Expression and localization of two-pore domain K⁺ channels in bovine germ cells

Chang-Gi Hur¹, Changyong Choe², Gyu-Tae Kim, Seong-Keun Cho¹, Jae-Yong Park, Seong-Geun Hong, Jaehee Han and Dawon Kang

Department of Physiology, College of Medicine and Institute of Health Sciences, Medical Research Center for Neural Dysfunction, Gyeongsang National University, 90 Chilam-dong, Jinju, Gyeongnam 660-751, South Korea, ¹CHO-A Biotechnology Research Institute, CHO-A Pharmaceutical Company Ltd, Seoul 150-992, South Korea and ²Animal Genetic Resources Station, National Institute of Animal Science, RDA, Namwon 590-832, South Korea

Correspondence should be addressed to D Kang; Email: dawon@gnu.ac.kr; J Han; E-mail: jheehan@gnu.ac.kr

Abstract

Two-pore domain K⁺ (K₂P) channels that help set the resting membrane potential of excitable and nonexcitable cells are expressed in many kinds of cells and tissues. However, the expression of K₂P channels has not yet been reported in bovine germ cells. In this study, we demonstrate for the first time that K₂P channels are expressed in the reproductive organs and germ cells of Korean cattle. RT-PCR data showed that members of the K₂P channel family, specifically KCNK3, KCNK9, KCNK2, KCNK10, and KCNK4, were expressed in the ovary, testis, oocytes, embryo, and sperm. Out of these channels, KCNK2 and KCNK4 mRNAs were abundantly expressed in the mature oocytes, eight-cell stage embryos, and blastocysts compared with immature oocytes. KCNK4 and KCNK3 were significantly increased in eight-cell stage embryos. Immunocytochemical data showed that KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 channel proteins were expressed at the membrane of oocytes and blastocysts. KCNK10 and KCNK4 were strongly expressed and distributed in oocyte membranes. These channel proteins were also localized to the acrosome sperm cap. In particular, KCNK3 and KCNK4 were strongly localized to the post-acrosomal region of the sperm head and the equatorial band within the sperm head respectively. These results suggest that K₂P channels might contribute to the background K⁺ conductance of germ cells and regulate various physiological processes, such as maturation, fertilization, and development.

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Introduction

Potassium (K⁺) channels, which form the largest family of ion channels, play a pivotal role in a variety of physiological processes in mammalian germ cells (Darszon et al. 1999, Everill & Kocsis 1999, Trimarchi et al. 2002, Tosti & Boni 2004, Winston et al. 2004, Yang et al. 2004). Ion channels are involved in electrical modification of plasma membrane of gametes. In bovine oocytes, the levels of K⁺ and L-type Ca²⁺ channels that are preponderant at the germinal vesicle stage are decreased at the metaphase II (MII) stage oocytes. At fertilization, sperm induce Ca²⁺ release from the intracellular stores that gate the Ca²⁺-activated K⁺ channels (Tosti & Boni 2004). These ion channels are also involved in electrical modification of sperm plasma membrane during chemotaxis and acrosome reaction processes (Tosti & Boni 2004). A voltage-dependent inward rectifying K⁺ channel that shows high conductance and the ether-a-go-go-related K⁺ channels control the cell cycle during mouse embryonic development (Day et al. 1993, 1998, Winston et al. 2004). The high-conductance K⁺ channels are active in oocytes, but their activity was decreased during mouse embryonic development with membrane depolarization (Day et al. 1993, Kang et al. 2006). Other K⁺ channels could be also involved in reproductive physiology. Two-pore domain K⁺ (K₂P) channels are responsible for K⁺ efflux during apoptotic volume decreases (AVD) in mouse zygotes (Trimarchi et al. 2002). Quinine sensitive-, TEA insensitive-, and calcium independent-K⁺ channels regulate the volume and motion of human sperm (Yeung & Cooper 2001), indicating that these channels possess K₂P channels. K₂P channels are sensitive to quinine and insensitive to TEA and calcium, and a K⁺ channel reported by Day et al. (1993) also shows similar single-channel kinetics and pharmacological properties to KCNK10, a member of the K₂P channel family. These reports give rise to study of the physiological roles and function of K⁺ channels.
Among K⁺ channels, K₂P channels set and stabilize the resting membrane potential and behave as leak K⁺ channels when they are expressed in *Xenopus* oocytes and mammalian cells (Kim 2005). These channels are expressed in both excitable and nonexcitable cells and regulated by a variety of biologically relevant stimuli, such as receptor ligands, temperature, lipids, pressure, oxygen tension, volatile anesthetic agents, pH, and neurotransmitters (Kim 2003). Recent studies using RT-PCR, immunostaining, and western blotting have demonstrated that K₂P channels are expressed in mammalian reproductive systems. KCNK3, KCNK5, KCNK17, KCNK15, and KCNK2 are detected in human cytotrophoblast cells, placental villous tissue and trophoblast cells, myometrium, and placental vascular system (Bai *et al.* 2005a, 2005b, 2006). KCNK2 and KCNK4 are also expressed in human uterine smooth muscle, and KCNK2 expression is significantly increased in pregnant term tissues (Tichenor *et al.* 2005). KCNK5, KCNK2, and KCNK4 are expressed by and localized specifically to monkey sperm (Chow *et al.* 2007).

Numerous studies have suggested that the K₂P channels could regulate cell excitability and a wide range of physiological processes in mammalian cells (Kim 2003, 2005, Talley *et al.* 2003, Besana *et al.* 2005, Kang & Kim 2006, Sanders & Koh 2006). In reproductive cell types, the presence and function of K₂P channels were also reported (Bai *et al.* 2005a, 2005b, 2006, Tichenor *et al.* 2005, Wareing *et al.* 2006, Chow *et al.* 2007). However, the expression and localization of the K₂P channels in bovine germ cells have not yet been reported. In this study, we identified the expression and localization of the K₂P channels in germ cells of Korean cattle, known as Hanwoo.

**Results**

**Alterations in two-pore domain K⁺ (K₂P) channel mRNA expression during bovine embryonic development**

To identify whether the K₂P channels are expressed in bovine germ cells, RT-PCR was performed. RT-PCR data showed that KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9, members of the K₂P channel family, were expressed in oocytes, immature and mature (metaphase II, MII) oocytes, eight-cell stage embryos, and blastocysts of Hanwoo (Fig. 1A). The mRNA levels of these channels were quantified using real-time PCR in immature oocytes, MII oocytes, eight-cell stage embryos, and blastocysts (Hanwoo) (Fig. 1A). The mRNA expression level was normalized to GAPDH. (C) Alteration in mRNA levels of KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9, during bovine embryonic development. The mRNA expression of KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 was quantified by means of real-time PCR in immature oocytes, MII oocytes, eight-cell stage oocytes, and blastocysts. The mRNA expression level was normalized to GAPDH. Each data point is the mean ± s.e.m. of 10 repeated experiments. *Significantly different from the corresponding control value obtained in immature (IM) oocytes (P<0.05). MII, eight cell, and BL indicate mature oocytes arrested at the time metaphase II, eight-cell stage embryo, and blastocyst respectively.

Figure 1 Expression of the K₂P channels in bovine reproductive organs and female germ cells. (A) The expression of KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 in ovary, oocytes, and embryos. RT-PCR products were separated on a 1.2% standard agarose gel and stained with ethidium bromide. (B) mRNA levels in the ovary and testis. Each bar is the mean ± s.e.m. of 10 repeated experiments. The y-axis shows relative mRNA levels on a log scale. The mRNA expression level was normalized to GAPDH. Each data point is the mean ± s.e.m. of 10 repeated experiments. *Significantly different from the corresponding control value obtained in immature (IM) oocytes (P<0.05). MII, eight cell, and BL indicate mature oocytes arrested at the time metaphase II, eight-cell stage embryo, and blastocyst respectively.

To identify whether the mRNA expression of K₂P channels changes during bovine embryonic development, real-time PCR was also performed (Fig. 1C). In female germ cells, KCNK2 and KCNK4 mRNA levels increased by approximately fourfold in MII oocytes compared with immature oocytes. KCNK4 and KCNK3 were increased in MII oocytes and eight-cell stage embryos but decreased in blastocysts, while KCNK2, KCNK10, and KCNK9 were slightly increased in MII oocytes but did not show significant changes in eight-cell stage embryos and blastocysts.
**K2P channel expression and localization in bovine germ cells**

The presence of mRNA in cells, however, does not necessarily imply the translation of mRNA into protein. The expression of K2P channels identified by RT-PCR was further studied at the protein level. The specificity and sensitivity of antibodies used in this study were previously confirmed and proved in this study. Each antibody specifically detected its own antigen (Kang et al. 2007a, 2007b, Supplementary Figures 1 and 2, which can be viewed online at www.reproduction-online.org/supplemental/). Immunocytochemical data stained with specific antibodies for the K2P channels showed that KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 were expressed and localized at the plasma membrane of oocytes and blastocysts. Oocytes tended to express highly KCNK10 and KCNK4 proteins, compared with other channels (Fig. 2). However, KCNK10 showed low expression in dorsal root ganglion (DRG) neurons, human breast cancer cells, and HaCaT cells, indicating that anti-KCNK10 antibody could specifically detect KCNK10 antigen in the bovine oocytes (Supplementary Figure 2). As shown in Fig. 2, KCNK4 was significantly decreased in MII oocytes and blastocysts compared with immature oocytes, while KCNK10 was increased in MII oocytes but decreased in blastocysts. KCNK9 was increased in MII oocytes and blastocysts. KCNK2 and KCNK3 were decreased in MII oocytes but increased in blastocysts.

In addition to female germ cells, K2P channel expression was analyzed in mature sperm, a male germ cell. KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 were detected in bovine sperm by immunocytochemistry. Negative and positive controls were checked by omitting the primary antibody and transfecting the K2P channel cDNA respectively. No staining was observed on any of the negative control cells. These channel proteins were distributed at sperm head, including acrosomal cap (Fig. 3). Specifically, KCNK3 and KCNK4 were predominantly expressed in the post-acrosomal region of the sperm head and at the equatorial band within the sperm head respectively.

![Figure 2](https://www.reproduction-online.org)
**Discussion**

*K_{2p} channel proteins* are expressed in many kinds of cells and tissues and regulated by various biophysical and chemical stimuli, and thus they could be involved in a variety of cellular functions. This study demonstrates for the first time that bovine reproductive tissues (ovary and testis) and germ cells (oocyte, embryo, and sperm) express the *K_{2p} channels*, and suggests that these channels could be involved in the regulation of oocyte maturation, fertilization, and development. These channels were distributed on the membranes of oocytes and blastocysts, and were localized to a special region of the bovine sperm.

As shown in Fig. 1, the mRNA levels of *KCNK3* and *KCNK4* were dramatically increased in eight-cell stage embryos after fertilization. On average, *KCNK3* showed low expression in immature and MII oocytes. In eight-cell stage embryos, the increase of *KCNK3* mRNA might be the result of a fertilization event induced by sperm *KCNK3*, which is strongly expressed in the post-acrosomal region. A marked increase of *KCNK4* in eight-cell stage embryos may also include *KCNK4* originating from the fertilizing sperm. Sperm depends on ion channels to rapidly exchange information with the outside world and to fertilize the MII oocyte (Darszon et al. 2007). The equatorial band and post-acrosomal region of sperm head, which in the study predominantly expressed *KCNK4* and *KCNK3*, are important during and after fertilization (see Fig. 3). Specifically, the equatorial band that is located on the middle of the sperm head is first bound with the oocyte membrane at fertilization (Mahony & Gwathmey 1999, Travis et al. 2001, Turner 2003). We further compared the channel expression pattern between intact sperm and acrosome-reacted sperm. In the acrosome reacted sperm, *KCNK3* and *KCNK4* proteins were still expressed at the post-acrosomal and equatorial regions respectively, although the levels of the *K_{2p} channel* proteins expressed in the acrosome cap were reduced (Supplementary Figure 3, which can be viewed online at www.reproduction-online.org/supplemental/). Further studies are needed to analyze the expression pattern of the *K_{2p} channel* proteins in more detail. However, one of the limitations of dual staining is the difference in fluorescence intensity. The intensity of TRITC-conjugate antibody (red) is lower than that of FITC-conjugated PSA (green). This limitation of dual staining should be considered in the comparison of protein expression. Nonetheless, the expression and distribution of the *K_{2p} channel* proteins in mammalian sperm give insight into the physiological role of these channels in acrosome reaction and fertilization.

As shown in Figs 1 and 2, the *K_{2p} channel* expression is changed during embryonic development, although the alteration in mRNA and protein levels showed different patterns among the channels. In mRNA expression, the channels were generally increased in MII oocytes compared with immature oocytes, and *KCNK4* and *KCNK3* were significantly increased in eight-cell stage embryos (P<0.05), suggesting that the expression of these channels may affect maturation, fertilization, and development. In another study, we observed that inhibitors of the *K_{2p} channels* significantly...
reduced maturation, fertilization, and development rate in bovine and mouse oocytes, and embryos (data not shown).

Voltage-dependent K⁺ and Ca²⁺ channels are widely recognized as critical players in the physiological functions of gametes and embryos, such as maturation, fertilization, cell cycle, and development (Mitani 1985, Day et al. 1993, 1998, Tosti & Boni 2004, Winston et al. 2004, Boni et al. 2007). In addition to voltage-dependent channels, voltage-independent K₂P channels could control reproductive cell functions as a modulator that controls voltage-dependent mechanisms in reproductive cells. Gametes and embryos are excitable cells in which transmembrane ion currents cause rapid metabolic responses (Tosti & Boni 2004, Cuomo et al. 2006). Various biological and physiological events due to a dramatic ion flux appear or disappear during gamete maturation, fertilization, cell division, and early embryonic development. The formation of the blastocyst, which is required for implantation and establishment of pregnancy, requires the ion transport and channel system (Barcroft et al. 2003). K₂P channels were also expressed in bovine blastocysts. However, the expression levels of these channels can be affected by in vitro culture conditions, thus the developmental rate to blastocyst can change during in vitro culture or in vivo. The KCNK2, KCNK10, and KCNK4 channels are regulated by the changes in temperature (Maingret et al. 2000, Kang et al. 2005, 2007a, 2007b) and reactive oxygen species (Kim et al. 2007) that may occur during in vitro maturation (IVM), fertilization (IVF), and development. Increased ambient temperature (heat shock) during IVM and/or IVF significantly reduced oocyte maturation, fertilization rates, and subsequent embryonic development (de Castro & Hansen 2007, Sugiyama et al. 2007). During in vitro manipulation including IVF procedures, germ cells tend to exposure to lower temperature than 37 °C or 39 °C. The low temperature induces close of KCNK2, KCNK10, and KCNK4 channels. The changes in membrane potential by thermosensitive channels are likely to affect to in vitro embryonic development. Also, oxygen (O₂) concentration is a factor that modulates the K₂P channels. Earlier studies have shown that in vitro cultured embryos could not develop normally because O₂ concentration in in vitro is higher than that in oviduct (Dalvit et al. 2005, Nagai et al. 2006). Hydrogen peroxide (H₂O₂) and nitric oxide that may be induced by high O₂ concentration activate KCNK2 and KCNK10 channels (Koh et al. 2001, Kim et al. 2007). These culture conditions are likely to increase or reduce the activity of K₂P channels that may affect in vitro embryonic development.

The identification of the K₂P channels expressed in reproductive organs and germ cells may help the understanding of ion channel-related function in reproductive physiology. Further study is needed to study the function and current recording of the K₂P channels expressed in mammalian germ cells. This should be helpful for research to improve bovine embryo technologies.

Materials and Methods

Oocyte recovery and in vitro embryo production

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory in PBS at 35–39 °C. All experiments were performed with the approval of Animal Ethics Committee of Gyeongsang National University. Cumulus-oocyte-complexes (COCs) were aspirated from antral follicles of 2–6 mm diameter with an 18 G needle fitted to a 10 ml syringe. After washing twice with Dulbecco’s PBS (D-PBS) containing 1% (w/v) polyvinyl alcohol (PVA), oocytes were examined under a 40× stereomicroscope (SZ60, Olympus, Tokyo, Japan). Oocytes with multiple layers of compact cumulus cells and evenly granulated cytoplasm were selected for this study. Fifteen COCs were transferred into a 50 μl drop of maturation medium under mineral oil. The COCs were then incubated at 39 °C in a humidified atmosphere with 5% (v/v) CO₂ in air. The medium used to mature COCs was TCM-199 containing Earle’s salts, 10% (v/v) fetal bovine serum (FBS, Invitrogen), 25 mmol/1 HEPES, 2.5 mmol/1 sodium pyruvate, 1 mmol/1 L-glutamine, and 0.1% (w/v) penicillin–streptomycin (Pen–Strep, Invitrogen). The pH and osmolality of the media were adjusted to 7.4 and 285 mOsm/kg respectively. At 22 h of maturation, cumulus cells were partially removed from the oocytes by vortexing for 10 s in Tyrode’s albumin lactate pyruvate medium containing 2% (w/v) BSA (Fraction V) and 10 mM HEPES (washing-TALP medium). Five COCs were transferred into a 50 μl fertilization-TALP droplet containing 10 μg/ml heparin, 2.5 mmol/1 caffeine, and 0.3% (w/v) BSA. The oocytes were then inseminated with sperm at a final concentration of 2×10⁶ sperm/ml. The cumulus cells were removed after 6 h of culturing post-insemination by vortexing for 2 min in washing-TALP medium. Cumulus-free zygotes were washed several times in washing-TALP medium and TCM-199 medium supplemented with 10% (v/v) FBS. After transferring 15 presumptive zygotes into a 50 μl droplet of TCM-199 medium, the embryos were cultured for 9 days.

Sperm isolation

The testes of Korean cattle were collected from a slaughterhouse and transported to the laboratory within 1 h on ice. Sperm collected from cauda epididymis were treated with the swim-up procedure. Briefly, sperm were washed once in 10 ml washing-TALP medium by centrifugation at 300 g for 10 min. From 0.5 ml sperm pellet, five 0.1 ml aliquots were transferred into five 2-ml round-bottom test tubes; then, each was overlaid with 0.5 ml fertilization-TALP medium. With the tubes tilted at a 45° angle, incubation at 39 °C in a humidified atmosphere of 5% CO₂ in air for 1 h allowed the motile sperm to swim-up. The top 0.4 ml of the supernatant was harvested and further centrifuged at 300 g for 10 min to yield a sperm pellet, which was resuspended in 0.6 ml fertilization-TALP medium. The resuspended sperm were used for immunostaining.
Reverse transcriptase (RT-PCR) analysis

First-strand cDNAs were synthesized from total RNA isolated from Hanwoo ovary and testis using oligo dT (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen). In vitro produced cells (60 immature oocytes, 60 mature oocytes, 35 eight-cell stage embryos, and 15 blastocysts) were lysed, and the lysates were used for cDNA synthesis. cDNA was generated from the lysate in a single tube using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). First-strand cDNA was used as a template for PCR amplification. Specific primers for each K_2P channel were used for PCR with Taq polymerase (Takara, Otsu, Japan). Table 1 lists the DNA sequences of primers used to detect the expression of each K_2P channel. PCR conditions were initial denaturation at 94°C for 4 min, then 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s, and a final extension step at 72°C for 10 min.

The DNA fragments obtained from the ovary, testis, and germ cells by RT-PCR were directly sequenced with the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, CA, USA).

Real-time PCR

K_2P channel expression identified by RT-PCR was quantified by means of real-time PCR with FastStart DNA Master SYBR Green 1 (Roche Applied Science) using the LightCycler System (LightCycler 2.0 instrument, Roche). The mRNA expression of each of the K_2P channels was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR primers were designed by using GenScript (https://www.genscript.com/ssl-bin/app/primer). Table 2 lists the DNA sequences of primers used to detect the expression of each K_2P channel. PCR conditions were consisted of a denaturing cycle (95°C for 10 min), 50 cycles of PCR (95°C for 7 s, 56°C for 7 s, and 72°C for 10 s), a melting cycle (95°C for 0 s and 65°C for 60 s) including a step cycle (starting at 65°C up to 95°C with a 0.1°C/s transition rate), and a cooling cycle (40°C for 30 s). Melt-curve analysis was conducted to confirm the homogeneity of each product, and the products were electrophoresed on a 1.2% (w/v) agarose gel to check product size.

Immunostaining of germ cells

Germ cells (oocytes, embryo, and sperm) were washed in PBS containing 0.1% PVA and fixed with 4% (w/v) paraformaldehyde in 0.1 M PBS for 2 h. The germ cells were washed in PBS, permeabilized in PBS containing 0.1% (v/v) Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA) for 10 min, and preincubated in a blocking buffer containing 2.0% (v/v) normal goat serum (NGS) for 3 h at room temperature under gentle rotation. The oocytes and embryos were incubated with affinity-purified polyclonal antibodies for KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9, which were diluted 1:150 in PBS containing 0.1% (w/v) Triton X-100. Sperm were smeared on slides and then fixed in –20°C methanol for 30 min. Fixed slides were exposed to affinity-purified polyclonal antibodies for KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 that were diluted 1:150 in PBS containing 0.1% gelatin (w/v), 0.05% NaN_3, and 1% (v/v) NGS for 3 h at room temperature under gentle rotation.

Table 1 Bovine primer sequences used for reverse transcriptase PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5'–3')</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
</table>
| GAPDH     | Forward: CAGCCGACACTCAGTCTCCAC  
Reverse: GGAATTCAGGAGATTTCTAGT | NM_001034034 |
| KCNK2     | Forward: CTGATTGTGGATCTGAAG | NM_174686 |
| KCNK10    | Forward: CTGATTGATGAGAGTTT  
Reverse: GTGAAGAGGTGTTT | XM_603455 |
| KCNK4     | Forward: GAGTTGACAGATGTTGAG  
Reverse: AGATGACAGAGATGTTGAG | XM_869390 |
| KCNK3     | Forward: GTGACCAGCAGAAGTAGAT | XM_597401 |
| KCNK9     | Forward: GTGACCAGCAGAAGTAGAT | XM_588194 |

Table 2 Bovine primer sequences used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5'–3')</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
</table>
| GAPDH     | Forward: ATGATCTACATGTCTCCA  
Reverse: GAGTATGAGGAGATTTCTAGT | NM_001034034 |
| KCNK2     | Forward: AGATGCTATATGGATTTT | NM_174686 |
| KCNK10    | Forward: ATAAATGGCGTCCAGG  
Reverse: CTGACATCGCTGAGAGA | XM_603455 |
| KCNK4     | Forward: GGATGATAGAGAGTAGAT  
Reverse: GTGACCAGCAGAAGTAGAT | XM_869390 |
| KCNK3     | Forward: CGAAGAGGAGATGTTGAG  
Reverse: AGATGACAGAGATGTTGAG | XM_597401 |
| KCNK9     | Forward: CTGACCAGCAGAAGTAGAT | XM_588194 |
Capacitation and the acrosome reaction of bovine sperm

Fresh sperm (10^6) isolated by a swim-up preparation were incubated at 39 °C and 5% CO_2 for 4 h in washing-TALP medium containing heparin (15 μg/ml) to achieve capacitation. After capacitation, the acrosome reaction was induced by the addition of 10 μM ionomycin (Calbiochem, CA, USA) for 20 min. Sperm suspension isolated by a swim-up preparation was used as the control sample (without heparin and ionomycin).

Detection of an acrosome reaction of capacitated bovine sperm

Sperm smeared onto a slide were dried and fixed with cold methanol (−20 °C), then washed thrice in PBS. The sperm smears were incubated with FITC-conjugated Psuma sativum agglutinin (FITC-PSA, 100 μg/ml) in a dark chamber for 30 min at room temperature. After washing thrice in distilled water, the sperm were subjected to propidium iodide (PI, 50 μg/ml) and wet mounted on glass slides. Stained cells were observed with a confocal laser scanning microscope equipped with a 40× objective (IX-81, Olympus). Primary antibodies were purchased from Chemicon (Temecula, CA, USA; KCNK9) and Alomone Labs (Jerusalem, Israel; KCNK3, KCNK2, KCNK10, and KCNK4). All other chemicals were purchased from Sigma Chemical Co., unless specified.

Statistical analysis

Light Cycler Software 4.0 (Roche) and Flouview (FV1000, v. 1.5, Olympus) were used for real-time PCR data and immunofluorescence analysis respectively. Student’s t-test was used with P < 0.05 as the criterion for significance. Data are represented as mean ± S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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