviductal stromal cells in both passages was <1% (data not shown).

### *2.2. Isolation and culturing of bacteria*

The *T. pyogenes* strain (TP2) used in this study was isolated from the uterus of a Holstein-Friesian dairy cow on day 15 postpartum (pp) at a commercial dairy farm in Schleswig-Holstein, Germany [21]. This primiparous cow showed vaginal discharge with more than 50% pus on day 21 pp and was classified accordingly as a cow developing clinical endometritis.

*B. pumilus* was isolated from the uterus of a healthy dairy cow on days 40–90 pp at a commercial dairy farm in Brandenburg, Germany [6].

The preparation of both bacterial strains for co-culturing was carried out as described previously [6,21]. Briefly, TP2 was cultivated in brain heart infusion (BHI) broth (Fluka, Steinheim, Germany) supplemented with 5% heat-inactivated (HI) FCS (Biochrom) at 37 °C for 48 h. *B. pumilus* from a stored glycerol stock was grown for 24 h in LB broth (according to Luria and Bertani; Carl Roth, Karlsruhe, Germany) at 37 °C. Bacteria were harvested by centrifugation at 3800 x g for 10 min, washed once with PBS to remove toxins, resuspended in 80% (v/v) PBS and 20% (v/v) glycerol (TP2) or in LB broth containing 20% (v/v) glycerol (*B. pumilus*), and subsequently stored in aliquots at -80 °C for further *in vitro* experiments.

Some *T. pyogenes* and *B. pumilus* aliquots were subjected to plate counting on sheep blood agar (Oxoid, Hampshire, United Kingdom) and LB agar (Carl Roth) respectively to determine the number of colony forming units (CFU)/ml.

### *2.3. Viability assay*

The influence of both bacterial species on the viability of BOEC was evaluated during the first 24 h of co-culture. Bovine oviductal epithelial cells in P0 and P3 were cultured until cells reached full confluence. Cells were thoroughly washed twice with PBS in order to remove any traces of antibiotics. Fresh BOEC culture medium containing no antibiotics was added to BOEC. Prior to the co-culture, the bacteria were thawed and diluted in BOEC culture medium without antibiotics to the desired multiplicity of infection (MOI). Different MOI of *T. pyogenes* (MOI 0.01, 0.05, 0.1 and 1) and of *B. pumilus* (MOI 1 and 10) were used. After 8 h or 24 h co-incubation with *T. pyogenes* or *B. pumilus*, respectively, the medium was removed; BOEC were washed twice with PBS and were stained with a 1:2 dilution of 0.5% (w/v) Trypan blue (Biochrom) in PBS. Viability was assessed after 8 h in the case of *T. pyogenes* so as to be sure that the cells were alive prior to collection for transcriptome studies. In each assay, cultured epithelial cells with the same BOEC culture medium without bacteria served as controls. Various optical fields were considered and pictures were taken using a microscope (Axiovert 25; Carl Zeiss, Göttingen, Germany) with 200 × magnification. Each assay was carried out with BOEC isolated from four cows.

### *2.4. BOEC co-cultured with bacteria for mRNA expression analysis and evaluation of IL8 and $PGE_2$ release*

Particular MOI of *T. pyogenes* and *B. pumilus* that would not affect BOEC viability during 24 h of *in vitro* co-culture were chosen to reveal their short-term influence on oviductal cells. Therefore, BOEC in P0 and P3 as well as the bacteria were prepared as described above. Bovine oviductal epithelial cells were co-cultured with either *T. pyogenes* (MOI 0.01) or *B. pumilus* (MOI 1 and 10) in BOEC medium containing no antibiotics. The medium was removed at 2 h, 4 h and 8 h, respectively. Cells were lysed with lysis buffer RLT (Qiagen, Hilden, Germany) and were stored at -80 °C for further use. Epithelial cells incubated with cell culture medium without antibiotics served as controls for each time point. An additional control was also included by lysing cells at 0 h. After 24 h, the cell culture supernatants of the different treatments for each bacterial strain and the controls were collected for IL8 and $PGE_2$ release and stored at -20 °C until analysis.

### *2.5. Total RNA extraction and cDNA synthesis*

The resultant cell lysates of the co-culture experiments were thawed and subjected to total RNA extraction using an InviMag Universal RNA Mini Kit (Stratec Molecular, Berlin, Germany) on a KingFischer Flex machine (Thermo Scientific, Langenselbold, Germany) following the manufacturer's instructions. The yield of extracted total RNA was estimated with a NanoDrop ND-1000 UV-VIS spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany). The quality control assessment of total RNA was carried out using an RNA 6000 Nano Chip on an Agilent 2100 Bioanalyzer (both Agilent Technologies, Waldbronn, Germany).

cDNA was synthesized as described previously [20]. Briefly, 0.15 µg total RNA was subjected to reverse transcription (RT) in a total volume of 60 µl by adding 2.5 µM random hexamer primers,

0.66 mM dNTPs, 1 x of the supplied RT buffer, and 200 U RevertAid Moloney-Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT; all Fermentas, St. Leon-Rot, Germany). Prior to RT, DNAse I (Fermentas) treatment was performed to remove any potential genomic DNA contamination [22]. M-MLV RT was not included in selected samples in order to monitor the absence of genomic DNA or any other contaminants. The generated cDNA was stored as 20 µl aliquots at -20 °C until further use.

### *2.6. Quantitative PCR*

The quantitative PCR (qPCR) for selected pro-inflammatory factors was performed following the Minimum information for publication of quantitative PCR experiments (MIQE) guidelines [23] in the presence of SYBR Green I using the Rotor Gene 3000 (Corbett Research, Mortlake, Vic, Australia) as described previously [20]. Each qPCR included 1 µl cDNA, 0.4 µM of forward and reverse primer each (details are given in Table 1; synthesized by Eurofins MWG, Ebersberg, Germany) and 1 x SensiMix SYBR Low-ROX (Bioline, Luckenwalde, Germany) in a total volume of 10 µl. The cycling conditions were as follows: 95 °C for 10 min, 45 cycles of 15 s at 95 °C, annealing at the temperature indicated in Table 1 for 20 s, 72 °C for 30 s, and a final step of cooling to 40 °C. The melting curve was assessed by constant fluorescence reading to confirm amplification specificity. A set of serial dilutions from distinct amplicons with known concentrations was generated in a conventional PCR and applied as a standard curve for gene quantification. The mRNA quantity of genes of interest was obtained by comparing the values of the standard curve with the samples using Rotor Gene 6.1 software (Corbett Research). The specificity of all amplicons was determined by sequencing (GATC Biotech, Konstanz, Germany) and all showed 100% homology to the published bovine sequences.

### *2.7. ELISA*

Commercially available human CXCL8/IL8 DuoSet and $PGE_2$ parameter assay kits (all R&D Systems, Wiesbaden, Germany) were used to determine the amount of IL8 and $PGE_2$ in cell culture supernatants, respectively. All procedures were performed following the manufacturer's instructions with slight modifications. Briefly, each sample for the IL8 assay was used undiluted. For the $PGE_2$ assay, each sample was threefold diluted with the same BOEC culture medium containing no antibiotics. All supernatants were centrifuged at 13,000 x g for 5 min in order to remove any epithelial cells and bacteria before conducting the assays. The optical density of each well was determined by using a microtiter reader (iMark Bio Rad, Bio-Rad Laboratories, Munich, Germany) in the presence of standard curves to estimate the concentrations of IL8 and $PGE_2$. IL8 and $PGE_2$ contents were measured as duplicates using BOEC culture supernatants from four and three different cows, respectively. The absorbance readings of one of the animals for $PGE_2$ were not within the standard range, therefore all data from this animal at P0 and P3 were excluded. The cross-reactivity of the human CXCL8/IL8 antibody set with bovine IL8 has been reported previously [24]. The limits of detection were 15.6–2000 pg/ml and 39–2500 pg/ml for IL8 and $PGE_2$, respectively.

**Table 1** Selected gene transcripts, primer sequences and annealing temperatures (Tm) used for quantitative PCR and its normalization with resulting amplicon length.

| Gene | Sequence of nucleotide | Accession no. | Product size (bp) | Tm (°C) |
|---|---|---|---|---|
| **IL1A** | **for** 5' -TCA TCC ACC AGG AAT GCA TC- 3'<br>**rev** 5' -AGC CAT GCT TTT CCC AGA AG- 3' | [47] | 300 | 59 |
| **IL1B** | **for** 5' -CAA GGA GAG GAA AGA GAC A- 3'<br>**rev** 5' -TGA GAA GTG CTG ATG TAC CA- 3' | [48] | 236 | 56 |
| **IL6** | **for** 5′ -TCC AGA ACG AGT ATG AGG- 3′<br>**rev** 5′ -CAT CCG AAT AGC TCT CAG- 3′ | [48] | 263 | 56 |
| **IL8** | **for** 5′ -CGA TGC CAA TGC ATA AAA AC- 3′<br>**rev** 5′ -CTT TTC CTT GGG GTT TAG GC- 3′ | [49] | 153 | 56 |
| **TNFA** | **for** 5′ -CAA GTA ACA AGC CGG TAG CC- 3′<br>**rev** 5′ -GCT GGA AGA CTC CTC CCT G- 3′ | [50] | 354 | 60 |
| **CXCL1/2** | **for** 5′ -GAC CTT GCA GGG GAT TCA CCT C- 3′<br>**rev** 5′ -CGG GGT TGA GAC ACA CTT CCT G- 3′ | [6] | 125 | 60 |
| **CXCL3** | **for** 5′ -GCC ATT GCC TGC AAA CTT- 3′<br>**rev** 5′ -TGC TGC CCT TGT TTA GCA- 3′ | [6] | 189 | 56 |
| **CXCL5** | **for** 5′ -TGA GAC TGC TAT CCA GCC G- 3′<br>**rev** 5′ -AGA TCA CTG ACC GTT TTG CG- 3′ | [49] | 193 | 61 |
| **PTGS2** | **for** 5' -CTC TTC CTC CTG TGC CTG AT- 3'<br>**rev** 5' -CTG AGT ATC TTT GAC TGT GGG AG- 3' | [20] | 359 | 60 |
| **PTGES** | **for** 5′ -TGC TGG TCA TCA AAA TGT ACG- 3′<br>**rev** 5′ -GCA GTT TCC CCA GGT ATG C- 3′ | [47] | 300 | 58 |
| **SDHA** | **for** 5′ -GGG AGG ACT TCA AGG AGA GG- 3′<br>**rev** 5′ -CTC CTC AGT AGG AGC GGA TG- 3′ | [6] | 219 | 60 |
| **RPL19** | **for** 5′ -GGC AGG CAT ATG GGA ATA GG- 3′<br>**rev** 5′ -CCT TGT CTG CCT TCA GCT TG- 3′ | NM_001040516.1 | 232 | 60 |
| **HDAC1** | **for** 5' –CCA GTG CAG TTG TCT TGC AG- 3'<br>**rev** 5' –TTA GGG ATC TCC GTG TCC AG- 3' | [19] | 217 | 60 |

### *2.8. Statistical analysis*

In order to quantify the mRNA expression of selected pro-inflammatory factors, the absolute value of each gene for each sample was divided by the normalization factor calculated using the mRNA expression of the reference genes succinate dehydrogenase complex, subunit A (SDHA), 60S ribosomal protein L19 (RPL19) and histone deacetylase1 (HDAC1) using geNorm [25]. The normalized mRNA expression data were log transformed in order to deal with skewness and to improve the normal distribution of data [26]. The normalized mRNA expression data for all genes in controls and co-cultured BOEC were calculated relative to the mRNA expression data in control cells at 0 h, which was set equal to one. Bar charts were generated from the relative values with bars

representing the mean ± SEM (n=4). Non-log transformed data were used to generate bar charts using GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA). ANOVA with repeated measurement via a general linear mixed effect model approach was conducted to determine two fixed effects (bacteria and co-culture time points), while animals were included as random effect.

The values of pro-inflammatory factors in cell culture supernatants after 24 h co-culture were estimated relative to the concentration of these components in control samples at the same time point, which was defined as one. Each bar chart displays the means ± SEM (n=4 for IL8 and n=3 for $PGE_2$). Paired-sample *t*-test was conducted to compare BOEC treated with bacteria with the corresponding control.

All statistical analyses were performed with SPSS Statistics for Windows Version 20 (IBM, Ehningen, Germany) and the level of significance was set at $P \leq 0.05$. A tendency was considered when $P < 0.10$.

## 3. Results

### *3.1. Viability assay*

Cultured BOEC in P0 remained viable in the presence of *T. pyogenes* during 24 h of co-culturing only at a MOI of 0.01 (Fig. 1A). However, BOEC were dead within 24 h, and even after 16 h, in the presence of *T. pyogenes* at a higher MOI. However, more than 80% of BOEC in P3 were dead after incubation with the same MOI 0.01 of *T. pyogenes*, either being positively stained with trypan blue or detached from the bottom of the well (Fig. 1B). The viability of BOEC in both P0 and P3 8 h after co-culture with *T. pyogenes* at MOI 0.01 was similar to that of the control (data not shown).

More than 90% of the co-cultured BOEC in P0 were viable after 24 h incubation with *B. pumilus* at both MOI (Fig. 1C), which was similar to the control (Fig. 1D). Therefore, pictures are presented that were obtained from BOEC after 24 h co-incubation with *B. pumilus* at MOI 10 (Fig. 1C and E). The same viability was also observed in BOEC in P3 with the same percentage of viable cells when co-cultured with *B. pumilus* at both MOI (Fig. 1E) in comparison with control cells (Fig. 1F).

### *3.2. mRNA expression of selected IL and TNFA*

Cultured BOEC incubated with *T. pyogenes* showed similar IL1A mRNA expression pattern over the time-course in P0 (P= 0.59) and P3 (P= 0.69) compared with the controls (data not shown). However, *B. pumilus* affected the transcription of IL1A in BOEC at different time points (Fig. 2A-B). BOEC co-cultured with both MOI of *B. pumilus* after 2 h and 8 h showed higher ($P < 0.05$) IL1A mRNA expression in P0 compared with the controls (Fig. 2A). In detail, a seven- and 17-fold increase ($P < 0.05$) in IL1A mRNA expression was observed after 2 h in cells co-cultured with *B. pumilus* at a MOI of 1 and 10 respectively compared with the controls. Only MOI 1 of the bacteria tended to induce higher IL1A mRNA expression in co-cultured BOEC after 4 h. Oviductal cells in P3 showed a significant seven-fold increase in mRNA expression ($P < 0.05$) of IL1A only after 8 h incubation with *B. pumilus* at a MOI 1 (Fig. 2B). BOEC co-cultured with *B. pumilus* at a MOI of 10 showed higher IL1A mRNA expression after 2 h (> four-fold) and 8 h (eight-fold) than the controls ($P < 0.05$).

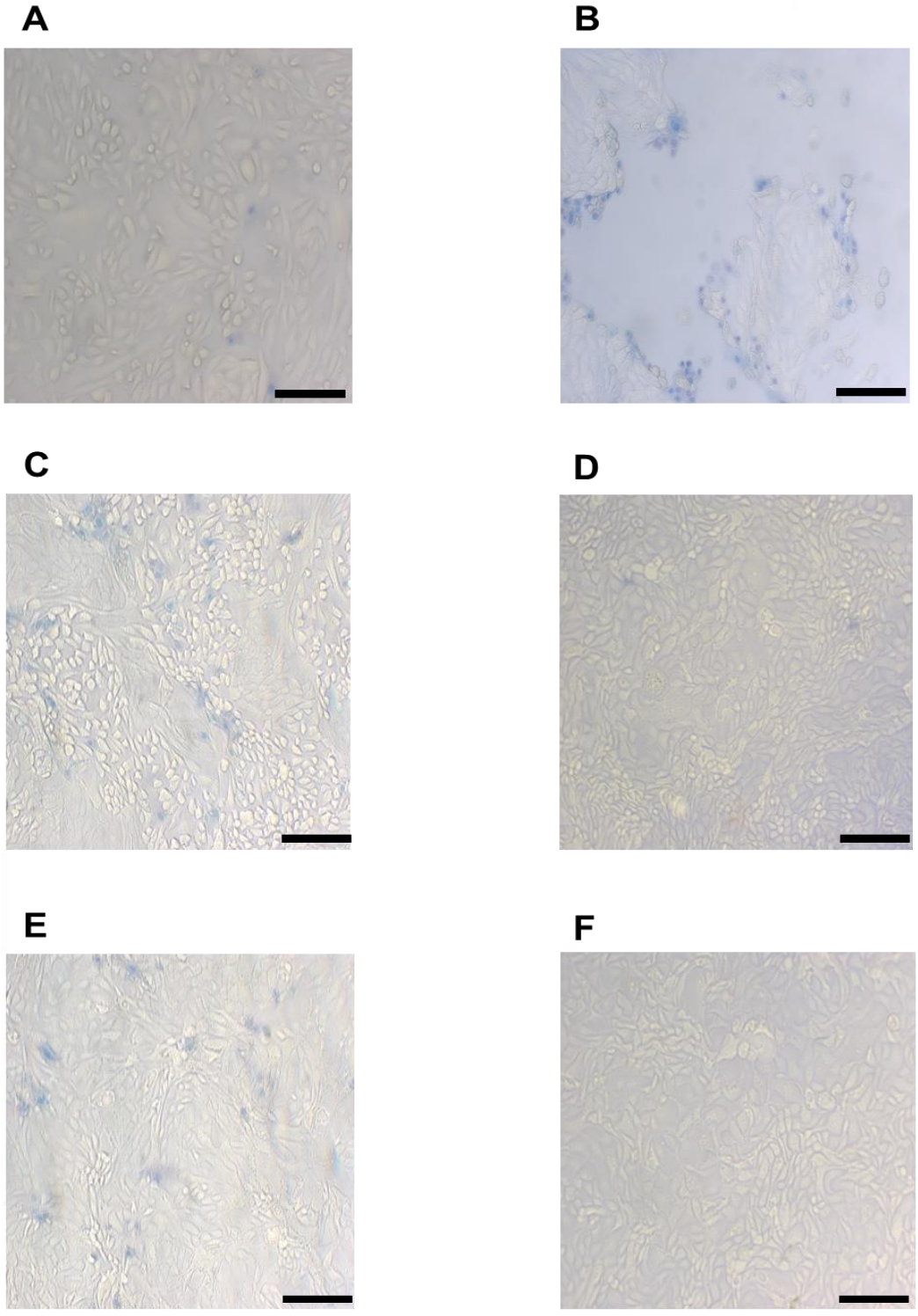


**Fig. 1.** Trypan blue staining of BOEC after 24 h co-culturing with: (A) *T. pyogenes* at a MOI 0.01 in P0; (B) *T. pyogenes* at a MOI 0.01 in P3; (C) *B. pumilus* at a MOI 10 in P0; (E) *B. pumilus* at a MOI 10 in P3; (D) and (F) control in P0 and P3, respectively. Magnification = 200 X.

A treatment * time interaction (P= 0.001) was observed due to higher IL1A mRNA expression throughout incubation with *B. pumilus*.

Cells showed a similar level of transcription of IL1B during co-culturing with *T. pyogenes* and in controls in both passages and at all time points (P-value of 0.70 and 0.48 in P0 and P3, respectively; data not shown). In contrast to *T. pyogenes*, IL1B mRNA expression in P0 BOEC cells co-cultured with *B. pumilus* at MOI 1 was higher, with the highest amount after 8 h (seven-fold increase; Fig. 2C). BOEC incubated with *B. pumilus* at a MOI of 10 showed higher IL1B mRNA expression ($P < 0.05$) after 2 h and 8 h (> six- and four-fold higher, respectively) compared with the control. Relative IL1B mRNA expression did not change in P3 BOEC co-cultured with *B. pumilus* (P= 0.18; Fig. 2D).

TNFA mRNA expression was relatively similar in BOEC during incubation with *T. pyogenes* and the control in P0 (P= 0.74) and P3 (P= 0.77; data not shown). The presence of *B. pumilus* affected the transcription rate of TNFA in P0 and P3 BOEC (Fig. 2E-F). BOEC in P0 showed a significant seven-fold increase in TNFA mRNA expression after 2 h in co-culture with *B. pumilus* regardless of the bacterial MOI. Also, cells expressed seven-fold higher levels of TNFA mRNA after 8 h incubation with *B. pumilus* at both MOI compared with the control ($P < 0.05$). P3 BOEC co-cultured with *B. pumilus* showed a significant increase in TNFA mRNA expression after 2 h. BOEC incubated with both MOI of *B. pumilus* showed six-fold higher transcription of TNFA in comparison with controls after 8 h ($P < 0.05$). A gradual increase in TNFA mRNA expression was observed during the incubation of P3 BOEC with *B. pumilus*.

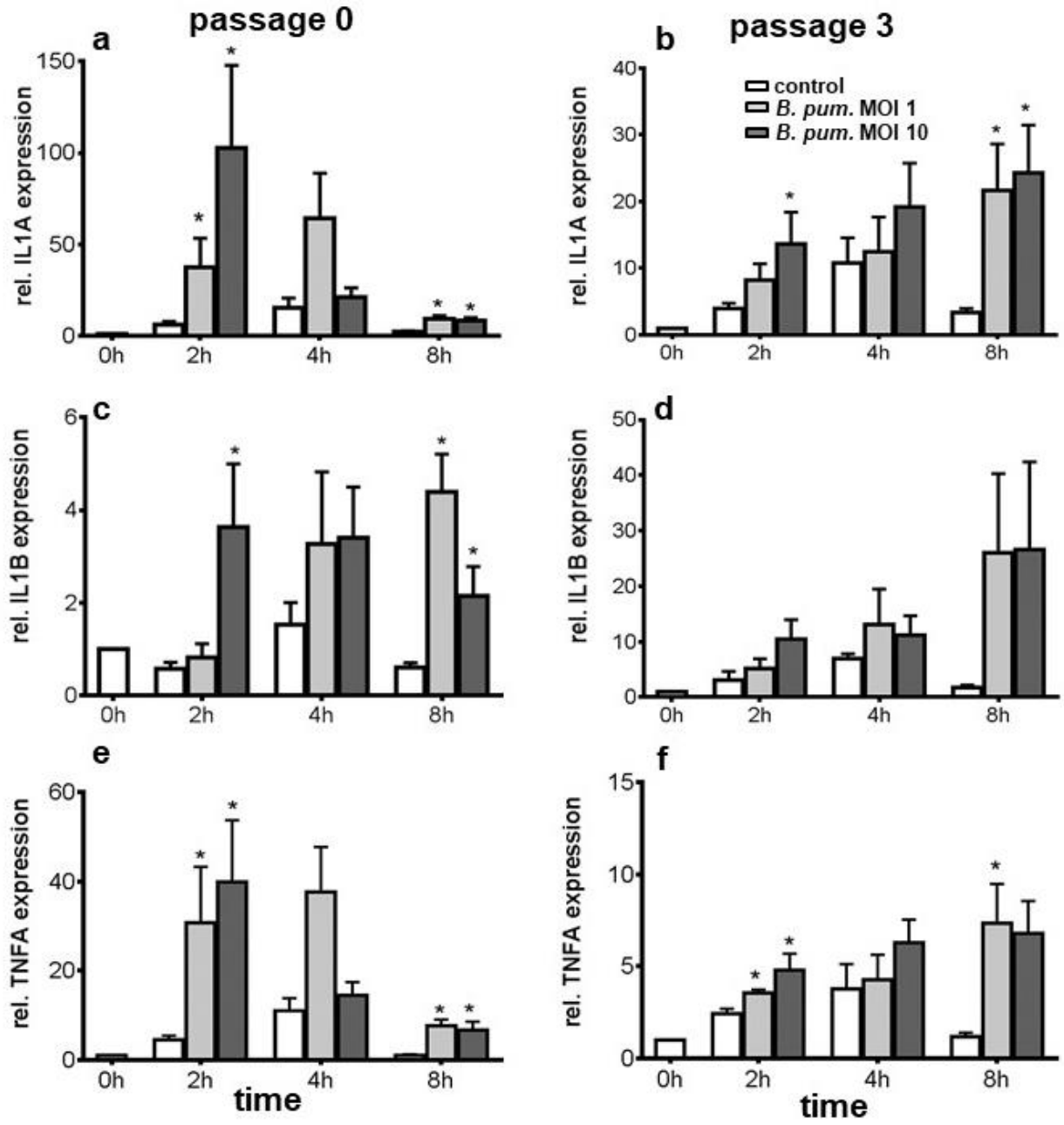


**Fig. 2.** Relative mRNA expression of IL1A (A-B), IL1B (C-D) and TNFA (E-F) in P0 and P3 BOEC co-cultured with *B. pumilus* (MOI 1 and 10) for up to 8 h, respectively (n=4). Each bar chart represents data as means ± SEM normalized to individual mRNA expression of RPL19, SDHA and HDAC1 as the internal control. All mRNA expression values were scaled relative to the mRNA expression data in control cells at 0 h, which was set equal to one. Values from the bacteria co-cultured cells were compared with the control at the same time point. An asterisk indicates a significant difference ($P < 0.05$) compared with untreated cells. The relative mRNA expression scale of individual studied gene from P0 to P3 is different. P-value for time and treatment * time for studied genes in P0 were as follows: IL1A ($P < 0.001$, $P < 0.001$), IL1B ($P= 0.01$, $P= 0.001$) and TNFA ($P < 0.001$, $P < 0.001$). P-value for time and treatment * time for studied genes in P3 were as follows: IL1A ($P= 0.01$, $P= 0.001$), IL1B ($P= 0.31$, $P= 0.58$) and TNFA ($P= 0.02$, $P < 0.001$).

*3.3. mRNA expression of selected chemokines*

Oviductal cells incubated with *T. pyogenes* apparently expressed similar levels of IL8 mRNA in comparison with the controls (P= 0.84 and 0.79 in P0 and P3, respectively; data not shown). In contrast, BOEC co-cultured with *B. pumilus* at a MOI 10 showed higher IL8 mRNA expression in P0 after 2 h (ten-fold) and 4 h (17-fold; Fig. 3A). MOI 1 of *B. pumilus* induced 20-fold higher IL8 mRNA expression in P0 BOEC after 4 h compared with the controls. Oviductal cells in P3 showed increased IL8 mRNA expression during the co-culture but this did not reach significance compared with the controls (Fig. 3B).

As the sequences for CXCL1 and -2 were 99% homologous, the primers CXCL1/2 were specific for CXCL1 and CXCL2. The sequences obtained for the amplicons were 100% homologous to CXCL1 and -2. The expression of CXCL1/2 mRNA in P0 (P= 0.90) and P3 (P= 0.72) BOEC during incubation with *T. pyogenes* was similar to that in control cells (data not shown). However, cells responded to *B. pumilus* with a higher rate of transcription of CXCL1/2 mRNA in comparison with the controls (Fig. 3C-D). BOEC in P0 showed significantly higher expression of CXCL1/2 mRNA during the entire period of co-culturing with *B. pumilus* at both MOI ($P < 0.05$). BOEC in P3 displayed two-fold higher CXCL1/2 mRNA expression 2 h after incubation with *B. pumilus* at a MOI of 10 compared with the control. Furthermore, BOEC showed a six-fold increase in CXCL1/2 mRNA after 8 h of co-culture with *B. pumilus* at both MOI in comparison with control cells. A treatment * time interaction was observed ($P < 0.001$) due to higher CXCL1/2 mRNA expression in BOEC during the co-culture period.

The mRNA expression of CXCL3 was similar in BOEC incubated with *T. pyogenes* and controls (P-value of 0.48 and 0.52 in P0 and P3, respectively; data not shown). However, BOEC in P0 showed five-fold higher CXCL3 mRNA expression after 2 h and 4 h incubation with *B. pumilus* at a MOI of 1 compared with the control (Fig. 4A). The presence of *B. pumilus* at a MOI of 10 resulted in a significant eight-fold increase in CXCL3 mRNA expression after 2 h co-culturing. Incubated BOEC showed higher expression of CXCL3 mRNA after 8 h regardless of *B. pumilus* MOI ($P < 0.05$). BOEC in P3 co-cultured with *B. pumilus* at a MOI of 1 only showed higher CXCL3 mRNA expression (> seven-fold increase) after 8 h (Fig. 4B). Furthermore, significantly higher transcription of CXCL3 was detected in BOEC after 2 h (two-fold) and 8 h (> seven-fold) of co-culturing with *B. pumilus* at a MOI of 10.

As observed for the other factors, CXCL5 mRNA expression in the presence of *T. pyogenes* was similar in both P0 (P= 0.62) and P3 (P= 0.42) BOEC and control cells (data not shown). However, BOEC in P0 expressed significantly higher levels of CXCL5 mRNA after 2 h incubation with *B. pumilus* at both MOI **(**Fig. 4C**)**. A significant two-fold increase in CXCL5 mRNA was only detected in BOEC co-cultured with *B. pumilus* at a MOI of 10 after 8 h. At this time point, MOI 1 of the bacteria tended to induce higher CXCL5 mRNA expression in cells. Furthermore, transcription levels of CXCL5 in BOEC in P3 during co-culture with *B. pumilus* were similar (P= 0.34) to those in controls **(**Fig. 4D**)**.

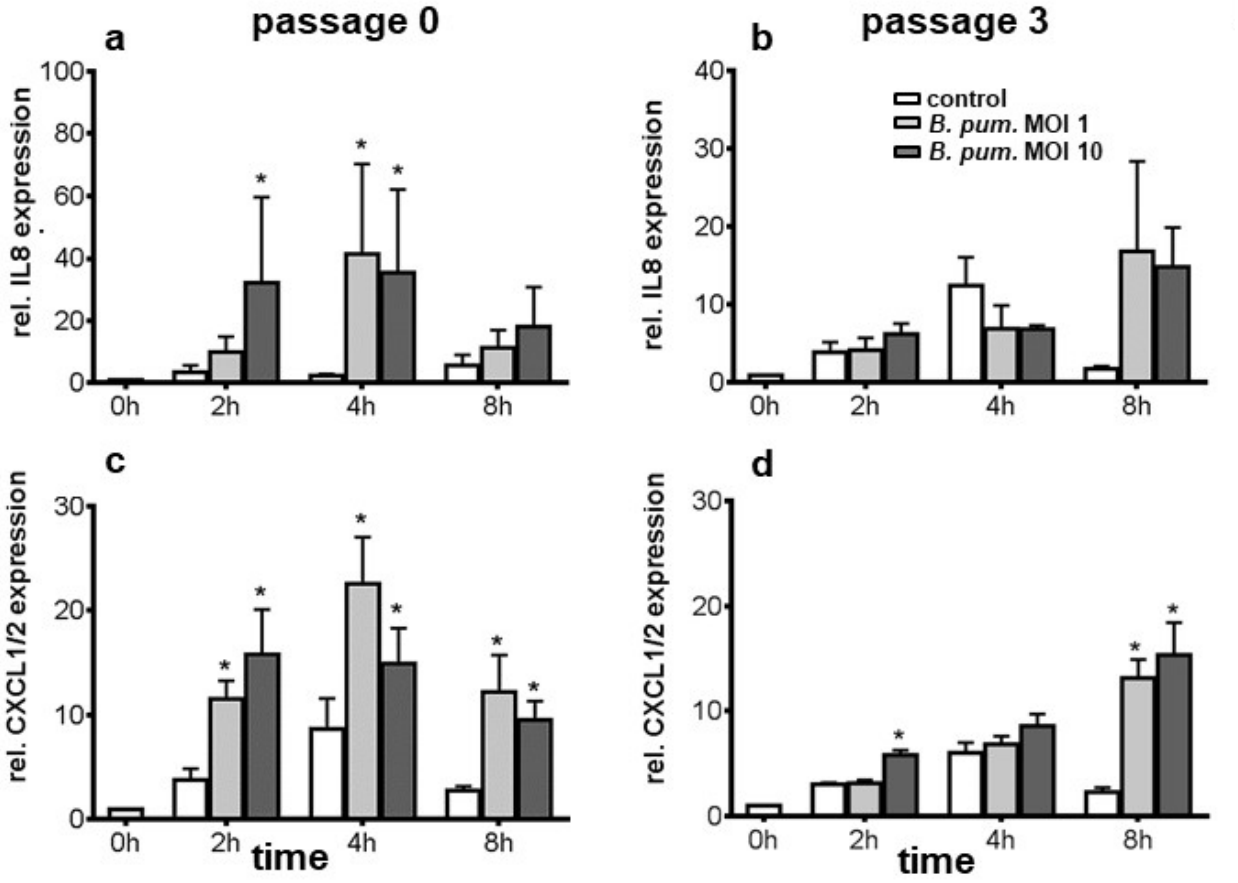


**Fig. 3.** Relative mRNA expression of IL8 (A-B) and CXCL1/2 (C-D) in P0 and P3 BOEC co-cultured with *B. pumilus* (MOI 1 and 10) for up to 8 h, respectively (n=4). Each bar chart represents data as means ± SEM normalized to individual mRNA expression of RPL19, SDHA and HDAC1 as the internal control. All mRNA expression values were scaled relative to the mRNA expression data in control cells at 0 h, which was set equal to one. Values from the bacteria co-cultured cells were compared with the control at the same time point. An asterisk indicates a significant difference ($P < 0.05$) compared with untreated cells. The relative mRNA expression scale of individual studied gene from P0 to P3 is different. P-value for time and treatment * time for studied genes in P0 were as follows: IL8 ($P= 0.13$, $P=0.3$) and CXCL1/2 ($P < 0.001$, $P < 0.001$). P-value for time and treatment * time for studied genes in P3 were as follows: IL8 ($P= 0.104$, $P= 0.07$) and CXCL1/2 ($P= 0.005$, $P < 0.001$).

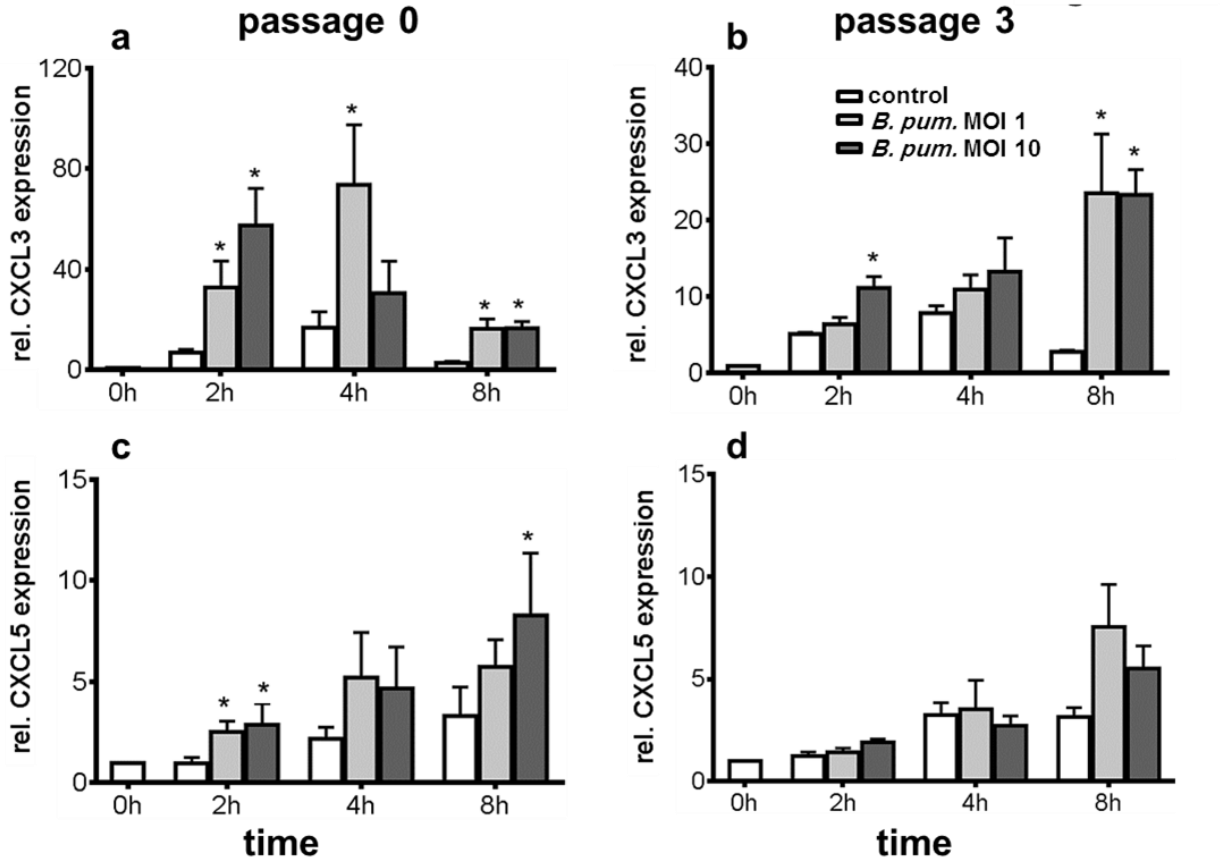


**Fig. 4.** Relative mRNA expression of CXCL3 (A-B) and CXCL5 (C-D) in P0 and P3 BOEC co-cultured with *B. pumilus* (MOI 1 and 10) for up to 8 h, respectively (n=4). Each bar chart represents data as means ± SEM normalized to individual mRNA expression of RPL19, SDHA and HDAC1 as the internal control. All mRNA expression values were scaled relative to the mRNA expression data in control cells at 0 h, which was set equal to one. Values from the bacteria co-cultured cells were compared with the control at the same time point. An asterisk indicates a significant difference ($P < 0.05$) compared with untreated cells. The relative mRNA expression scale of individual studied gene from P0 to P3 is different. P-value for time and treatment * time for studied genes in P0 were as follows: CXCL3 ($P < 0.001$, $P < 0.001$) and CXCL5 ($P = 0.001$, $P < 0.17$). P-value for time and treatment * time for studied genes in P3 were as follows: CXCL3 ($P = 0.02$, $P < 0.001$) and CXCL5 ($P < 0.001$, $P= 0.06$).

### *3.4. PTGS2 and PTGES mRNA expression*

Similar levels of PTGS2 mRNA expression were found in P0 ($P = 0.79$) and P3 ($P = 0.72$) BOEC incubated with *T. pyogenes* and controls (data not shown). mRNA expression of PTGS2 showed a tendency to be higher in P0 BOEC co-cultured with *B. pumilus* at a MOI of 10 after 2 h and 4 h

incubation compared with the controls (Fig. 5A). BOEC in P3 tended to show increased levels of PTGS2 mRNA expression after 8 h co-culture (five-fold increase) with *B. pumilus* at a MOI of 10 (Fig. 5B).

Similar levels of PTGES transcription were observed in co-cultured BOEC with *T. pyogenes* (P-value of 0.80 and 0.64 in P0 and P3, respectively) and control cells (data not shown). BOEC co-cultured with *B. pumilus* in both passage numbers showed an increase in PTGES mRNA expression (Fig. 5C-D). The treatment * time interaction detected in both passages (P= 0.03 and 0.009 for P0 and P3, respectively) depicts a gradual increase of this gene in BOEC incubated with bacteria over time. A minimum five-fold increase in the mRNA expression of PTGES was detected in BOEC in P0 after 8 h of incubation with *B. pumilus* at a MOI 1 and 10 ($P < 0.05$). BOEC in P3 expressed four- and six-fold more PTGES mRNA ($P < 0.05$) after 8 h co-cultured with *B. pumilus* at the two MOI, respectively.

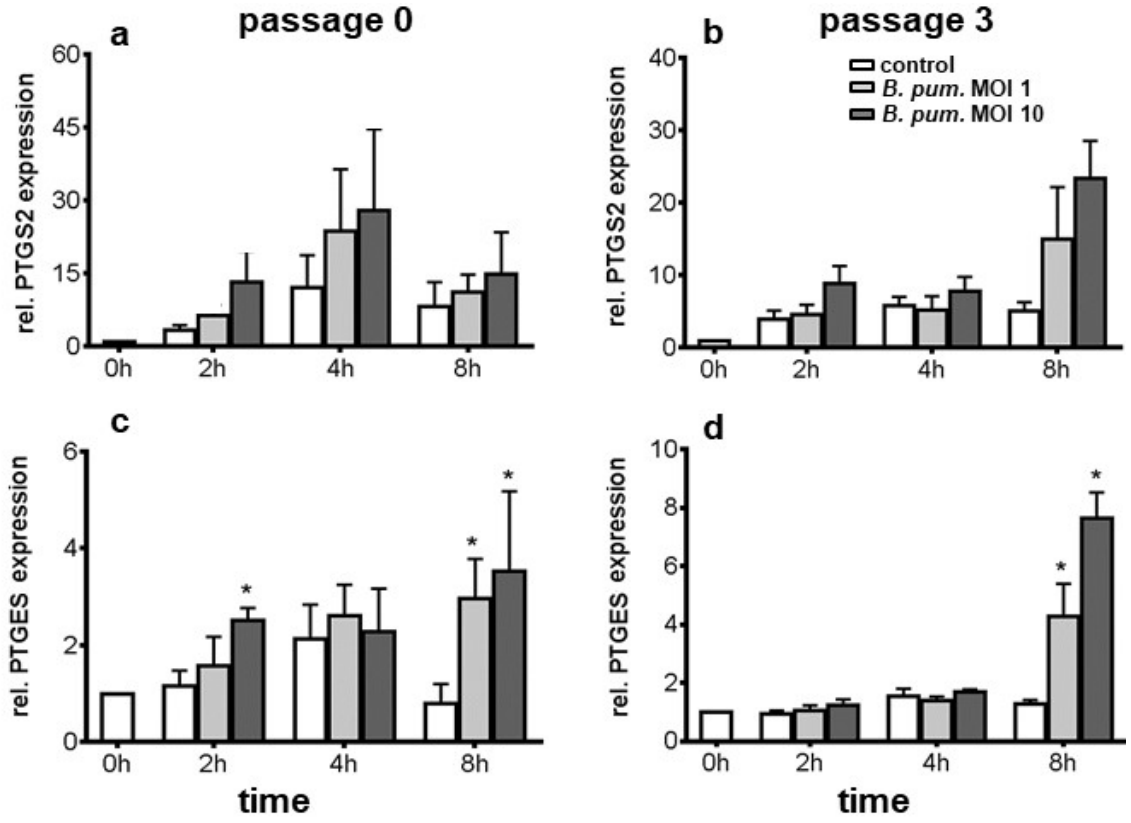


**Fig. 5.** Relative mRNA expression of PTGS2 (A-B) and PTGES (C-D) in P0 and P3 BOEC co-cultured with *B. pumilus* (MOI 1 and 10) for up to 8 h, respectively (n=4). Each bar chart represents data as means ± SEM normalized to individual mRNA expression of RPL19, SDHA and HDAC1 as the internal control. All mRNA expression values were scaled relative to the mRNA expression data in control cells at 0 h, which was set equal to one. Values from the bacteria co-cultured cells were compared with the control at the same time point. Asterisk indicates a significant difference ($P < 0.05$) compared with untreated cells. The relative mRNA expression scale of individual studied gene from P0 to P3 is different. P-value for time and treatment * time for studied genes in P0 were as follows: PTGS2 ($P = 0.13$, $P= 0.40$) and PTGES ($P = 0.16$, $P= 0.03$). P-value for time and treatment * time for studied genes in P3 were as follows: PTGS2 ($P = 0.01$, $P= 0.24$) and PTGES ($P < 0.001$, $P= 0.009$).

*3.5. Release of IL8 and $PGE_2$ from BOEC*

There was a relatively similar rate of release of IL8 and $PGE_2$ after 24 h from P0 BOEC incubated with *T. pyogenes* and controls (data not shown).

Incubation of P0 oviductal cells with *B. pumilus* resulted in a three-fold higher ($P < 0.05$) amount of IL8 in cell culture supernatants after 24 h co-culture at both MOI (Fig. 6A). BOEC in P3 released a similar amount of IL8 after co-culture with *B. pumilus* to that released by the control (data not shown).

Both MOI of *B. pumilus* elicited an increase in $PGE_2$ release from BOEC in P0 after 24 h of incubation (Fig. 6B). *B. pumilus* did not affect $PGE_2$ release of co-cultured BOEC in P3 (data not shown).

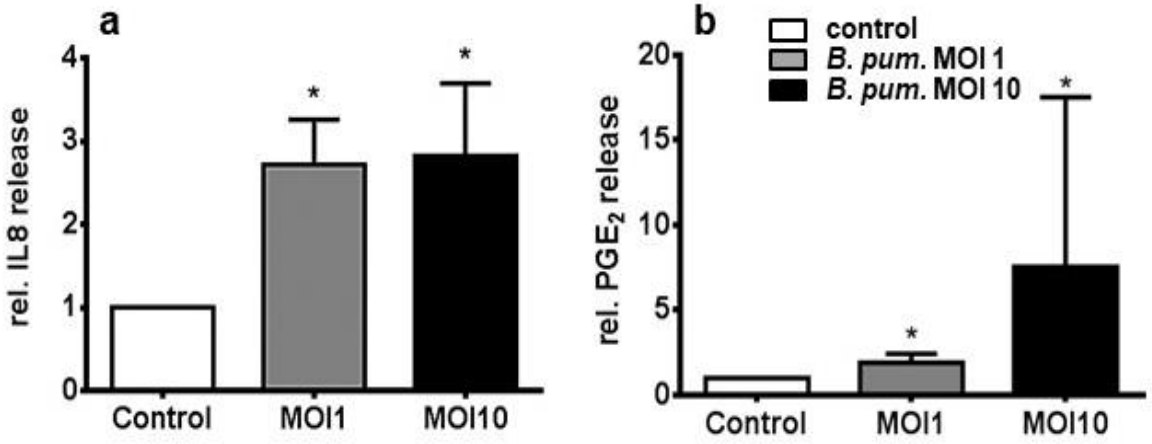


**Fig. 6.** Release of IL8 and $PGE_2$ determined in supernatants of BOEC co-cultured with MOI 1 and 10 of *B. pumilus* by ELISA: (A) IL8 release from P0 BOEC co-cultured with *B. pumilus* after 24 h and (B) $PGE_2$ release in P0 BOEC co-cultured with *B. pumilus* after 24 h. All release data were scaled relative to the concentration in supernatants of control cells after 24 h, which was represented equal to one. Each bar chart represents data as means ± SEM. An asterisk indicates a significant difference ($P < 0.05$) compared with untreated cells. The relative release scale of IL8 and $PGE_2$ from P0 to P3 is different.

## 4. Discussion

The bovine oviduct is in permanent contact with the normal bacterial flora in the reproductive tract. Therefore, pathogens can migrate from the uterus or the follicular fluid into the oviduct [27]. This requires a local immune response system within the oviduct, which is established via different epithelial cell secretions, recently termed the ‘epimmunome’ [28], against foreign microorganisms.

Results from the present study indicate different cellular inflammatory responses of BOEC in the presence of two bacterial species possessing different pathogenic characteristics.

The presence of *T. pyogenes* did not induce any clear pro-inflammatory response in BOEC at either the transcriptional or protein level. The deleterious influence of *T. pyogenes* on cell viability at later points during incubation proves the pathogenicity of this species. Pathogenic microbes have developed advanced mechanisms to elude the epithelial defense system through compounds that inhibit cellular signaling and pathogen recognition [29]. Studies have shown particular mechanisms used by pathogens by which they impair the mRNA encoding of pro-inflammatory factors in order to suppress the host's immune response [30]. In addition, different bacterial species have the ability to synthesize particular forms of endotoxins that are not recognized by the host's pathogen recognition receptors [31]. *T. pyogenes* possesses a large group of virulence factors that allow this bacterium to manipulate the inflammatory responses and cause cell death [32]. Pyolysin obtained from *T. pyogenes* was shown to be more lethal for cultured bovine endometrial stromal cells than for the epithelium [7]. In contrast to these findings, in the present study it was observed that *T. pyogenes* at a MOI >1 disrupted BOEC viability 16 h after incubation.

It is important to mention that the normalized non-relative mRNA expression of various candidate pro-inflammatory factors such as PTGES together with several mucin genes (data not shown) in BOEC in P3 was obviously lower than in P0. Moreover, the present investigation showed the crucial impact of *in vitro* cell passaging on the ability of BOEC to generate inflammatory responses, with an impaired immune response in the higher passage cultures. Previous work has demonstrated the negative effects of cell passaging on mRNA expression and the secretion rate of several pro-inflammatory factors in human gingival fibroblasts [33].

The results of this study indicate that the presence of *B. pumilus* triggered a pro-inflammatory response in oviductal cells. A few studies have investigated the immune responses of BOEC, but only against commercially available lipopolysaccharide (LPS) of *Escherichia coli* (a Gram-negative bacterium) [34,35]. However, many bacterial pathogens have been shown to establish a complex interaction with epithelial cells far beyond that of simple secreted exotoxins [36]. Thus, applying the pathogen as a whole organism in culture with epithelial cells, particularly BOEC, might help generate cellular responses similar to those *in vivo*. The need for studies on the effects of pathogenic microbes or their related endotoxins on cellular immune responses remains crucial [8]. In addition, bacterial species with potentially pathogenic characteristics such as *B. pumilus* do not cause such rapid cell death and allow longer incubation with cells. Such bacterial species could be an alternative to pathogenic bacteria to elucidate and understand cellular mechanisms during inflammation [6].

Higher mRNA expression of both selected IL1 members, particularly IL1A, was detected in oviductal cells exposed to *B. pumilus*. Both IL1A and IL1B are pivotal for generating rapid and widespread pro-inflammatory activity in epithelial cells of the uterus [6,37]. The present data support a similar role of these cytokines in oviductal epithelial cells. Moreover, IL1B causes a systematic host response in epithelial cells against invading pathogens by enhancing the expression of other pro-inflammatory factors, e.g. IL6 and TNFA [38,39]. The increased mRNA expression of TNFA during incubation with *B. pumilus* in the present study is consistent with these findings. The results of the present investigation support previous findings of higher transcription level of TNFA in cultured bovine oviductal cells

treated with different doses of LPS [40]. The expression of TNFA leads to the progression of adaptive immunity through activation of T-cells and differentiation of B-cells, further evolving the immune action of cells towards distinct pathogens [41,42].

The higher mRNA expression of CXC chemokines provides oviductal epithelial cells with the ability to attract leukocytes to challenge invading pathogens. A higher rate of transcription of IL8 in primary oviductal cells after 24 h treatment with bacterial endotoxins has been observed previously [34]. Besides, IL8 and CXCL1/2 and -3 are mainly neutrophil attractants, while CXCL5 attracts monocytes to the site of infection [43]. The lower expression of IL8 and CXCL1/2 and -3 along with the higher amount of CXCL5 mRNA at a later time point during *in vitro* co-culture may indicate a shift in BOEC to phagocytic cell recruitment, which enhances the inflammatory response of oviductal cells.

The higher transcription of selected PG synthesis enzymes in the present study indicates their importance in the progression of the inflammatory response from epithelial cells in the oviduct. PTGS2 is an isoform of this enzyme that is expressed more highly under inflammatory conditions in different cell types [44]. The concomitant early higher mRNA expression of PTGS2 with both IL1A and -1B supports previous findings, which have detected the prolonged effect of these cytokines through the higher transcription level and synthesis of this enzyme [38]. Higher mRNA expression of both PTGS2 and PTGES along with higher amounts of $PGE_2$ in BOEC supernatants shows the importance of both these enzymes in the production of $PGE_2$ in the oviduct. These findings contradict a previous study that emphasized the regulatory role of PTGES but not of PTGS2 [34]. The higher release of $PGE_2$ during bacterial co-culture could imply an anti-inflammatory role in oviductal cells, aiding in balancing the innate immune response of these cells.

The inflammatory response of BOEC in P3 against *B. pumilus* appeared to be less efficient. The pro-inflammatory reaction of cells in P0 was more rapidly triggered by the presence of bacteria, particularly at lower MOI, than in P3 BOEC. This might indicate an alteration in oviductal cell function that should not be underestimated. Consecutive cell passaging has been reported to significantly alter the transcription profile of cells in higher passage numbers than lower ones [45]. The release of pivotal pro-inflammatory cytokines was clearly reduced in primary cells derived from repeated passaging [46].

## 5. Conclusion

The present study reveals the detrimental impact of *T. pyogenes* on oviductal cells due to the lack of an early immune response, resulting in rapid cell death. The particular mechanism of action of *T. pyogenes*, which disturb the early pro-inflammatory response of BOEC, remains elusive. Cultured BOEC clearly recognized the presence of *B. pumilus* by displaying higher levels of transcription of selected IL (IL1A, -1B), TNFA and chemokines (CXCL1/2, -3, -5 and IL8) for up to 8 h of incubation. The higher mRNA expression of these factors and the higher rate of release of IL8 and $PGE_2$ from BOEC show the ability of oviductal cells to trigger a strict and rapid reaction to imminent danger caused by pathogenic microorganisms. The lower ability of cells in the later passage to trigger pro-inflammatory responses towards bacteria indicates that researchers conducting epithelial-bacterial *in vitro* co-culture should not underestimate the influence of culturing conditions on epithelial cellular responses.

**Conflict of interest statement**

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content

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# 5. Discussion

## 5.1. *In vitro* culturing of BOEC and associated conditions influence different aspects of cells functionality

*In vitro* culture systems are vital for of studying the mechanisms of different cell populations. However, in order to obtain valid and reliable information on cellular responses after induction of various treatments, cell populations should be as close as possible in the *in vitro* state to the *in vivo* conditions found in the organ environment.

As soon as the oviductal cells were cultured in the new ‘*’in vitro*’’ environment, the mRNA expression pattern of all selected genes within this study showed clear differences. As the cellular environment plays a crucial regulatory role in mRNA expression, altering culture conditions to make them more similar to the ‘’*in vivo* environment’’ may reduce differences between the *in vitro* and *in vivo* mRNA expression profiles. Data from the present work supports previous findings in other reproductive tract tissues, where high number of mRNA abundance rates including that for estrogen receptor-alpha and progesterone receptors were evidently different between *in vivo* and cultured cells (Zaitseva *et al.* 2006). Transcription profiling of human airway epithelial cells revealed significant number of differentially expressed genes mostly with increased expression in cultured cells compared with *in vivo* (Dvorak *et al.* 2011). Analyses of the functional categories of the differentially expressed genes revealed increased expression of clusters of genes related to proliferative processes in cultured cells, whereas *in vivo* cells had relatively higher expression of the categories related to the cytoskeleton and immune response.

The same issue has been reported for cultured hepatocytes where there was a small percentage of overlap between mRNA expression modification in the liver *in vivo* and in cultured hepatocytes (Kienhuis *et al.* 2009). It has been speculated that epithelial to mesenchymal transition and resistance to apoptosis via distinct molecular pathways are responsible for this dissimilar transcription level (Schug *et al.* 2008, Godoy *et al.* 2010). In our study, cytokeratin staining of cultured BOEC proved their epithelial purity throughout the cell passages. Therefore, another reason could be that monolayer culture of epithelial cells mostly results in the loss of the cells’ *in vivo* histological structure and composition, as well as the disruption of their polarity (Massart *et al.* 1996). Nevertheless, this model is widely used and seems a useful attempt to understand the roles of the oviduct during gamete interaction and early embryo development (Ulbrich *et al.* 2010). The *in vitro* model used in the present project is cost-effective and less time-consuming for generating cell confluency in comparison with other present BOEC culturing methods. Moreover, that the cells in this model can be subcultured makes it suitable for studying the effects of different culturing conditions on cellular responses.

Along with the isolation process, a new *in vitro* environment imposes high level of oxidative stress on the cells, generating high levels of reactive oxygen species (ROS) and cellular antioxidant defense malfunction (Halliwell 2014). The extent and temporal ROS production might increase cell proliferation or lead to senescence, cell death, or adaptation (Davies 1999). The higher transcription levels of both PTGS along with MUC1 and IL8 from *in vitro* BOEC could be a potent cellular

inflammatory response to compensate the magnitude of this oxidative shock for further survival during culture.

Furthermore, there was obvious alteration in the transcription levels of genes involved in the cellular metabolic pathways in the transition from *in vivo* to *in vitro* conditions. Others have shown abnormal activity of metabolic enzymes such as cytochrome P-450 from primary *in vitro* liver cells compared with its levels in animal tissue (Nebert 2006). Also, it has been indicated that the activity of phenylalanine hydroxylase, a rate-limiting enzyme in protein catabolism during normal physiological conditions (Flydal & Martinez 2013), vanished during the first few minutes of trypsinization of mammalian liver before hepatocytes are even plated out on dishes. Data from the abovementioned studies along with that of the present work, demonstrate that normal culturing environment has profound effects on gene expression. Our results indicate that although BOEC cultures are an important tool for studying the oviduct, *in vitro* studies must be carefully planned and evaluated to provide meaningful results.

The influence of cell passaging on mRNA expression capability of BOEC was clearly observed throughout the cell passages conducted in this study. It appears that BOEC functionality can also be affected to some extent by *in vitro* cell passaging. Findings of this investigation supported the findings of previous work pinpointing the consequences of cell passaging on growth rate, protein level and mRNA expression profile alteration in other primary cultured cells (Arkell & Jackson 2003, Shi *et al.* 2004, Nam *et al.* 2014). A recent study found that the transcription levels of senescence-related gene, p21, and oncogene, c-Myc, were significantly upregulated in the higher passage number cells, which was closely related with the obviously increased cell senescence (Lian *et al.* 2016). Januszyk *et al.* (2015) took it one step further and elucidated the effect of primary cell passaging on single cell mRNA expression capability. They successfully demonstrated evident differences in the transcription profile of passage 0 and passage 1 primary cultured cells along with remarkable changes in the cellular subpopulation dynamics. Early passages of cell cultures are frequently considered as heterogeneous. Most probably the majority of changes occur in the first few passages. Also, continuous maintenance of cells in culture may lead to selection of subpopulations with improved growth properties due to genetic instability. As early passage cells might represent heterogeneous cell populations from the tissue or tumor, they could be thought of as reflecting their origins.

Selected MUC mRNA expression pattern altered particularly in the later passaged cells. This decrease in transcription level was accompanied with lower WGA staining intensity of these compounds in BOEC cultured at higher number of cell passage. Indeed, researchers have opted to profile mucin gene expression in primary and passage-1 cultures of lung epithelium (Bernacki *et al.* 1999). That study has demonstrated that at least nine MUC genes (MUC1–4, 5AC, 5B, and 6–8) were expressed in cell cultures, however only MUC4, -5AC, and -5B protein could be detected. In the present project, the mRNA expression of MUC5AC, -12 and -15 was also investigated, however, the mRNA abundance of these mucins in cultured BOEC were below the detection limit (data not shown). It is unclear what controls MUC mRNA expression in culture. Interestingly, specific culture conditions, such as an air–liquid interface culture with collagen gel substratum, can elevate MUC mRNA expression. The molecular mechanism underlying this elevation is still unknown (Boei *et al.* 2017).

MUC predominantly are epithelium coating agents that act as physical barriers limiting damage to the epithelium and attenuating the activation of innate and adaptive immune responses (Lang *et al.* 2007). Lower expression of MUC in later passaged BOEC could be an indicator of more fragile oviductal cells with weaker immune system capability. This was more evident when the effect of *in vitro* culturing in the second part of the project was followed up. It appears that primary cell passaging solely influences the inflammatory response capability of oviductal cells. The lower inflammatory response in higher passage number cells was more obvious when passage 3 (P3) oviductal cells noticed to be more vulnerable during incubation with *T. pyogenes*. Furthermore, passage 0 (P0) incubated cells recognized the presence of *B. pumilus* more rapidly and engaged a more potent inflammatory response to the bacteria than P3 BOEC. These results support previous studies describing the negative impact of cell passaging on cellular function in other tissues, particularly their expression of different inflammatory factors at both transcription and proteomic level (Kent *et al.* 1996, Izadpanah *et al.* 2008). Taking the impact of *in vitro* culturing condition into consideration would allow researchers to generate results that are unambiguous and more physiologically relevant.

The drastic decrease in OVGP1 mRNA abundance and to some extent its secretion from lower passage number (P0) cells to higher passage number (P3) cells might reveal the undeniable change that occurs in the specific functions of oviductal cells. This coincided with a change in BOEC morphology from P0 to P3. The morphological change of cultured cells may eventually end up in cellular dedifferentiation, which can occur in monolayer epithelial culture, affecting their secretory activity and physiological interaction (Walter 1995, Sostaric *et al.* 2008).

BOEC metabolic activity is potentially influenced by cell passaging. *In vitro* sub-culturing strongly affects the metabolic and biochemical activity of the primary cells from other tissues (Rattner *et al.* 1997, Sambuy *et al.* 2005). Aerobic and anaerobic metabolism are the primary means of deriving cellular energy, and much more energy is derived aerobically than anaerobically. Hence, it is assumed that the prevailing metabolic state of the cells will have a significant effect on their terminal differentiation. Evidence has shown that metabolic state can influence both protein activation and protein conformation (Gatlik-Landwojtowicz *et al.* 2004, Vishwasrao *et al.* 2005), both of which are crucial during cell differentiation. This may also affect the ability of growth factors and other stimuli to induce the desired outcomes *in vivo* (Deorosan & Nauman 2011).

Higher HK and GAPDH mRNA abundance in the early cell passage number BOEC supports previous works indicating pyruvate production as a potential ROS scavenger for minimizing the impact of oxidative stress on cells (O'Donnell-Tormey *et al.* 1987, Long & Halliwell 2009). Further work should be carried out to evaluate the transcription levels of other important genes directly involved in cell mitochondrial activity. Cell culture metabolomic analysis could be used as a potent approach to assess the metabolic pathways and identify the characteristic metabolites. However, culture conditions may induce variations in metabolic profiles of primary cells in *in vitro* culture, which present an obvious problem for comparative metabolomic analysis. This renders it highly difficult to compare cell metabolic profiles with different *in vitro* culturing standards directly, and to determine whether the transformation of cellular metabolic profiles is related to the changes in biological characteristics of the cells or the differences among the used culture medium or other supplements. Therefore, the need for well-structured global *in vitro* culturing ‘’standardization’’ for primary tissues is inevitable.

It is well known that cellular metabolic profiles are extremely sensitive to various environmental factors, such as the used culture medium, dissolved oxygen level in the culture medium and temperature (Johnson & Gonzalez 2012). The culture medium is a crucial component influencing cell growth and survival, the expression of products, and the metabolic profile (Dietmair *et al.* 2012, Mohmad-Saberi *et al.* 2013). Therefore, it exerts different biological effects on different cell types. Very few studies have evaluated the effects of culture media on cellular responses, which might lead to inaccurate and unreliable results (Huang *et al.* 2015). Culturing medium is an indispensable component of *in vitro* models, and its composition is pivotal to achieving good experimental reproducibility. The concentrations of glucose in various cell culture media are higher than its circulatory concentration in the animal. Herein we attempted to perform BOEC culture exposing the cells to a medium with glucose concentration (5.5 mM) that resembled the circulating glucose concentration in dairy cattle more closely (Garverick *et al.* 2013). The present work indicated the possibility of obtaining pure BOEC with media composing a glucose content closely to *in vivo*. Additionally, the transcription capability of BOEC cultured with 5.5 mM glucose-content-medium did not differ drastically from that of cells exposed to the conventional medium, at least for most of the candidate genes in this study. Higher transcription level of both PTGS2 and IL8 in cells cultured in 25 mM glucose-content-medium in BOEC could indicate a cellular immune response to a hyperglycemic milieu. Previous studies have indicated that hyperglycemia induced ROS production leading to generation of a potent oxidative stress in other cell types (Patel *et al.* 2013).

The possible impact of bovine estrous cycle stage on the transcription capability of BOEC was also observed in the present study. Most changes in BOEC mRNA expression pattern of selected genes occurred in the later passage number (>2). Others revealed drastic influence of bovine reproductive cycle on morphology and developmental patterns of BOEC (Thibodeaux *et al.* 1991). Findings of the present study contradicts that of Thibodeaux *et al.* (1991), which persuade the use of sub-cultured oviductal cells for investigations such as *in vitro* embryo development. Different mRNA expression rates of studied genes in later passage BOEC indicates that cells under influence of estrus cycle might release dissimilar components that eventually elicits oviductal responses greatly different from *in vivo*.

## 5.2. BOEC pro-inflammatory actions were distinct in response to bacteria with different pathogenic attribute

The focus of the present investigation was to understand the potential response of oviductal cells to distinct bacterial pathogens. Inflammation plays a vital role during microbial infections of the reproductive tract, affecting pregnancy in mammals (Sheldon *et al.* 2014). The inflammatory processes predominantly cause endometritis, perturb ovarian follicle development, suppress the endocrine activity of the hypothalamus and pituitary in cattle and decrease the pregnancy rate significantly. Major pro-inflammatory cytokines such as IL1 and -8 can mimic the tissue destruction seen in *Chlamydia*-infected oviducts (Hvid *et al.* 2007). Females with premature ovarian failure express high levels of major histocompatibility complex antigens, which are primarily induced by TNFA and interferons (Hill *et al.* 1990). Results from this part of the project indicated that the pro-inflammatory reactions of BOEC are apparently dependent on the pathogenicity characteristic of the studied bacterial species.

Oviductal cells did not show pro-inflammatory responses during incubation with *T. pyogenes*. The co-culture method used in this study was standardized to better imitate *in vivo* condition, where invaded pathogens adhere to the host cells, multiply and colonize host tissues and inflict damage. Therefore a specific MOI of *T. pyogenes* was selected for the 24 h incubation of bacteria and epithelial cells. Specific-strain of *T. pyogenes* that was used in the co-culture method has been shown by our group to be more important in developing bovine reproductive diseases (Ibrahim *et al.* 2017). The oviductal cells apparently did not release significantly higher levels of the selected pro-inflammatory factors after 24 h *in vitro* incubation, yet the bacteria seemed to trigger cell death particularly in later passage number BOEC. It is of capital importance to mention that regardless of BOEC passage number, presence of the bacteria with MOI> 1 induced cell death after 16 h of co-culture.

Gram-positive bacteria can produce various enterotoxins such as superantigens. These proteins bind the antigen receptors of a very large group of T cells inducing rapid proliferation and apoptosis, leaving behind generalized immunosuppression together with the removal of many peripheral T cells (Janeway 2001). To avoid the host defense mechanism, i.e. phagocytic cells, these bacteria employ antiphagocytic virulence factors such as the matrix (M1) protein and the hyaluronic acid capsule (Moses *et al.* 1997). They are also able to increase their survival by expressing the pore-forming cytolysin, streptolysin O, which inhibits the transport of bacteria to lysosomes and facilitates escape from lysosomes (Nakagawa *et al.* 2004, Hakansson *et al.* 2005). Secreted proteases from Gram-positive bacteria species also play a key role in blunting chemokine activities. In humans, a particular proteinase interferes with neutrophil recruitment by cleaving and inactivating the neutrophil chemoattractants IL8, as well as CXCL1 and CXCL2 (Hidalgo-Grass *et al.* 2006). Another immune evasion strategy is the blockage of the complement system. Pathogens impede complement deposition by cleaving complement component 5a (C5a), an important inducer of neutrophil recruitment, through C5a peptidase as well as the anchorless surface dehydrogenase (Terao *et al.* 2006). *T. pyogenes* also might engage a different set of toxins particularly dedicated to epithelial cell inflammatory response malfunction or suppression.

The lack of pro-inflammatory response of BOEC could partially be explained by the absence of immune cells in the monolayer culture system. The positive effect of hematopoietic cells driven effectors on the rate of epithelial cell cytokine production has been shown in other organs such as intestine (Haller *et al.* 2000). The main advantage of *in vitro* models based on single cell types lies in their ability to provide key information on a particular cell type's reaction to an infectious microbe. Despite their widespread use and noted benefits monoculture systems are not an ideal foundation for *in vitro* modelling of infectious processes. An *in vitro* alternative to monoculture is co-culture of multiple cell types, most commonly including epithelial cells. This approach is being used to bridge the gap between the overly simplistic single lineage *in vitro* models and the dynamic biological processes that occur *in vivo*. Co-culturing epithelial cells with other cell types in proportional levels approximating known tissue constituent has been used to mimic the *in situ* interactions of various body systems. Such models have been used to study immune defense and have highlighted the effects of neutrophils, eosinophils, monocytes, and lymphocytes on epithelial cell function (Berin *et al.* 1999).

Meanwhile, incubation with *B. pumilus* triggered an inflammatory response from oviductal cells by increasing the rate of different studied pro-inflammatory factors at both transcriptome and protein level.

Higher mRNA abundance of cytokines, particularly IL1A, -6 and TNFA, was observed after early time points of the incubation with the bacteria. Similar inflammatory responses after *B. pumilus* incubation with bovine endometrial epithelial cells have been observed (Gärtner *et al.* 2016). Results of the present study support previous statement pinpointing IL1A as an early phase moderator of sterile inflammation (Kurt-Jones *et al.* 1985). Di Paolo and Shayakhmetov (2016) have conceptualized the role of IL1A into an 'inflammatory loop' model, in which inflammation is initiated by stressed or damaged cells via IL1A-dependent activation of chemokines that recruit inflammatory hematopoietic cells to the site of damage or stress. These hematopoietic cells respond to the IL1A-containing milieu, where pro IL1A can be either released from damaged cells or exposed as membrane-bound IL1A on the surface of cells undergoing oxidative or metabolic stress. This in turn will activate cells' IL1A and -1B production downstream of the IL1 receptors. The initial IL1A and its receptor signaling therefore initiates a loop of sustained and self-perpetuating inflammation that results in extensive tissue damage that occurs until the receptor signaling is either exhausted or suppressed.

IL1A precursor is constitutively present and active in epithelial layers, including the reproductive tract, and act as an ''alarmin'' through rapid activation of an inflammatory cytokines cascade (Rider *et al.* 2011). Expression of the N-terminal amino acids of IL1A stimulates IL8 production in the presence of complete blockade of the IL1 receptors on the cell surface (Werman *et al.* 2004). Moreover, IL1A nuclear function seems to be a transcription inducer of other pro-inflammatory genes (Dinarello 2011), and it apparently plays the same role in cultured oviductal cells. IL1B is mainly produced by hematopoietic cells, e.g. monocytes and unlike IL1A its precursor is not active (Dinarello 2011). Lower transcription levels of IL1B in this study could be due to the absence of immune cells in the culture. Higher mRNA abundance of IL6 and TNFA is in accordance with other findings indicating a systemic host response from IL1B in epithelial cells by stimulating the production of other vital pro-inflammatory factors (Dinarello *et al.* 1986, King *et al.* 2002). Nevertheless, cytokines are induced in a stronger manner by IL1A precursor than by mature IL1B (Rider *et al.* 2011).

Higher expression of IL8 mRNA and its encoded protein proves the importance of this factor in cellular inflammatory response. Others have also shown the IL8 production in both bovine oviductal and endometrial epithelial cells in response to treatment with commercially available Gram-negative bacterial endotoxins, i.e. lipopolysaccharides (Kowsar *et al.* 2013, MacKintosh *et al.* 2013). IL8 mainly functions as distinct chemoattractant for neutrophils and its synthesis is strongly regulated by IL1 and TNFA in many different cell types (Bickel 1993). Over-expression of IL1A precursor is evidently sufficient for triggering IL8 production (Werman *et al.* 2004). It has been also determined that IL1A contributes to *Chlamydial* induction of IL8 through a mechanism independent of the functional IL1 receptors (Cheng *et al.* 2008). Moreover, increased levels of TNFA in damaged liver cells directly promote the generation of C-X-C motif (CXC) chemokines, primarily IL8 (Colletti *et al.* 1998). TNFA apparently regulates IL8 production through the NF-κB and phosphatidyl-inositol 3-kinases (PI3K) mediating pathways.

IL8 is frequently detected at sites of infections. This may be because various microbes and their products can induce IL8 production in a variety of cell types (Mukaida 2000). Controlled induction of IL8 is for maintaining homeostatic balance. For example, elevated IL8 induction can lead to exacerbated inflammation in chronic inflammatory diseases (Kinane *et al.* 2008). On the contrary,

inhibition of IL8 secretion may delay neutrophil influx creating an advantage for pathogen survival and leading to chronic infection (Benakanakere *et al.* 2016).

The present work elucidates for the first time the higher mRNA expression of CXC chemokine genes in primary cultured BOEC during incubation with bacteria. CXCL5 chiefly attracts monocytes to the site of infection (Hurst *et al.* 2001). Neutrophils are the main responsive cell type, as they express the receptors CXC ligand receptor 1 and -2 (CXCR1 and -2), which can bind to CXCL1-5 (Palomino & Marti 2015). Moreover, bacterial toxin stimulation of mast cells leads to immediate release of CXCL1 and CXCL2 containing granules, but not histamine-containing granules, as well as transcriptional activation of CXCL1 and CXCL2 (Griffith *et al.* 2014). Neutralization of TNFA appears to eventually decrease levels of CXCL expression during inflammation. Members of the IL1 family are able to alter the expression of the chemoattractant CXCL1, -3 and -5 during bacterial infection. Additionally, CXCL9 and -10, which have chemotactic activity for lymphocytes, are target genes of IL17 (O'Quinn *et al.* 2008). CXCL1, -2 and -5 mRNAs are induced by IL-17 and/or TNFA weakly and are subject to rapid degradation, but their stability is significantly enhanced in the presence of both IL17 and TNFA (Hartupee *et al.* 2007). Generally, chemokines CXCL1, CXCL2, CXCL3 and CXCL5, are classified as inflammatory, while CXCL4 is plasmatic or platelet related (Le *et al.* 2004). Lower transcription level of IL8 coinciding with the higher CXCL5 mRNA expression at later time points of bacterial co-culture might show a transition of BOEC in phagocytic cell recruitment, which enhances the inflammatory response of oviductal cells.

In addition to cytokines, AA metabolites, particularly $PGE_2$, have significant roles in initiating and/or terminating the inflammatory processes (Samuelsson 1991). Higher release rate of $PGE_2$ along with transcription level of its two crucial synthesis enzymes, PTGS2 and PTGES, reveals its pivotal role in the oviductal cellular inflammatory response. Results of the present investigation confirm the work of others, which showed that PTGS2 is a dominant source of PG formation during inflammation (Mardini & FitzGerald 2001) playing a dual role in the inflammatory process, i.e., initially triggering the action and later helping to resolve the process (Ricciotti & FitzGerald 2011). The higher release of $PGE_2$ by the BOEC during incubation might implicate the homeostatic function of this metabolite on the cellular immune response. Previous *in vitro* studies have depicted $PGE_2$ involvement in regulating balance between different forms of inflammatory responses and their associated cytokines (Snijdewint *et al.* 1993).

# 6. Conclusion

The present work was able to demonstrate the pivotal role of cell culturing, particularly cell passaging, in altering the mRNA expression levels of genes in BOEC and some of the released products that are involved in various physiological functions of oviductal cells. Results from this study could be used as a paradigm for further studies on oviductal epithelial cells *in vitro*. In order to have more knowledge on the scale of impact of cell culture on oviductal cells different aspects of an *''in vitro''* cell culture have to be covered. The type of growth media and serum type and percentage used on cells are among such factors, which might be taken into consideration and further improve the standard quality of the cell culture.

During this project, we incubated BOEC with live bacterial species of the bovine reproductive tract that encompass different characteristics of pathogenicity and were able to demonstrate differential inflammatory responses of the cells. Inflammatory response of cells particularly to *B. pumilus* shows that oviductal cells are capable to engage a strict reaction to imminent danger mainly caused by pathogenic microorganisms. This in turn enables the oviduct to provide an optimal environment for fertilization and early embryo development that are crucial for a successful reproduction. Further work could evaluate the immune interactions of these cells with pathogenic strains of Gram-negative bacteria such as *Escherichia coli*. Revealing the impact of these pathogens on the embryo during its developmental stage in polarized cultured BOEC would be also an interesting topic for further research. The very first stage of embryo development, roughly up to 4 days, occurs within the lumen of oviduct. Very little is known about the detrimental effect of bacteria or their endotoxins on bovine embryo development and on the interaction between the oviductal cells and the developing embryo.

# 7. Summary

The mammalian oviduct is crucial for the gamete maturation and supporting the early embryo development. The *in vitro* culturing of bovine oviductal epithelial cells (BOEC) allows researchers to study these cells without the systemic variation that occur in animals. Various *in vitro* culturing conditions require careful optimization.

The pathophysiological mechanisms of bacterial species in the bovine reproductive tract leading to postpartum diseases and sub-optimal fertility have become an important topic for research. *Trueperella pyogenes* is a commonly isolated pathogen highly associated with abnormal vaginal discharge in infected animals. Other bacterial species such as *Bacillus pumilus* could be involved in the development of postpartum diseases. Little is known about pathogens or their endotoxins interaction with BOEC. The present work was set up to understand cellular responses of BOEC under different culturing conditions and further in co-culture with the mentioned bacteria.

In the first part of the project, oviducts were collected at the slaughterhouse and classified into non-luteal and luteal stage. Monolayer culture of BOEC was conducted to determine cellular transcription and functional capability of BOEC 1) in transition from *in vivo* state to *in vitro* state; 2) during three consecutive cell culture passages; 3) affected by the impact of LOW (5 mM) and HIGH (25 mM) glucose content media; and 4) influenced by the effect of different phases of the estrous cycle *in vitro.* Total RNA was extracted from *in vivo* and *in vitro* samples and subjected to quantitative polymerase chain reaction. Moreover, collected supernatants from passage 0 (P0) and passage 3 (P3) BOEC were measured for prostaglandin $E_2$ ($PGE_2$), oviduct-specific glycoprotein 1 (OVGP1) and interleukin (IL) 8 release to observe the influence of *in vitro* culturing on BOEC functionality. Almost all candidate genes mRNA expression pattern (prostaglandin synthases, cell metabolism enzymes and mucins) were evidently changed from *in vivo* to *in vitro*. The mRNA expression pattern of microsomal prostaglandin $E_2$ synthase 2 (PTGES2), mucin 1 (MUC1), -4 and OVGP1 was influenced by the number of cell culture passages. The mRNA expression of most candidate genes was not affected by the concentration of glucose in the culture media. The estrous cycle stage altered candidate genes mRNA expression in BOEC *in vitro*, notably at later passages (>P2), but not *in vivo*. MUC1 was the only selected gene whose mRNA expression pattern was influenced by estrous cycle stage at P1. The release rate of $PGE_2$ and OVGP1 between P0 and P3 cells was not significantly different, yet BOEC in P3 released evident higher amounts of IL8 compared with cells in P0.

The second part of the project entirely focused on inflammatory responses of *in vitro* cultured BOEC under the standardized milieu, based on the outcome from the initial phase of the project, during incubation with either *T. pyogenes* or *B. pumilus*. Trypan blue staining determined BOEC viability during 24 h co-culture with different multiplicity of infection (MOI) of *T. pyogenes* or *B. pumilus*. Cells remained viable with *T. pyogenes* at a MOI of 0.01 and with *B. pumilus* at a MOI 1 and 10. Transcription level and released rate of candidate pro-inflammatory factors was not evidently changed in BOEC co-cultured with *T. pyogenes* compared with the controls. Meanwhile, higher mRNA expression of IL1A, -1B, -6, tumor necrosis factor alpha (TNFA), chemokine (C-X-C motif) ligand (CXCL) 1/2, -3, -5 and IL8 along with PG synthesis enzymes in BOEC co-cultured with *B. pumilus* was observed. Addition of *B. pumilus* released higher amount of IL8 and $PGE_2$ from BOEC than

controls. The impact of culturing condition on cellular immune response was followed up and it was noticed that viability and early pro-inflammatory response of P3 BOEC incubated with bacteria was clearly lower than from P0 BOEC.

In conclusion, the present work showed that conventional oviductal cell culturing highly influences the physiological and immunological responses of the cells prior to any treatment. This is very important and has to be considered during research on BOEC at *in vitro* level. Furthermore, this work successfully revealed a differential inflammatory response of oviductal cells against live strains of pathogenic/potential pathogenic bacteria isolated from the bovine reproductive tract. These results can contribute to a better understanding of these microorganisms interactions with the oviductal cells.

# 8. Zusammenfassung

**Evaluierung der Reaktionen von bovinen primären Oviduktepithelzellen unter physiologischen und pathophysiologischen Bedingungen**

Die Ovidukte von Säugetieren spielen eine wichtige Rolle bei der Reifung der Gameten und unterstützen die Entwicklung des frühen Embryos. Die *in vitro* Kultivierung von bovinen Oviduktepithelzellen (BOEC) ermöglicht es Wissenschaftlern, diese Zellen zu untersuchen, ohne dass die Zellen den systemischen Einflüssen des tierischen Organismus unterliegen. Verschiedene *in vitro* Kultivierungsbedingungen erfordern eine sorgfältige Optimierung.

Pathophysiologische Mechanismen, die durch Bakterienspezies im bovinen Reproduktionstrakt verursacht werden, führen zu nachgeburtlichen Krankheiten und zu suboptimaler Fruchtbarkeit, und sind daher zu einem wichtigen Forschungsthema geworden. *Trueperella pyogenes* ist ein häufig nachgewiesener Krankheitserreger, der eine hohe Korrelation zu abnormem Vaginalausfluss bei erkrankten Tieren zeigt. Andererseits können andere Bakterienspezies, wie *Bacillus pumilus*, bei der Entstehung von nachgeburtlichen Erkrankungen ein Rolle spielen. Wenig ist bislang bekannt über die Wechselwirkung von Krankheitserregern oder ihren Endotoxinen mit BOEC. Die vorliegende Arbeit soll zum besseren Verständnis zellulärer Antworten von BOEC bei verschiedenen Kultivierungsbedingungen und weiterführend bei Ko-Kultivierung mit den erwähnten Bakterien beitragen.

Im ersten Teil des Projekts wurden Ovidukte auf dem Schlachthof gewonnen und nachfolgend in die Nicht-Luteal- bzw. Lutealphase eingeteilt. Monolayerzellkulturen wurden von BOEC angelegt, um die zelluläre Transkription und die Funktionalität der Zellen zu bestimmen 1) beim Übergang vom *in vivo*-Zustand in den *in vitro*-Zustand; 2) während drei nacheinander folgenden Zellkultur-Passagen; 3) zwischen Nährmedien mit niedrigen (LOW; 5 mM) und hohen (HIGH; 25 mM) Glukosegehalt; und 4) unter dem Einfluss verschiedener Phasen des Sexualzyklus *in vitro*. Die Gesamt-RNA wurde aus *in vivo*- und *in vitro*–Proben extrahiert und einer quantitativen Polymerase-Kettenreaktion zugeführt. Darüber hinaus wurden Zellkultur-Überstände von BOEC in Passage 0 (P0) und P3 gewonnen und diese auf Prostaglandin $E_2$ ($PGE_2$)-, oviduktspezifisches Glykoprotein 1 (OVGP1)- und Interleukin 8 (IL8)-Freisetzung bestimmt, um den Einfluss der *in vitro* Kultivierung auf die Funktionalität der BOEC zu untersuchen. Die mRNA-Expressionsmuster fast aller Kandidatengene (Prostaglandin-Synthasen, Enzyme des Zellmetabolismus und Muzine) änderten sich deutlich beim Übergang vom *in vivo*– in den *in vitro*-Zustand. Das mRNA-Expressionsmuster von Prostaglandin $E_2$-Synthase 2 (PTGES2), Muzin (MUC) 1, -4 und OVGP1 wurde durch die Zahl der Zellkulturpassagen beeinflusst. Die mRNA-Expression der meisten Kandidaten-Gene wurde durch die Glukosekonzentration des Zellkulturmediums nicht beeinflusst. Die Phase des Sexualzyklus veränderte die mRNA-Expression der Kandidaten-Gene in BOEC *in vitro*, besonders in späten Passagen (>P2), aber nicht *in vivo*. MUC1 war das einzige Kandidatengen, dessen mRNA-Expression in P1 vom Stadium des Sexualzyklus beeinflusst wurde. Die Freisetzungsrate von $PGE_2$ und OVGP1 war zwischen Zellen der P0 und P3 nicht signifikant unterschiedlich, aber BOEC setzte IL8 in höheren Mengen in Zellen der P3 frei als in P0.

Der zweite Teil des Projekts befasste sich mit den Entzündungsreaktionen in den *in vitro* kultivierten BOEC während der Inkubation mit *T. pyogenes* oder *B. pumilus* unter den Standardbedingungen

basierend auf den Ergebnissen des ersten Teils. Die Funktionalität der BOEC wurde durch Trypanblau-Färbung nach 24 h Ko-Kultivierung mit verschiedenen „Multiplicity of infection“ (MOI) von *T. pyogenes* oder *B. pumilus* untersucht. Die Zellen blieben mit *T. pyogenes* bei einer MOI 0.01 und mit *B. pumilus* bei einer MOI 1 und 10 vital. Die Transkription der Entzündungsfaktoren und deren Freisetzung änderte sich nicht signifikant in den BOEC bei Ko-Kultivierung mit *T. pyogenes* im Vergleich zu den Kontrollen. Dennoch wurde eine höhere mRNA-Expression von IL1A, -1B, -6, Tumornekrose-Faktor alpha (TNFA), Chemokin-(C-X-C-Motiv)-Ligand 1/2 (CXCL1/2), -3, -5 und IL8 wie auch von PG-Synthasen in BOEC bei Ko-Kultivierung mit *B. pumilus* beobachtet. Bei Zugabe von *B. pumilus* setzten BOEC höhere Mengen von IL8 und $PGE_2$ frei als in den Kontrollen. Ebenso wurde der Einfluss der Kultivierungsbedingungen auf die zelluläre Immunreaktion verfolgt, wobei sich herausstellte, dass die Funktionalität und die Entzündungsreaktion bei mit Bakterien inkubierten BOEC in P3 offensichtlich niedriger lag als bei BOEC in P0.

Zusammenfassend lässt sich feststellen, dass die vorliegende Arbeit zeigen konnte, dass konventionelle Ovidukt-Zellkulturbedingungen die physiologischen und immunologischen Zellantworten in großem Maße beeinflussen, bevor eine Behandlung der Zellen vorgenommen wird. Dies ist sehr wichtig und muss beachtet werden, wenn BOEC auf *in-vitro*-Ebene untersucht werden. Weiterhin konnte diese Arbeit erfolgreich eine unterschiedliche Entzündungsreaktion von Oviduktzellen gegenüber lebenden pathogenen Bakterienspezies aus dem bovinen Reproduktionstrakt zeigen. Diese Ergebnisse tragen zu einem besseren Verständnis der Wechselwirkung zwischen diesen Mikroorganismen und den Oviduktzellen bei.

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# 10. Publications and scientific activity

## Manuscripts

**Danesh Mesgaran, S.,** J. Sharbati, R. Einspanier and C. Gabler. **mRNA expression pattern of selected candidate genes differs in bovine oviductal epithelial cells *in vitro* compared with the *in vivo* state and during cell culture passages.** Journal of Reproductive Biology and Endocrinology; DOI 10.1186/s12958-016-0176-7

**Danesh Mesgaran, S.,** Gärtner MA, Wagener K, Drillich M, Ehling-Schulz M, Einspanier R and Gabler C. **Different inflammatory responses of bovine oviductal epithelial cells in vitro influenced by bacterial species with distinct pathogenicity characteristics and passage number** (Re-submitted after reviewer comments-Journal of Theriogenology)

## Publications and Presentations

**Danesh Mesgaran, S.,** K. Wagener, M. A. Gärtner, M. Drillich, M. Ehling-Schulz, R. Einspanier and C. Gabler. Inflammatory responses of bovine oviductal epithelial cells *in vitro* depended on the pathogenic characteristics of different bacterial species. 18th International Conference on Animal Reproduction (ICAR). Accepted as poster presentation.

**Danesh Mesgaran, S.,** J. Sharbati, R. Einspanier and C. Gabler. 2016. Number of cell culture passage influences the mRNA expression pattern of several mucins in bovine oviductal epithelial cells in vitro. 49. Jahrestagung Physiologie und Pathologie der Fortpflanzung, 26. Accepted as poster presentation.

**Danesh Mesgaran, S.,** R. Einspanier and C. Gabler. 2014. Expression pattern of various genes in bovine oviductal epithelial cells in vitro in different cell passages. 47. Jahrestagung Physiologie und Pathologie der Fortpflanzung, 24. Accepted as oral presentation

**Danesh Mesgaran, S**. 2015. Differential innate immune responses of bovine oviductal epithelial cells towards pathogenic bacteria. Invited speaker at Institute of Veterinary Anatomy, Freie Universität Berlin.

## Honors and awards

- Speaker at "Physiology and Biochemistry" conference, Berlin, Germany (2016)
- Recognized among top 3 best abstracts at 47. Jahrestagung Physiologie und Pathologie der Fortpflanzung, Giessen, Germany (2014)

## 11. Acknowledgment

Earning a PhD has been a lifelong goal and I am fortunate to have had an inspiring group of advisors along the way. First and foremost I would like to thank **Dr. Christoph Gabler**, my PhD advisor and chairman of my doctoral committee for guiding me through my graduate career. It was my lucky day when **Dr. Gabler** accepted me in his research group where I could learn valuable lab techniques applicable to reproductive physiology and immunology in bovine. Through all the twists and turns of graduate school I could always count on **Dr. Gabler** to help guide my direction. I would also like to express my appreciation and thanks to my doctoral committee members: **Dr. Jennifer Schön** and **Professor Johanna Plendl** for their advice and support during my PhD projects and allowing me to grow as a research scientist.

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## 12. Selbständigkeitserklärung

Hiermit bestätige ich, **Sadjad Danesh Mesgaran**, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den **31.01.2018** **Sadjad Danesh Mesgaran**

Detailed assessment of contribution of Mr. **Sadjad Danesh Mesgaran** to the research projects presented under this cumulative doctoral thesis

| Contribution | Research project #1 (Effect of culturing conditions on bovine ovicutal cell responses) | Research project #2 (Inflammatory response of bovine oviductal cells to pathogenic bacteria) |
|---|---|---|
| Study design | 30% | 30% |
| Data collection | 85% | 65% |
| Data analysis | 75% | 80% |
| Manuscript writing | 85% | 85% |
| Manuscript editing | 55% | 55% |

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