Revised

Expression and function of TRPC channels in the female bovine reproductive tract

Maryam Ghavideldarestani*, Stephen L. Atkin[1], Henry J. Leese, Roger G. Sturmey

Centre for Cardiovascular and Metabolic Research, Hull York Medical School, University of Hull, Cottingham Road, Hull, HU6 7RX, UK

[1] Current address: Weill Cornell Medical College Qatar, Qatar Foundation, Education City, PO Box 24144, Doha, Qatar.

Abbreviated title: TRPC in bovine oviduct epithelium

Key terms: TRPC channels, sex hormones, calcium, Epithelium, Oviduct, Bovine

Word count: 4525

Abstract count: 201

Number of figures: 7

* ***Author for correspondence:***

Maryam Ghavideldarestani, PhD

Centre for Cardiovascular and Metabolic Research,

Hull York Medical School, University of Hull,

Cottingham Road, Hull, HU6 7RX, UK

Phone: +98-9111325394

Email: maryam.ghavideldarestani@gmail.com

Disclosure statement:

All authors have no potential financial conflicts of interest.

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Abstract

The epithelium lining the oviduct/fallopian tube is critical for early reproductive events, many of which are mediated via intracellular calcium ions. Despite this, little is known about the regulation of calcium homeostasis in the oviductal epithelium. Epithelial Transient Receptor Potential Channels (TRPCs) modulate calcium flux in other tissues and their expression and functional regulation have therefore been examined using the bovine oviduct as a model for the human. The effects of FSH, LH, 17β-estradiol (E2) and progesterone on TRPCs expression and intracellular calcium flux were determined. TRPC1, 2, 3, 4 and 6 were expressed in the bovine reproductive tract and their gene expression varied throughout the estrous cycle. In more detailed studies undertaken on TRPC1 and 6 we show that protein expression varied through the estrus cycle; specifically, E2, FSH and LH individually and in combination up-regulated TRPC1 and 6 expression in cultured bovine oviduct epithelial cells (BOECs), whilst progesterone antagonized these effects. Functional studies showed changes in calcium mobilization in BOECs were dependent on TRPCs. In conclusion, TRPC 1, 2, 3, 4 and 6 are present in the epithelium lining the bovine oviduct and TRPC 1and 6 vary through the estrous cycle suggesting an important role in early reproductive function.

Key words: TRPC channels, sex hormones, calcium, Epithelium, Oviduct, Bovine

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1. Introduction

Calcium is an important intracellular second messenger that has been shown to have a significant role in the early events of mammalian reproduction including oocyte activation [1] and oviduct contraction required for the transit of the ovulated egg from the ovary to the site of fertilisation [2]. Calcium transport across epithelial cells occurs by a number of mechanisms, including transit across tight junctions, Na^{+}/Ca^{2+} exchangers (NCXs), voltage-dependent Ca^{2+} channels (VDCCs) and members of the transient receptor potential (TRP) channel superfamily[3-5].

The TRP superfamily comprises 28 proteins, characterised by six transmembrane domains unique to the family, intracellular N- and C-terminals and a pore domain located between the fifth (S5) and sixth (S6) segments. Members of the mammalian TRP superfamily may be divided into seven families based on amino acid homologies: TRPC (*C*anonical); TRPV (*V*anilloid); TRPM (*M*elastatin); TRPP (*P*olycystin); TRPML (*M*uco*L*ipin); TRPA (*A*nkyrin) and; TRPN (*N*OMPC) [6, 7]. Despite a wealth of knowledge of calcium transport at the molecular level in a wide variety of tissues and cell types, very few studies have investigated the potential involvement of TRP channels in calcium transport across uterine and oviductal epithelia [3, 8-10], which is surprising since calcium dysregulation has been implicated in follicular arrest and menstrual disturbances [11, 12].

The epithelial cells of the female reproductive tract have critical roles in early development. In the oviduct, the epithelium facilitates gamete transport [13], and fertilization [14] and the cleavage stages of embryo development [15] while; the cells of the uterus are closely involved in pregnancy recognition [16] and blastocyst implantation [17]. A major mechanism by which the epithelia of the female reproductive tract support early development is through the regulation of the composition of the fluid environment in which these events occur [15].

The bovine estrous cycle begins with ovulation as a result of the preovulatory Luteinizing hormone (LH) surge which in turn triggers nuclear and cytoplasmic maturation of the oocyte [18]. The tissue of the recently ovulated follicle which express both FSH and LH receptors [19] undergoes transformation under the effect of FSH and LH produced in gonadotrophs of the anterior pituitary gland [20], and differentiates to form small and large luteal cells, respectively that secrete progesterone. Formation of a functional corpus Luteum (CL) requires LH. Progesterone is the dominant hormone for the major part of the bovine estrous cycle. The concentration of progesterone increases from day 3-4 of the estrous cycle, and then, dramatically until day 8 when a plateau is reached [18]. A decrease in progesterone concentration, the result of rapid regression of the CL induced by $PGF_{2\alpha}$ secreted by the endometrium [21] is the key event in the estrous cycle. Regression of the CL begins 1-4 days before estrous and is completed within 2 days [18].

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The primary aim of this study was therefore to identify the TRPC isoforms present in epithelial cells lining the oviduct of bovine, used as model system due to its physiological similarities to the human [22, 23]. The focus of this study was on TRPC1 and TRPC6 as the main candidates for Store-Operated Channels (SOC) and Receptor-Operated Channels (ROC), respectively [24, 25]. We decided to focus attention on these two isoforms in bovine oviduct epithelial tissue throughout the estrous cycle including, their gene and protein regulation by sex hormones, and the role of TRPCs in regulating intracellular calcium flux.

2. Material and methods

2.1 Bovine tissue

Fresh female bovine reproductive tracts obtained from a local abattoir were transported to the laboratory within 2 hours of slaughter in Hanks Balanced Salt Solution without CaCl2 and MgCl2 (HBSS; Gibco Invitrogen) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gibco Invitrogen), and 1 µM Aprotinin (Sigma Aldrich), a competitive serine protease inhibitor that inhibits trypsin, chymotrypsin, kallikrein and plasmin. The stage of estrous determined according to the gross morphology of the ovary [26]. Since the experiments on bovine tissue were carried out on the waste material obtained from animals after slaughter in a local abattoir no institutional committee approval was required.

2.2 Isolation and culture of bovine oviduct epithelial cells

Oviducts were dissected from the reproductive tract and connective tissue carefully removed. Bovine Oviduct Epithelial Cells (BOECs) were harvested by squeezing the oviduct from isthmus to infundibulum. Cells were collected in HBSS and centrifuged at 2500 x *g* for 5 minutes. The supernatant was discarded and the cells washed twice more by this process. The cell pellet was then re-suspended in 1 ml of culture medium (1:1 ratio of Dulbecco's Modified Eagle's Medium and Nutrient Mixture F-12 Ham, supplemented with 270 U/ml PenStrep, 20 µg/ml Amphotericin B, 2 mM L-Glutamine, 2.5% v/v Newborn Calf Serum, 2.5% v/v Foetal Calf Serum, 0.1% w/v Albumin from Bovine Serum (essentially fatty acids free). Cell viability and number were assessed using Trypan Blue Exclusion test on a hemocytometer. Cells were seeded into a T25 culture flask at a density of 5x106/ml and maintained at 39˚ C in a 5% CO2 incubator. Culture medium was first changed after 24 hours and then every 48 hours until the cells reached the confluence stage after 7days.

2.3 RNA extraction and Quantitative Real-Time PCR

Total RNA was extracted using NucleoSpin® RNA II isolation kit (Macherey- Nagel). RNA concentration and purity were assessed by measuring 260/280 nm absorbance on a nanospectrophotometer (Implen, Germany). Isolated RNA with a 260/280 ratio of ~2 was used for

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further experiments. Isolated RNA was reverse-transcribed to cDNA using EZ-First Strand cDNA Synthesis Kit (Geneflow, Isreal). 1µg RNA was used in all reverse transcription experiments. Gene expression was determined by quantitative real-time PCR using SYBR green. β- actin was chosen as a housekeeping gene and used as an internal comparator in parallel with the control sample (primer sequences supplemental tables 1 and 2). Relative gene expression was analyzed using StepOne software V2.0 and the baseline and threshold were set manually. RT-qPCR data were analysed using the ΔΔCt method.

2.4 Immunohistochemistry and confocal microscopy

Immunostaining for TRPC1 and TRPC6 was performed on frozen 10 µm sections of bovine oviduct biopsies. The tissue sections were either permeabilized (ice cold Methanol and 0.1% Triton X-100) to detect intracellular localization of TRPC1 and 6, or used non-permeabilized to examine cell surface localization of TRPC1 and 6. The oviduct was divided into infundibulum, ampulla and isthmus based on the morphology of the tube, prior to the sectioning.

Non-specific binding sites were blocked with 2% donkey serum (Sigma Aldrich) in PBS for 30 minutes at room temperature (RT). Samples were then incubated with 1µg/ml of each of TRPC1 goat polyclonal IgG (Santa Cruz) and TRPC6 rabbit polyclonal IgG (Abcam) primary antibodies diluted in PBS containing 1% fetal calf serum (FCS) in the humidified chamber at 4°C overnight. Primary antibodies were removed and the slides washed with PBS containing 0.25% Tween 20 (Sigma Aldrich). Secondary antibodies, 4µg/ml Alexa Four 647 donkey anti goat (Invitrogen) (against TRPC1 primary) and 4µg/ml Alexa Flour 488 donkey anti rabbit (Invitrogen) (against TRPC6 primary), were diluted in PBS containing 1% FCS. Tissue sections were incubated with secondary antibodies in a dark humidified chamber at RT for one hour. Slides were washed with 0.25% Tween 20 in PBS. Specimens were mounted in Vectashield containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Samples were visualized using a laser scanning confocal microscope (LSM 710-Zen2008; Carl Zeiss, Oberkochen, Germany) equipped with an argon/krypton laser source. A single wavelength of 568 nm was used for excitation, and the emitted fluorescence at 603 nm (Alexa Fluor 488, emitted at 519 nm) was collected through an oil-immersion 100x objective.

Semi-quantitative determination of fluorescent staining was measured from the apical, basal and lateral membranes using ImageJ.

2.5 Western Blotting

Cultured BOECs were lysed in radioimmunoprecipitation assay (RIPA) buffer. Protein concentration was measured using DC Protein Assay Reagents Package (BioRad, USA). Equal amount of 30 µg of

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total protein was loaded per well. Proteins were separated on a 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blot was incubated at 4°C overnight with 0.2 µg/ml anti- rabbit TRPC1or TRPC6 antibodies (ALomone labs) in TBS-T buffer containing 2% BSA. The membrane was washed with TBS-T and incubated with 2 µg/ml polyclonal donkey to rabbit IgG conjugated to horseradish peroxidise (Abcam) in TBS-T buffer containing 2% BSA at RT for 60 minutes. Visualization was carried out using ECL reagents and developed on a film.

2.6 Sex hormone treatment

Confluent BOECs were incubated with 10 ng/ml Progesterone (P4), 2 pg/ml 17β- estradiol (E2), 0.5 ng/ml FSH, and LH [27] individually and in combination for 24 hours prior to the mRNA and protein extraction.

2.7 Intracellular Calcium assay

BOECs were seeded at a density of $2x10^5$ cells/ml into sterile black polystyrene 96 well plates (Nalge Nunc, Fisher Scientific). Confluence, as determined visually, was regained 7 days after being seeded into the 96 well plates.

The culture medium was removed and confluent BOECs were washed with calcium free solution (130 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 10 mM HEPES, 8 mM Glucose, 0.4 mM EGTA, pH 7.4). Cells were then incubated with calcium free solution containing 10µM Fura PE 3-AM (Sigma Aldrich) for 30 minutes at 39˚C in a 5% CO2 incubator. Fura PE 3-AM was removed from the wells and cells washed 3 times with calcium free solution. Cells were kept in the dark after treatment with Fura PE 3-AM to avoid non-specific bleaching. The 96 well plate containing the BOECs was placed in an Infinite M200 Tecan plate reader (Tecan). Cells were maintained at 39˚ C in the plate reader. After measuring the basal intracellular calcium, calcium free solution was replaced with calcium-containing solution (130 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 10 mM HEPES, 8 mM Glucose, 1.5 mM CaCl2, pH 7.4). Depending on the number of agonists and antagonists used and their required time of action, different numbers of kinetic cycle (5 sec each) without intervals were used for each experiment.

Changes in intracellular calcium concentration were measured using 25 µM Hyperforin for minimum of 60 sec, a TRPC6 channel activator, 25 µM SKF96365 for minimum of 60 sec, a general TRP channel blocker, and 15 µM 2.5-Di-t-butylhydroquinone (DBQ), a sarcoplasmic/endoplasmic reticulum Ca2+ -ATPase (SERCA) inhibitor. DBQ depletes the intracellular calcium stores which consequently activate the present Store-Operated Channels. Each agonist or antagonist was added to the 96 well plate after removal of the previous one.

2.8 Statistics

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Each experiment was performed using at least 3 samples in triplicate (n = 3) and expressed as ± 1 standard deviation, and Student's *t-test* performed using Origin 6.1 software (OriginLab Corporation, Northampton, Massachusetts).

3. Results

3.1 Expression of TRPC genes in bovine oviduct tissue throughout the estrous cycle

From 7 isoforms of TRPC subfamily TRPC1, TRPC2, TRPC3, TRPC4 and TRPC6 were expressed in bovine oviduct epithelium (Supplemental Figure 1). TRPC1 gene expression in the bovine oviduct was down-regulated at the stage 2 and 4 by 0.25 ($p<0.001$) and 0.35 ($p<0.001$) fold respectively compared to the stage 1 of the estrous cycle. However, at stage 3, oviduct expression of TRPC1 was up-regulated by a small, but significant amount (1.49 fold, $p<0.05$) (Figure 1).

TRPC2 expression was down-regulated in bovine oviduct epithelial tissue by 0.1 ($p<0.001$), 0.7 ($p<0.01$), 0.3 ($p<0.01$) fold at stage 2, 3 and 4 respectively compared to the stage 1(Figure 1).

In bovine oviduct epithelial tissue, expression level of TRPC3 was down-regulated by 0.7($p<0.001$), 0.65 ($p<0.01$) and 0.15 ($p<0.001$) fold at stage 2, 3 and 4 respectively relative to the stage 1 (Figure 1).

Expression of TRPC4 in bovine oviduct epithelial tissue was down-regulated by 0.05 ($p<0.001$), 0.6 ($p<0.01$) and 0.15 ($p<0.001$) fold at stage 2, 3 and 4 relative to the stage 1 (Figure 1).

TRPC6 expression in the oviduct fell by 0.5 ($p<0.01$), 0.8 ($p<0.01$), 0.4 ($p<0.001$) fold at stage 2, 3 and 4 relative to the stage 1 (Figure 1).

3.2 *Localization and abundance of TRPC1 and TRPC6 in bovine oviduct epithelial tissue*

Various physiological events occur in each part of the oviduct. Localization and abundance of TRPC1 and TRPC6 was studied in each section of the oviduct throughout the estrous cycle (Figure 2 and 4). In the infundibulum, membrane abundance of TRPC1 was equal at stage 1 and 2. However, it was increased by 7 ($p<0.001$) and 8.85 ($p<0.001$) fold at stage 3 and 4. Membrane abundance of TRPC1 in ampulla was equal at 1, 2 and 3 of the estrous cycle. However, TRPC1 was slightly more abundant (1.7 fold ($p<0.05$)) at stage 4. In the isthmus, membrane abundance of TRPC1 was highest at stage 1 and lowest at stage 4 of the estrous cycle. Membrane abundance of TRPC1 in isthmus was generally decreased by 0.8 ($p<0.01$), 0.85 ($p<0.05$) and 0.55 ($p<0.001$) fold at stage 2, 3 and, respectively. (Figure 2 and 3A).

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Cytosolic abundance of TRPC1 in infundibulum was higher at stage 2 and 4 and lowest at stage 3 of the estrous cycle. In the ampulla, cytosolic abundance of TRPC1 was equal at stage 1 and 3 and was higher than that of stage 2 and 4 of the estrous cycle. Cytosolic abundance of TRPC1 was decreased by 0.12 ($p<0.01$) and 0.13 ($p<0.01$) fold at stage 2 and 4 compared to the stage 1 of the estrous cycle. Cytosolic abundance of TRPC1 in isthmus at stage 1 was higher than that of stage 2, 3 and4 (Figure 4 and 5A).

Membrane abundance of TRPC6 in the epithelium lining infundibulum was markedly lower at stage 1 compared to other stages of the estrous cycle. Membrane abundance of TRPC6 in infundibulum was increased by 18325 ($p<0.01$), 19.7 ($p<0.001$) and 18.75 ($p<0.01$) at stage 2, 3 and 4, respectively. In ampulla, membrane abundance of TRPC6 was equal at stage 1 and 2. Whereas, at stage 3 and 4 TRPC6 was slightly more abundant by 1.3 ($p<0.05$) and 1.6 ($p<0.05$) fold respectively, relative to that of stage 1 and 2. In isthmus, membrane abundance of TRPC6 was dramatically higher at stage 1 compared to stage 2, 3 and 4. TRPC6 membrane abundance was reduced by 0.1 ($p<0.01$), 0.2 ($P<0.01$) and 0.15 ($p<0.01$) at stage 2, 3 and 4 respectively. (Figure 2 and 3B).

In infundibulum, cytosolic abundance of TRPC6 was equal at stage 1, 2 and 4. Cytosolic abundance of TRPC6 in epithelium lining infundibulum was lowest at stage 3 of the estrous cycle and it was decreased by 0.75 fold ($p<0.01$). Cytosolic abundance of TRPC6 in epithelium lining ampulla was highest at stage 3 and equally lowest by 0.05 ($p<0.01$) fold at stage 2 and 4. In isthmus, cytosolic abundance of TRPC6 was highest at stage 1 and it was equal at stage 2, 3 and 4 (Figure 4 and 5B).

3.3 Hormonal regulation of TRPC1 and 6 gene/protein expression in BOECs

Having identified variation in gene and protein expression at different stages of the estrous cycle, we measured the impact of hormone addition on expression of TRPC1 and 6 in the bovine model. Addition of P4, E2, FSH, and LH individually and in combination[27] to the BOECs culture system for 24 hours induced significant changes in expression of TRPC1 and 6 in cells derived from reproductive tracts throughout the estrous cycle (Figure 6; Supplemental Table 3A and 3B): Expression of TRPC1 in BOECs treated with E2 was down-regulated at stage 1 and 3, while an up-regulation was observed at stage 2 and 4 compared to the control. FSH and LH generally led to increased expression of TRPC1 at all stages of the estrous cycle. By contrast, P4 induced a down-regulation in expression of TRPC1 at stage 1 and 3. However, an up-regulation in expression of TRPC1 was observed at stage 2 and 4 of the estrous cycle in response to P4 treatment. When added together, P4 and E2 did not induce any changes in expression of TRPC1 at stage 1 and 3. However, TRPC1 expression was up-regulated at stage 2 and 4 as a result of P4 and E2 treatment. A similar pattern of TRPC1 expression was observed when BOECs were treated with a combination of P4, FSH and LH. This combination led to increased expression of TRPC1 at all stages of the estrous cycle.

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When P4 was added to the mixture of E2, FSH and LH, the up-regulatory effect of this mixture was abolished at stage 1 and dramatically decreased at stage 2, 3 and 4 (Figure 6A and supplemental Table 3A).

TRPC6 gene expression level in BOECs was not altered by E2 at stage 1, 2 and 3. However, increased expression of TRPC6 was detected in stage 4 BOECs in response to E2-treatment. FSH and LH generally up-regulated the expression of TRPC6 at all stages of the estrous cycle whereas P4-treatment of BOECs resulted in an up-regulation in TRPC6 expression at all stages of the estrous cycle except stage 1. When added in combination, P4 and E2 increased the expression of TRPC6 at all stages of the estrous cycle. Treatment of BOECs with a mixture of P4, FSH and LH did not alter TRPC6 gene expression at stage 1. However, expression of TRPC6 at stage 2, 3 and 4 was increased in response to P4, FSH and LH treatment. Concurrent treatment of BOECs with E2, FSH and LH up-regulated the expression of TRPC6 dramatically at all stages of the estrous cycle. However, this up-regulatory effect was reduced when P4 was added to this mixture (Figure 6B and supplemental Table 3B).

After confirming the response of TRPC 1 ad 6 at the gene level, we next attempted to map this onto protein levels. TRPC1 protein levels were lower in cells collected from oviducts at stages 1, 2 and 3 of the estrous cycle after exposure to E2 (Figure 6C). After treatment with FSH and LH- the amount TRPC1 protein was increased in BOECs collected from tissue at stage 4 of the estrous cycle compared to the control group (Figure 6C; Table 3). In BOECs treated with P4, the amount of TRPC1 protein was lower at stage 3 compared to the untreated BOECs. However, this decrease was significantly greater than that at stage 4 of the estrous cycle (Figure 6C). Protein expression of TRPC1 at all 4 stages of the estrous cycle was significantly decreased in BOECs treated with a mixture of E2, FSH, LH and P4. By contrast, TRPC6 protein expression did not change in response to P4 exposure. Addition of FSH and LH led to a reduction of TRPC6 protein expression in cells collected from stage 1 tissue and a rise in stage 3 compared to the control group (Figure 6C). Protein expression of TRPC6 was strongly reduced in E2-treated BOECs at all stages of estrous cycle; more significantly at stage 3 and 4 compared to the untreated BOECs. When added in combination, E2, FSH, LH and P4 led to a slight rise in protein expression of TRPC6 at stage 1 of the estrous cycle in BOECs (Figure 6C).

3.4 Intracellular calcium concentration in the BOECs throughout the estrous cycle

Finally, we examined the activity of TRPC 1 and 6 in epithelial cells from the female reproductive tract, using the bovine oviduct model (Figure 7). Using Hyperforin, an activator of TRPC6, intracellular Ca^{2+} mobilization changes in $[Ca^{2+}]i$ in stage 2 BOECs was 1.2 fold higher than that of the stage 1 cells ($p< 0.001$). The increase in $[Ca^{2+}]_i$ induced by Hyperforin was higher by 1.5 ($p<$

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292 0.01) and 1.3 fold ($p< 0.001$) respectively in stage 3 and 4 BOECs relative to the stage 1 BOECs.
293 When the calcium channel antagonist SKF96365 was included, $[Ca^{2+}]_i$ in BOECs was lower by 0.89
294 ($p< 0.01$), 0.76 ($p< 0.01$) and 0.34 ($p< 0.001$) fold respectively at stage 2, 3 and 4 of the estrous cycle
295 compared to that of the stage 1 (Figure 7A and D). Treatment of BOECs with SKF96365 without
296 activation of TRPC6 resulted in an inhibition in Ca^{2+} influx (Figures 7B and E). Changes induced by
297 SKF96365 in stage 2 BOECs were not significantly different to that of the stage 1. Furthermore, no
298 significant difference was observed in $[Ca^{2+}]_i$ after SKF96365 treatment in stage 3 and 4 BOECs
299 relative to that of stage 1 (Figure 7B and E).

300 When Hyperforin was added to the SKF96365-treated BOECs, $[Ca^{2+}]_i$ increased at all stages of the
301 estrous cycle (Figure 7B and E). No significant difference was observed in the response of BOECs to
302 Hyperforin at stage 2, 3 and 4 compared to stage 1 of the estrous cycle (Figure 7B and E). The effect
303 of 2.5DBQ which causes intracellular calcium store depletion, is shown in Figures 7C and F. The
304 DBQ-induced transient increase in $[Ca^{2+}]_i$ at stage 2 was not significantly different to that of the stage
305 1; however, the DBQ-induced effect in BOECs was lower at stage 3 and 4 by 0.75 ($p< 0.001$) and
306 0.49 ($P< 0.001$) fold respectively compared to stage 1 of the estrous cycle. Replacing the Ca^{2+} free
307 solution with extracellular solution containing 1.5 mM Ca^{2+} after depleting the intracellular store
308 resulted in an increase in $[Ca^{2+}]_i$. This increase was not significantly different at stage 2, 3 and 4
309 compared to the stage1 (Figure 7C and F). Addition of SKF96365 to the extracellular solution led to a
310 fall in $[Ca^{2+}]_i$ in BOECs. The effect of SKF96365 was greater at stage 4 compared to the other stages
311 of the estrous cycle. Effect of SKF96365 was greater at stage 2, 3 and 4 by 1.18 ($p< 0.05$), 1.28 ($p<$
312 0.001) and 2.09 ($p< 0.001$) fold respectively relative at stage 1 (Figure 7C and F).

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4. Discussion

These studies report the first detailed exploration of TRPC channels in the epithelium lining the bovine oviduct and show that gene expression of TRPC1,2, 3, 4 and 6 are present and vary throughout the estrous cycle. TRPC1 and 6 protein expression determined by IHC also varied throughout the estrous cycle and were functionally active and hormonally regulated.

In general, expression of all the TRPC isoforms present in the bovine oviduct epithelium was highest at stage 1 of the estrous cycle, corresponding to when 17β-estradiol (E2), FSH and LH are at their highest levels and progesterone (P4) is at its lowest level. A notable exception to this pattern was TRPC1 whose expression was highest at stage 3. Stage 1 (day 1-4) of the estrous cycle starts immediately after ovulation, when the oocyte is transported into the oviduct. Transport of the oocyte is dependent on the ciliary beat frequency which is calcium-dependant [28]. Although the regulatory effect of E2 on TRPC genes expression has not been investigated previously, it has been reported that TRPV5, TRPV6 and TRPM2 genes are up-regulated by E2 [29, 30].

17β- estradiol, which is at its highest level just before the stage 1 of the estrous cycle, stimulates NF-κB activation in bovine granolusa cells [31]. Moreover, FSH triggers the NF-κB activity in rat granolusa cells leading to expression of the X-linked inhibitor of apoptosis (XIAP) and inhibition of apoptosis [31]. Inhibition of NF-κB activation suppresses the FSH-stimulated follicle growth in vitro [32]. By contrast, progesterone reduces the activation of toll-like receptor 4 (TLR4) and the NF-κB signaling pathway in the brain of male rats after subarachnoid hemorrhage [33]. The promoter region of TRPC1 contains an NF-κB binding site [34]. Furthermore, expression of TRPC1 in human vascular endothelial cells [34] and TRPC3 in human airway smooth muscle cells [35] is up-regulated in response to TNF-alpha; which is an activator of NF-κB pathway [36]. I kappa B Kinase (IKK) which phosphorylates the NF-κB inhibitor (IKB) is activated by TNF-α. Phosphorylation of IKB at serine 32 and 36 leads its ubiquitination and degradation by 26S proteasome. This in turn results in the release of the nuclear localization signal of NF-κB and translocation of NF-κB to the nucleus. Consequently, binding of NF-κB to its binding sites on DNA is likely to result in transcription of NF-κB-linked proteins such as TRPC1 and TRPC3. However, FSH-induced activation of NF-κB is independent of IKB phosphorylation [32].

The mechanism underlying the effect of sex hormones on TRPC gene expression may involve the TNF-α and NF-κB pathways. The promoter region of TRPC1 contains an NF-κB binding site ENREF_40 [37-40] and it is recognized that estradiol, progesterone and FSH can all act through the this pathway [28, 41]. Binding of NF-κB to DNA is likely to result in transcription of NF-κB-linked proteins such as TRPC1. However, further studies are required to support this.

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Immunostaining for TRPC1 and 6 protein expression and abundance showed variation throughout the estrous cycle in bovine oviduct epithelial tissue, supporting the notion that hormones may play a role in the regulation of these proteins. In general, TRPC6 was more abundant than TRPC1in bovine oviduct epithelium, more specifically on the apical membrane of the tissue indicating the possible role of this TRPC isoform in secretion [22, 23]. In infundibulum and ampulla, TRPC1 protein was present on the cytoplasmic membrane at highest levels at stage 4 when the concentration of P4 is very low or absent and E2, and FSH and LH are the dominant hormones. However, in isthmus, TRPC1 membrane abundance was highest at stage 1 and lowest at stage 4. Changes in cytosolic abundance of TRPC1 in bovine oviduct epithelial tissue throughout the estrous cycle was similar to that of the TRPC6.

Abundance of both TRPC1 and TRPC6 was variable from the infundibulum to the isthmus end of the oviduct throughout the estrous cycle which might indicate the involvement of these channels in various physiological functions of the infundibulum (oocyte transport) [42], ampulla (fertilization) [43] and isthmus (spermatozoa reservoir and early embryo transport) [44, 45] throughout the estrous cycle.

TRPC channels are functional in STIM-dependent and STIM-independent mode indicating their role as Store- Operated Channels (SOC) and Receptor- Operated Channels (ROC). Changes in TRPC1 abundance might be due to its physiological role in association with STIM and the complex of STIM and Orai proteins [46]. STIM1 regulates TRPC1, 3, 4, 5 and 6 [47]. However, TRPC1, 4 and 5 are gated directly by STIM1 whereas, the regulatory effect of STIM1 on TRPC3 and TRPC6 is via the heteromultimerization of TRPC1-TRPC3 and TRPC4-TRPC6 [47].

Intracellular calcium measurements illustrated that TRPCs were physiologically active in BOECs, since Hyperforin significantly increased calcium influx. Furthermore, depletion of intracellular calcium with DBQ increased the basal calcium uptake in BOECs at all stages of the estrous cycle suggesting that depletion of intracellular calcium resulted in activation of Store-Operated Channels (SOC) of which TRPCs are components. Hence, SKF96365-induced decrease in intracellular calcium concentration was higher in BOECs pre-treated with DBQ. This indicates that Store-Operated Calcium channel (SOC) activation is occurring in BOECs and that the TRPC isoforms are functional, similar to SOCs and TRPC1 in prostate epithelial cells [48].

In conclusion, TRPC1, 2, 3, 4 and 6 were expressed in bovine oviduct epithelial tissue and their gene and protein expression varied throughout the estrous cycle, suggesting a role in bovine reproductive events via regulation of calcium homeostasis. Furthermore, changes in TRPC gene and protein expression and functional activation were likely due to hormonal changes through the estrous cycle as shown from the studies on TRPC1 and 6. Such cyclical regulation suggests a possible role(s) of these

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channels in the female reproductive tract during the cyclical physiological remodeling associated with the estrous or menstrual cycles.

Acknowledgment

This work was financially supported by the Diabetes Centre Charitable funds, Hull and East Yorkshire Hospitals trust.

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Author Contributions

MG conducted all the experiments and prepared all the figures. MG and SA wrote the main manuscript text. SA, RG and HL supervised this project. All authors reviewed the manuscript.

Declaration of competing financial interests

We declare that the authors have no competing interests as defined by Nature Publishing Group, or other interests that might be perceived to influence the results and/or discussion reported in this article.

Figure legends

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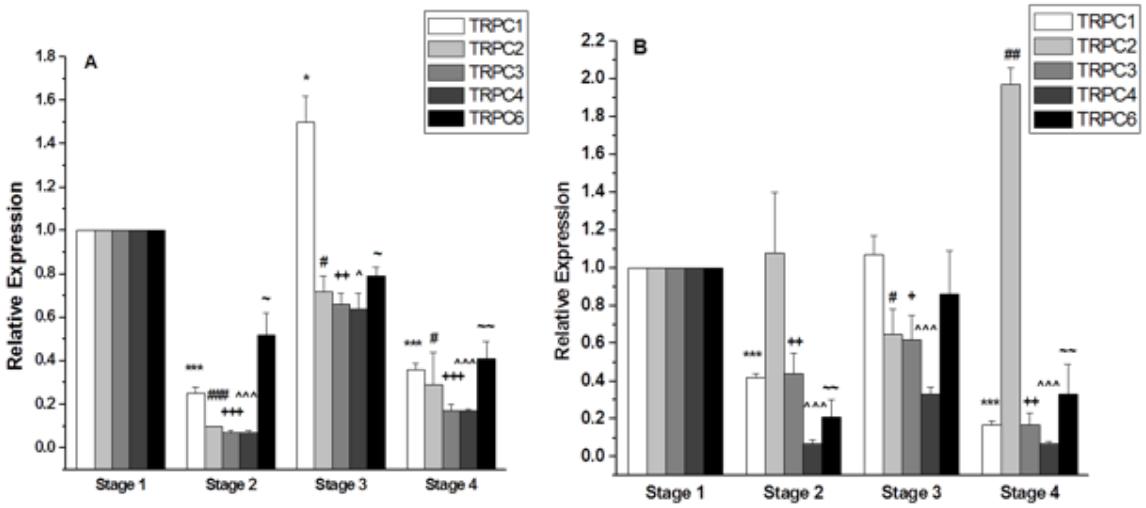

516

517 **Figure 1.** Patterns of gene expression of TRPC isoforms in bovine oviduct epithelial tissue (A) and
518 bovine oviduct epithelial cultured cells (BOECs) (B) throughout the estrous cycle. A, Expression of
519 all TRPC isoforms in bovine oviduct epithelial tissue was highest at stage 1 of the estrous cycle.
520 However, gene expression of TRPC1 was highest at stage 3 of the estrous cycle. B, Expression of
521 TRPC isoforms in bovine oviduct epithelial cultured cells throughout the estrous cycle was different
522 to that in the tissue. *, #, +, ^ and ~ represent the P value, comparing the changes in the TRPC1,
523 TRPC2, TRPC3, TRPC4 and TRPC6 genes expression at different stages of the estrous cycle to the
524 stage 1 in bovine oviduct epithelial tissue and cultured cells, respectively. Data are expressed as mean
525 3 independent experiments (n=3) ± 1 standard deviation. Statistical analysis was carried out using
526 Student's t-test (*, #, +,^ and ~; $p<0.05$; **, ##, ++,^^ and ~~ $p<0.01$; *** , ###, +++, ^^^ and ~~~
527 $p<0.001$).

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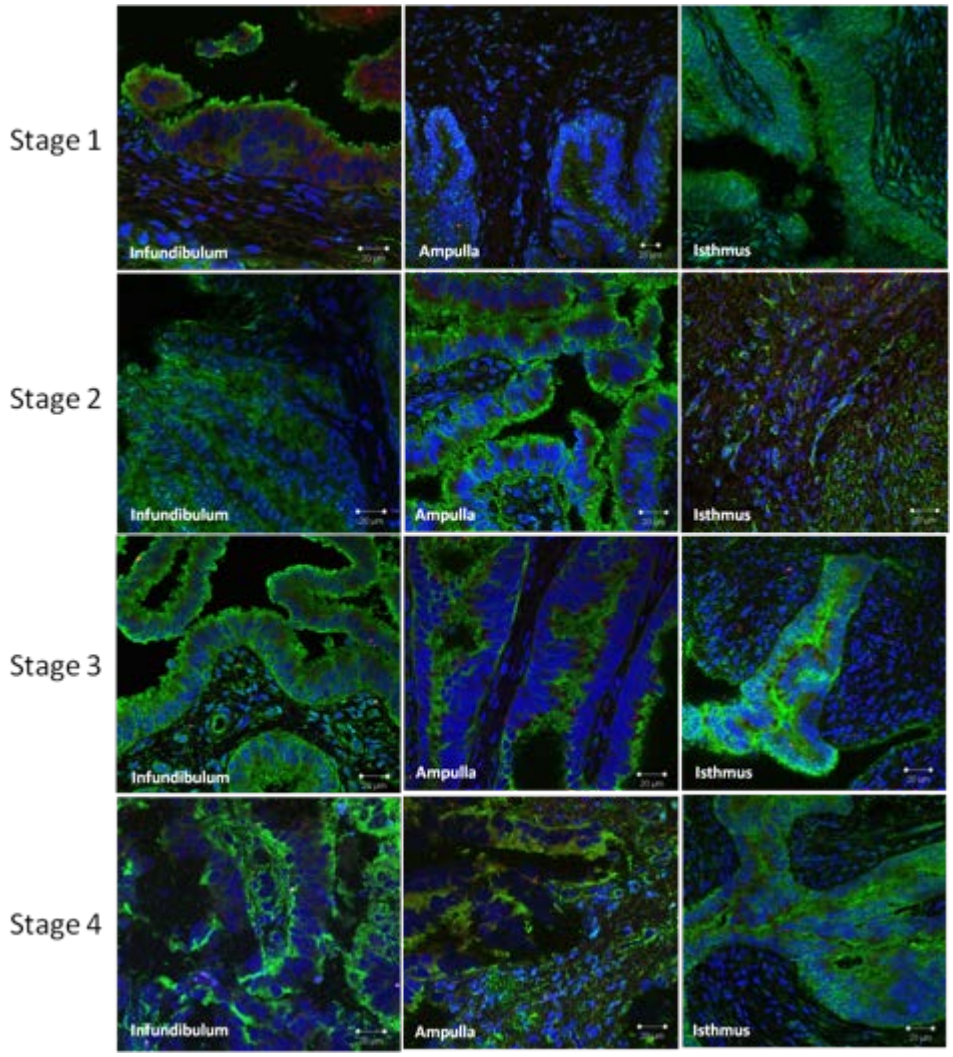

528

529 **Figure 2**. Membrane localization of TRPC1 and TRPC6 in bovine oviduct epithelial tissue (Non-
530 Permeabilized) during stages of the estrous cycle. TRPC1 with Alexa Four 647 FITC conjugated
531 (Red), TRPC6 with Alexa Flour 488 (Green) and nuclei are labelled with DAPI (Blue). Images are
532 representative examples from samples analysed in triplicate.

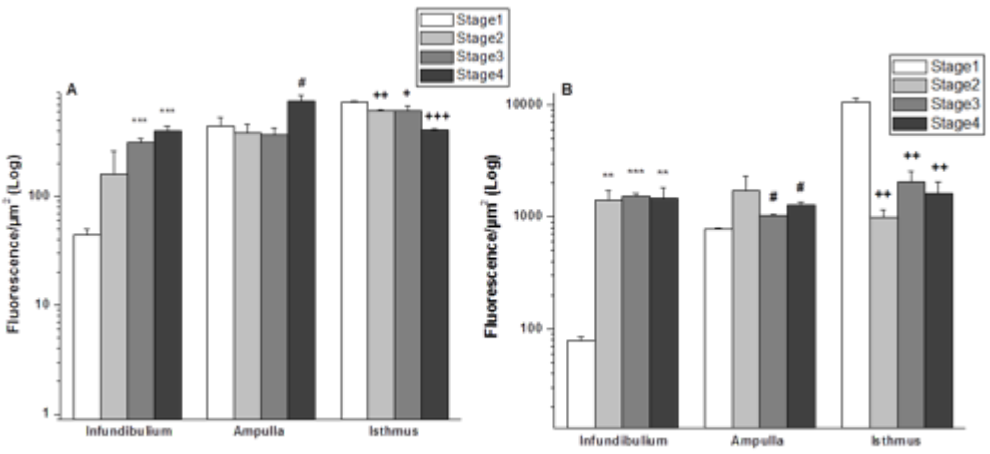

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Figure 3. Membrane abundance of TRPC1 and TRPC6 in bovine oviduct epithelial tissue (Non-Permeabilized) during stages of the estrous cycle. (A), membrane abundance of TRPC1 in infundibulum did not differ between stage 1 and stage 2 . However, relative membrane abundance of TRPC1 was increased at stage 3 and 4 relative to the stage 1. In ampulla, membrane abundance of TRPC1 was equal at stage 1, 2 and 3. However, it was increased at stage 4. Membrane abundance of TRPC1 in isthmus was generally decreased at stage 2, 3 and 4. (B), membrane abundance of TRPC6 in infundibulum was lowest at stage 1 and highest at stage 3. In ampulla, TRPC6 was equally abundant at stage 1 and 2. However, membrane abundance of TRPC6 was increased at stage 3 and 4 relative to the stage 1. Membrane abundance of TRPC6 was highest at stage 1 in isthmus and was reduced at stage 2, 3 and 4. Semi quantitative data are presented as mean (n=3) ± 1 standard deviation. The graphs are plotted on a logarithmic scale for ease of interpretation. *, # and + represent P value, comparing the abundance of each TRPC1 and TRPC6 in bovine infundibulum, ampulla and isthmus epithelial tissue respectively, obtained from stage 2, 3 and 4 of the estrous cycle to that in the stage 1. Statistical analysis was carried out using Student's t-test (* , # and +; $p<0.05$; ** , ## and ++$p<0.01$; *** , ### and +++ $p<0.001$).

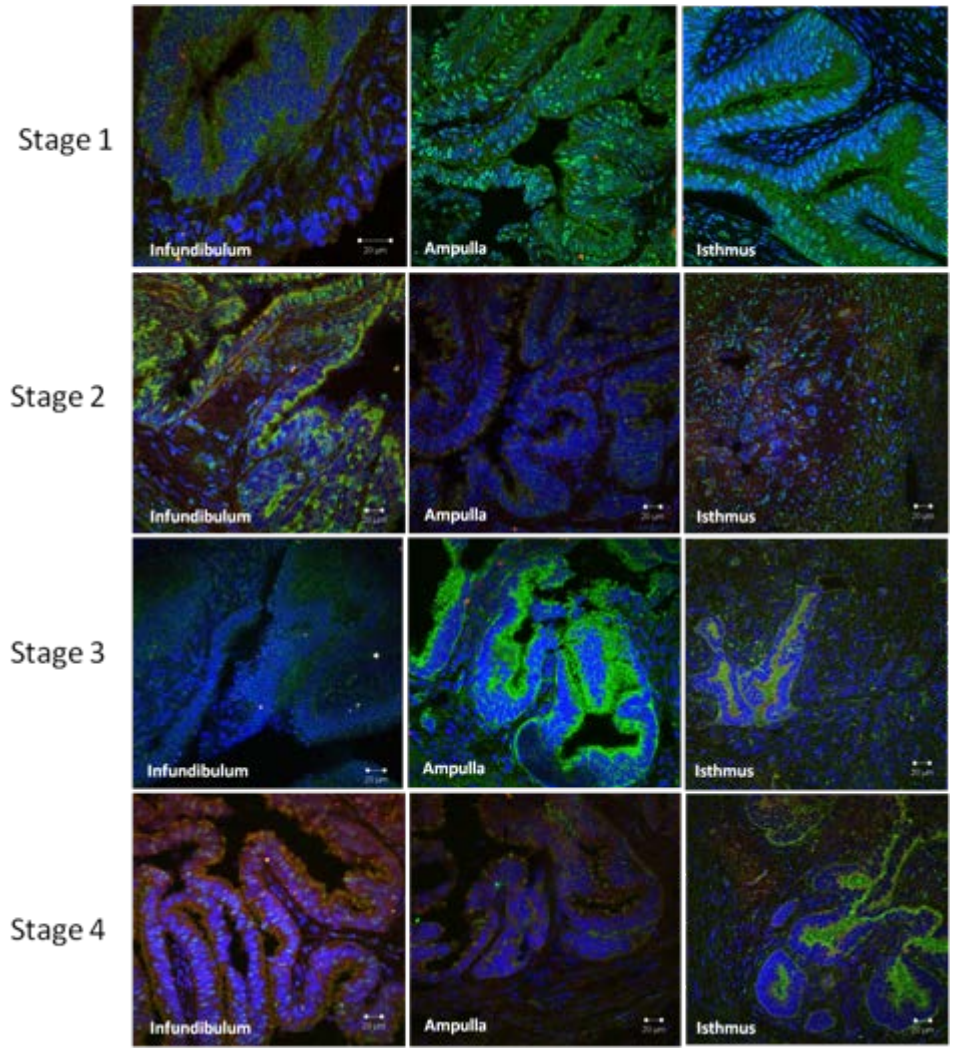

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Figure 4. Cytosolic localization of TRPC1 and TRPC6 in bovine oviduct epithelial tissue (Permeabilized) during stages of the estrous cycle. TRPC1 with Alexa Four 647 FITC conjugated (Red), TRPC6 with Alexa Flour 488 (Green) and nuclei are labelled with DAPI (Blue). Images are representative examples from samples analysed in triplicate.

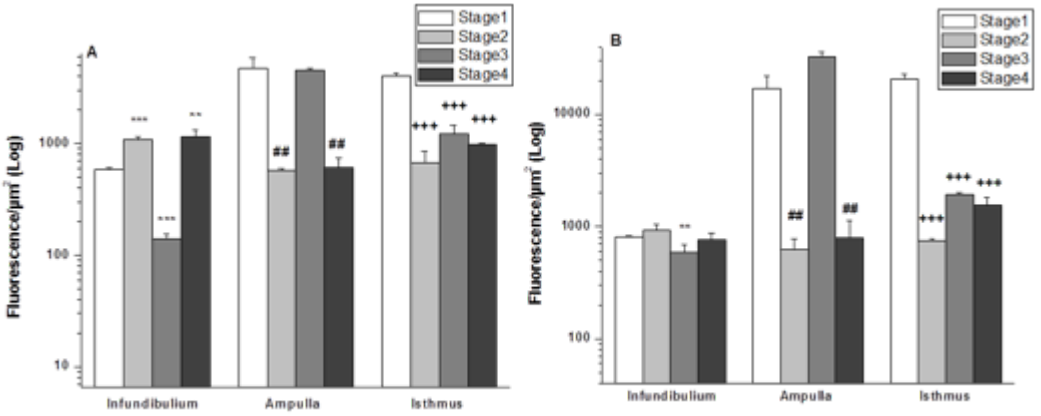

Figure 5. Cytosolic abundance of TRPC1 and TRPC6 in bovine oviduct epithelial tissue (Permeabilized) during stages of the estrous cycle. (A), cytosolic abundance of TRPC1 in infundibulum was increased at stage 2 and 4 compared to the stage 1. However, a decrease in cytosolic abundance of TRPC1 was detected at stage 2. In ampulla, membrane abundance of TRPC1 was equal at stage 1 and 3. However, it was decreased at stage 2 and 4 compared to the stage 1. Membrane abundance of TRPC1 in isthmus was generally decreased at stage 2, 3 and 4 compared to stage 1. (B), membrane abundance of TRPC6 in infundibulum was equal at stage 1, 2 and 4. However, it was decreased at stage 3. In ampulla, TRPC6 was equally abundant at stage 1 and 2. However, membrane abundance of TRPC6 was decreased at stage 3 and 4 relative to the stage 1. Membrane abundance of TRPC6 was highest at stage 1 in isthmus and was reduced stage 2, 3 and 4. Semi quantitative data are presented as mean (n=3) ± 1 standard deviation. The graphs are plotted on a logarithmic scale for ease of interpretation. *, # and + represent P value, comparing the abundance of each TRPC1 and TRPC6 in bovine infundibulum, ampulla and isthmus epithelial tissue respectively, obtained from stage 2, 3 and 4 of the estrous cycle to that in the stage 1. Statistical analysis was carried out using Student's t-test (* , # and +; $p<0.05$; ** , ## and ++$p<0.01$; *** , ### and +++ $p<0.001$).

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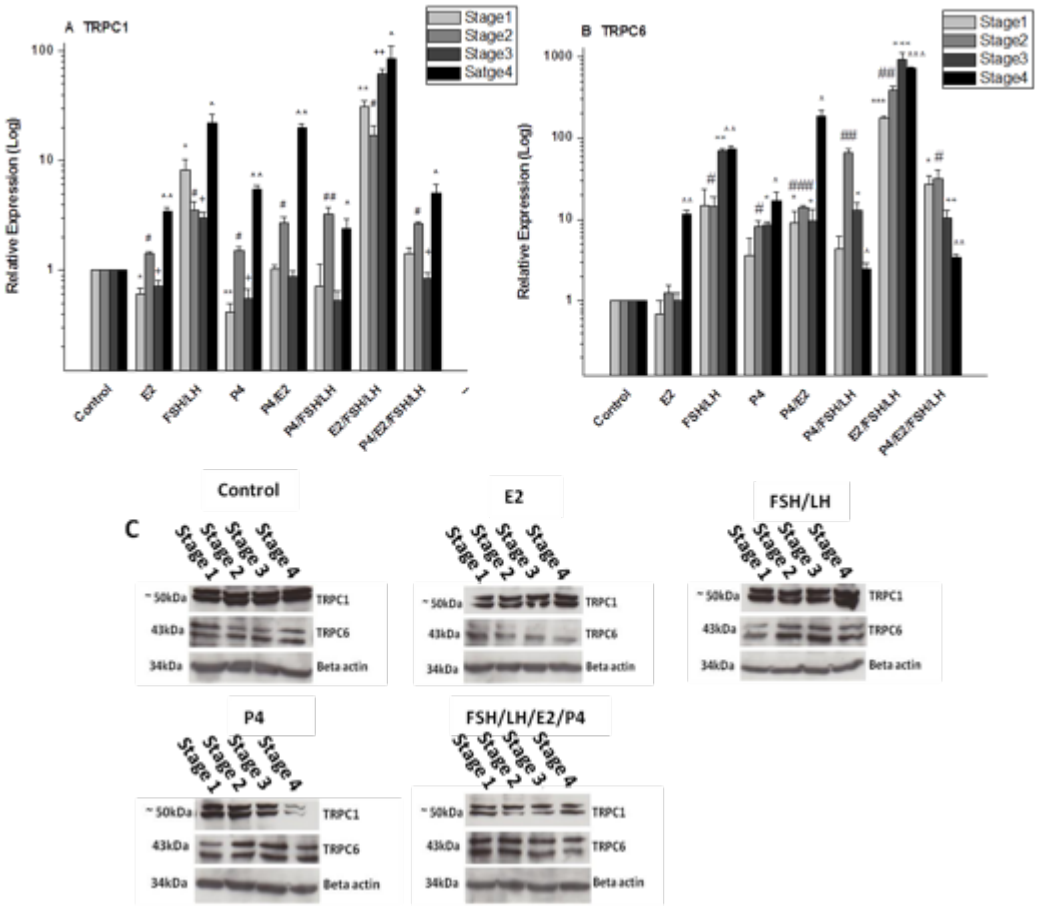

Figure 6. The effect of sex hormones on the gene expression of TRPC1 (A) and TRPC6 (B) throughout the estrous cycle. The expression of both TRPC1 and TRPC6 in BOECs harvested from oviducts at stages 1, 2, 3 and 4 of the estrous cycle was altered by each of the sex hormones individually and combined. (A) However, combination of E2/P4 and P4/FSH/LH did not induce any significant effect on the expression of TRPC1 in stage 3 BOECs. (B) At stage 2 and 3, expression of TRPC6 was altered in BOECs treated with each of the sex hormones individually, with the exception of E2, and their combination. The graphs are plotted on a logarithmic scale for ease of interpretation. Changes induced in gene expression in BOECs are expressed as a fold of that of the untreated BOECs. Data are expressed as mean 3 experiments (n=3) ± 1 standard deviation. (*/#/+/^ = $p<0.05$; **/##/++/^^ = $p<0.01$; ***/###/+++/^^^ = $p<0.001$).(C) Effect of sex hormones on TRPC1 and TRPC6 protein expression in BOECs. Protein expression level of TRPC1 and TRPC6 was altered by E2, FSH and LH, P4 and the mixture of E2, FSH, LH and P4 individually and combined compared to the untreated BOECs (n=1). E2: Estrogen; P4: Progesterone.

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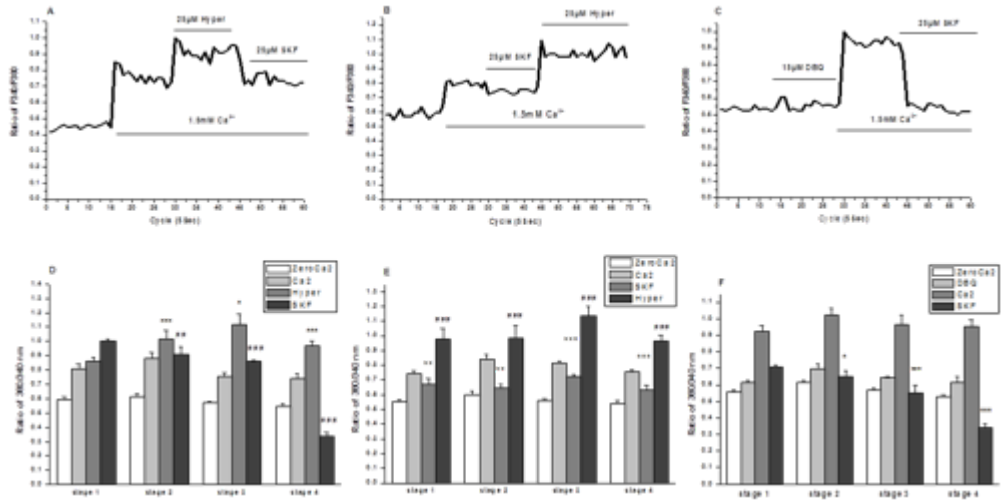

585

586 **Figure 7.** Calcium mobilisation in oviduct epithelial cells. **A** shows a representative trace of
587 intracellular calcium concentration, which is an average signal of a 96 well, induced by Hyperforin
588 followed by SKF96365 in BOECs harvested from tissue throughout the estrous cycle. (* and #
589 represent the P value, comparing the changes in calcium influx induced by Hyperforin and SKF96365
590 respectively, at different stage of the estrous cycle to the stage 1 in BOECs.). **B** is a representative
591 trace of intracellular calcium concentration, which is an average signal of a 96 well, induced by
592 SKF96365 (* and # represent the P value, comparing the changes in calcium influx induced by
593 SKF96365 and Hyperforin post SKF96365 treatment respectively, at different stages of the estrous
594 cycle to the intracellular calcium level before the treatment in BOECs.) **C** shows depleting the
595 intracellular calcium store using DBQ enhanced the inhibitory effect of SKF96365 on TRP channels
596 present in BOECs throughout the estrous cycle (* indicates the P value comparing the effect of
597 SKF96365 on calcium influx at stage 2, 3 and 4 relative to that of the stage 1 of the estrous cycle).
598 Figures 7D, 7E and 7F show mean changes in calcium influx All data are expressed as a mean of 6
599 experiments (n=6) ± 1 standard deviation. Statistical analysis was carried out using Student's *t-test* (*
600 = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). ZeroCa2+: Zero Calcium; Ca2+: Calcium; Hyper:
601 Hyperforin; SKF: SKF96365; DBQ: 2.5-Di-t-butylhydroquinone.

602

603 **Supplemental data legend**

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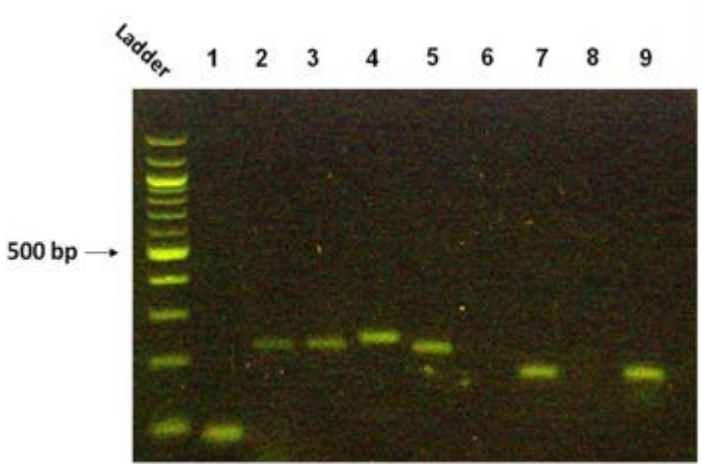

Supplemental Figure 1. TRPC isofoms expressed in bovine oviduct epithelium. PCR Products eletrophoresed on a 2% agarose gel, indicating positive expression of TRPC 1, 2, 3, 4 and 6 in bovine oviduct tissue. Expression of TRPC5 and TRPC7 was not detected. PCR products were loaded on the gel as following: lane 1; β actin (100 bp), lane 2; TRPC1 (232 bp), lane 3; TRPC2 (233bp), lane 4; TRPC3 (244 bp), lane 5; TRPC4 (227 bp), lane 6 ; TRRPC5 (179 bp), lane 7; TRPC6 (183 bp), lane 8; TRPC7 (168 bp) and lane 9; Cytokeratin18 (181 bp).

Gene	Primer	Sequence	Tm (°C)
Bovine β-actin	β actin F	TTCAACACCCCTGCCATG	59.64
	β actin R	CACCGGAGTCCATCACGAT	59.73
Bovine	bCytkr18E3E4F	TGAGATCGAGGCTCTCAAGG	60.63
cytokeratin18	bCytkr18E3E4R	TGAGCCAGCTCGTCATACTG	60.16
Bovine TRPC1	bTRPC1E5E7F	CTCGTGGAGGTGGAATTCAG	60.65
	bTRPC1E5E7R	TGGACTGGGAAACAAACTCC	59.94
Bovine TRPC2	bTRPC2E3E4F	TCATCCTGACTGCCTTCCTC	60.35
	bTRPC2E3E4R	ATGAGCATGTTGAGCAGCAC	60.02
Bovine TRPC3	bTRPC3E2E4F	CAAAAAGTTCGTGGCTCACC	60.67
	bTRPC3E2E4R	GCCCAGGAAGATGATGAAAG	59.63
Bovine TRPC4	bTRPC4E6E7F	GACCAATGTCAAAGCACAGC	59.30
	bTRPC4E6E7R	CATTGAAGGGGGTAGGAAGG	60.67
Bovine TRPC5	bTRPC5E6E7F	TGATCGCCATGATGAACAAC	60.49
	bTRPC5E6E7R	TTGTTGAACCAGTTGCCAAG	59.73
Bovine TRPC6	bTRPC6E6E7F	TGCTTGATTTTGGAATGCTG	59.81
	bTRPC6E6E7R	AGGGGTCCCACTTTATCCTG	60.18
Bovine TRPC7	bTRPC7E3E4F	TCCTGGCTGTCTTTGGAGTC	60.39
	bTRPC7E3E4R	CTGATGCGTTCAGAACCAAC	60.16

Supplemental Table 1. Primers used for TRPCs gene detection in bovine oviduct epithelium using conventional PCR

Gene	Primer	Sequence	Tm (°C)
Bovine TRPC1	QbTRPC1E6E7F	CCGGCAGTGTAAAATGTTTGC	59
	QbTRPC1E6E7R	CATTGGATGTATGGTTTAGGATAACTTC	58
Bovine TRPC6	QbTRPC6E4F	CCCATCCAAACTGCCAACAG	60
	QbTRPC6E4R	GCGAGGACCACAAGGAACTT	59

Supplemental Table 2. Primers used in RT- qPCR reaction for detecting the changes in TRPC genes expression in bovine oviduct epithelium. The RT-q PCR conditions consisted of 95°C for 10 minutes

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followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute and a cycle of melt curve consisted of 95°C for 15 seconds, 60ºC for 1minutes and 95°C for 15 seconds.

A

TRPC 1	Control	E2	FSH/LH	P4	P4/E2	P4/FSH/LH	E2/FSH/LH	P4/E2/LH/FSH
Stage 1	-	0.60f ↓	8.12f ↑	0.60f ↓	-	-	32.00f ↑	-
Stage 2	-	1.30f ↑	3.49f ↑	1.50f ↑	2.70f ↑	2.63f ↑	16.82f ↑	3.24f ↑
Stage 3	-	0.72f ↓	3.00f ↑	0.55f ↓	-	-	61.31f ↑	0.53f ↓
Stage 4	-	3.44f ↑	22.12f ↑	5.44f ↑	20.00f ↑	5.03f ↑	86.00f ↑	2.40f ↑

B

TRPC 6	Control	E2	FSH/LH	P4	P4/E2	P4/FSH/LH	E2/FSH/LH	P4/E2/LH/FSH
Stage 1	-	-	14.95 ↑f	-	9.18f ↑	-	175.00 f↑	27.10f ↑
Stage 2	-	-	14.43f ↑	8.10f ↑	14.20f ↑	65.60f ↑	382.00 f↑	31.40f ↑
Stage 3	-	-	70.40f ↑	8.60f ↑	9.71f ↑	13.00f ↑	933.00 f↑	10.50f ↑
Stage 4	-	11.50 f↑	74.40f ↑	17.10f ↑	183.00 f↑	2.40f ↑	726.00 f↑	3.39f ↑

Supplemental Table 3. (A) Effect of sex hormones on TRPC1 gene expression in BOECs throughout the estrous cycle. n=3; No significant changes:- ; Fold change: f ; E2: Estrogen; P4: Progesterone. (B) Effect of sex hormones on TRPC6 gene expression in BOECs throughout the estrous cycle. n=3; No significant changes: - ; Fold change: f ; E2: Estrogen; P4: Progesterone.

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