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# Expression and function of transient receptor potential channels in the female bovine reproductive tract

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The epithelium lining the oviduct 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, and progesterone on TRPCs expression and intracellular calcium flux were determined. Transient receptor potential channels 1, 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, 17β-estradiol, FSH, and LH individually and in combination upregulated TRPC1 and 6 expression in cultured bovine oviduct epithelial cells although progesterone antagonized these effects. Functional studies showed changes in calcium mobilization in bovine oviduct epithelial cells were dependent on TRPCs. In conclusion, TRPC1, 2, 3, 4, and 6 are present in the epithelium lining the bovine oviduct, and TRPC1 and 6 vary through the estrous cycle suggesting an important role in early reproductive function.

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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 fertilization [2]. Calcium transport across epithelial cells occurs by a number of mechanisms, including transit across tight junctions, $Na^{+}/Ca^{2+}$ exchangers, voltage-dependent $Ca^{2+}$ channels and members of the transient receptor potential (TRP) channel superfamily [3–5].

The TRP superfamily comprises 28 proteins, characterized 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 (Canonical); TRPV (Vanilloid); TRPM (Melastatin); TRPP (Polycystin); TRPML (Mucolipin); TRPA (Ankyrin); and TRPN (NOMPC) [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 oviduct epithelia [3,8–10], which is surprising because 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], fertilization

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[14], and the cleavage stages of embryo development [15], whereas 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 LH surge, which in turn triggers nuclear and cytoplasmic maturation of the oocyte [18]. The tissue of the recently ovulated follicle which expresses 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 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 to 4 of the estrous cycle, and then dramatically until Day 8 when a plateau is reached [18]. A decrease in progesterone concentration and 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 to 4 days before estrous and is completed within 2 days [18].

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 because of 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 transient receptor potential channels (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 (HBSS) without $CaCl_2$ and $MgCl_2$ (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 is determined according to the gross morphology of the ovary [26]. Because 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 (BOECs)

Oviducts were dissected from the reproductive tract and connective tissue carefully removed. Bovine oviduct epithelial cells were harvested by squeezing the oviduct from isthmus to infundibulum. Cells were collected in HBSS and centrifuged at 2500 × *g* for 5 minutes. The supernatant was discarded, and the cells washed twice more by this process. The cell pellet was then resuspended 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% newborn calf serum [v:v], 2.5% fetal calf serum [v:v], 0.1% albumin from bovine serum [w:v; 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 5 × 106/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 7 days.

### 2.3. RNA extraction and quantitative real-time polymerase chain reaction (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 nano spectrophotometer (Implen, Germany). Isolated RNA with a 260/280 ratio of ∼2 was used for further experiments. Isolated RNA was reverse-transcribed to complementary DNA using EZ-First Strand cDNA Synthesis Kit (Geneflow, Isreal). One microgram 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 Supplementary Tables 1 and 2). Relative gene expression was analyzed using StepOne software v2.0, and the baseline and threshold were set manually. Quantitative real-time polymerase chain reaction data were analyzed 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 nonpermeabilized to examine cell surface localization of TRPC1 and 6. The oviduct was divided into infundibulum, ampulla, and isthmus on the basis of morphology of the tube, before the sectioning.

Nonspecific binding sites were blocked with 2% donkey serum (Sigma–Aldrich) in PBS for 30 minutes at room temperature. Samples were then incubated with 1 μg/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 in the humidified chamber at 4 °C overnight. Primary antibodies were removed, and the slides were washed with PBS containing 0.25% Tween 20 (Sigma–Aldrich). Secondary antibodies, 4 μg/mL Alexa Four 647 donkey anti goat (Invitrogen; against TRPC1 primary) and 4 μg/mL Alexa Flour 488 donkey anti rabbit (Invitrogen; against TRPC6 primary),

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