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Fertility impairment by maternal stress: impact of cortisol on oviduct epithelium functions

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4 List of abbreviations

2D	two-dimensional
3D	three-dimensional
4Rs	Replacement, Reduction, Refinement, and Rehabilitation
ACTH	adrenocorticotropic hormone
ALI	air-liquid interface
ATP1A1	ATPase Na+/K+ transporting subunit α 1
BDNF	brain-derived neurotrophic factor
CBG	corticosteroid binding globulin
CCLV-RIE270	a cell line derived from the porcine oviduct epithelium
CRH	corticotrophin-releasing hormone
CXCL8	C-X-C motif chemokine ligand 8
d	day
DBD	DNA binding domain
E2	(17)estradiol
ENaC	epithelial Na+ channel subunit α
ESR	estrogen receptor
ESR1	estrogen receptor 1
FGT	female genital tract
FKBP4	FKBP Prolyl Isomerase 4
FKBP5	FKBP prolyl Isomerase 5
GCs	glucocorticoids
GILZ	glucocorticoid-induced leucine zipper
GPER	G protein-coupled estrogen receptor 1
GR	glucocorticoid receptor
h	hours
H_2O_2	hydrogen peroxide
HF/HS	high-fat/high-sugar
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HSD11B1	hydroxysteroid 11-beta dehydrogenase 1
HSD11B2	hydroxysteroid 11-beta dehydrogenase 2
IGF1	insulin-like growth factor 1
IL6	interleukin 6
IL8	interleukin 8
KLF4	KLF transcription factor 4

LBD	ligand binding domain
NANOG	Nanog homeobox
NR3C1	nuclear receptor subfamily 3 group c member 1
NRs	nuclear receptors
NTD	N-terminal domain
OE-E6/E7	immortalized human fallopian tube cell line
OVGP1	oviduct-specific glycoprotein 1
P4	progesterone
PGR	progesterone receptor
POEC	porcine oviduct epithelial cells
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
SCNN1A	sodium channel epithelial 1 subunit α
SGK1	serum- and glucocorticoid-regulated kinase 1
TEER	transepithelial electrical resistance
TEPD	transepithelial potential difference
TFF1	trefoil factor 1
TGFB1	transforming growth factor beta 1
TNF-α	tumor necrosis factor alpha
TSC22D3	TSC22 domain family member 3
UTJ	uterine-tubal junction

5 Introduction

5.1 Preimplantation stress-related fertility impairment

5.1.1 Maternal stress and its influence on reproduction

Stress is defined as a state in which an individual's internal homeostasis is disrupted by a real or potential threat (Herman 2013). The neuroendocrine response to stress, one of the key component of the body's stress response system, is characterized by secretion of a cascade of hormones. In response to the disruption of homeostasis, the hypothalamus-pituitary-adrenal (HPA) axis is activated and corticotrophin-releasing hormone (CRH) is released by the hypothalamus, driving the body to initiate a complex and coordinated response involving cognitive, behavioral, and physiological reactions. Subsequently, adrenocorticotropic hormone (ACTH) is produced and released by the pituitary into the general circulation, which further leads to the secretion of species-specific glucocorticoids (GCs, cortisol in most mammals and corticosterone in rodents) from the adrenal cortex (Herman 2013;Mcewen 2017) (Figure 1). GCs are the main stress-related hormones and able to inhibit the release of CRH from the hypothalamus (the negative feedback loop of the HPA axis) (Miller and Auchus 2011). The main physiological action of GCs is mediated by binding to glucocorticoid receptors (GRs) that belong to the nuclear receptor superfamily, are found in almost all cell types, and regulate the transcription of 10-20% of the genome among species (Galon et al. 2002;Lu et al. 2007;Ren et al. 2012). The consequences of stress, which may be beneficial or detrimental to the body, depend on the type, timing, intensity, and duration of stress as well as the hormonal reactions (Axelrod and Reisine 1984;Mcewen 1998). In general, the adaptive response induced by the hormonal signaling cascade serves to restore homeostasis and to improve the chances of survival for the organism. However, stress becomes detrimental to the organism and leads to many functional disorders when the stress is severe or persists chronically (Sato et al. 2008;Eisenmann et al. 2016).

The influence of stress on the health and well-being of humans and animals has drawn increasingly scientific and public attention over the last few decades. Accumulating evidence suggests that maternal stress leads to elevated levels of endogenous GCs and adverse reproductive outcomes in mammals (Mwanza et al. 2000;Whirledge and Cidlowski 2010;Zhai et al. 2020). The activated GC signaling has been reported to favor gluconeogenesis, the mobilization of amino acids, and cardic output over other functions less essential for survival, including reproduction (Whirledge and Cidlowski 2010;Whirledge and Cidlowski 2013b). Reproduction in females involves a high demand for energy, nutrients, and metabolic adjustment, and thus is particularly vulnerable to stressors (Speakman 2008;Bobba-Alves et al. 2022). Regardless of stress types and species differences, recent publications have

indicated that maternal stress has profound influences on almost every aspects of reproductive performance, including the development and maturation of gametes, fertilization, early embryonic development, implantation, gestation, and lactation (Tilbrook et al. 2000;Einarsson et al. 2008;Whirledge and Cidlowski 2010;Fernandez-Novo et al. 2020;Zhai et al. 2020). Furthermore, maternal stress can also lead to long-lasting side effects across generations, and recent publications have suggested stress in the parental generation impacts postnatal growth, development of the sex organs, puberty onset, sexual manner, and life expectancy of offspring (Merlot et al. 2017;Nath et al. 2017;Al-Khaza'leh et al. 2020;Eberle et al. 2021). Women suffering from physiological or psychological stress are susceptible to stress-related reproductive disorders, including the loss of fertilized embryos and delayed delivery (Ilacqua et al. 2018;Stickel et al. 2019). Further studies suggest that excessive cortisol production in women under chronic stress is highly associated with disrupted hormonal profiles, which are fundamental for reproductive processes, and ultimately lead to infertility (Damti et al. 2008). It has been well recognized that GCs affect the reproductive function by interfering with all levels of the hypothalamus-pituitary-gonadal (HPG) axis (Whirledge and Cidlowski 2010). However, the direct action of GCs on the local organs and cells of the female genital tract (FGT) remains poorly understood, except for some reports in the uterus indicating that GCs disrupt the hormonal action of estrogens (Whirledge and Cidlowski 2010;Whirledge et al. 2013).

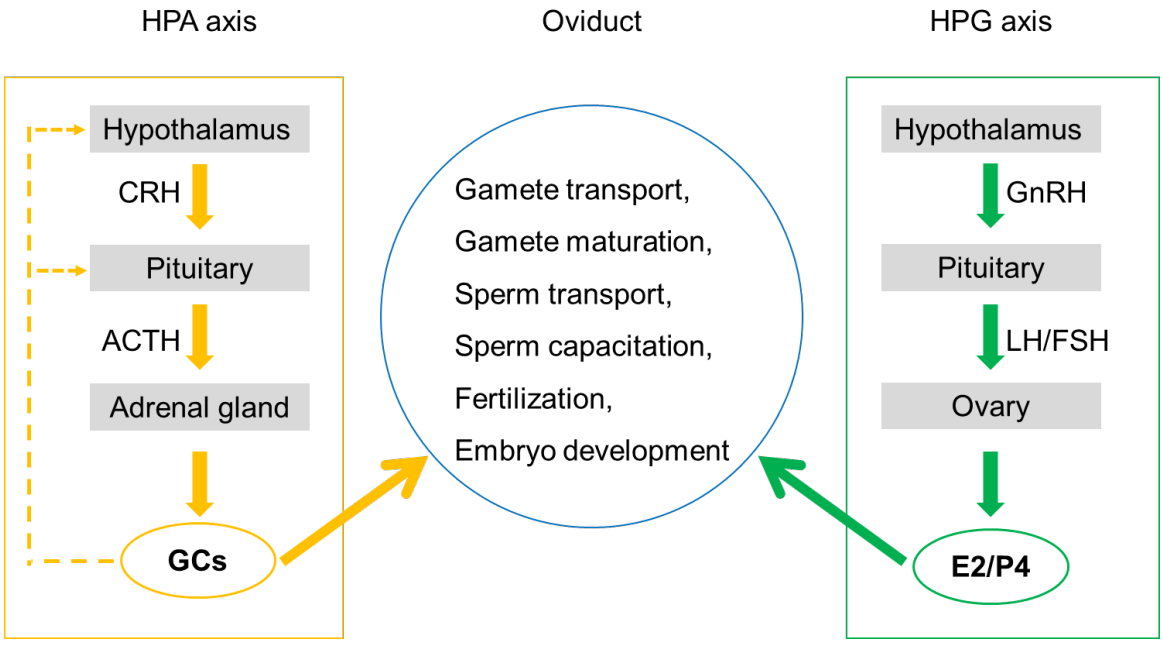

Figure 1. *The hormonal cascades of HPA and HPG axis and potentially impacted reproductive functions of the oviduct. Hypothalamus-pituitary-adrenal (HPA); hypothalamus-pituitary-gonadal (HPG); corticotrophin-releasing hormone (CRH); adrenocorticotropic hormone (ACTH); glucocorticoids (GCs); gonadotropin-releasing hormone (GnRH); luteinizing hormone (LH); follicle-stimulating hormone (FSH); (17)estradiol (E2); progesterone (P4).*

5.1.2 Important roles of the oviduct in mammalian reproduction

In the female reproductive system, the oviduct (termed fallopian tube in humans) is a tube-like structure connecting the ovary and the uterus. Even though there are some differences in the anatomy and morphology between species, the oviduct in most mammals is grossly divisible into distal (infundibulum and ampulla) and proximal (isthmus and uterine-tubal junction (UTJ)) segments and three histological layers (mucosa, muscularis, and serosa). The oviduct epithelium is the innermost and foremost mucosal lining of oviduct and the first communicative site with ovulated oocytes, sperms, and early embryos and plays a fundamental role in supporting reproduction (Agduhr 1927;Coy et al. 2012).

In vivo and *in vitro* studies have suggested that the region-dependent ultrastructure of the oviduct is likely associated with its specific roles in the reproductive process (Coy et al. 2012;Avilés et al. 2015;Li and Winuthayanon 2017). Across the whole oviduct, the epithelial layer folds and extends into the luminal side of the oviduct with a distinct pattern and degree. Generally, there are larger and more branched mucosal folds in the infundibulum and ampulla, and an evident reduction in size and number in the isthmus region (Abe 1996;Barton et al. 2020). In the funnel-shaped infundibulum, the widest segment of the oviduct, a fringe of finger-like structures (fimbriae) is commonly associated with catching and transporting the released oocytes into the oviduct after ovulation (Talbot et al. 1999;Yuan et al. 2021). The regions of ampulla and isthmus are considered to be the sites of fertilization and sperm reservoir, respectively (Avilés et al. 2015).

The oviduct epithelium contains two major cell populations, ciliated and non-ciliated (secretory) cells, and the region-specific functions in reproduction are linked to the unique and variable cell composition of the oviduct epithelium. Previous studies have demonstrated that the proportion of secretory cells in the region of ampulla is greater than in the region of isthmus; while the ciliated cells have an opposite trend across the whole oviduct regions (Ghosh et al. 2017;Ford et al. 2020). Secretory cells are mainly responsible for the production of oviduct fluid and providing growth factors, nutrients, and antimicrobial factors, while ciliated cells are involved in sperm binding and transport, oocyte pickup, embryo transport, and the flow of oviduct fluid.

To maintain the proper biological function and microenvironment for developing gametes and embryos, the oviduct epithelium undergoes multi-scale transformations in morphology, bioelectricity, immune response, and secretion of oviduct fluid in a stage- and region-related manner. The cellular subpopulations and microstructure of the oviduct epithelium, including epithelial height and cilia density, show evident transformations depending on the stage of estrous cycle and the region of oviduct epithelium (Abe and Oikawa 1993b;Shirley and Reeder

1996;Ito et al. 2016). Previous data in mice have demonstrated that functional motile cilia in the infundibulum are indispensable for the pick-up of ovulated oocytes at ovulation. The motile cilia in other regions of the oviduct play a significant role in the bidirectional transport of gametes and embryos through the oviduct, which indicates a region-specific function of motile cilia (Yuan et al. 2021). Apart from the direct contact with the oviduct epithelium, the developing gametes and preimplantation embryos require an optimal microenvironment when residing in the oviduct fluid. Molecular movement across the oviduct epithelium is mainly determined by the intracellular junctional integrity and active secretion of oviduct fluid from the oviduct epithelial cells (Lyons et al. 2006;Saint-Dizier et al. 2019). Tight junctions play a significant role in the maintenance of epithelial polarity and allow for the selective passage of electrolytes (e.g., Na^+, K^+, Ca^{2+}, and Cl^- ions), solutes, and macromolecules across the epithelium. Previous studies have shown that the bidirectional flow of electric current accompanied by fluid transportation across the epithelium leads to variations in osmotic gradients across the oviduct epithelium, which in turn are related to formation and control of oviduct fluid (Gott et al. 1988;Downing et al. 1997). It has been shown that the ion composition of the bovine oviduct fluid is distinct from the serum levels and linked to the modulation of sperm storage (Burkman et al. 1984;Hugentobler et al. 2007). In the bovine oviduct, delayed transportation and reduced motility of sperm were reported when tight junctions between epithelial cells were impaired (Owhor et al. 2019). Therefore, proper tight junctions of the oviduct epithelial cells are essential to the functional establishment of the oviduct epithelium and are responsive to distinct prenatal developmental stimuli in mammals.

As a pivotal site for allogeneic sperm and semi-allogeneic embryos, the oviduct epithelium possesses an efficient immune response system, precisely regulating the local immune environment, while simultaneously supporting the reproductive development that occurs in the oviduct (Schjenken and Robertson 2014;Marey et al. 2016;Schjenken and Robertson 2020). In several species, an inflammation-like reaction to sperm has been described, including the rapid recruitment of neutrophils and macrophages to the site of sperm deposition, which leads to phagocytosis of post-capacitated spermatozoa (Eisenbach 2003;Robertson 2005;Schjenken and Robertson 2014). Throughout the estrous cycle in cattle, a region- and cycle-specific number of neutrophils was detected in the oviduct milieu. Phagocytic activity of neutrophils towards sperm was suppressed by oviduct epithelial cells and oviduct fluid, thereby supporting the survival of sperm and ensuring the occurrence of fertilization (Marey et al. 2014;Yousef et al. 2019). Likewise, cyclic changes of apoptotic activity in the oviduct are linked to the process of fertilization with regard to oocyte competence, sperm storage and release in bitch (Urhausen et al. 2011). In addition, previous studies reported that typical cytokines responsible for modulating inflammation activity were expressed in the oviduct epithelium (e.g.,

interleukin 6 (IL6), interleukin 8 (IL8), and tumor necrosis factor alpha (TNF-α)), regulating the development of oocytes and the transportation of gametes in the oviduct (Wijayagunawardane and Miyamoto 2004;Liu et al. 2009). In humans, the transcription of genes related to immunomodulatory and inflammatory functions are significantly suppressed when embryos are transported through the ampulla region of the oviduct (Hess et al. 2013).

5.1.3 Preimplantation maternal stress-related reproductive disorders

Before implantation, early mammalian embryos undergo a wide range of cellular, metabolic, and epigenetic changes, and are more fragile and vulnerable than the later stages of developing embryos (Hobel and Culhane 2003). Therefore, the preimplantation period, which extends from fertilization and early embryo development occurring inside the oviduct to the onset of implantation in the uterus, is considered a highly “sensitive window” to various stressors (Einarsson et al. 2008;Eckert and Fleming 2011;Zhai et al. 2020). As the first contact zone, the oviduct provides an ideal microenvironment, encompassing the oviduct epithelium and oviduct fluid, interacting with gametes and embryos via direct cell contact or the exchange of signal molecules (Saint-Dizier et al. 2019;Bastos et al. 2022).

Recently, a growing number of reports emphasizes the critical consequences of stress on reproductive development especially through disturbing the homeostasis of the oviduct. The reproductive capacity of female mice was significantly suppressed after 48 hours (h) of restraint stress at the stage of embryo development within oviduct (Zheng et al. 2016). In comparison to the control group, the cortisol level in blood rises substantially, the total cell numbers per blastocyst and birth rate are both reduced notably in stressed mice, and apoptotic damage occurs in embryos and oviduct (Zheng et al. 2016). When recovered zygotes from unstressed mice are cultured *in vitro* with the same high level of corticosterone as in stressed mice, the development capacity of embryos is not affected, suggesting that GCs exposure alone causes no direct damage to embryos (Tan et al. 2017;Yuan et al. 2022). Another *in vitro* study suggests that corticosterone treatment impairs the development of embryos when co-cultured with oviduct cells, while a corticosterone antagonist blocks the damage to embryos. Thus, it is speculated that maternal stress and higher levels of GC damage embryos not directly but via oviduct cells, as apoptosis was induced and growth factor secretion by the oviduct cells was reduced (Tan et al. 2017). When female sows exposed to 48 h of food deprivation, a much lower number of viable spermatozoa were observed in the oviductal sperm reservoir after artificial insemination, followed by a decreased cleavage rate of fertilized embryos compared with the control group (Mwanza et al. 2000). In pigs, researchers have suggested that stress due to food deprivation and specifically the stress hormone cortisol is highly associated with suppressed reproductive activity of the oviduct and subsequent delayed ova transport within oviduct (Mwanza et al. 2000;Razdan et al. 2001). Furthermore, chronic (4, 8, and 12 weeks)

stressors in the forms of daily restraint and forced swimming significantly increased serum corticosterone levels in female rats, decreased the relative weight of oviduct, and impaired its antioxidant defense mechanisms (Divyashree and Yajurvedi 2018). As the antioxidant capability of the oviduct has been found to participate in protecting sperm and embryos against stress-induced damage in rats and cows, it is also likely that chronic stress leads to infertility through an imbalanced antioxidant environment of the oviduct (Harvey et al. 1995;Ribeiro et al. 2021).

5.2 Potential actions of increased cortisol on oviduct epithelium

The GR is encoded by the nuclear receptor subfamily 3 group c member 1 (NR3C1) gene and the main receptor of GCs, regulating GC-induced stress responses (Ramamoorthy and Cidlowski 2016). In stressed conditions, a high level of cortisol (the main GC in most mammals) reaches the uterine and ovarian arteries surrounding the oviduct tissue and binds to the GR expressed in the oviduct epithelial cells. Upon binding, the cortisol-GR complex is translocated into the nucleus to activate or repress gene transcription, and mediate the reproductive function of the oviduct (Andersen 2002;Ruiz-Conca et al. 2020). Many factors determine the final effects of cortisol on the oviduct epithelium, including NR3C1 expression level and bioavailable cortisol within the oviduct microenvironment. Three possible ways in which stress-induced cortisol might affect the reproductive function of oviduct have been suggested (Figure 2).

5.2.1 Direct cortisol action on oocyte/sperm/embryo

Within the oviduct microenvironment surrounded by oviduct epithelial cells, viable gametes and embryos in the oviduct fluid are the essential prerequisites for successful reproduction. The oviduct fluid contains various molecular effectors, including hormones and growth factors. Components of the complex fluid are mainly secreted by the oviduct epithelial cells while others are selectively transported from the circulating blood (Gatien et al. 2019). The contribution of follicular fluid to the oviduct fluid has not yet been fully understood, and some researchers have argued that only a negligible amount of follicular fluid is present in the oviduct fluid, since less than 1% of the P4 in follicular fluid was recovered from the oviduct fluid in sows (Hansen et al. 1991). In humans, evidence suggests that the ovulated egg is transported into the oviduct accompanied by a fluid wave, indicating that a sufficient amount of follicular fluid travels to the oviduct and is a major constituent of the oviduct fluid immediately after ovulation (Lyons et al. 2006). In addition, reports have also demonstrated that components of follicular fluid can be drawn into the oviduct through ciliary flow or the adjacent blood vessels (Kurzrok et al. 1953;Hunter et al. 2007;Mulley 2019). Few studies have reported the cortisol concentrations in the oviduct fluid collected *in vivo*. In bovine, Lamy et al. has determined that there is a much

higher (5-8 times) level of cortisol in oviduct fluid collected at a commercial slaughterhouse than the baseline level in plasma in the resting condition (Lamy et al. 2016). In contrast to the cortisol level in follicular fluid collected from the slaughterhouse, cortisol level in oviduct fluid is much higher on average (Spicer and Zinn 1987). Considering high level of cortisol has been observed in follicular fluid shortly before ovulation and the ruptured follicle may continue secreting follicular fluid, it is likely that eggs and sperms are bathed in a cortisol-enriched oviduct fluid due to the accumulated cortisol in plasma during stress conditions (Koninckx et al. 1980;Yong et al. 2000). Therefore, stress-induced high levels of cortisol reach the oviduct fluid and potentially bind to the GR expressed in developing oocytes, sperm, and embryos within the oviduct, finally affecting their development (Korgun et al. 2003;Gong et al. 2017;Ruiz-Conca et al. 2020).

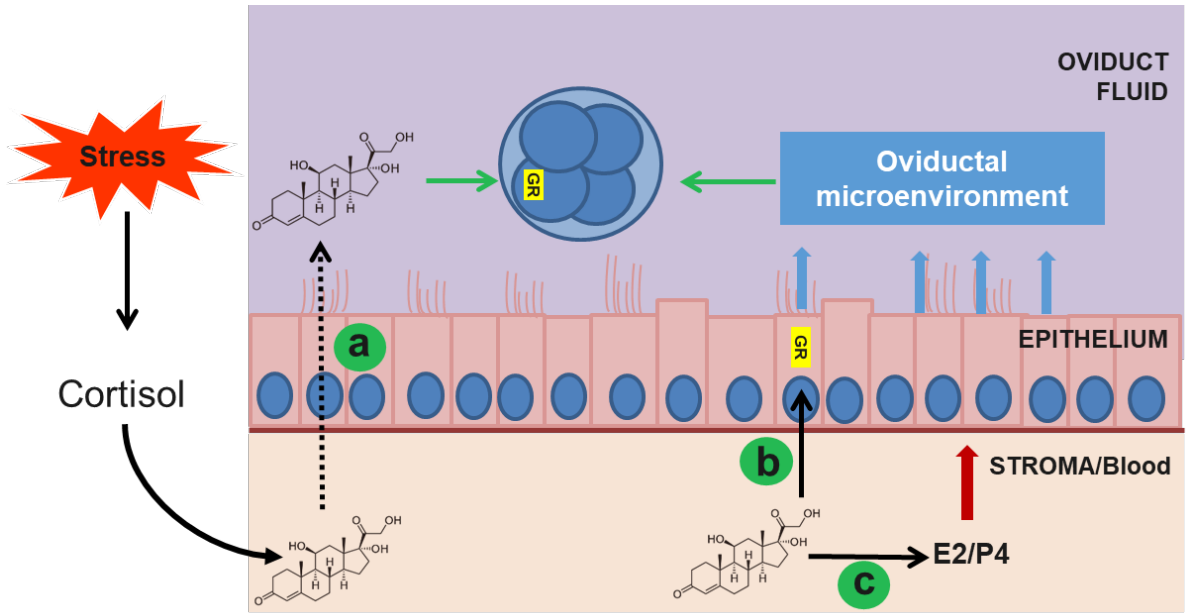

Figure 2. *Possible actions of the stress hormone cortisol on early embryos during oviduct transition. (a) stress-induced cortisol directly interacts with glucocorticoid receptor (GR) expressed in early embryos. Moreover, the oviduct microenvironment suitable for early embryonic development can be affected by (b) direct ligand-receptor binding in oviduct cells and (c) indirectly by an interplay of cortisol and ovarian steroids upon stress. Glucocorticoid receptor (GR); progesterone (P4); (17)estradiol (E2). (Figure originally created by Prof. Jennifer Schön and Dr. Shuai Chen and modified with permission)*

5.2.2 Stress exposure induced atypical function of oviduct epithelial cells

Recently, it has been proposed that GCs and the NR3C1 gene are associated with oviduct function potentially. In rabbits, seminal plasma triggered differential expression of the NR3C1 gene in oviduct tissue, showing a spatial and temporal profile matched to the corresponding segments and time points for various reproductive events (Ruiz-Conca et al. 2020). In pigs,

expression of NR3C1-related genes in UTJ region of the oviduct showed a significant reduction in response to natural mating and artificial insemination. The reduced expression of NR3C1-related genes and downregulated cytokine immune response in the UTJ region, functionally recognized as the oviduct sperm reservoir, may promote immune tolerance toward sperm (Ruiz-Conca et al. 2020). The overall evidence suggests that NR3C1-mediated cortisol action is essential to maintaining the functional regulation of the oviduct epithelium *in vivo*. Increasing evidence suggests that severe or prolonged stress has negative effects on the reproductive activities occurring within the oviduct, which are potentially associated with high levels of cortisol. The activation of GR signaling within oviduct epithelial cells is involved in the detrimental effects on reproduction induced by excessive cortisol. In mice, exposure to stress corticosterone induced oxidative stress and apoptosis of the oviduct epithelial cells *in vitro* through elevation of hydrogen peroxide (H_2O_2) and activation of Fas signaling. Additionally, several important growth factors (IGF1, transforming growth factor beta 1 (TGFβ1), and brain-derived neurotrophic factor (BDNF)), essential for embryo development and secreted by oviduct epithelial cells were also notably suppressed by corticosterone treatment (Tan et al. 2017). Hydrocortisone (synthetic preparation of cortisol) treatment modified the expression of genes related to the tight junctions of immortalized epithelial cells of human fallopian tube (Zhaeentan et al. 2018).

5.2.3 Stress exposure disturbs the signaling of ovarian steroids in the oviduct epithelium

The cellular signaling of cortisol and ovarian steroid hormones (i.e., E2 and P4) is similar and mediated by binding to the corresponding receptive nuclear receptors (NRs). As E2 and P4 are the main driving forces that regulate the oviduct function via estrogen receptor (ESR) and progesterone receptor (PGR), hence the stress-related disorders of ESR and PGR signaling may indirectly affect the reproductive performance of the oviduct (Okada et al. 2003;Akison and Robker 2012). As members of the NR superfamily, the core structure of GR, ESR, and PGR are composed of three modular domains with distinct functions: the N-terminal domain (NTD), DNA binding domain (DBD), and C-terminal ligand binding domain (LBD) (Moras and Gronemeyer 1998;Pecci et al. 2022). Studies have shown that the amino acid sequences of DBD and LBD regions are highly conserved amongst all NRs in different mammalian species, while they are less conserved in the NTD region (Fuller 1991;Papageorgiou et al. 2021). The conservation of the protein sequence and functional motif in the DBD region strongly suggests that steroid hormone receptors may have specific and similar DNA-binding preferences for final transcription of targeted genes (Papageorgiou et al. 2021). It has been found out that ~90% of protein sequence are identical in the DBD regions of PGR and GR, suggesting the interactive cross-talk between NRs regarding the genomic regulation (Fuller 1991;Ogara et al.

2019;Pecci et al. 2022). Recent data have revealed that almost half (46.5%) of the genomic binding sites of ESR and GR overlap upon simultaneous induction with E2 and dexamethasone (GR agonist) in endometrial cells (Vahrenkamp et al. 2018). Upon co-occurrence of steroid hormones (GCs, E2, and P4), the individual receptor-dependent selection of genomic binding sites are altered significantly, which triggered a unique hormone-dependent transcriptional outcome (Vahrenkamp et al. 2018;Pecci et al. 2022). In addition, both ligand-activated GR and ESR can bind to the promoter elements of specific genes (e.g., C-X-C motif chemokine ligand 8 (CXCL8) and trefoil factor 1 (TFF1)), and the competitive binding leads to opposing effects of cortisol and E2 on the transcription of the same responsive genes (Cvoro et al. 2011;Karmakar et al. 2013). The cortisol- and E2-induced co-regulation of glucocorticoid-induced leucine zipper (GILZ, encoded by TSC22D3 gene) expression, an important cortisol target, suggests that the immune response of the uterus is potentially mediated directly by both cortisol and E2 *in vivo* (Whirledge and Cidlowski 2013a). Additionally, previous studies have shown that the DNA response element of E2 can be converted to a GC-responsive element by the alteration of three acidic residues (Green et al. 1988;Mader et al. 1989). In the uterus, the E2-dependent regulation of cellular proliferation and inflammatory response were both modified by cortisol, showing antagonistic effects on the biological response to E2 in the uterus (Whirledge et al. 2012;Whirledge and Cidlowski 2013a). Similarly, it has been reported that cortisol interplays with P4, and the LBD regions of PGR and GR have ~55% sequence identity. Therefore, P4 can bind to both receptors with distinct affinity (Kontula et al. 1983). In turn, cortisol has been showed promoting or antagonizing effects on P4 action in varied target cells as cortisol is able to cross-bind to PGR (Leo et al. 2004;Zhang et al. 2019a;Pecci et al. 2022).

5.3 Pig as an animal model for reproductive biology research

As the source of the most consumed meat globally, the pig industry has been substantially challenged by stress-related consequences due to intensive farming conditions. Pigs housed at high density are more vulnerable and frequently exposed to potential stress factors, such as poor living environments, handling practices, weaning, and food deprivation (Barnett et al. 2001;Martinez-Miro et al. 2016). During the seasons with high temperature, heat stress occurs easily in pigs of all ages and leads to increase in body temperature and plasma cortisol, a reduction in feed intake, and poor animal welfare (Hao et al. 2014). Additionally, grouping of female pigs in a limited space easily leads to aggressive behavior, food deprivation, and subsequent stress with elevated cortisol concentrations, thereby decreasing fertility (Coutellier et al. 2007;Martinez-Miro et al. 2016). Therefore, a better understanding of the underlying mechanisms of stress in pigs will favor animal husbandry and welfare. In addition, growing clinical evidence and experimental studies on animal models have demonstrated that

environmental and psychological stress are highly associated with reproductive failure in humans (Nargund 2015;Bala et al. 2021). A prospective study has reported that procedural stress associated with undergoing assisted reproductive treatment reduces the number of oocytes retrieved and transferred in infertile women (Klonoff-Cohen et al. 2001). Because pigs and humans have close genomic, anatomical, and physiological similarities, advances in the pig reproduction area will also provide fundamental and alternative knowledge for human research (Bendixen et al. 2010;Swindle et al. 2012). With regard to female reproductive organs and tissues, the microstructures and epithelial layers of the FGT are very similar between pigs and humans despite some differences in gross anatomy. The profiles of reproduction-related hormones and the length of reproductive cycle are closely comparable. In addition, the mucosal immune system in the porcine FGT shares similarities to that of in human (Mcanulty et al. 2011;Swindle et al. 2012). The majority of porcine immune-related genes are also expressed in humans, along with analog fluctuation levels of mucosal antibodies within the FGT throughout the estrous cycle (Meurens et al. 2012;Mordhorst and Prather 2017). Finally yet importantly, porcine tissue samples for research are easily accessible and manageable from commercial pig slaughterhouses, which avoids the use of laboratory animals. Therefore, using *in vitro* models of primary cells isolated from the porcine FGT as abattoir byproducts is highly compliant with the principles of the 4Rs principle for animal use in science (Replacement, Reduction, Refinement, and Rehabilitation) (Mandal and Parija 2013) .

5.4 Porcine oviduct epithelial cells cultured at the Air-Liquid Interface (ALI-POEC)

It has been proven that the oviduct epithelium supports many important reproductive events. Within the oviduct, subtle and dynamic signal exchanges occur locally between the maternal reproductive system and the gametes and embryos at different stages, and the proper communication and responses are fundamentally required for successful reproduction (Almiñana and Bauersachs 2020;Kölle et al. 2020). However, the search for factors affecting the signal exchanges is challenging, as the oviduct *in vivo* is not easily accessible. To better elucidate the basic regulatory mechanisms within the oviduct, a challenge for the establishment of the *in vitro* approaches is to model the microenvironment as closely as possible to the *in vivo* conditions.

Conventionally, primary oviduct epithelial cells are cultured in a two-dimensional (2D) system where cells adhere to a plastic or glass surface and are submerged in culture medium. However, 2D *in vitro* models lack polarized cell architecture, mucociliary differentiation, and barrier integrity (Danesh Mesgaran et al. 2016). Moreover, the 2D cultured oviduct epithelial cells showed aberrant transcriptional profiles, comprising the ability to bind sperm and support fertilization *in vitro* (Sostaric et al. 2008;Lawrenson et al. 2013;Ferraz et al. 2017). For the purpose of modeling the maternal-gamete or -embryo interaction *in vitro*, directional signals

from the gametes or embryos and mother are not applicable in 2D culture systems due to the stereoscopic structure of oviduct *in vivo*. Therefore, the conventional 2D culture models of oviduct epithelial cells are less biologically relevant when studying the normal physiology of the oviduct epithelium *in vivo*. Recently, the three-dimensional (3D) culture of oviduct has been established and used in many studies, including models of organoids, microfluidics, ALI culture, and 3D printing (Ferraz et al. 2017;Chen and Schoen 2021).

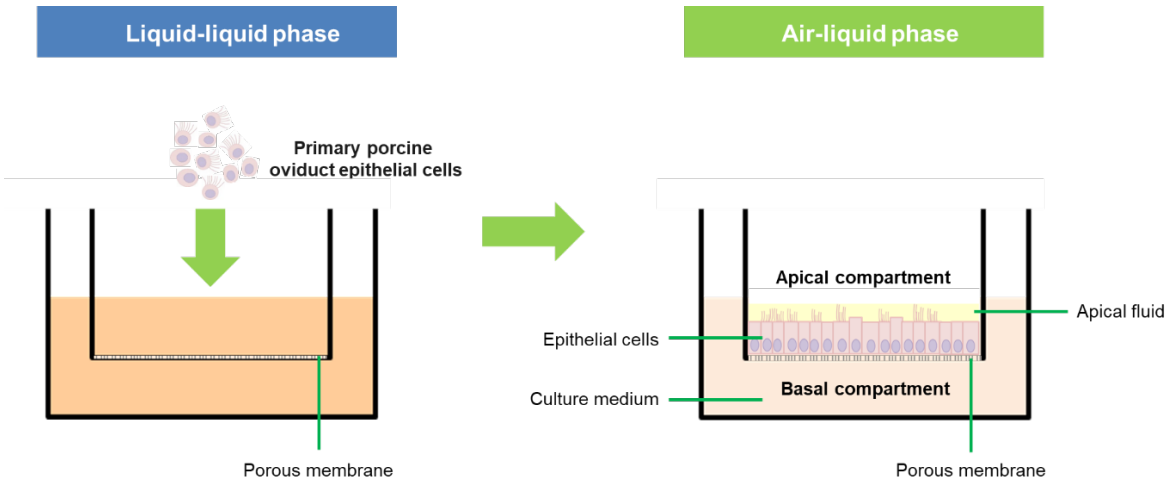

***Figure 3**. Schematic illustration of the air-liquid interface (ALI) culture with primary porcine oviduct epithelial cells (POEC). The confluent POEC grow on a hanging porous membrane, forming a distinct apical compartment (equivalent to the luminal cavity of the oviduct epithelium) and basal compartment (equivalent to the tissue and blood supply below the epithelium). Upon mucociliary differentiation, cells secrete visible and constant apical fluid into the apical compartment. (Figure provided by Dr. Shuai Chen and modified with permission)*

In many mammalian species, ALI culture has been widely used in FGT epithelia *in vitro*, which not only possesses differentiation capacity but also re-establishes well-functioning epithelia that are responsive to hormonal stimuli and produce *in vivo*-like apical fluid or mucus (Miessen et al. 2011;Chen et al. 2017;Zhu et al. 2020;Leemans et al. 2022). Generally, the ALI culture model utilizes porous membranes supporting the proliferation and differentiation of primary oviduct epithelial cells with *in vivo*-like basolateral supply of culture medium (Figure 3). Due to the highly differentiated phenotypes of ciliated and secretory cells, the ALI model promotes apical-basolateral polarity of the oviduct epithelial cells and allows the separation of apical and basal compartments representing the oviduct lumen and sub-epithelial structures, respectively. The ALI model of primary oviduct epithelial cells exhibits a well-maintained epithelial polarity and constant directional secretion of oviduct fluid surrogates containing protein content comparable to *in vivo* (Chen et al. 2017). When compared with other 2D and 3D models, the ALI culture is easily accessible and affordable and can be used for evaluating ciliary function and the barrier integrity of epithelium, and for determining the properties of the oviduct fluid

(Ferraz et al. 2017;Chen and Schoen 2019;Zhu et al. 2020). With manipulation from the apical and basal compartments, the ALI model of oviduct epithelial cells replicates the native environment *in vitro* and provides a closer perspective to examine the response signals from gametes and embryos, and the maternal side of the oviduct epithelial cells.

6 Aims of the study

In summary, maternal stress and subsequent elevated GCs during the preimplantation phase have been demonstrated to interfere with the reproductive function of the oviduct. However, the underlying mechanisms and especially the effect of the stress hormone cortisol on the oviduct microenvironment *in vivo* are largely unclear. With the compartmentalized ALI culture of porcine primary oviduct epithelial cells, the morphological and physiological setting of the oviduct epithelium was rebuilt *in vitro.* The aims of this study are to:

6.1 Characterize the direct action of cortisol on the oviduct epithelium

One of the main interests is to reveal the direct regulatory role of cortisol in the physiology and function of the oviduct epithelium. For mimicking the local stress situation, the ALI-POEC model was employed to basolaterally apply cortisol for 12 h, 72 h or 21 d representing short-, middle- and long-term stress conditions. Levels of cortisol and its inactive metabolite cortisone were monitored in both apical and basolateral compartments to understand the hormonal distribution pattern and cellular metabolization. Phenotypic and molecular assessments were employed to explore the impact of maternal stress on the microenvironment created by the oviduct epithelium.

6.2 Assess the effects of ovarian steroids on the oviduct epithelium *in vitro*

Using the same stimulation model, we also focused on the individual role of ovarian steroids (i.e., E2 and P4) in regulating oviduct function. POEC were exposed to peak levels of either E2 or P4 for different durations (12 h and 72 h) to explore and compare the hormone-dependent effects on the oviduct epithelium.

6.3 Explore the interaction between cortisol and ovarian steroids in the oviduct epithelium

Our last objective was to investigate whether the stress hormone cortisol and ovarian steroid hormones mutually affect each other in terms of oviduct function. Co-administration of cortisol with either ovarian steroid was carried out in ALI-POEC culture for different durations (12 h and 72 h), and cultures were assessed for epithelial microstructure, bioelectrical properties, and gene expression responses (steroid hormone signaling, oviduct function, immune response, and apoptosis).

7 Research Publications in Journals with Peer-Review

Publication 1

7.1 Does Maternal Stress Affect the Early Embryonic Microenvironment? Impact of Long-Term Cortisol Stimulation on the Oviduct Epithelium

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Does Maternal Stress Affect the Early Embryonic Microenvironment? Impact of Long-Term Cortisol Stimulation on the Oviduct Epithelium

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Abstract: Maternal stress before or during the sensitive preimplantation phase is associated with reproduction failure. Upon real or perceived threat, glucocorticoids (classic stress hormones) as cortisol are synthesized. The earliest "microenvironment" of the embryo consists of the oviduct epithelium and the oviductal fluid generated via the epithelial barrier. However, to date, the direct effects of cortisol on the oviduct are largely unknown. In the present study, we used a compartmentalized in vitro system to test the hypothesis that a prolonged stimulation with cortisol modifies the physiology of the oviduct epithelium. Porcine oviduct epithelial cells were differentiated at the air–liquid interface and basolaterally stimulated with physiological levels of cortisol representing moderate and severe stress for 21 days. Epithelium structure, transepithelial bioelectric properties, and gene expression were assessed. Furthermore, the distribution and metabolism of cortisol was examined. The polarized oviduct epithelium converted basolateral cortisol to cortisone and thereby reduced the amount of bioactive cortisol reaching the apical compartment. However, extended cortisol stimulation affected its barrier function and the expression of genes involved in hormone signaling and immune response. We conclude that continuing maternal stress with long-term elevated cortisol levels may alter the early embryonic environment by modification of basic oviductal functions.

Keywords: stress; cortisol; preimplantation period; oviduct; air–liquid interface

1. Introduction

The beginning of life takes place in the oviduct, which is also called the "fallopian tube". Upon successful sperm–oocyte recognition, fertilized embryos reside a few days within the oviduct developing (depending on the species) mostly up to the morula or even blastocyst stage before entering the uterus. The "microenvironment" of the early embryo consists of the oviduct epithelium, and the oviductal fluid generated via active epithelium secretion as well as passive and active transport across the epithelial barrier. This microenvironment guarantees optimal temperature, oxygen tension, PH, and nutrients to ensure embryo survival [1,2]. These early embryonic stages are considered a "sensitive window", as alterations in environmental conditions have profound consequences on embryo development and an individual's health in later life [3].

If a mammalian organism's homeostasis is under real or perceived threat [4], the hypothalamic–pituitary–adrenal (HPA) axis is activated to synthesize glucocorticoids (GCs), i.e., classic stress hormones, in order to cope with the challenges. It has long been recognized that stress and elevated GCs disrupt reproduction and fertility at multiple levels [5,6]. In contrast to the

well-characterized central interference of the HPA axis with the hypothalamic–pituitary–gonadal (HPG) system [6,7], the local actions of GCs in the female reproductive tract (FRT) remain largely unclear except for a few reports on ovaries [8].

Recent literature has reported in vivo and in vitro evidence that preimplantation maternal stress, specifically during the period when the embryo is hosted and transported by the oviduct, is closely associated with infertility and reproduction failure. In sows, stress evoked by food deprivation or repeated injection of adrenocorticotropic hormone (ACTH) during the postovulatory stage has been observed to lead to delayed ova transport, aberrant oviductal activity [9–11], endocrine profile changes, and impaired embryonic development [11]. Pregnant mice suffering from restraint stress during the embryonic oviduct transport period, as measured by elevated peripheral corticosterone levels (main rodent GC), have shown reduced embryo quality, conception rate, and litter size; furthermore, the negative consequences in this case were extended to behavior and physiology in postnatal life [12–15]. In vitro studies which exclude actions through the nervous system have revealed that zygotes co-cultured with mouse oviductal epithelial cells treated with high doses of corticosterone or corticotropin-releasing hormone (CRH) show reduced developmental competence (decreased blastocyst rate and blastocysts with decreased numbers of blastomeres) [16], while direct corticosterone/CRH exposure to mouse zygotes does not compromise embryo development. This altogether suggests that the effect of maternal GC on early embryos might be transmitted indirectly by the oviduct [16].

However, to date, almost no information is available on the short- or long-term effects of cortisol on the functionality of the oviduct epithelium. The pig shares some similarities in physiology, genetics, and metabolism with humans. Furthermore, pig sample materials are easily available as they are used for meat production; therefore, the pig is becoming increasingly popular as a model organism in biomedical research [17,18]. Especially for the study of female reproduction, in comparison to rodents, the pig is more analogous to humans in terms of hormonal status and estrous cycle length, although the pig has an estrous cycle and humans have a menstrual cycle [19]. We know that stressors affect the reproductive performance of female pigs during early pregnancy and that sows are especially susceptible to sustained elevation of cortisol and long-term stress [20,21]. Recently, we established a compartmentalized air–liquid interface (ALI) culture procedure which allows long-term culture of differentiated porcine oviduct epithelial cells (ALI-POEC) [22,23]. The cells are grown on porous filter supports which separate apical and basolateral compartments. The formed tissue-like epithelial layer actively produces an oviductal fluid surrogate on its apical side, while effectors (e.g., hormones or metabolites) can be administrated from the basolateral cell surface, mimicking in vivo conditions [24,25].

In the present study, we use this compartmentalized long-term culture system to test the hypothesis that long-term elevation of cortisol influences the early embryonic microenvironment by modifying the physiology of the oviduct epithelium. Hence, ALI-POEC were initially maintained to reach full differentiation. Then, physiological levels of cortisol representing moderate and severe stress were administrated basolaterally for a prolonged period of 21 days. We assessed the effect on epithelium structure, transepithelial bioelectric properties, gene expression related to oviduct functionality, and inflammation. Furthermore, the extracellular environment was examined from both apical and basal compartments for markers of cell damage, distribution, and metabolism of cortisol and cortisone.

2. Results

2.1. Effect of Long-Term Cortisol Stimulation on Morphology of ALI-POEC

The stimulation of the ALI-POEC system by basolateral administration of cortisol is illustrated in Figure 1A. Long-term exposure to cortisol caused no obvious effects on the morphology of ALI-POEC. Upon stimulation with 100 and 250 nM cortisol for 21 days, cells exhibited highly polarized structure, columnar shape, presence of cilia, and protrusions in a manner highly identical to the control cultures (0 nM, Figure 1B). Further quantification revealed that the percentages of secretory cells remained

unaffected ($p > 0.05$; Figure 1C). Neither the total cell numbers ($p > 0.05$; Figure 1D) nor cellular height showed any significant changes by any level of cortisol treatment ($p > 0.05$; Figure 1E).

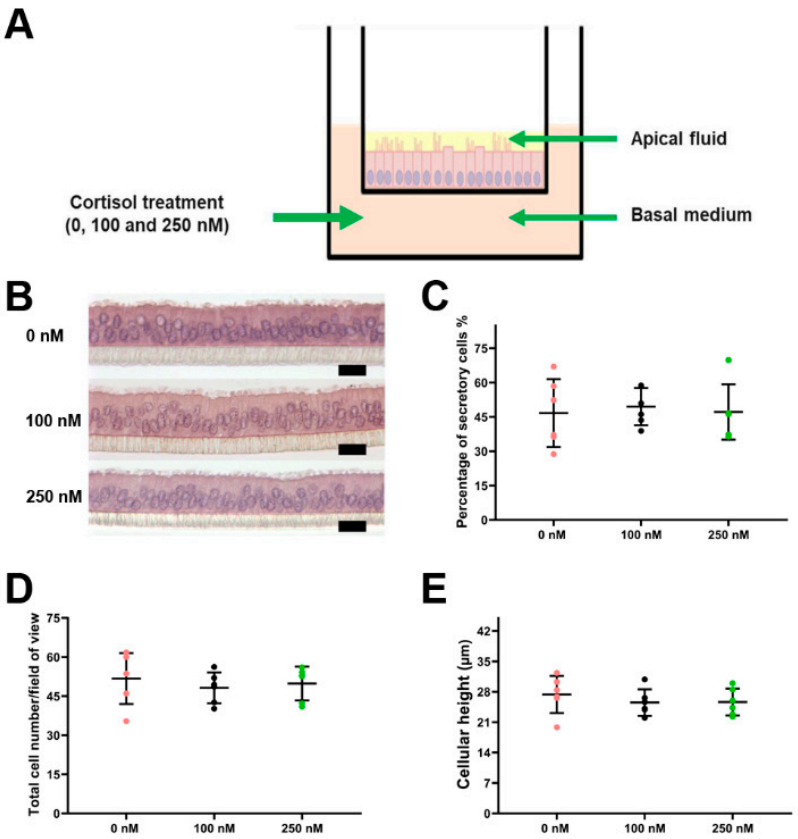

Figure 1. ALI-POEC morphology in response to long-term cortisol (100 and 250 nM) stimulation. (**A**) Schematic illustration of cortisol treatment in porcine oviduct epithelial cells grown at the air–liquid interface (ALI-POEC). (**B**) Representative cross-sections of ALI-POEC, hematoxylin–eosin (HE) staining, scale bar = 20 µm; (**C**) percentage of secretory cells; (**D**) total cell number/field of view; (**E**) cellular height. Data are shown as mean with standard deviation (SD). *n* = six animals.

2.2. Long-Term Cortisol Stimulation Triggers the Canonical Glucocorticoid Receptor (GR) Pathway

The mRNA expression of *NR3C1* (encoding GR proteins) and its dominating subtype *NR3C1*α were both found to be significantly down-regulated after 21 days of treatment with 250 nM cortisol ($p < 0.05$, Figure 2A,B). The transcriptional levels of FK506 binding protein 51 (*FKBP5*), a co-regulator of the GR signaling pathway, as well as TSC22 domain family member 3 (*TSC22D3*), a typical cortisol-inducible gene, were strongly elevated by cortisol in a dose-dependent manner ($p < 0.05$, Figure 2C,B).

The localization of GR protein was visualized by immunofluorescence. The results revealed that GR was mainly centered around the nucleus in the control group, which, however, became less evident upon cortisol stimulation (Figure 2E). Moreover, the fluorescence signal of GR protein was stronger in the control than the treated groups (Figure 2E), which was in line with the mRNA expression.

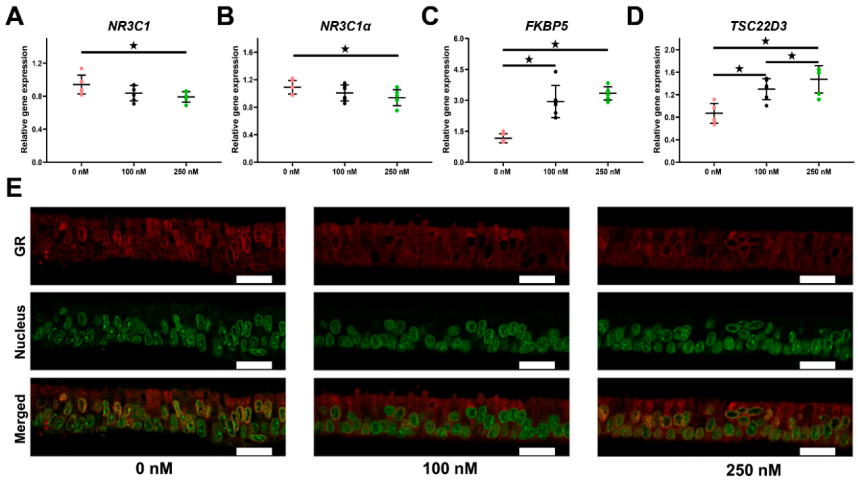

Figure 2. Activation of the glucocorticoid receptor (GR)-signaling pathway by cortisol in ALI-POEC. Differential marker gene expression of (**A**) *NR3C1*, (**B**) *NR3C1α*, (**C**) *FKBP5*, and (**D**) *TSC22D3*. Data are shown as mean with SD. Asterisks indicate a significant difference at $p < 0.05$. n = six animals. (**E**) Immunofluorescence staining of GR (red fluorescence) in ALI-POEC, nuclei stained with SYBR Green I; scale bar = 20 µm.

2.3. Long-Term Cortisol Treatment Alters Oviductal Functionality

2.3.1. Transepithelial Bioelectric Properties

To assess the barrier function and ionic transport of the oviduct epithelial layer, transepithelial electrical resistance (TEER) and transepithelial voltage assessments were carried out. All samples developed proper TEER falling into the range of good quality cultures [26], reflecting full confluence and differentiation of the epithelial layer (Figure 3A). Stimulation with 250 nM cortisol significantly increased the electrical resistance in comparison to the 100 nM group ($p < 0.05$, Figure 3A). Likewise, the transepithelial voltage was also significantly elevated in the 250 nM cortisol group ($p < 0.05$, Figure 3B).

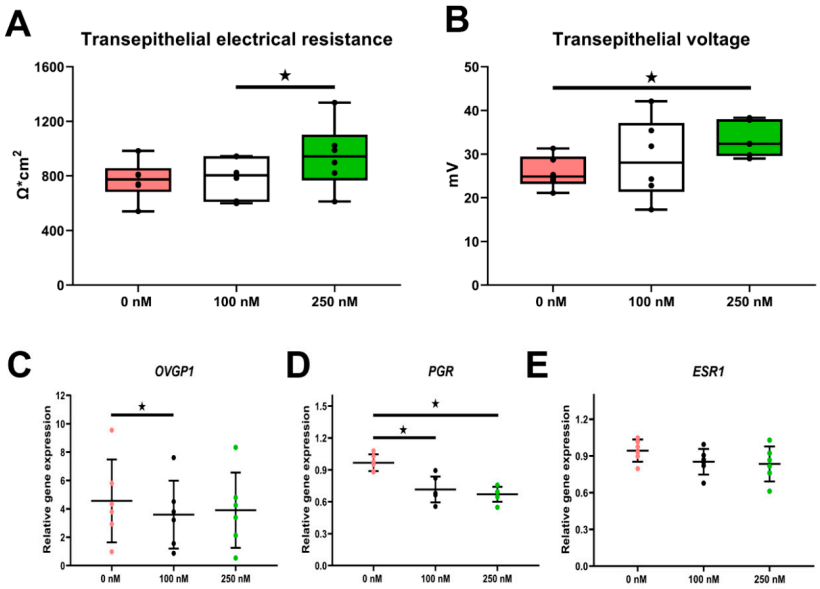

Figure 3. Effect of cortisol stimulation on oviductal functionality parameters in ALI-POEC. Elevation of transepithelial electrical resistance TEER (**A**) and transepithelial voltage (**B**); relative mRNA abundance of (**C**) oviduct-specific glycoprotein 1 (*OVGP1*), (**D**) progesterone receptor (*PGR*), and (**E**) estrogen receptor 1 (*ESR1*). Data are shown as mean with SD. Asterisks indicate a significant difference at $p < 0.05$. n = six animals.

2.3.2. Long-Term Cortisol Stimulation Down-Regulated Expression of Oviductal Marker Genes

We further quantified the expression of key oviduct functional genes, including steroid hormone receptors. Oviduct-specific glycoprotein 1 (*OVGP1*) was significantly decreased by cortisol treatment ($p < 0.05$, Figure 3C). Similarly, both levels of cortisol remarkably down-regulated the transcription of progesterone receptor (*PGR*, $p < 0.05$, Figure 3D), while the expression of estrogen receptor 1 (*ESR1*) was not affected ($p > 0.05$, Figure 3E).

2.4. Impact of Long-Term Cortisol on Inflammation and Apoptosis

2.4.1. Expression of Genes Related to Inflammation

Considering the immunosuppressive effect of cortisol in vivo, we assessed the expression of immune-related genes after long-term cortisol stimulation. The pro-inflammatory cytokine *IL6* was significantly down-regulated by both cortisol doses ($p < 0.05$, Figure 4A). No regulation on C-X-C motif chemokine ligand 8 (*CXCL8*) or prostaglandin-endoperoxide synthase 2 (*PTGS2*) was observed ($p > 0.05$, Figure 4B,C).

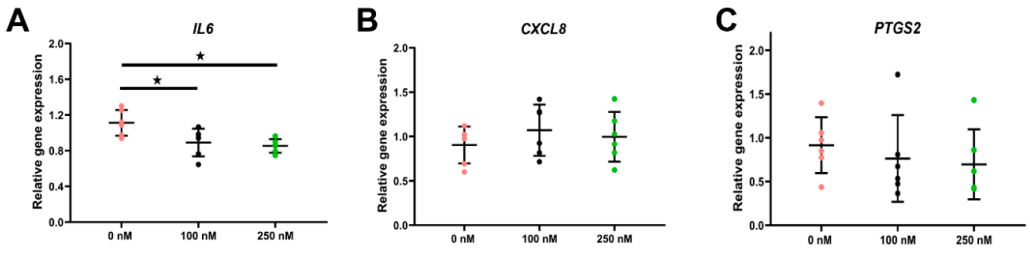

Figure 4. Effect of cortisol on inflammatory marker gene expression in ALI-POEC. Relative mRNA abundance of (**A**) *IL6*, (**B**) C-X-C motif chemokine ligand 8 (*CXCL8*), and (**C**) prostaglandin-endoperoxide synthase 2 (*PTGS2*). Data are shown as mean with SD. Asterisks indicate a significant difference at $p < 0.05$. n = six animals.

2.4.2. Long-Term Cortisol Treatment Does Not Trigger Apoptosis in ALI-POEC

We measured lactate dehydrogenase (LDH) activity in the apical and basolateral compartments of ALI-POEC. Although the cells had been exposed to cortisol for a prolonged period of 21 days, the release of LDH protein into both compartments revealed no significant differences ($p > 0.05$, Figure 5A). The LDH signal in the apical compartment, in general, was stronger than the basal compartment (Figure 5A).

The expression of genes related to DNA damage (*GADD45G*, Figure 5G) and cell death (*CASP3*, *BAX*, and *NFKBIA*) did not differ between the control and cortisol-treated groups (Figure 5D–F). Cortisol stimulation slightly suppressed the expression of *TP53* and *DDB2* (Figure 5B,C).

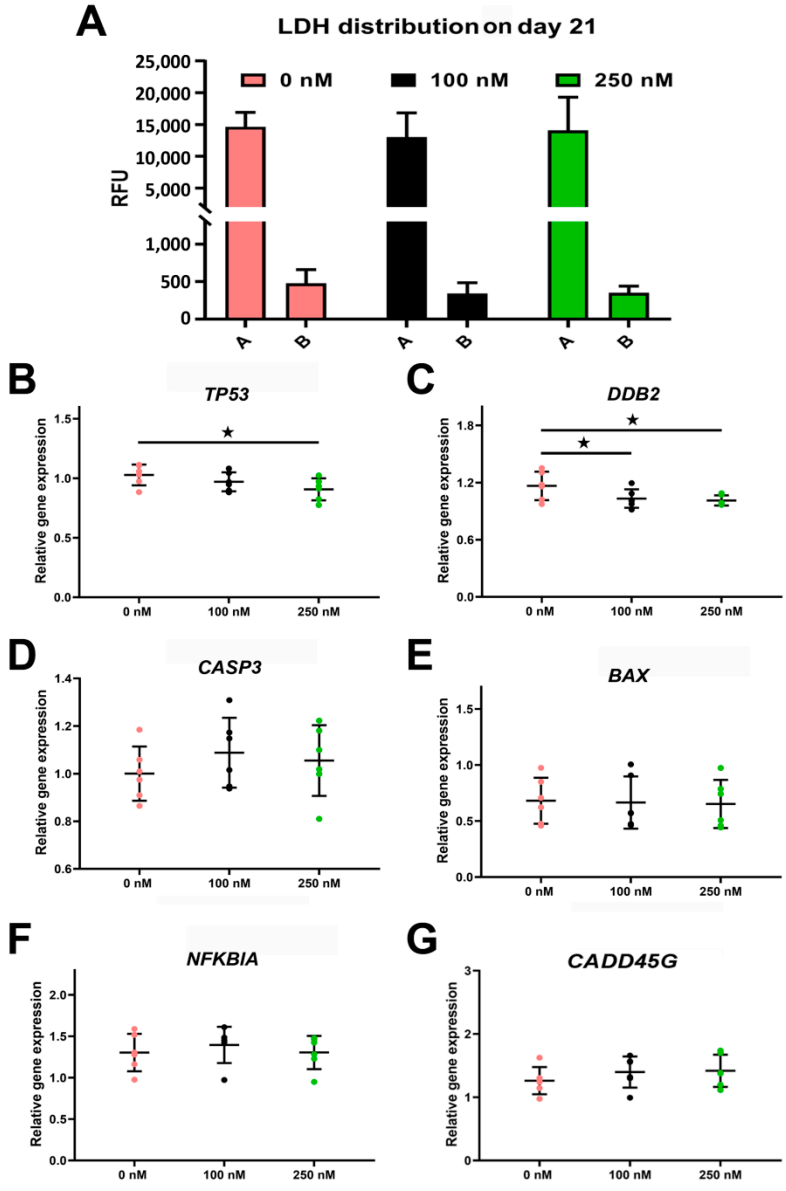

Figure 5. Apoptosis biomarkers in response to cortisol stimulation in ALI-POEC. (**A**) Constant lactate dehydrogenase (LDH) activity in apical and basal compartment. (**B**–**G**) Relative mRNA abundance of cell death marker genes. Data are shown as mean with SD. Asterisks indicate a significant difference at $p < 0.05$. n = six animals. Legend: RFU, relative fluorescence units.

2.5. Distribution and Metabolism of Cortisol and Cortisone in the ALI-POEC System

In the control group, cortisol and cortisone were detected neither in the apical fluid nor in the basal medium from day 0 to 21 (Figures 1A and 6A,B). On day 0, 122.22 ± 2.04 nM and 222.38 ± 12.31 nM cortisol were detected in the basal medium of cultures treated with 100 nM and 250 nM cortisol, respectively (Figure 6A), while cortisone remained undetectable (Figure 6B). On day 21 (12 h after the last cortisol application), cortisol concentration in the basal medium was 30.79 ± 5.45 nM (100 nM group) and 69.51 ± 11.26 (250 nM group). Cortisol in the apical fluid increased to 13.61 ± 1.36 nM (100 nM group) and 23.34 ± 7.38 nM (250 nM group), respectively, on day 21. The cortisol level in the medium (basal cell side) remained higher than in the oviductal fluid surrogate on the apical side of ALI-POEC (Figure 6A). Inversely, on day 21, the cortisone level grew evidently in both the apical fluid and basal medium, reaching 101.68 ± 25.14 nM (apical) and 110.92 ± 34.47 nM (basal) in the 100 nM group and 221.79 ± 59.02 nM (apical) and 238.07 ± 77.79 nM (basal) in the 250 nM group (Figure 6B).

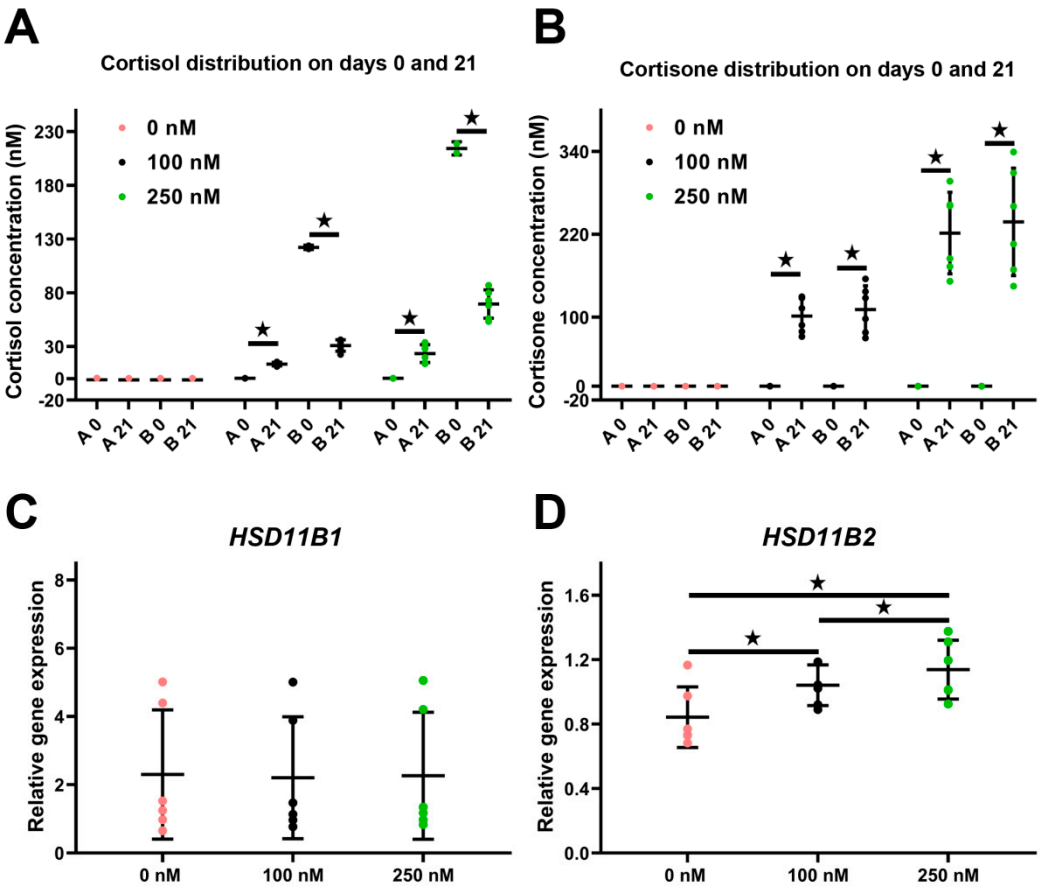

Figure 6. Distribution and metabolism of cortisol and cortisone in ALI-POEC. (**A**) Directional changes of cortisol distribution during treatment; (**B**) rising cortisone levels in apical and basal compartment. Relative mRNA abundance of (**C**) *HSD11B1* converting cortisone to active cortisol and (**D**) *HSD11B2* inactivating cortisol to cortisone. Data are shown as mean with SD. Asterisks indicate a significant difference at $p < 0.05$. n = six animals.

The expression of hydroxysteroid 11-beta dehydrogenase 1 (*HSD11B1*) and 2 (*HSD11B2*), the enzymes converting cortisone to cortisol and vice versa, were assessed by RT-qPCR. Cortisol treatment did not change the expression of *HSD11B1* but did induce a significant dose-dependent upregulation of *HSD11B2* (Figure 6C,D).

3. Discussion

Activation of the stress axis during early pregnancy disrupts fertilization and early embryo development inter alia via elevated cortisol [27,28]. However, the direct local actions of cortisol at the upper FRT are largely unknown. In this study, we initially applied the ALI-POEC culture system to investigate the effect of sustained cortisol elevation on the early embryonic microenvironment. The applied compartmentalized culture system holds serval unique features. It faithfully remodels the structure and functionality of oviduct epithelium tissue in vivo. It allows stimulation of the epithelium from the basolateral cell pole, therefore better simulating the cortisol supply from the arterial vascular bed. The compartmentalized system furthermore permits simultaneous monitoring of physiological situations, in both the apical (corresponding to the oviductal lumen) and basal (corresponding to sub-epithelial maternal tissue) compartments. As the culture system is applicable for long-term culture, we were able to mimic a prolonged stress period of 21 days without cell passaging or any sign of cell dedifferentiation.

In general, upon the 21 days of cortisol administration, neither the dose that mimics moderate stress (100 nM) nor the one simulating severe stress (250 nM) provoked any obvious alternation to the gross morphology of the in vitro oviductal epithelium, as revealed by histomorphometry. Cells maintained their fully differentiated status with constant composition of ciliated and secretory subtypes, though a slight but not significant decline in cell numbers and epithelium height was noticed. However, TEER, a highly sensitive parameter used to assess epithelium integrity that is largely determined by tight junctions [26], showed a significant increase in electrical resistance in the "severe stress" group. In line with our findings, Zhaeentan et al. have recently reported that cortisol treatment in human fallopian tube epithelial cells regulates the expression of tight junction genes [29]. Similarly, a TEER increase after cortisol stimulation has been observed in an intestinal epithelial cell line (Caco-2/Bbe cells) [30]. The movement of charged ions across the epithelium layer is essential for oviductal fluid formation [31]. Active transport of solutes like chloride (Cl^-) and sodium (Na^+) produces a potential difference (PD), which could be measured as transepithelial voltage. We found that long-term cortisol stimulation leads to a marked increase in PD, suggesting enhanced unidirectional trafficking of certain ions across the oviductal epithelium. Collectively, this indicates an effect of long-term cortisol stimulation on the oviductal barrier function.

Additionally, the expression of oviductal marker gene *OVGP1* and hormone receptors was altered by cortisol stimulation. *OVGP1*, which plays multi-functional roles in sperm–zona pellucida binding, fertilization, early embryo cleavage, and development [32], was notably suppressed. It is well known that oviduct functionality is regulated by ovarian-derived hormones, namely estrogen (E2) and progesterone (P4), which fluctuate during the estrous cycle [33]. The level of *ESR1* remained unaffected, whereas expression of *PGR* was markedly down-regulated by both moderate and high levels of cortisol, hinting at a profound impairment of the oviduct epithelium responsiveness to P4 after a long-term cortisol challenge.

Major cortisol action takes place through the activation of the GR (encoded by the *NR3C1* gene). Hence, we assessed the effect of long-term cortisol stimulation on the classical GR signaling pathway. Long-term cortisol treatment slightly down-regulated the mRNA of the main subtype *NR3C1α* in oviductal cells. Expression of the GR inducible gene *FKBP5* sharply increased in a dose-dependent manner, which could adversely modulate GR signaling by causing less efficient nuclear translocation of the receptor complex [34]. Likewise, *TSC22D3* (an indicator of GR pathway sensitivity) was also strongly up-regulated in a dose-dependent manner [35]. Recent evidence suggests that GCs hold both anti- and pro-inflammatory effects related to the complex mechanisms of GR signal transduction [36].

Cortisol is well known for its immune suppressive functions. In luminal epithelial cells of the bovine endometrium cortisol down-regulated mRNA expression of pro-inflammatory cytokines, like *IL6* and *CXCL8* [37]. Accordingly, long-term cortisol stimulation also suppressed IL6 expression in ALI-POEC. However, *CXCL8* expression was not affected, pointing at an oviduct specific pattern of pro-inflammatory cytokine regulation.

Previous studies in mice have reported that preimplantation restraint stress triggers apoptosis in oviducts, thereby leading to embryo mortality [14]. In our study, we quantified the amount of LDH, a cytoplasmic enzyme released to the cell environment when the plasma membrane is damaged during apoptosis, necrosis, and other forms of cellular damage, in both the apical secretion and basolateral medium after long-term cortisol stimulation [38]. Surprisingly, our results revealed that the amount of dead or damaged cells was not affected on either side, which was further supported by the expression of apoptosis markers, e.g., *TP53*, *CASP3*, and others. This finding is inconsistent with the in vivo study. We hypothesize that apoptosis is not caused by direct cortisol action on the oviduct epithelium but may be rather regulated by the HPG axis, which was not mimicked in our culture approach.

There are not many studies available which report cortisol content within the oviductal fluid. The cortisol levels measured in ALI-POEC apical fluid after long-term cortisol stimulation are roughly in accordance with recent data from bovine oviductal fluid [39]. The oviductal cortisol levels measured in this in vivo study were surprisingly high compared to the physiological plasma cortisol levels in

cattle. However, the oviductal fluid was gained from slaughterhouse by-products and so probably from animals highly stressed by transport and slaughter.

The ALI-POEC system revealed the massive cortisol metabolizing capacity of the oviduct epithelium. Even after three weeks of repeated cortisol application, the majority of bioactive cortisol in the culture system was converted to its inactive metabolite cortisone. Thus, the epithelial cells actively prevented high cortisol concentrations in the apical fluid. In line with this finding, long-term cortisol stimulation led to an upregulation of *HSD11B2* mRNA expression, the enzyme that converts cortisol to cortisone, in the oviduct epithelium.

In conclusion, we investigated the effect of maternal stress on the early embryonic microenvironment by long-term stimulating highly differentiated oviduct epithelial cells with cortisol concentrations measured in vivo during moderate and severe stress. The oviduct epithelium was able to metabolize cortisol and thereby stabilize the cortisol concentration reaching the embryonic microenvironment. However, extended cortisol stimulation affected the barrier function and marker gene expression of the in vitro oviduct epithelial tissue. The expression of genes involved in the hormone responsiveness and immunological functions of the oviduct were impaired. This supports our hypothesis that long-lasting maternal stress associated with long-term elevated cortisol levels may affect the early environment of the embryo by modification of basic oviductal functions. However, in this study we only analyzed the long-term effects of cortisol in a rather static system (cortisol application every 12 h). The time-dependent adaptation of ALI-POEC to the cortisol stimulus as well as the effect of steady cortisol concentrations (e.g., via perfusion of the culture system) needs further investigation. Finally, embryo co-culture experiments will prove if and how cortisol affects embryonic development via the luminal epithelial tissue.

4. Materials and Methods

4.1. Media and Reagents

DMEM/Ham's F-12, fetal bovine serum (FBS), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and amphotericin B were purchased from Merck Millipore (Billerica, MA, USA). Other reagents were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated.

4.2. Tissue Collection and Cell Isolation

The collection of porcine tissue and cell isolation was performed following our previously published protocol [23,40]. Briefly, porcine oviducts of healthy gilts (approximately six months old) were collected from a local slaughterhouse (Danish Crown Teterower Fleisch GmbH, Teterow, Germany) where these animals were slaughtered for meat production purposes. Only oviducts of non-cycling gilts were included in the study. The oviduct epithelial cells were isolated by digestion with collagenase 1A and accutase. Isolated cells were seeded on hanging inserts or cryopreserved in liquid nitrogen for long-term storage.

4.3. ALI-POEC Culture and Cortisol Stimulation Experiments

4.3.1. ALI-POEC Culture

ALI-POEC cultures using cells from individual animals (from n = six donors, one 24- and one 12-well insert/group/animal) were carried out as recently described by our group [23] with slight modifications. From day 0 to day 6, cells were cultured in proliferation-inducing medium (M1) under liquid–liquid interface condition at 37 °C, 5% CO_2, and 5% O_2. After day 7, the cells were switched to ALI condition using serum-free medium (M2a) which contained neither cortisol nor cortisone. Medium change and apical fluid removal were performed twice a week. After three weeks of culture, cells were used for cortisol stimulation.

4.3.2. Cortisol Preparation and Stimulation

Two concentrations of cortisol (100 and 250 nM), as measured in the plasma of sows under moderate and severe stressors, were employed for the stimulation experiment [41,42]. The 10 mg/mL cortisol stock solution was prepared in 100% ethanol and stored in aliquots at −20 °C until use. Serum-free M2a medium [23] with the desired cortisol concentrations (0, 100, or 250 nM) was administrated to the basolateral compartment of ALI-POEC for a period of 21 days (Figure 1A). Our pretest revealed that cortisol levels in the medium of ALI-POEC dramatically dropped after 12 h. Thus, for the purpose of mimicking chronic stress over a long time period with a sustained high level of cortisol, the stimulation medium was refreshed at 12 h intervals. Accordingly, the medium in the control group, which contained solvent only, was also changed every 12 h.

4.4. TEER and Transepithelial Voltage Assessment

Before harvesting the 24-well ALI culture, TEER and transepithelial voltage were measured using an EVOM2 Epithelial Voltohmmeter (WPI, Sarasota, FL, USA). To minimize any offset, the electrodes were equilibrated by soaking them in 100 mM KCl for 24 h before measurement. The apical compartment of the ALI culture system was refreshed with 200 µL of preequilibrated DMEM/Ham's F-12, and then the ohmic resistance and voltage of samples along with a blank sample (culture insert without cells) were measured within 5 min to ensure a sustained temperature. The final unit area resistance (Ω*cm^2) and transepithelial voltage were calculated per the manufacturer's instructions.

4.5. Determination of Cortisol, Cortisone, and LDH in the Apical and Basal Compartments

Previously, we have reported that ALI-POEC produces an oviductal fluid surrogate in the apical compartment [23]. Generally, within 3 days cells cultured in a 24- or 12-well insert are able to produce approximately 10–30 µL fluid in the apical compartment. A timespan of at least 3 days is therefore needed to collect and analyze components of the apical fluid. Therefore, for the cortisol/cortisone and LDH assay, on day 18 of cortisol stimulation, the apical side of cells was gently rinsed three times with prewarmed DMEM/Ham's F-12 to remove any dead cells or debris. As mentioned in Section 4.3.2, the stimulation medium in the basolateral side was changed every 12 h. On day 21 of stimulation, apical fluid (generated from day 18–21) and basal medium (12 h after medium change) were both recovered (see Figure 1A) and centrifuged at 13,000 × *g* at 4 °C for 10 min, after which supernatants were collected and stored at −70 °C until further use.

The concentrations of cortisol were assessed by an enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences Inc., New York, USA; ADI-900-071), while levels of cortisone were measured using a commercially available chemiluminescent immunoassay (CLI) kit (Abbor Assays, Ann Arbor, MI, USA; K017-C1). The fluorometric LDH activity assay kit (Abcam, Boston, MA, USA; ab197004) was used to determine cellular cytotoxicity. For all assays, samples were measured in duplicate.

A FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, BW, Germany) was used for the optical density (wavelength at 405 nm for cortisol), luminescence (for cortisone), and fluorescence (excitation/emission = 535/587 nm for LDH) reading within 5 min after plate preparation. The concentrations of hormones were calculated against the corresponding standard curves prepared in the same matrix, utilizing BMG Omega Mars software (BMG LABTECH, Ortenberg, BW, Germany). Relative fluorescence units (RFU), which directly correlate to the number of damaged cells, estimated the LDH activity.

4.6. Histomorphometry and Immunofluorescence

The histological processing followed our previously described procedure [25]. Paraplast embedded samples were cut into 4 µm sections for hematoxylin–eosin (HE) or immunofluorescence staining. For histomorphometric analysis, HE stained images (5× images/sample) were taken at 400× magnification

to measure the cellular height (5× positions/image), total cell number, and the number of secretory cells using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) [43].

Immunofluorescence staining was performed to identify the subcellular localization of the GR protein. For antigen retrieval, sections were immersed and cooked in 100 mM citrate buffer for 3 min. Sections were blocked with 3% BSA for 30 min at RT and incubated with anti-GR antibody (Abcam; ab3578; 1:200) overnight at 4 °C. Antigen was detected by Alexa Fluor 647 conjugated polyclonal goat anti-rabbit antibody (Thermo Fisher Scientific, Dreieich, HE, Germany; A-21245; 1:200) and the nucleus was counterstained with SYBR Green I (Thermo Fisher Scientific, Dreieich, HE, Germany; S-7563; 1:200). The slides were imaged at 400× magnification by a confocal laser scanning microscope LSM 800 equipped with Zen software (Carl Zeiss, Oberkochen, BW, Germany).

4.7. Gene Expression Analysis

Gene expression of ALI-POEC was quantified by RT-qPCR as recently reported [33]. All primer sequences, annealing temperatures and PCR efficiencies are listed in Table S1. Briefly, total RNA was prepared using the NucleoSpin RNA kit (Macherey-Nagel, Dueren, NW, Germany) and quantified using NanoDrop ND-1000 (Thermo Fisher Scientific, Dreieich, HE, Germany). ALI-POEC of the six donor animals (biological replicates) were used in each treatment group. cDNA was synthesized from 1 µg of total mRNA by RevertAid reverse transcriptase (Thermo Fisher Scientific, Dreieich, HE, Germany). Each qPCR analysis was performed in duplicate (for the six biological replicates) using SensiFast™ SYBR No-ROX reagents (Bioline Reagent, Ltd., London, UK) and a LightCycler 96 system (Roche, Mannheim, BW, Germany).

The threshold cycle (CT) value was automatically determined for each reaction with the analysis software LightCycler 96 (Roche). For each primer pair, standard qPCR analysis was performed to determine the CT values for a 10-fold dilution series of the cDNA template. Calibrated amplification curves were generated to evaluate the PCR efficiency. Specificity of the RT-qPCR reactions was determined by melting curve analysis and sequencing of the amplified products.

The CT values were then converted into relative quantities in comparison to one randomly chosen control sample using the $2^{-\Delta\Delta CT}$ method and corrected by the corresponding PCR efficiency.

The stability of four reference genes, including beta-actin (*ACTB*), succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and transforming growth factor β-stimulated clone 22 domain family member 2 (*TSC22D2*) were determined using the geNorm algorithm [44]. The normalization factor was generated based on the geometric mean of the two most stable endogenous reference genes (*TSC22D2* and *SDHA*).

4.8. Statistical Analysis

Data were analyzed by SPSS Statistics 25 for Windows (IBM Corp., Armonk, NY, USA). The normality of the data was tested by the Schapiro-Wilk method. Data were analyzed by repeated-measures analysis of variance (ANOVA) followed by post hoc comparisons with the Fisher least significant difference (LSD) test. For data that did not follow a normal distribution, the Friedman rank sum test was conducted, followed by the Wilcoxon rank sum test with LSD correction. In all experiments, $p < 0.05$ was considered as significant.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/2/443/s1.

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Abbreviations

HPA	Hypothalamic–pituitary–adrenal
GCs	Glucocorticoids
HPG	Hypothalamic–pituitary–gonadal
ACTH	Adrenocorticotropic hormone
CRH	Corticotropin-releasing hormone
ALI	Air–liquid interface
ALI-POEC	Porcine oviduct epithelial cells grown at the air–liquid interface
GR	Glucocorticoid receptor
TEER	Transepithelial electrical resistance
LDH	Lactate dehydrogenase

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Publication 2

7.2 Regulation of Porcine Oviduct Epithelium Functions via Progesterone and Estradiol Is Influenced by Cortisol

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8 Discussion

The present study explored the individual and interactive effects of cortisol and sex steroid hormones on polarized POEC *in vitro*. Using the ALI-POEC model, physiological concentrations of hormones were employed to examine the response of the oviduct epithelium to a cortisol stimulus as a function of time and interaction with sex steroids. Both divergent and overlapping modifications of the physiological features of POEC were demonstrated in response to individual hormone stimulations (cortisol, E2, and P4). Individual cortisol, representing part of the maternal stress response, modified the oviduct functionality by regulating the barrier function, expression of genes related to hormone signaling and immune response. The oviduct epithelium seems to protect gametes and embryos from the effects of cortisol, as it demonstrates a remarkable ability to metabolize cortisol into its biologically inactive form (cortisone). Additionally, cortisol also affects the signaling of sex steroids, and the disruption of the E2- or P4-dependent regulation of oviduct function potentially influences the oviduct microenvironment of gametes and embryos.

8.1 Mechanisms of cortisol action on the oviduct epithelium

8.1.1 Regulatory effects of the oviduct epithelium on cortisol access to gametes and embryos

Over the past few decades, severe stress and the increased level of cortisol have been widely associated with adverse effects on reproductive activities occurring in the oviduct (Andersen 2002;Einarsson et al. 2008;Liu et al. 2012;Zheng et al. 2016;Zhai et al. 2020;Yuan et al. 2022). Cortisol, a classic stress hormone, is lipid-soluble and assumed to enter target cells freely by simple diffusion through the lipid bilayer of the cellular membrane. Studies have demonstrated that cortisol level increases systemically within a short time period, and the regulatory actions of cortisol have been detected within seconds to a few minutes, indicating that bioactive cortisol in the bloodstream is capable of reaching and acting on the target cells rapidly after the onset of stress (Tasker et al. 2006;Schwartz et al. 2016). As previously discussed, the elevation of cortisol in circulating bloodstream has been frequently used as a biomarker to evaluate stress levels in animal models and determine their correlation with adverse effects on gametes, embryos, and future offspring (Dobson et al. 2003;Maccari et al. 2003;Whirledge and Cidlowski 2017;Zhai et al. 2020). However, emerging evidence has suggested that the sole measurement of cortisol in blood samples may not be sufficient to reflect the local cortisol in targeted tissues and cells, probably due to the reduction of cortisol secretion mediated by the negative feedback of the HPA axis and the tissue-specific cortisol binding and metabolizing capacity (Cohen et al. 2012;Russell et al. 2012;Gong et al. 2015;Lee et al. 2015;Van Den Heuvel et al. 2019). Therefore, measurement of cortisol level in target tissues, biological fluid,

and local blood vessels will assist in developing a better understanding of cortisol action on target tissues and cells.

Oviduct fluid plays a critical role in the maturation of spermatozoa and oocytes, and development of early embryos. Recent studies have shown that cortisol is present in oviduct fluid and may affect reproductive processes (Andersen 2002;Lamy et al. 2016;Teteau et al. 2022). As discussed previously, steroid hormones in the oviduct fluid may have several origins, including the local vascular transport towards the oviduct (Hunter et al. 2007;Hunter 2012;Nelis et al. 2015). It has been suggested years ago that the concentrations of cortisol do not differ in peripheral blood (a prominent ear vein) and the ovarian arterioles supplying blood to left and right isthmus regions of the oviduct in pigs shortly before ovulation (Hunter et al. 1983). Comparable levels of cortisol have been detected in blood samples collected from ovarian (a localized blood vessel in the oviduct) and jugular (a peripheral blood vessel) veins of cows with superovulation (Acosta et al. 2005). The averaged baseline level of cortisol in peripheral blood (jugular vein) is approximately 2-8 ng/mL in cows (Nakao et al. 1994;Hopster et al. 1999). Under the extreme stress of slaughtering, the average cortisol levels are approximately 32 and 49 ng/mL in ipsilateral and contralateral (to the corpus luteum) oviduct fluid, whereas cortisol level in the peripheral blood can reach 93 ng/mL (Dunn 1990;Lamy et al. 2016).

With the application of ALI culture system, the polarized POEC allow the separation of two compartments, including apical compartment that is equivalent to the oviduct fluid and basal compartment that is equivalent to the sub-epithelial oviductal tissue and blood supply to the oviduct. In our study, two levels of cortisol reflecting moderate and severe stress were basolaterally administrated to the polarized porcine oviduct epithelium *in vitro*, and the stimulation media were refreshed every 12 h. On day (d) 21 (12 h after the last cortisol administration), the cortisol measurement showed an evident concentration gradient of cortisol (basal compartment > apical compartment). In line with this, previous report in sheep has shown that the level of cortisol in peripheral blood (16-26 ng/mL) is higher than in oviduct fluid (< 2 ng/mL) (Teteau et al. 2022). When mice exposed to 2 d of restraint stress, cortisol levels increased approximately five-fold in the blood but only two-fold in the oviduct tissue (Zheng et al. 2016). Even though there is limited number of reports on the cortisol gradient between the blood and oviduct, it is notable that the fold changes of cortisol level in peripheral blood and oviduct was not the same. Considering that the blood cortisol has to cross a series of cellular barriers within the oviduct tissue, these findings may suggest that *in vivo* and *in vitro* oviduct tissue could function as a barrier to maintain the cortisol gradient between the oviduct fluid and blood.

To the best of our knowledge, the cortisol gradient in microenvironment of oviduct (oviduct fluid, oviduct tissue, and neighboring blood) are likely attributed to the hypothetical passive diffusion

of protein-free cortisol, as most of cortisol in the blood is protein-bound, and only protein-free cortisol can freely diffuse across cell membranes (Mcmanus et al. 2019). In rats upon forced-swim stress, the rapid increase of corticosteroid-binding globulin (CBG, a binding protein of cortisol) in the peripheral blood of the jugular vein may correlate with the delayed (20-30 min) rise of free GC in the subcutaneous tissue and brain as compared with the earlier increase of total GC in peripheral blood (Qian et al. 2011). In addition, the data in our study demonstrated that the majority of bioactive cortisol was transformed into inactive metabolite cortisone, suggesting the ALI-POEC has massive cortisol metabolizing capacity even after 21 d of repeated high-dose cortisol stimulation. In line with this finding, the gene expression of HSD11B2 in ALI-POEC responsible for transforming cortisol into cortisone was also increased in response to long-term cortisol stimulation. Together, the data may indicate that the porcine oviduct epithelium *in vivo* is able to regulate the hormonal environment within the oviduct lumen even under stressed conditions due to its metabolizing capacity.

8.1.2 Cortisol action on the oviduct epithelium through GR binding

In response to various stressors, the classical genomic effects of cortisol are mainly activated by binding to GR in target cells (Timmermans et al. 2019). The divergent effects of cortisol in different tissues and cells are determined by many factors, including the cellular bioavailability of cortisol, number and modification of GR, binding sites of the DNA response elements, and activity of chaperones and TFs, as recently reviewed (Sevilla et al. 2021). NR3C1 transcription has been extensively used as a biomarker in the context of stress responses (Lee et al. 2010;Mourtzi et al. 2021). However, subsequent studies have reported conflicting results with regard to the effects of stress on NR3C1 expression in different brain regions of rodents, which was either unaffected (Mizoguchi et al. 2003;Raone et al. 2007;Gray et al. 2014), increased (Bourke et al. 2013;Li et al. 2015), or decreased (Raone et al. 2007;Mifsud et al. 2017) under various stressful events. In rats, four weeks of chronic stress induced by water immersion and restraint led to decreased and increased expression of NR3C1 gene in the prefrontal cortex and hippocampus, respectively (Mizoguchi et al. 2003). However, expression of NR3C1 gene in the hippocampus, hypothalamus, and blood cells of mice were reduced upon four weeks of GC administration (Lee et al. 2010). Previous studies have suggested that there is a tight association between high DNA methylation of NR3C1 gene and reduced NR3C1 mRNA and protein expression (Weaver et al. 2004;Mcgowan et al. 2009;Gatta et al. 2021). Supposedly, the reduced expression of NR3C1 gene, at least in part, could impair the sensitivity of negative feedback of the HPA axis, which leads to delayed cortisol restoration in humans exposed to social stress (Van Der Knaap et al. 2015). In our study, the polarized POEC were exposed to short-, middle-, and long-term cortisol stimulation. The transcriptional reduction of NR3C1 and subtype NR3C1α were exclusively induced by long-term (21 d) cortisol exposure, suggesting

that overall GR activity may be reduced under long-term stressful conditions. The disruption of corticosterone recovery in rodents due to 10 d of physical stress or corticosterone injection has been reported to decrease the protein level of GR in specific regions of intestinal epithelium, leading to impairment of the epithelial tight junctions (Zheng et al. 2013). In our study, the cortisol-induced regulation of transepithelial electrical resistance (TEER, a parameter related to tight junction properties) was correlated with the duration of cortisol exposure. Specifically, middle-term (72 h) cortisol exposure led to a minor reduction in TEER, while long-term (21 d) treatment resulted in a slight increase in TEER, replicating previously published reports in intestinal and lung epithelial cells when chronically exposed to GC (Fischer et al. 2014;Kielgast et al. 2016;Zheng et al. 2017). As reported previously, tight junctions are also involved in the paracellular passage of ions and water, thereby affecting the molecule exchange driven by the electrical gradient across the epithelium (Li et al. 2004;Sassi et al. 2020). Transepithelial potential difference (TEPD) between apical and basal compartments of the ALI-POEC probably resulted from overall transport of ions, including Na^+, K^+, and Cl^- across the epithelium (Tran et al. 2013;Saw et al. 2022). The present data showed that the TEPD of POEC was modified in response to cortisol exposure, which is consistent with previous publications that GC agonists modulate the ionic channel activity in various epithelia (Sayegh et al. 1999;Mansley et al. 2016;Ivy et al. 2019;Ahsan et al. 2020). Along with controlling ion transport, oviduct epithelial cells also produce many secretory glycoproteins, contributing to the proper maturation of gametes and development of early embryos. For instance, the beneficial effects of oviductal glycoprotein 1 (OVGP1) on the fertilization process and early embryo development have been reviewed recently (Zhao et al. 2022). Our current data show stress hormone cortisol leads to decreased mRNA abundance of OVGP1, which is in line with a report in 2D-cultured POEC (An et al. 2022). In addition, mRNA expression of IL6, an immune response regulator involved in oocyte maturation, embryonic development, and implantation, was suppressed by cortisol exposure in our study (Robertson et al. 2010;Yang et al. 2020). Taken together, prolonged cortisol stimulation did affect the regulation of the oviduct microenvironment (barrier function, ionic transport, and expression of genes involved in secretory activity and immune response), which may alter the suitability of this microenvironment for gametes and embryos and lead to reduced fertility and reproduction failure.

In general, our current data suggest that cortisol exposure, reflecting the long-term severe stress *in vivo*, did not induce substantial damage to oviduct epithelial cells compared to the decreased weight and activated apoptosis of oviductal cells published in other reports (Zheng et al. 2016;Divyashree and Yajurvedi 2018;An et al. 2022). The first and simplest explanation for these findings is that different species and experimental designs were used, leading to mild, moderate or strong responses to various stressful stimuli. Second, the transcriptional and

translational reduction of GR has been regarded as an adaptive mechanism against the damaging effects of chronic stress (Spies et al. 2021). In accordance with this, our data show that chronic (21 d) exposure to cortisol suppressed transcriptions of NR3C1 and NR3C1α, which indicates that the sensitivity and reaction of ALI-POEC might decline due to chronic stress exposure. Moreover, the majority of applied cortisol was metabolized and transformed into cortisone by induction of HSD11B2 gene, limiting the cortisol bioavailability in POEC. In addition, the co-chaperone FKBP prolyl isomerase 5 (FKBP5, encoded by FKBP5 gene) has inhibitory effect on GR signal transduction, which depends on the activation of GR itself and is a negative intracellular feedback loop (Zannas et al. 2016). In our study, the expression of FKBP5 gene in ALI-POEC was significantly increased by cortisol exposure, indicating the corresponding GR-dependent expression of genes could be inhibited, as FKBP5 protein can reduce GR binding affinity and delay the nuclear translocation of GC-GR ligand (Wochnik et al. 2005). Hence, prolonged cortisol exposure seems to modulate the responsiveness of ALI-POEC through reduced cellular access to bioactive GR and cortisol along with delayed nuclear translocation of GR. Undoubtedly, further studies are required to dissect the underlying mechanisms governing the sensitivity and response of POEC to elevated cortisol under stressful situations.

8.1.3 Cortisol action on the oviduct epithelium by affecting E2 and P4 signaling

Ovarian steroid hormones (i.e., E2 and P4) have been considered as the driving forces of functional regulation of the oviduct epithelium through corresponding NRs. Studies have already shown that GR, ESR and PGR (all members of the NR superfamily) are composed of three similar and conserved structural domains that function as ligand-dependent TFs regulating diverse functions in target cells. Canonically, the transcriptional regulation of NR-responsive genes is directly activated when NRs bind to specific DNA response elements or indirectly when they bind to other TFs. Numerous studies have explored the signaling pathway of individual NRs, as they have diverse effects on physiological processes and more than 15% of commercial drugs are intended to bind to the NRs superfamily (Santos et al. 2017). Emerging evidence suggests that cross-talk among NR signaling has been reported, and the preconceived regulatory effects of individual NRs can be challenged by unknown signaling interactions (Ogawa et al. 2005;De Bosscher et al. 2020). Estrogen receptor alpha (encoded by the ESR1 gene) is the major subtype of ESR in the oviduct. Activation of the GR pathway has an antagonistic effect on ESR1-mediated growth and differentiation in the uterus (Rhen et al. 2003). Conversely, activation of the ESR1 pathway blocks the GR-dependent repression of the inflammatory response in human cancer cells (Cvoro et al. 2011;Vahrenkamp et al. 2018).

In our current study, the interplay between cortisol and sex steroids, essential for regulating oviduct function, was explored and assessed at various functional parameters of POEC.

Overall, the E2- and P4-induced functional variations at the phenotypic and molecular levels were partially antagonized or augmented by concurrent treatment with cortisol. Regarding POEC morphology, exposure to cortisol alone had no effect on cellular height, while the height-reduction induced by P4 was attenuated when cells were co-treated with cortisol probably due to the competitive binding of cortisol to PGR, which is concordant with previous findings in mammary gland and placenta cells (Karalis et al. 1996;Patel and Challis 2002;Leehy et al. 2016;Ruiz et al. 2020). However, the addition of cortisol to P4 also reinforced the P4-promoted expression of FKBP5, TSC22D3, and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A) genes, exhibiting P4-like effects, which coincides with previous findings (Haslam et al. 1981;Leo et al. 2004;Buser et al. 2011). On the other hand, cortisol alone and in conjunction with P4 evidently decreased mRNA expression of PGR, which may lead to the inhibition of PGR signaling, as P4-induced effects are mediated through the induction of PGR expression (Diep et al. 2016). Similar to FKBP5-induced negative feedback of GR signaling, increased mRNA and protein expression of FKBP5 have shown inhibitory effects on PGR activity and P4 actions in human endometrial cells (Guzeloglu Kayisli et al. 2015;Schatz et al. 2015;Guzeloglu-Kayisli et al. 2021). Hence, cortisol-induced mRNA reduction of PGR and FKBP5 may reduce the P4 effects on ALI-POEC. Collectively, our current results reveal that cortisol interplays with P4 action on oviduct epithelial cells at the phenotypic and genomic levels.

Previously, cross-talk between E2 and cortisol in reproductive tissues has been reported in the uterus of humans and rodents, and cortisol showed opposing effects on E2 actions at both phenotypic and molecular levels (Rabin et al. 1990;Rhen and Cidlowski 2006;Whirledge et al. 2013). In line with this, the current data show that addition of cortisol to E2 blocked E2-responsive mRNA reduction of PPARGC1A and sodium channel epithelial 1 subunit alpha (SCNN1A) genes in POEC. Apparently, further investigation is still required to unravel the underlying mechanism in oviduct epithelial cells, as the E2 pathway is essential for reproduction. Previously, the studies on the interactive cross-talk between cortisol and E2 have emerged in cancer cells because GC, frequently used as an adjuvant therapy against the side effects of chemotherapy in ESR1-positive breast cancer, was found to promote cancer progression and metastasis in women (Yager and Liehr 1996;Yager and Davidson 2006;Obradovic et al. 2019;Mayayo-Peralta et al. 2021). There are several possible pathways by which cortisol can interfere with E2 signaling: cortisol reduces free E2 level in blood by increased estrogen sulfation, GR limits the access of ESR and its coactivator to specific DNA binding sites, and ligand bound GR suppresses the association between ESR and chromatin (Gong et al. 2008;Karmakar et al. 2013;Miranda et al. 2013;Truong and Lange 2018;Tonsing-Carter et al. 2019;Paakinaho and Palvimo 2021;Butz and Patocs 2022). In mice, it was shown

that GR protein is essential for GC-induced inhibitory effects on E2-mediated increase of uterine weight and expression of target genes (Gong et al. 2008;Wang et al. 2016). Although the mRNA expression of only few investigated genes indicated the interaction of cortisol and E2, the data show that individual E2 or along with cortisol significantly reduced the expression of NR3C1 gene, which may disturb the GR-dependent suppressive effect of cortisol on E2. Additionally, our data suggest that cortisol has additive effects on mRNA abundance of E2-responsive genes, promoting FKBP5 and suppressing IL6, in line with previous observations that GR has notable overlapping DNA binding regions with ESR-responsive genes (Cvoro et al. 2011;West et al. 2016). Previous reports in cancer cells have shown that GC and E2 inhibit gene expression of a subset of immune regulators (e.g. IL6 and CXCL8) through respective activation of GR and ESR (Cvoro et al. 2008;Cvoro et al. 2011). In contrast, mRNA expression of IL6 but not CXCL8 was suppressed by treatment with individual cortisol or E2 in our study. Our current data showed that E2 alone did not induce the expression of ESR1, but of G protein-coupled estrogen receptor 1 (GPER), a plasma membrane receptor of E2 that plays a key role in E2 actions (Pietras and Szego 1977;Vivacqua et al. 2006;Arnal et al. 2017). The activation of GPER, previously known as GPR30, could modify the classical nuclear ESR signaling in some cases, as previously reviewed (Prossnitz and Barton 2009;Prossnitz and Maggiolini 2009;Maggiolini and Picard 2010;Luo and Liu 2020)). Therefore, known antagonistic or agonistic effects of cortisol on E2 in different tissues and cells might be altered when co-activation of GPER occurs. Even though there were only limited interactions between cortisol and E2/P4 in our cell model, the current data suggest that cortisol affects E2 and P4 signaling in the oviduct epithelium in varying pathways, which could result in aberrant responsiveness of oviduct epithelium to fluctuating sex steroids.

8.2 Comparison of E2- and P4-mediated actions on the oviduct epithelium

E2 and P4 have been recognized as two pivotal regulators of oviduct function via the activation of corresponding receptor pathways. The level of E2 rises in follicular phase of the estrous cycle, reaches the peak shortly before ovulation, starts to decline after ovulation, and remains at the baseline level during the luteal phase. In contrast, the circulating P4 in blood is at baseline level in the follicular phase and remains significantly higher during the luteal phase. The roles of E2 and P4 in oviduct functionality have been recently reviewed, demonstrating that both hormones are essential and indispensable, and they mediate the oviduct epithelium collaboratively (Bhatt et al. 2004;Lee et al. 2012;Barton et al. 2020;Mcglade et al. 2022;Slayden et al. 2022).

8.2.1 E2- and P4-mediated morphological remodelling of the oviduct epithelium

The morphological and ultrastructural regulation of the oviduct epithelium has been linked to varying levels of E2 and P4 in several species (Binelli et al. 2018;Barton et al. 2020;Slayden et al. 2022). In the present study, high levels of E2 and P4 corresponding to the follicular and luteal phase of the porcine estrous cycle were applied individually to compare E2- and P4-induced actions on POEC. Our current data show that the primary POEC form a polarized monolayer with appropriate differentiation (columnar shape, dense apical cilia, and cytoplasmic protrusions). In comparison with vehicle control, the ultrastructure of POEC was measurably affected only by 72 h of treatment with E2 or P4, as indicated by histological investigations.

In the current study, 72 h of treatment with E2 led to significant increase in the average epithelial height and proportion of ciliated cells. These E2-induced alterations of the ultrastructure and cell population of POEC are in line with previous studies indicating that E2 stimulates the process of differentiation in oviduct epithelial cells (Abe and Oikawa 1993a;Comer et al. 1998;Winuthayanon et al. 2010;Eddie et al. 2015). However, there was no morphological alteration in our previous work, which is likely due to the divergent treatment strategies and distinct activation of E2 receptors (Chen et al. 2013). It is speculated that the activation of GPER instead of ESR1 may lead to E2-dependent differentiation of polarized POEC, which is in agreement with previous findings that ESR1, the dominating subtype of E2 receptor in the oviduct, is not essential for differentiation and ciliogenesis of the oviduct epithelial cells (Wang et al. 2000;Okada et al. 2004;Chen et al. 2013;Chen et al. 2018;Zhu et al. 2019). Further extensive research should be performed to elucidate E2-dependent signaling pathways in oviduct epithelial cells. Taken together, our findings indicate that E2-induced actions on the oviduct epithelium are probably determined by the activation of corresponding E2 receptors, and the precise E2-induced subtype-specific physiological function on the oviduct epithelium requires further investigations. In contrast to E2, P4 led to reductions of both epithelial height and the ratio of ciliated cells in polarized POEC when compared to vehicle control, which correlates with recent reviews that P4 resulted in the degeneration of ciliated cells in oviduct epithelium (Li and Winuthayanon 2017;Mcglade et al. 2022;Slayden et al. 2022). Furthermore, the decrease of epithelial height and the ciliated POEC subpopulation have been observed in the current and previous studies, and are irrelative to the concentration of P4 and presence of E2, suggesting that P4 may be the major driver of morphological remodelling of porcine oviduct epithelium (Chen et al. 2013;Chen et al. 2018). Additionally, studies have suggested that estrogen receptor β (encoded by ESR2 gene) is expressed in the oviduct epithelium and has a distinct impact on the regulation of oviduct epithelium in comparison with estrogen receptor α (Ulbrich et al. 2003;Shao et al. 2007). Hence, replication of such findings

and an in-depth molecular examination of the hormone-specific actions are required to gain insight into the mediating effects of steroid hormones on the morphology of the oviduct epithelium.

8.2.2 E2- and P4-mediated secretory and bioelectric properties of the oviduct epithelium

As the first maternal environment for gametes and early embryos, dynamic regulations of the oviduct microenvironment occurs during the estrous cycle, including the production and composition of oviduct fluid. It is widely accepted that oviduct fluid is mainly composed of secretions by oviduct epithelial cells and transudate from sub-epithelial cells and blood vessels, providing an optimal environment for every stage of the developing gametes and early embryos (reviews in (Leese 1988;Ferraz et al. 2017;Saint-Dizier et al. 2019)). As a polarized epithelial layer, the fluid formation in the luminal cavity of oviduct epithelium largely depends on the bidirectional transport (secretion and absorption through either paracellular or transcellular pathways) of ions and fluid (reviews in (Leese 1988;Leese et al. 2001;Ferre-Dolcet and Rivera Del Alamo 2023)). The active or passive transcellular routes of ions and fluid are mainly governed by the apical- and basolateral-specific expressed transmembrane pumps, channels, and exchangers, which maintain the homeostatic electrochemical gradient across the epithelium. In contrast, the paracellular transport is passively driven by the electrochemical gradient, or selectively modulated by the molecular components of the junctional complex (tight and adhesion junctions) between epithelial cells (Anderson 2001;Leese et al. 2001;Tang and Goodenough 2003;Mirihagalle et al. 2022;Ferre-Dolcet and Rivera Del Alamo 2023).

Using a similar ALI model of bovine oviduct epithelial cells, researchers have reported that individual and combined steroid hormones (E2, P4, and testosterone) modelling the physiological and pathophysiological conditions *in vivo* affect the amino acid content of the *in vitro*-derived bovine oviduct fluid (Simintiras et al. 2016;Simintiras and Sturmey 2017). In our study, we assessed the amount of oviduct fluid and the regulators affecting its formation, without analysing its content. Clearly, exposure to E2 significantly increased the amount of oviduct fluid, which is in line with reports stating that E2 has a stimulating effect on the secretion and composition of oviduct fluid (Mcdonald and Bellve 1969;Roberts et al. 1975;Gott et al. 1988;Saint-Dizier et al. 2019). Surprisingly, there were no statistical variations in the amount of oviduct fluid in our current study when POEC was exposed to physiologically peak level of P4, which is inconsistent with the well-known suppressive effect of P4 on oviduct fluid secretion (Leese et al. 2001;Barton et al. 2020). Undoubtedly, various factors affect the fluid formation in the luminal cavity of the FGT as previously reviewed (Leese 1988;Leese et al. 2001;Saint-Dizier et al. 2019). However, to our best of knowledge, the indicative knowledge of the P4-

dependent reduced secretion of oviduct fluid is mainly due to early *in vivo* studies, in which the secretion of oviduct fluid is reduced in the P4-dominant phase of animals (Mcdonald and Bellve 1969;Kavanaugh et al. 1992;Wiseman et al. 1992). These studies, however, could not disentangle the individual effects of E2 and P4. Furthermore, possibilities of contaminations (dead cells, secretion of uterine and stroma cells) and inflammation due to the invasive sampling methods may have existed, which could confound the results as reviewed recently (Leese et al. 2001;Leese et al. 2008;Ballester et al. 2014;Aguilar and Reyley 2018). Compared to previous *in situ* and *in vitro* studies, the ALI-POEC model pinpoints the effects of oviduct epithelium on the formation and regulation of oviduct fluid. Thus, the results of the current study provide valuable information that ovarian steroid hormones regulate formation of oviduct fluid, and further studies are required to examine the underlying molecular mechanisms.

In permeable mucosa containing endothelium and epithelium, TEER is a well-accepted indicator to assess intercellular tight junctions and responsive to treatment with steroid hormones (Wilson et al. 2008;Chen et al. 2015;Zihni et al. 2016;Van Der Giessen et al. 2019). In our study, E2 stimulation led to a significant decrease and P4 caused a slight increase in TEER, which is inconsistent with a similar bovine study where individual E2 and P4 did not affect TEER (Simintiras et al. 2016). The divergent results are likely due to differences in cell population and differentiation status of the cultured cells, since different types of oviduct epithelial cells can respond to steroids distinctly (Binelli et al. 2018;Barton et al. 2020;Mcglade et al. 2020). The ALI-POEC used in the current project seems to be composed of differentiated ciliated and secretory cells, but also stem cell marker genes (KLF transcription factor 4 (KLF4) and Nanog homeobox (NANOG)) are still expressed and no fibroblasts are observable in the ALI culture system (Chen et al. 2018). In contrast, the *in vitro*-reproduced bovine oviduct epithelium is made up of oviduct epithelial cells, as well as fibroblast cells (less than 5%) (Simintiras et al. 2016). The second notable factor is the hormonal treatment regime, including divergent steroid concentrations and treatment duration, which could result in differential response of oviduct epithelia as reported previously (Chen et al. 2013;Simintiras et al. 2016;Simintiras and Sturmey 2017;Chen et al. 2018). Despite the aberrant observations related to hormonal effects on TEER in our POEC and earlier bovine study, a negative correlation between a reduced TEER and an increased amount of oviduct fluid was observed (Simintiras et al. 2016). In our study, TEER was notably decreased by exposure to E2, which was likewise negatively correlated with the E2-induced increase in the oviduct fluid. Several E2- and P4-dependent pathways are involved in the regulation of oviduct fluid, including the cellular population of oviduct epithelium, blood flow to the oviduct, and ion transport and expression of water channels (Leese et al. 2001;Leese et al. 2008;Saint-Dizier et al. 2019). Correlative connection between altered TEER and modification of paracellular permeability

has been reported in other polarized epithelia (Zheng et al. 2013;Srinivasan et al. 2015;Zong et al. 2019); hence, our data suggests that E2-induced increase of oviduct fluid might be, at least in part, attributed to the increased paracellular permeability of porcine oviduct epithelium.

Apart from the regulatory effects on oviduct fluid secretion, ovarian steroid hormones modulate the ion composition of oviduct fluid (Hugentobler et al. 2007;Leese et al. 2008;Hugentobler et al. 2010). In secretory epithelia, fluid movement is normally driven by the force of electrochemical gradient across the epithelium (Leese et al. 2001;Skowronski et al. 2011). As discussed in chapter 8.1.2, TEPD results from transepithelial transport of ions and is frequently used as an indicator of the overall gradient of electrochemical potential. In this regard, our current data demonstrated that treatment with E2 or P4 alone provoked obvious variations in TEPD, suggesting that ion flux across POEC was potentially altered. Along with this, the mRNA abundance of serum- and glucocorticoid-regulated kinase 1 (SGK1) of POEC was increased in the E2- and P4-treated groups (12 h and 72 h), which is in line with reports that SGK1 is a transcriptional target of steroid hormones (Itani et al. 2002;Godbole et al. 2018;Wu et al. 2022). SGK1 is a well-defined regulator of ENaC (encoded by SCNN1A gene) activity, and the co-regulated expression of SGK1 and SCNN1A genes upon steroid hormones in our study correlates with previous findings that SGK1 and ENaC play important roles in hormone-regulated Na^+ transport (Pearce 2001;Faletti et al. 2002;Pearce 2003). In addition, the mRNA expression of Na^+-K^+-ATPase, a pump of Na^+ and K^+ and encoded by ATP1A1 gene, was only downregulated by P4 treatment (Chinigarzadeh et al. 2015;Zhang et al. 2019b). Collectively, we could propose that ovarian steroid hormones are involved in the regulation of fluid amount and ionic contents at least partially via modulating tight junction properties and expression of ionic channels.

9 Limitations and outlook

Overall, the findings of the current study provide valuable information to understand the effects of stress hormone cortisol on the oviduct epithelium. However, caution should be exercised in their interpretation, as the results were obtained on only one research model and one species. Undoubtedly, numerous factors affect hormonal actions *in vivo*, and the current *in vitro* models are unable to reproduce the complex *in vivo* situation. For this reason, other research models and species are definitely required to investigate and verify the biological effects of steroid hormones on oviduct epithelial cells. For instance, in this study, the first limitation of the steroid stimulation model is that the administered steroid hormones in the ALI-POEC system lack their respective specific binding proteins. *In vivo*, the majority of steroid hormones in the bloodstream are not free but bound to general (albumin) or specific (globulin) binding proteins, and only a small fraction of unbound steroid hormones are presumed to freely circulate in the bloodstream and enter the target cells through free diffusion. Besides, circadian rhythmicity of mammals should be taken into consideration in further experiments, as the production of GC is directly under the control of circadian rhythmicity and stressful stimuli. In addition, cortisol-mediated rhythmic oscillations of the central nervous system and the FGT are critical to ensure the success of reproduction. Therefore, *in vitro* models including specific binding proteins (e.g. CBG) and daily fluctuations of steroids (via perfusion or microfluidic devices) could more closely resemble the physiological condition of steroids *in vivo*. As noted previously, the physiological function and cellular composition of the oviduct vary notably between the isthmus and ampulla regions. Therefore, the second limitation of this study is that only primary epithelial cells isolated from whole oviducts are employed for ALI culture. Hence, the complete region- and cell-type-specific separation of cells is of interest for further studies to explore the precise responsiveness of oviduct epithelial cells to steroid hormones. In addition, recent studies have highlighted that autocrine or paracrine factors, such as signal from the underlying stromal cells, are also involved in the steroid hormone-mediated regulation of oviduct epithelial cells, which suggests that co-culture of epithelial-stromal cells is required to provide new compelling evidence on this subject.

10 Summary

In both humans and animals, severe maternal stress and elevation of the stress hormone cortisol have been linked to reduced fertility. As a pivotal reproductive organ, the oviduct provides a suitable microenvironment for the maturation of gametes and early embryonic development. The early embryo is especially vulnerable to maternal health problems, including stress. Although many studies have demonstrated that reproductive function is impaired at multiple levels due to severe or prolonged maternal stress-activated HPA axis, the direct and local actions of the stress hormone cortisol on the oviduct epithelium are not yet fully understood. The aim of this study was to explore the potential effects of cortisol on the oviduct epithelium.

First, porcine primary oviduct epithelial cells were cultured and differentiated using the ALI culture model to replicate the native oviduct epithelium *in vivo*. To investigate the effect of long-term stress on the oviduct epithelium, physiological levels of cortisol representing moderate and severe stress in pigs were administered basolaterally for long-term (21 d). The expression of GR pathway-related genes in POEC was activated in response to cortisol treatment. Even though long-term exposure to cortisol had no effect on the overall morphology of the oviduct epithelium, the barrier function and mRNA expression of genes regulating oviduct function and immune response were modified by cortisol. Additionally, the *in vitro* oviduct epithelium constantly metabolized cortisol to biologically inactive cortisone, suggesting that the oviduct epithelium is able to modulate the hormonal environment of the oviduct even under the condition of chronic repeated stress.

Second, individual (cortisol, E2, P4) or combined (cortisol/E2, cortisol/P4) hormone stimulation was applied for 12 h and 72 h, and the morphological, bioelectrical, and transcriptional profile of the cultures were assessed to explore the specific effects of ovarian steroid hormones (i.e., E2 and P4) and the hormonal interactions between cortisol and ovarian steroid hormones. The results suggest that individual E2 and P4 have primary and complex effects on the function of the oviduct epithelium, and P4 is one of the driving force to regulate the morphological modification. In addition, E2 and P4 induced transcriptional regulation of genes involved in cortisol signaling (NR3C1, FKBP5, and TSC22D3), implying that ovarian steroid hormones affect cortisol action on oviduct epithelial cells. Cortisol, in turn, not only directly induces changes in the bioelectrical properties and gene expression of oviduct epithelial cells, but also appears to affect the response of cells to ovarian steroid hormones at the morphological, functional, and gene expression levels by altering the corresponding receptor-dependent signal transduction.

In summary, the results of the present study in pigs suggest that the oviduct epithelium is capable of regulating the hormonal environment of the oviduct and limiting the luminal accumulation of stress hormone cortisol under stressed conditions. The cortisol exposure not only affected the function of the oviduct epithelium directly but also modified the E2- and P4-induced regulation of oviduct epithelium functions. This implies indirect effects on the dynamic control of the oviductal microenvironment, which is essential for proper gamete maturation and especially early embryonic development. Thus, perturbation of the fine-tuned interplay between cortisol and sex steroids in the regulation of oviduct epithelium functions may be one of the mechanism by which an elevated maternal cortisol level contributes to the stress-induced impairment of fertility in pigs.

11 Zusammenfassung

Beeinträchtigung der Fruchtbarkeit durch maternalen Stress: Auswirkungen von Cortisol auf die Funktionen des Eileiterepithels

Sowohl bei Menschen als auch bei Tieren wurden schwerer mütterlicher Stress und ein erhöhter Spiegel des Stresshormons Cortisol mit einer verminderten Fruchtbarkeit in Verbindung gebracht. Als zentrales Fortpflanzungsorgan bietet der Eileiter eine geeignete Mikroumgebung für die Reifung der Keimzellen und die frühe Embryonalentwicklung. Der frühe Embryo ist besonders anfällig für mütterliche Gesundheitsprobleme, einschließlich Stress. Obwohl in vielen Studien nachgewiesen wurde, dass die Fortpflanzungsfunktion des Eileiters aufgrund der durch mütterlichen Stress aktivierten HPA-Achse auf mehreren Ebenen beeinträchtigt ist, sind die direkten und lokalen Auswirkungen des Stresshormons Cortisol auf das Eileiterepithel noch nicht vollständig geklärt. Ziel dieser Studie war es, die möglichen Auswirkungen von Cortisol auf das Eileiterepithel zu untersuchen.

Zunächst wurden primäre Eileiterepithelzellen vom Schwein kultiviert und mit Hilfe des ALI-Kulturmodells differenziert, um das native Eileiterepithel *in vivo* zu replizieren. Um die Auswirkungen von Langzeitstress auf das Eileiterepithel zu untersuchen, wurden physiologische Cortisolkonzentrationen, die mäßigen und schweren Stress bei Schweinen repräsentieren, basolateral über einen längeren Zeitraum (21 Tage) verabreicht. Die Expression von Genen, die mit dem GR-Signalweg zusammenhängen, wurde in den POEC als Reaktion auf die Cortisolbehandlung aktiviert. Obwohl die Langzeitexposition mit Cortisol keine Auswirkungen auf die Gesamtmorphologie des Eileiterepithels hatte, wurden die Barrierefunktion und die mRNA-Expression von Genen, die die Funktion des Eileiters und die Immunantwort regulieren, durch Cortisol verändert. Darüber hinaus wurde Cortisol im In-vitro-Epithel des Eileiters ständig in biologisch inaktives Cortison umgewandelt, was darauf hindeutet, dass das Epithel des Eileiters in der Lage ist, das hormonelle Umfeld des Eileiters selbst unter der Bedingung chronischer, wiederholter Belastung zu modulieren.

Anschließend wurde eine individuelle (Cortisol, E2, P4) oder kombinierte (Cortisol/E2, Cortisol/P4) Hormonstimulation für 12 und 72 Stunden durchgeführt und das morphologische, bioelektrische und transkriptionelle Profil der Kulturen untersucht, um die spezifischen Auswirkungen der ovariellen Steroidhormone (E2 und P4) und die hormonellen Wechselwirkungen zwischen Cortisol und ovariellen Steroidhormonen zu untersuchen. Die Ergebnisse deuten darauf hin, dass E2 und P4 grundlegende und komplexe Auswirkungen auf die Funktion des Eileiterepithels haben, wobei P4 die treibende Kraft für die Regulierung der morphologischen Veränderungen sein könnte. Darüber hinaus regulierten E2 und P4 die Transkription von Genen, die an der Cortisol-Signalübertragung beteiligt sind (NR3C1, FKBP5 und TSC22D3), was darauf hindeutet, dass ovarielle Steroidhormone die Wirkung von Cortisol

auf die Epithelzellen des Eileiters beeinflussen. Cortisol wiederum induziert nicht nur direkt Veränderungen der bioelektrischen Eigenschaften und der Genexpression von Eileiterepithelzellen, sondern scheint auch die Reaktion der Zellen auf ovarielle Steroidhormone auf morphologischer, funktionaler und Genexpressionsebene durch Veränderung der entsprechenden rezeptorabhängigen Signaltransduktion zu beeinflussen.

Zusammenfassend deuten die Ergebnisse der vorliegenden Studie darauf hin, dass das Eileiterepithel in der Lage ist, das hormonelle Millieu des Eileiters zu regulieren und die luminale Akkumulation des Stresshormons Cortisol unter Stressbedingungen zu begrenzen. Die Cortisol-Exposition beeinflusste nicht nur direkt die Funktion des Eileiterepithels, sondern modifizierte auch die E2- und P4-induzierte Regulation dieser Funktionen. Dies impliziert indirekte Auswirkungen auf die dynamische Kontrolle der Mikroumgebung des Eileiters, die für die optimale Reifung der Gameten und insbesondere für die frühe Embryonalentwicklung von wesentlicher Bedeutung ist. Somit könnte eine Störung des fein abgestimmten Zusammenspiels zwischen Cortisol und Sexualsteroiden bei der Regulierung der Funktionen des Eileiterepithels einer der Mechanismen sein, durch den ein erhöhter mütterlicher Cortisolspiegel zur stressbedingten Beeinträchtigung der Fruchtbarkeit beiträgt.

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13 List of publications

13.1 First authorship publications

Du S, Trakooljul N, Palma-Vera S, Murani E, Schuler G, Schoen J, Chen S. Regulation of Porcine Oviduct Epithelium Functions via Progesterone and Estradiol Is Influenced by Cortisol. Endocrinology, 2023, 164(1).

Du S, Trakooljul N, Schoen J, Chen S. Does Maternal Stress Affect the Early Embryonic Microenvironment? Impact of Long-Term Cortisol Stimulation on the Oviduct Epithelium. Int J Mol Sci, 2020, 21(2):443.

Du S, Zhao G, Shao J, Fang Y, Tian G, Zhang L, Wang R, Wang H, Qi M, Yu S. *Cryptosporidium* spp., *Giardia intestinalis*, and *Enterocytozoon bieneusi* in Captive Non-Human Primates in Qinling Mountains. Korean J Parasitol. 2015,53(4):395-402. **Work performed before Ph.D. study (College of Veterinary Medicine, Northwest A&F University, Yangling, China)*

13.2 Co-authorship publications

Zhang X, Zhang F, Li F, Hou J, Zheng W, **Du S**, Zhao Q, Zhu X. The presence of *Giardia intestinalis* in donkeys, *Equus asinus*, in China. Parasit Vectors, 2017,10(1):3. **Work performed before Ph.D. study (Qilu Animal Health Products Co., Ltd., Jinan, China)*

Zhao G, **Du S**, Wang H, Hu X, Deng M, Yu S, Zhang L, Zhu X. First report of zoonotic *Cryptosporidium* spp., *Giardia intestinalis* and *Enterocytozoon bieneusi* in golden takins (*Budorcas taxicolor bedfordi*). Infect Genet Evol. 2015, 34:394-401. **Work performed before Ph.D. study (College of Veterinary Medicine, Northwest A&F University, Yangling, China)*

13.3 First authorship abstracts

Du S, Palma-Vera S, Trakooljul N, Schoen J, Chen S. Regulation of oviduct epithelial functions via sex steroid hormones is influenced by cortisol.

19th International Congress on Animal Reproduction-ICAR 2020+2. June 26-30, 2022. Bologna, Italy.

Poster presentation

Du S, Schoen J, Trakooljul N, Kanitz E, Chen S. Mimicking maternal stress in vitro: Using a compartmentalized oviduct epithelium model to investigate transepithelial cortisol distribution.

53rd Annual Conference of Physiology and Pathology of Reproduction and 45th Joint Conference of Veterinary and Human Reproductive Medicine. February 26-28, 2020. Rostock, Germany.

Reprod Domest Anim. 2020 Feb;55 Suppl 1:3-35. doi: 10.1111/rda.13591

Oral presentation

Du S, Schoen J, Murani E, Trakooljul N, Chen S. Cortisol regulates oviduct epithelium marker gene expression *in vitro*.

52nd Annual Conference of Physiology and Pathology of Reproduction. February 20-22, 2019. Göttingen, Germany.

Reprod Domest Anim. 2019 Feb;54 Suppl 1:3-41. doi: 10.1111/rda.13387.

Oral presentation

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16 Conflict of interests

There are no conflicts of interests.

17 Selbständigkeitserklärung

Hiermit bestätige ich, Shuaizhi Du, dass ich die vorliegende Arbeit selbständig und ausschließlich unter Zuhilfenahme der genannten Quellen und Hilfen angefertigt habe. Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen und wurde bisher nicht veröffentlicht.

Berlin, am 08.08.2023 Shuaizhi Du