

Original Article

Onion peel extract and its constituent, quercetin inhibits human Slo3 in a pH and calcium dependent manner

Tharaka Darshana Wijerathne¹, Ji Hyun Kim¹, Min Ji Kim¹, Chul Young Kim², Mee Ree Chae³, Sung Won Lee^{3,*}, and Kyu Pil Lee^{1,*}

¹Department of Physiology, College of Veterinary Medicine, Chungnam National University, Daejeon 34134, ²College of Pharmacy, Hanyang University, Ansan 15588, ³Department of Urology, Samsung Medical Center, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul 06351, Korea

ARTICLE INFO

Received July 3, 2019
Revised August 2, 2019
Accepted August 2, 2019

*Correspondence

Kyu Pil Lee
E-mail: kplee@cnu.ac.kr
Sung Won Lee
E-mail: drswlee@skku.edu

Key Words

KSper
LRRC52
Phosphoinositides
Quercetin
Slo3

ABSTRACT Sperm function and male fertility are closely related to pH dependent K⁺ current (KSper) in human sperm, which is most likely composed of Slo3 and its auxiliary subunit leucine-rich repeat-containing protein 52 (LRRC52). Onion peel extract (OPE) and its major active ingredient quercetin are widely used as fertility enhancers; however, the effect of OPE and quercetin on Slo3 has not been elucidated. The purpose of this study is to investigate the effect of quercetin on human Slo3 channels. Human Slo3 and LRRC52 were co-transfected into HEK293 cells and pharmacological properties were studied with the whole cell patch clamp technique. We successfully expressed and measured pH sensitive and calcium insensitive Slo3 currents in HEK293 cells. We found that OPE and its key ingredient quercetin inhibit Slo3 currents. Inhibition by quercetin is dose dependent and this degree of inhibition decreases with elevating internal alkalization and internal free calcium concentrations. Functional moieties in the quercetin polyphenolic ring govern the degree of inhibition of Slo3 by quercetin, and the composition of such functional moieties are sensitive to the pH of the medium. These results suggest that quercetin inhibits Slo3 in a pH and calcium dependent manner. Therefore, we surmise that quercetin induced depolarization in spermatozoa may enhance the voltage gated proton channel (Hv1), and activate non-selective cation channels of sperm (CatSper) dependent calcium influx to trigger sperm capacitation and acrosome reaction.

INTRODUCTION

A spermatozoon is a cell specialized to deliver its genetic information to the ovum to generate an embryo. To mobilize and fertilize the egg, spermatozoa use complicated molecular mechanisms to respond to the environment and regulate their motility. When sperm is released into the female reproductive system, they undergo capacitation, resulting from a rise in internal pH and calcium concentrations [1-3]. Sperm capacitation triggers hyper-activation and sperm chemotaxis, preparing the sperm for the fusion with egg (acrosome reaction) [4-7]. Therefore, successful

fertilization of the egg by spermatozoa depends on intracellular pH, membrane voltage, and intracellular calcium concentration ($[Ca^{2+}]_i$) which are regulated by ion channels in the plasma membrane of spermatozoa. In spermatozoa, hyper-activation is controlled by rises in $[Ca^{2+}]_i$ that change flagella beat patterns into asymmetrical ones, an essential ability for mammalian fertilization. Additionally, $[Ca^{2+}]_i$ plays a pivotal role in the acrosome reaction. Thus, drugs that can raise the internal calcium concentration can be used as fertility enhancers.

Genetic ablation studies have suggested that only a few ion channels are functionally required for male fertility. The CatSper



This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License, which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. Copyright © Korean J Physiol Pharmacol, pISSN 1226-4512, eISSN 2093-3827

Author contributions: T.D.W., J.H.K., M.J.K., C.Y.K., M.R.C., S.W.L., and K.P.L. designed the research; T.D.W., J.H.K., and M.J.K. performed the experiments; all authors contributed to the analysis of data and preparation of the manuscript.

channel is a sperm specific calcium ion channel. It is activated by intracellular alkalization that can be provided by the voltage gated proton channel Hv1 (in human sperm; HSper) and the genital hormone progesterone [8,9]. Both CatSper and Hv1 require membrane depolarization in order to function, which can be achieved by the inhibition of potassium currents of sperm (in human sperm; KSper) [10,11]. Together, voltage, pH_i, and progesterone work collectively to regulate Ca²⁺ influx into spermatozoa, though other regulatory mechanisms may also exist. Therefore, drugs that inhibit KSper activity have the potential to enhance sperm function by depolarizing the sperm membrane to activate Hv1, alkalizing the cytosol and resulting in CatSper activation allowing calcium to enter the spermatozoa.

Exact ion channels that conduct KSper currents has been a topic of debate. Two ion channels, namely Calcium-activated potassium channel subunit alpha-1 (KCNMA1; Slo1) and potassium channel subfamily U member 1 (KCNU1; Slo3) have been proposed as the upstream candidates that affect KSper [9,12,13]. However, both Slo3 and Slo1 gating properties were quite different from native KSper currents. On the other hand, Slo3 null mice were infertile and lacked all pH sensitive KSper currents, strongly indicating that Slo3 could be the ion channel responsible for KSper currents [11,14]. Interestingly, it was recently discovered that the Slo3 auxiliary subunit, leucine-rich repeat-containing protein 52 (LRRC52), expressed in human sperm together with Slo3, shifts the gating properties of Slo3 to a level similar to that of native KSper. This led to a consensus that Slo3 rather than Slo1 is the ion channel behind KSper currents [12,14-17]. Thus, pharmaceuticals targeting Slo3 and its auxiliary subunit LRRC52 have a potential to be used as fertility enhancers.

Onion peel extracts (OPE) have been widely used in oriental traditional medicine as a fertility enhancer [18-20]. Quercetin is the major functional compound present in OPE [21]. It has been found that quercetin increases sperm motility and quantity, stimulates reproductive organs and enhances sperm storage lifetimes in cryopreservation [22-28]. However, quercetin has also been reported to have negative effects such as reducing the number of litters in female mice, and conflicting reports have also reported reduced sperm motility [29,30].

It has been shown that quercetin activates the Slo1 channel [31]. The effects of quercetin on sperm specific Slo3 however, are yet to be studied. Here, we ask whether quercetin affects Slo3 activity. We found that quercetin has a dose dependent inhibitory effect on Slo3. This inhibitory effect could be negated by internal alkalization and increases to internal calcium concentration. Furthermore, the degree of effectiveness of quercetin on Slo3 depends on internal and external pH levels. Interestingly quercetin demonstrates inhibitory activity against a wide range of biological targets including protein kinase C (PKC), protein kinase A (PKA), phosphatidylinositol (PI) kinase and phospholipase A [32-35]. This guided us to test the effects of quercetin on Slo3 in the presence of PKC, PKA, and PI kinase inhibitors. However, none

of the signaling mechanisms seem to explain the reason behind an inhibitory effect of quercetin on Slo3. Therefore, we conclude that quercetin inhibits Slo3 currents in a pH and calcium sensitive manner. Further studies are needed to reveal the exact mechanism behind the inhibitory effect of quercetin on Slo3.

METHODS

Cell culture and transfection

Cell culture and transfection protocols described in our previous work were adopted here with minimal modifications [36]. Briefly, human embryonic kidney 293 cells were grown in Dulbecco's Modified Eagle Medium (GIBCO; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1× antibiotic-antimycotic reagent (Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum. Cells grown to a confluency of 80%–90% were transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific) transfection reagent according to the manufacturer's specifications. 1 µg of cDNA and 3 µl of Lipofectamine each were separately mixed with 50 µl OptiMEM (Thermo Fisher Scientific) in two 1.5 ml tubes and incubated at RT for 5 min. Following the incubation, the two mixtures were mixed together by rapid trituration. The resulting DNA-Lipofectamine mixture was incubated for 20 min at RT and put into a selected well in a 12-well cell culture plate. Before adding the DNA-Lipofectamine mixture, the culture media in the 12-well cell culture plate was replaced with OptiMEM. Cells were seeded on to glass coverslips 24 h after the transfection and were used in patch clamping experiments 36–48 h after transfection. 0.45 µg pCDNA3-hSlo3 (NP_114016.1) was co-transfected with 0.45 µg hLRRC52 (NP_114016.1) and 0.1 µg of pCDNA3.1-GFP for Slo3 experiments.

Electrophysiology

Electrophysiological protocols and saline solutions described in our previous work are adopted in this work with slight modifications [36]. GC 150TF-7.5 Glass capillaries (Harvard Apparatus, Holliston, MA, USA) were pulled using a vertical pipette puller (PC-10; Narishige Group Products, Tokyo, Japan) with heat levels adjusted to 60.4 Ω in the first step and 50.0 Ω in the second step. These pipettes possessed a 3–4 MΩ resistance when filled with K-aspartate based pipette solutions described as follows. Responses of hSlo3 to internal pH was studied using the following pipette solutions: 130 mM K-aspartate, 10 mM NaCl, 1 mM ethylene glycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM N-(2-hydroxyethyl)piperazine-N'-2-ethansulfonic acid (HEPES), 15 mM D-glucose (pH adjusted using KOH/HCl). The effect of internal free calcium on Slo3 was studied using the following pipette solutions: divalent-free solution; 130 mM K-aspartate, 10 mM NaCl, 1 mM EGTA, and 20 mM HEPES, (pH

7.3 using KOH); intracellular solutions with different free Ca^{2+} concentrations; 130 mM K-aspartate, 10 mM NaCl, 5 mM EGTA, $XCaCl_2$ and 20 mM HEPES; (pH 7.3 using KOH). Free calcium concentrations were calculated using the MaxChelator tool [37]. External bath saline solutions were as follows. Standard extracellular solution; 140 mM NaCl, 5 mM KCl, 10 mM HEPES 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH adjusted to 7.4 using NaOH). Whole-cell voltage-clamp experiments were performed at RT using an Axopatch 200B capacitor-feedback patch-clamp amplifier (Mo-

lecular Devices, Sunnyvale, CA, USA) connected to a Digidata-1440A Digitizer (Molecular Devices). Voltage protocol used for step pulses was as follows. 400 ms long step pulses with +25 mV voltage increments were given from -125 mV with a one second interval at -100 mV holding potential (Fig. 1A). Continuous voltage ramps of +140 mV (400 ms long) every 10 sec were given with a holding potential of -100 mV to monitor continuous gradual changes in channel activity. Membrane potential was corrected for the liquid junction potential (LJP) by deducting the LJP from

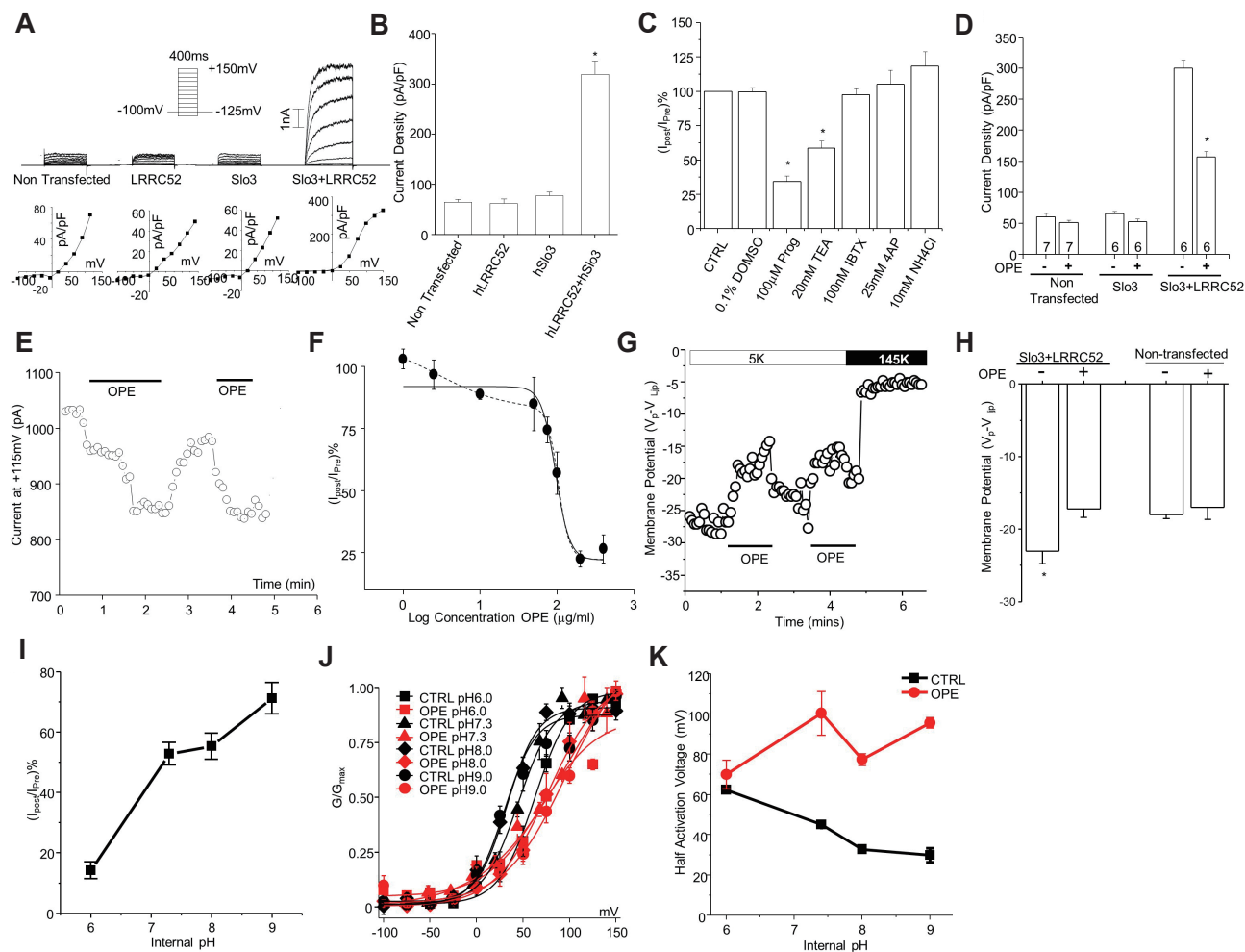


Fig. 1. Slo3 currents in HEK293 cells are inhibited by onion peel extract (OPE) in a pH dependent manner. Representative step pulse recordings (A, top) and IV curves (A, bottom) measured from cells transfected with different combinations of Slo3 and leucine-rich repeat-containing protein 52 (LRRC52) as indicated. (B) Current densities as calculated from non-transfected (n = 20), LRRC52 transfected (n = 8), Slo3 transfected (n = 8) and Slo3+LRRC52 co-transfected (n = 21) cells. (C) The percentage current remaining after the exposure to 0.1% DMSO (n = 15), 100 μ M progesterone (n = 7), 20 μ M tetraethylammonium (TEA) (n = 5), 100 nM Iberitoxin (bTX) (n = 5), 25 mM 4AP 4-aminopyridine (4-AP) (n = 10), and 10 mM NH4Cl (n = 6) for 60 sec. (D) Current density plot of non-transfected (n = 7), Slo3 transfected (n = 6) and Slo3+LRRC52 transfected (n = 6) cells before and after 60 sec of exposure to 100 μ g/ml OPE. (E) Continuous recording indicating the changes in Slo3+LRRC52 current upon exposure to 100 μ g/ml OPE perfused with external solution. (F) The inhibition of Slo3 currents by OPE at concentrations of 1 μ g/ml (n = 5), 2.5 μ g/ml (n = 6), 50 μ g/ml (n = 5), 75 μ g/ml (n = 5), 100 μ g/ml (n = 6), 200 μ g/ml (n = 5), 400 μ g/ml (n = 5), was dose-dependent that could be fitted to a single dose (continuous line) or double-dose response (dotted line). (G) Representative voltage recording indicating 100 μ g/ml OPE depolarize the membrane potential in Slo3 transfected cells in a reversible manner. (H) Average membrane potential shift due to OPE (n = 9) in non-transfected and Slo3 transfected cells. (I) Quercetin dependent inhibition of Slo3 diminishes with increasing internal pH. (J) Relative conductivity plot of Slo3 currents at mentioned internal pH values before and after exposure to the OPE. (K) Half activation voltages as calculated from GV curves at plate J at internal pH of 6 (n = 5) 7.3 (n = 5), 8 (n = 5) and 10 (n = 10). CTRL, control.

the measured pipette potential (V_p). LJP was calculated using the inbuilt LJP calculator in Pclamp 10.7.0.3 software (Molecular Devices).

Statistical analysis

Statistical analysis was performed according to the guidelines provided on experimental design and analysis in pharmacology [38]. Data acquired from Pclamp 10.7.0.3 software (Molecular Devices) were directly analyzed with Clampfit 10.7.0.3 software (Molecular Devices) or imported to Origin Pro 8.1 (OriginLab Corporation, Northampton, MA, USA) for further analysis. Generation of current voltage (IV) curves from step pulse data was done from IV plot tool in Clampfit. Other calculations and analysis were done using the integrated statistical analysis tools in Origin Pro 8.1 as described below. Conductance at different command voltages was measured by calculating the slope of the IV curves using a standard linear fit ($y = mx + c$). Calculated conductance values for each cell was normalized relative to the maximum conductance recorded in the same recording. The relative conductance values were plotted against voltage to calculate half activation voltage values using a Boltzman fit ($y = A2 + (A1 - A2) / (1 + \exp((x - x0) / dx))$).

Drug responses shown as percentage relative current remaining were calculated by dividing the current at +125 mV after treatment (I_{post}) by the current before treatment of drug (I_{pre}) in each cell, to avoid unnecessary variations arising from cell to cell differences. Current at +125 mV was calculated using the inbuilt IV tool in clampfit software by averaging the current between 350–400 ms at the +125 mV command step. These calculated relative percentage current values were compared to control values using the Wilcoxon signed-rank test. Non-normalized paired data were compared using a paired sample t-test. Other non-paired data were compared with a one-way ANOVA followed by a post hoc Tukey's mean comparison test. Dose response data were fitted using the dose response fit model ($y = A1 + (A2 - A1) / (1 + 10^{(\text{LOG}x0 - x)^p})$). All data are shown as mean \pm standard error of the mean represented using error bars, followed by the sample size in brackets. A level of probability below 0.05 was used as the threshold probability for statistical significance.

Materials

The brown outer layers of the onions were extracted three times with ethanol under reflux for 3 h. The combined filtrate was concentrated using a rotary evaporator, freeze dried, and stored at -20°C until further analysis. The HPLC profile of the onion extract and the compounds identified from it was standardized using a validated HPLC assay [21]. Stock solutions of OPE were made by dissolving them in the appropriate amount in DMSO to a final concentration of 100 mg/ml, which were then stored at -20°C . GF109203X (GFX), H-89 dihydrochloride, and wortman-

nin were purchased from Tocris bioscience (Bristol, UK). Quercetin, luteolin and morin were purchased from Cayman Chemical (Ann Arbor, MI, USA). Stock solutions of chemicals were made by dissolving the appropriate powder mass of each chemical in either DMSO or distilled water, which were then stored at -80°C until further use. Working solutions of chemicals were freshly made each day prior to experiments by dissolving the stock chemical solutions in electrophysiological saline solution.

RESULTS

OPE inhibits Slo3 K^+ current in heterologously expressed HEK293 cells

We first re-established a successful Slo3 expression system in HEK 293 cells for electrophysiological recording as previously reported [36] (Fig. 1A, B). The measured Slo3+LRRC52 current responded to classical Slo3 modulators such as progesterone and Tetraethylammonium (Fig. 1C). Furthermore, the measured Slo3 currents were sensitive to internal pH (Fig. 2F) but lacked the sensitivity to internal calcium (Fig. 3B) which is a previously been reported as a key feature of Slo3 channels that set them apart from calcium sensitive Slo1 channels [39]. After confirming the standard phenotype for Slo3 currents, we tested the effect of OPE on Slo3 currents under the same conditions. A concentration of 100 $\mu\text{g}/\text{ml}$ OPE inhibited Slo3 and LRRC52 current from 300.45 ± 12.69 pA/pF to 156.75 ± 8.96 pA/PF ($p < 0.001$). Furthermore, the inhibition of Slo3+LRRC52 currents by OPE was dose dependent with an EC_{50} of 95.75 $\mu\text{g}/\text{ml}$ (Fig. 1F). This dose response could be fitted to a double-dose response curve as shown by the dotted lines (Fig. 1F). The reason for double-dose response fitting in Slo3 could be due to the use of crude OPE: while quercetin was the main active ingredient, the mixture contains several other chemicals [21]. Furthermore, the inhibition of Slo3 by OPE was reflected by the reversible depolarization in membrane potential upon exposure to OPE (Fig. 1G, H). Membrane potential depolarized from -23.03 ± 1.69 mV to -17.22 ± 1.17 mV upon OPE administration ($p < 0.001$). This inhibition of Slo3 by OPE decreased with intracellular alkalization (Fig. 1I). The voltage-dependent activation of Slo3 currents was shifted to the left with intracellular alkalization (Fig. 1J, K), and OPE administration shifted the voltage-dependent activation of Slo3 towards the right, meaning that the voltage required to activate Slo3 moved towards more depolarized potentials (Fig. 1I, J). The half activation voltage value shifted from 62.35 ± 2.18 mV to 69.97 ± 15.77 mV at pH 6.0, 45.04 ± 3.00 mV to 100.42 ± 10.87 mV at pH 7.3, 32.76 ± 3.17 mV to 77.41 ± 6.59 mV at pH 8.0 and 29.92 ± 5.49 mV to 95.62 ± 7.59 mV at pH 9.0.

Current measured in non-transfected cells and those transfected with Slo3 alone (without LRRC52) was negligible ($p = 0.19$ and $p = 0.06$ respectively) (Fig. 1D), displaying a minimal response

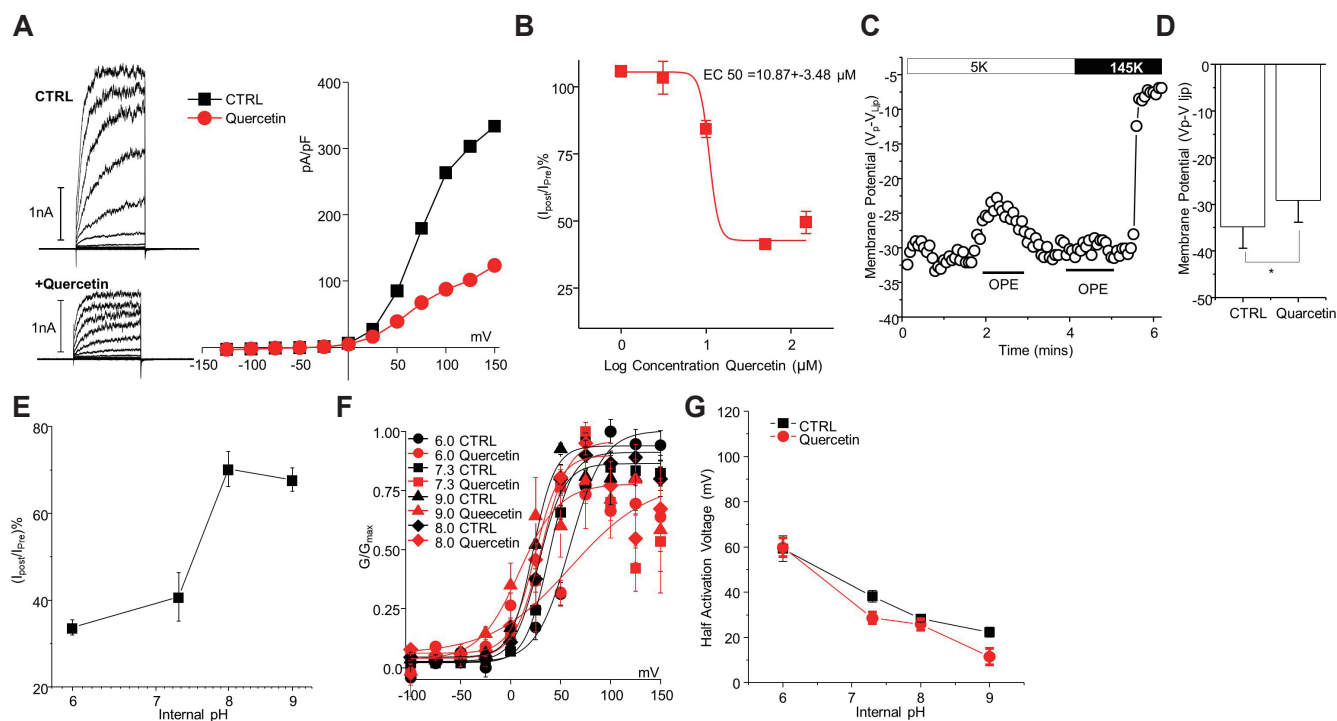


Fig. 2. Quercetin is the main component of onion peel extract (OPE) that inhibits Slo3 in a pH dependent manner. (A) Representative step pulses before (*top left*) and after (*bottom left*) exposure to 50 μM quercetin. (*Right*) IV curve generated from step pulses show at left. (B) Dose response of quercetin on Slo3 calculated at doses of 1 μM (n = 5), 3.12 μM (n = 5), 10 μM (n = 5), 50 μM (n = 7), and 150 μM (n = 5) reveals a EC₅₀ value of 10.87 ± 3.48 μM. Quercetin depolarizes the cell membrane in Slo3 over expressing cells as shown in continuous (C) recording and average membrane potential data (D) (n = 5). (E) Relative Slo3 current inhibition by quercetin at different internal pH levels of 6.0 (n = 8), 7.3 (n = 9), 8.0 (n = 7), and 9.0 (n = 7). (F) Normalized GV curves representing effect of quercetin on mentioned internal pH conditions. (G) Half activation voltages calculated from plate D, plotted against internal proton concentration. CTRL, control. *p < 0.05.

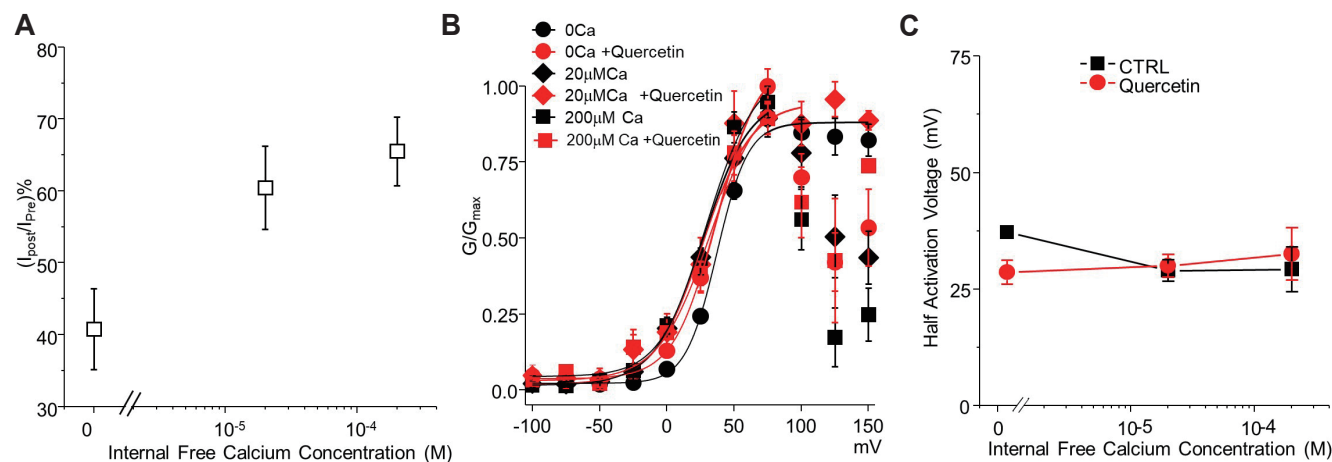


Fig. 3. Raising internal free calcium hinders the quercetin dependent inhibition of Slo3. Relative current remaining (A) and change in GV curves (B) after exposure to quercetin at internal free calcium concentrations of 0 μM (n = 8), 20 μM (n = 6), and 200 μM (n = 6) buffered by ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). (C) Half activation voltage calculated from the GV curves in plate B. CTRL, control.

to OPE. Similarly, the membrane potential of non-transfected cells slightly depolarized from -18.00 ± 0.51 mV to -17.00 ± 1.67 mV ($p = 0.50$) upon exposure to OPE (Fig. 1H). The combination of Slo3+LRRC52 yielded characteristic Slo3 currents, whereas the Slo3 alone cells and non-transfected HEK cells did

not. Therefore, from here onwards we refer to current measured from Slo3+LRRC52 co-transfected cells as ‘Slo3 current’. Taken together these results indicated that OPE inhibits Slo3 currents in a dose-dependent and internal pH dependent manner which depolarizes the cell membrane.

Quercetin, a major constituent of OPE, specifically inhibits Slo3

Quercetin is the major active component of OPE, which is a mix of several other active ingredients [21]. In order to investigate the contribution of quercetin, we tested its effects on Slo3 currents in isolation, without the other components of OPE. Quercetin, similarly to OPE, inhibits Slo3 current amplitude in a dose dependent and pH dependent manner with an EC_{50} value of $11.27 \mu\text{M}$ at internal pH 7.3 (Fig. 2A, B). As an example, quercetin inhibited Slo3 currents down to $33.17 \pm 1.71\%$ compared to current prior to exposure to queen at internal pH 6.0, whereas further pH increases to 7.3, 8.0 and finally to 9.0 (by to internal alkalization) gradually decreased the inhibitory effects of quercetin by increasing the level of remaining current to $40.78 \pm 5.60\%$, $70.23 \pm 4.04\%$, and $67.74 \pm 2.69\%$ respectively compared to the control (Fig. 2E). Unlike OPE however, quercetin did not alter the voltage dependence of Slo3 activation (Fig. 2E) indicating some involvement of other minor constituents of OPE in Slo3 regulation. The calculated half activation voltages from Boltzmann fitting of relative conductivity data in Fig. 2F at internal pH 6, 7.3, 8 and 9 were 59.30 ± 5.65 mV, 38.21 ± 2.58 mV, 28.40 ± 1.53 mV and 22.36 ± 2.23 mV before exposure to quercetin and 59.82 ± 4.04 mV, 28.62 ± 2.55 mV, 25.82 ± 2.66 mV and 11.52 ± 3.63 mV after exposure to quercetin, respectively (Fig. 2F, G). Furthermore, similarly to OPE, quercetin depolarized the cell membrane of Slo3+LRRC52 expressing cells (Fig. 2C, D). These results demonstrate that quercetin is the main component of OPE that inhibits Slo3 in a dose and pH dependent manner.

A rise in internal free calcium hinders the inhibitory effect of quercetin

In sperm, internal pH change regulates calcium influx via the pH sensitive CatSper channel. Therefore, we wanted to test whether internal calcium variation has an effect on the inhibitory action of quercetin on Slo3. Interestingly, we found that inhibition of Slo3 by quercetin is dependent on the internal free calcium concentration (Fig. 3A). Increasing the internal free calcium concentration hinders the inhibitory effect of quercetin on Slo3: when internal free calcium was buffered by EGTA at levels of $0 \mu\text{M}$, $20 \mu\text{M}$ and $200 \mu\text{M}$, relative current remaining after quercetin treatment was $40.78 \pm 5.60\%$, $60.45 \pm 5.78\%$ and $65.51 \pm 4.79\%$ respectively (Fig. 3A). Even though, inhibitory effects of quercetin was lowered due to increasing calcium, quercetin, however, did not change the voltage dependency of Slo3 at internal free calcium concentrations of $20 \mu\text{M}$ and $200 \mu\text{M}$ (Fig. 3B, C). Furthermore, increasing the internal free calcium had a minimal effect on the half activation voltage of Slo3 probably due to the fact that Slo3 lacks the calcium binding calcium bowl that is present in closely related calcium sensitive, Slo1 (BK) channels. Therefore, it is possible that the hindering of the inhibition of Slo3 by quercetin is

mediated through a calcium dependent signaling pathway rather than by calcium directly acting on Slo3.

Substituting moieties in the flavonoid ring of quercetin dictates its inhibitory efficacy on Slo3

As the inhibitory efficacy of quercetin on Slo3 was strongly dependent on internal pH, we wanted to test whether the external pH would have any influence on inhibitory effect of quercetin on Slo3 particularly because the external pH in the female reproductive tract is acidic, whereas it is alkaline in the male reproductive tract. Similarly, to the internal pH, raising the external pH reduced the inhibitory effect of quercetin on Slo3 (Fig. 4A). However, unlike the internal pH, external pH changes did not affect Slo3 currents in quercetin untreated cells. Therefore, it's likely that pH has a direct effect on quercetin besides its direct effect on channel gating. Furthermore, we noticed that the color of $50 \mu\text{M}$ quercetin containing saline solutions changed when the pH of the saline was changed (data not shown) indicating alteration to moieties association to the quercetin flavonoid ring [40]. In order to investigate this, we tested the effects of luteolin and morin on Slo3. Luteolin and morin are analogues of quercetin which share a common flavonoid ring with slight variations in functional moieties (Fig. 4D). $50 \mu\text{M}$ luteolin, which has the highest pKa value of 7.05, along with four hydrogen bond donors, displayed a $53.47 \pm 3.15\%$ inhibition. On the other hand $50 \mu\text{M}$ morin, which has a pKa value of 6.88 (closer to quercetin's pKa value of 6.90) and a total of 5 hydrogen bond donors (similar to quercetin), inhibited Slo3 currents up to $42.15 \pm 2.16\%$, which was not statistically different from that elicited by quercetin (Fig. 4B) [41]. The current-voltage relationship elicited by step pulses remained the same characteristic shape after exposure to the quercetin analogues (Fig. 4B). These results raised the question as to whether variations in the polyphenolic ring affect the function of quercetin on Slo3. As an example, quercetin's activity as a PI3 kinase inhibitor is strongly dependent on the moieties at the 2- and 3-positions of the polyphenolic ring [42]. Furthermore, allosteric regulation of Slo3 by phenolic rings contacting quinidine occurs via a hydrophobic binding site [43]. The hydrophobicity of quercetin can also be affected by alterations to patterns of substitution on the flavonoid ring. However, when we tested mutant Slo3 that lack the quinidine binding site, quercetin was still able to inhibit both Slo3 mutants and Slo3 wildtype to a similar degree (Supplementary Fig. 1).

Protein kinase inhibitors and phosphoinositide 3-kinase inhibitors strongly inhibited Slo3 currents but could not prevent further inhibition of Slo3 current by quercetin

Quercetin is a biologically active chemical that can inhibit both PKC and phosphoinositide 3-kinase (PI3K) activity [33,34,42].

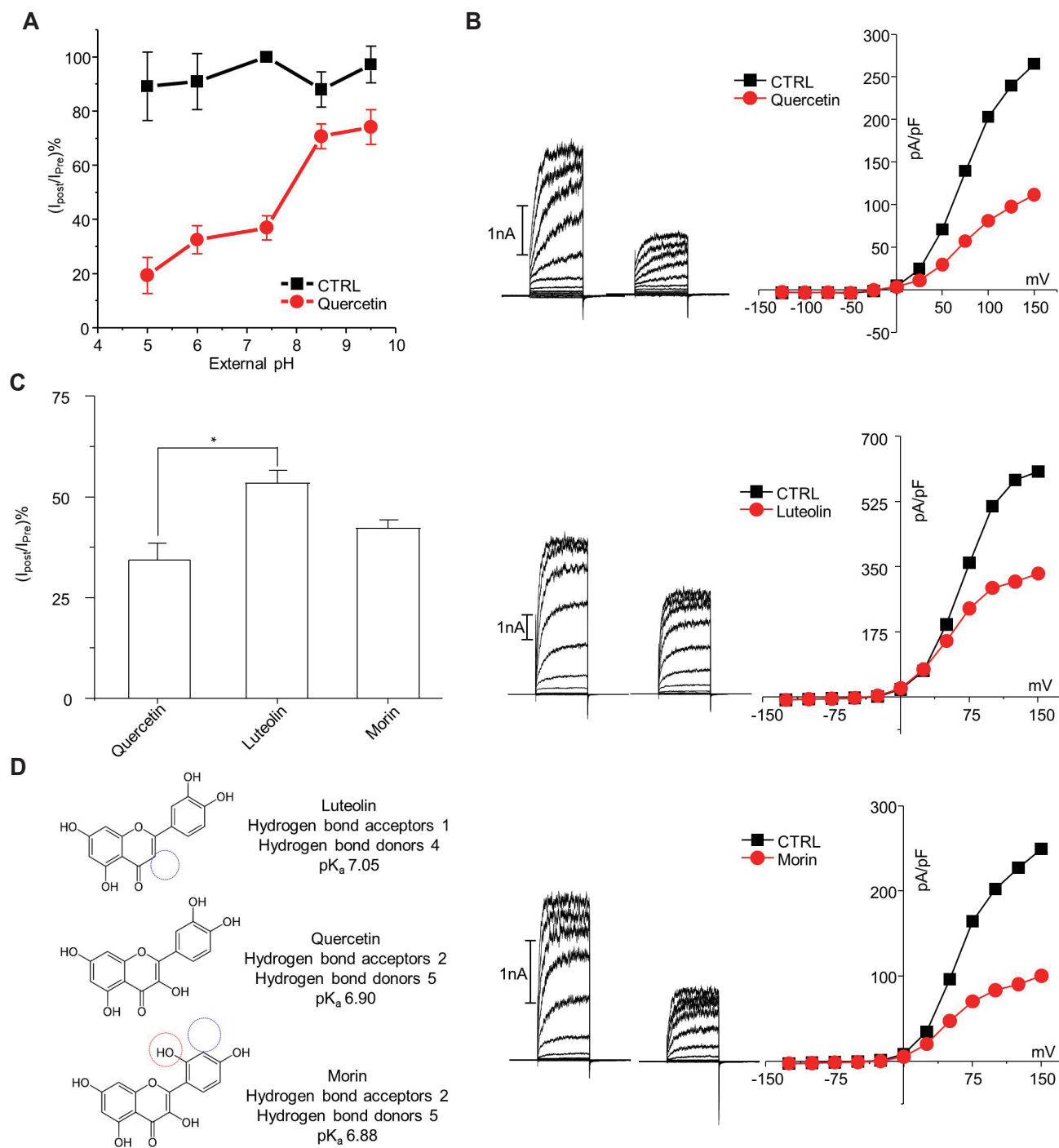


Fig. 4. Relative inhibition of Slo3 by quercetin is dependent on structural variations in quercetin. (A) Increasing in external pH decreases the inhibitory effects of quercetin. (C) Percentage current remaining after exposure to 50 μM quercetin (n = 7) compared to its structural analogues, 50 μM luteolin (n = 7) and 50 μM morin (n = 7). (B) Step recordings and IV curves representing the inhibition of current after exposure to quercetin (top), luteolin (middle) and morin (bottom). (D) Structure of each flavonoid with the different number of hydrogen bond acceptors in each chemical. Chemical structures and information on hydrogen bond acceptors of each chemical are adopted from *IUPHAR/BPS Guide to PHARMACOLOGY* [48]. CTRL, control. *p < 0.05.

The stimulatory effects of quercetin on sperm Hv1 occur via the inhibition of PKC [21]. Furthermore, we found that the inhibitory effects of quercetin decrease with increasing calcium concentration (Fig. 3) indicating a possibility that the promotion of calcium

dependent protein kinase activity C can negate the inhibitory effects of quercetin on Slo3. Therefore, we investigated whether Slo3 currents respond to protein kinase modulators. To do this, we tested the effects of protein kinase A inhibitor H-89 and protein

kinase C inhibitor GFX on Slo3. Both protein kinase inhibitors were applied with external perfusion at 5 μ M concentration, and both attenuated Slo3 currents (Fig. 5A, B). The relative current re-

maining was $48.27 \pm 2.66\%$ and $28.30 \pm 4.40\%$ after H-89 and GFX treatment respectively (Fig. 5C). However, introducing quercetin in the presence of H-89 or GFX further inhibited the Slo3 current

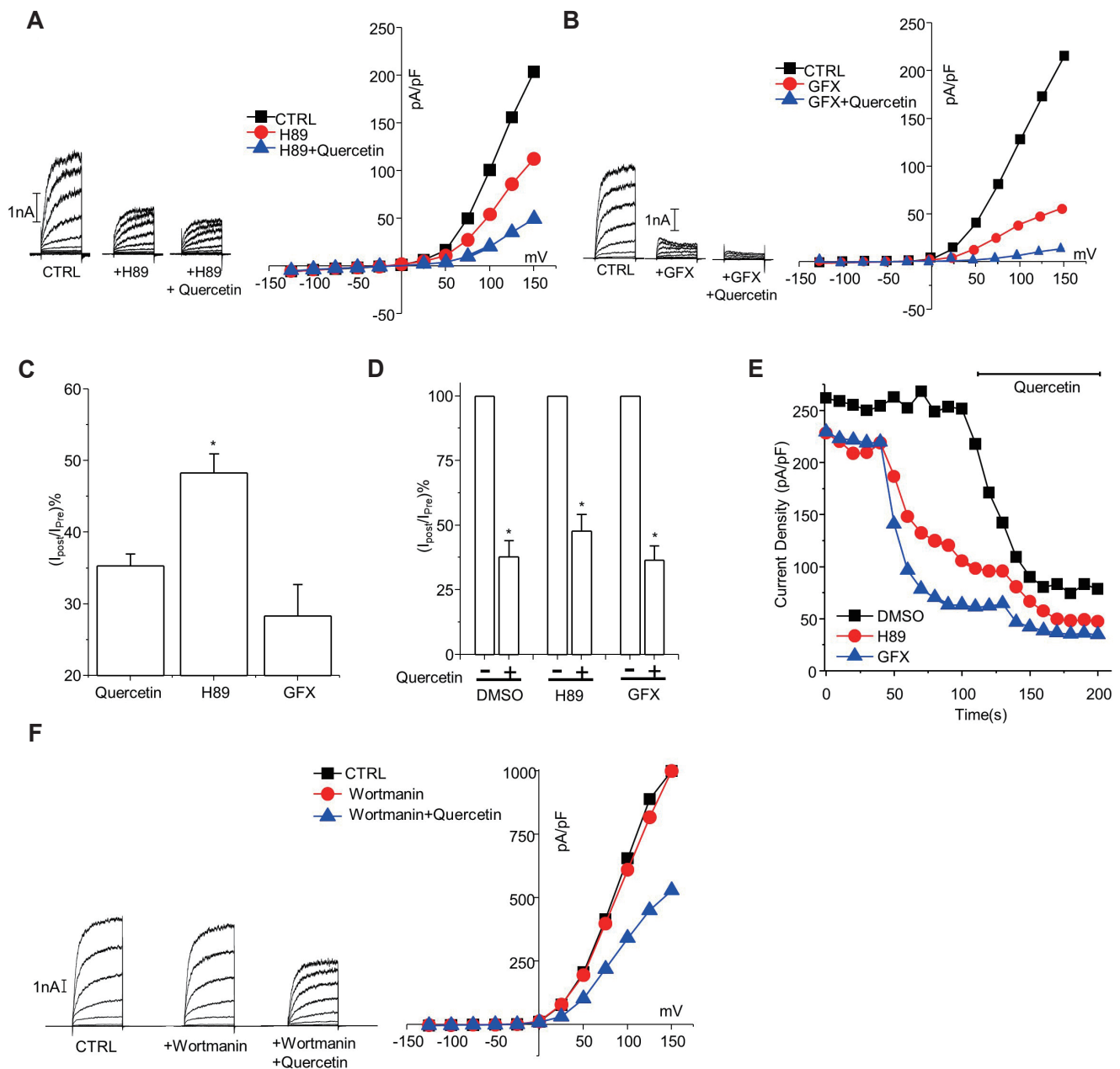


Fig. 5. Protein kinase or phosphoinositide 3 kinase (PI3K) inhibitory action of quercetin alone cannot explain the inhibitory effect of quercetin on Slo3. (A) Representative step pulses prior to treatment (left), after exposure to protein kinase A (PKA) inhibitor H89 (middle), after exposing to quercetin with H89 (right) and representative IV curve generated from those step pulses. (B) Representative step pulses prior to treatment (left), after exposure to protein kinase C (PKC) inhibitor GF109203X (GFX) (middle), after exposing to quercetin with GFX (right) and representative IV curve generated from those step pulses. (C) Relative current remaining after treatment of 50 μ M quercetin (n = 7) and 5 μ M protein kinase inhibitors H89 (n = 6) and GFX (n = 5). (D) Relative inhibition of Slo3 currents by quercetin in cells pre-treated with protein kinase inhibitor (n = 6). (E) Representative continuous trace indicating the further inhibition of Slo3 currents by quercetin in the cells pretreated with protein kinase inhibitors. (F) Representative step pulses prior to treatment (left), after exposure to PI3K inhibitor wortmanin (middle), after exposing to quercetin with wortmanin (right) and IV curve generated from those step pulses. (G) Relative current remaining after treatment of 50 μ M quercetin (n = 6) and 5 μ M PI3K inhibitors wortmanin (n = 5). (H) Relative inhibition of Slo3 currents by quercetin in cells pre-treated with PI3K inhibitor (n = 5) compared to control (n = 6). (I) Representative continuous trace indicating the further inhibition of Slo3 currents by quercetin in the cells pretreated with PI3K inhibitor wortmanin. Compared to untreated (DMSO) condition. Ctrl, control. *p < 0.05.

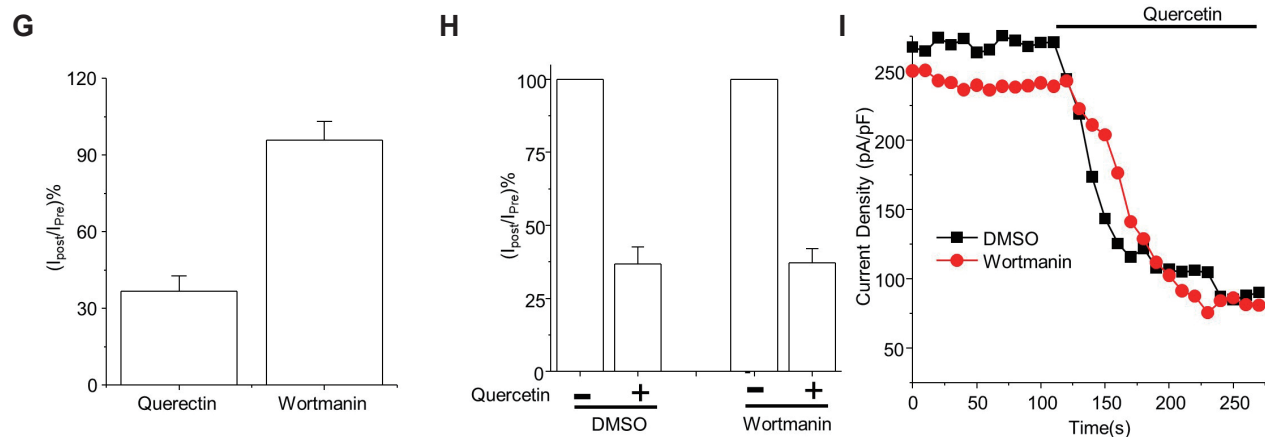


Fig. 5. Continued.

to $47.72 \pm 14.33\%$ and $36.40 \pm 12.57\%$ respectively (Fig. 5D) indicating that PKA inhibition or PKA inhibition does not explain the inhibitory effect of quercetin on Slo3. Other than PKC inhibitory function, quercetin is known also as a phosphoinositide kinase inhibitor [34,42]. The PI3K inhibitory action of quercetin is strongly dependent on moieties at the 2- and 3-positions of the polyphenolic ring which can be altered due to changes in pH [42]. Therefore, we tested whether Slo3 currents are inhibited by PI3K inhibitor wortmannin ($25 \mu\text{M}$) (Fig. 5F). The current remaining after wortmannin treatment was $95.14 \pm 6.87\%$. Quercetin treatment after wortmannin treatment inhibited the Slo3 current to a level similar to non-wortmannin treated cells, indicating a minimal effect of PI3K inhibition on Slo3 function (Fig. 5H, I). Therefore, there is not enough evidence to conclude that the inhibitory effect of quercetin on Slo3 is due to PKC or PI3K inhibition.

DISCUSSION

Onion peel extract and its constituent quercetin is known to be an effective infertility treatment due to its action on sperm motility. We studied the effects of quercetin on Slo3 in HEK293 cells co-expressing Slo3 and its sperm specific axillary subunit LRRC52. In this study, we found that quercetin has inhibitory action on Slo3 and therefore depolarizes the cell membrane. This depolarization can send protons to the outside of cell via Hv1 (HSper). Concurrent intracellular alkalinization and membrane depolarization can activate pH and depolarization sensitive CatSper currents (Supplementary Fig. 2) [44]. Calcium influx to sperm via CatSper can bind with calaxins [45]. Calcium bound calaxins inhibit dynein motors which in turn result in an asymmetrical whip like movement in flagellum, a common characteristic of sperm hyper-activation [46]. This boosts sperm movement in the female reproductive tract in order to reach and fertilize the egg. Furthermore, quercetin is a direct activator of Hv1 [21]. Therefore, quercetin has the potential to modulate sperm motility and

hyper-activation which in turn can affect the process of fertilization.

In our current work, we found that the effect of quercetin on Slo3 is calcium and pH dependent. Therefore, HSper and CatSper dependent calcium regulation can alter the effects of quercetin on Slo3. Further experiments revealed that two of the main biological functions of quercetin, inhibiting protein kinase and phosphoinositide kinase, may also be able to inhibit Slo3 currents. However, there was not enough evidence based on experiments performed here to conclude that protein kinase and phosphoinositide kinase inhibitory action play a role in the inhibitory effect of quercetin on Slo3 under the tested conditions (Fig. 5).

The pH dependency of the effect of quercetin on Slo3 reported here could be for two reasons: 1) alterations to quercetin structure due to both external and internal pH, and 2) modifications to channel gating due to changes in internal pH. As demonstrated by differential effects on Slo3 of quercetin analogues luteolin and morin (Fig. 4), the position of the hydroxyl group in the polyphenol ring is important. Luteolin, which only has a single hydrogen acceptor, has similar effects on Slo3 to quercetin, whereas morin, which has two hydrogen acceptors, had a reduced inhibitory effect on Slo3 [47]. Moreover, pH changes can cause structural changes to quercetin at the C3-OH group in the C-ring, the catechol moiety in the B-ring and the C2-C3 double bond in the C-ring, which can lead to changes in biological activity [40,48]. Increasing the pH deprotonates the hydroxyl rings in quercetin itself [48] thus hindering its lipid solubility and thereby reducing its membrane permeability. Changes to quercetin structure are made evident by color changes of quercetin containing saline solutions [40]. $50 \mu\text{M}$ quercetin containing pH 7.3 saline solutions are usually mild yellowish in color (data not shown). However, with increasing pH, the color of the quercetin solution intensifies and turns to an orange like shade. In addition to structural modifications to quercetin, pH also affects the gating properties of Slo3. Slo3 is a pH sensitive channel. The internal alkalinization depolarizes the gating potential to a level closer to the physiological

membrane potential making it more likely for the channel to activate [15,36]. Furthermore, as shown in Figs. 1 and 2, Slo3 gating potential is sensitive to internal pH. Therefore, it is possible that alterations to internal pH affect the channel's response to quercetin simply by altering channel gating properties in conjunction with other pH dependent effects on quercetin itself.

This pH dependence of quercetin's inhibitory effect on Slo3 is of importance since sperm experience a rapid change in external and internal pH during the process of fertilization [49,50]. As an example, during entry into the female reproductive tract from the male reproductive tract, the external pH switches from alkaline to acidic [3,51,52]. Furthermore, during capacitation, the internal pH of sperm raises due to Hv1 channels which efflux protons to the outside of the cell. Besides pH changes, sperm capacitation results in calcium influx via CatSper channels, which can alter the effect of quercetin on Slo3. Therefore, a proper understanding of the effects of quercetin on Slo3 at different pH conditions represents important information towards optimizing the use of quercetin as a pharmaceutical compound.

Results shown here pertaining to the inhibitory effects of quercetin on Slo3 support growing evidence suggesting that Slo3 rather than its close relative Slo1 (BK1) is the main contributor to KSper currents. Slo1 is activated by quercetin, while Slo3 is inhibited by quercetin [31]. The activation of KSper would not promote sperm hyper-activation, since hyper-polarized sperm membrane potential inhibits Hv1 and CatSper currents. Quercetin promotes sperm motility and function [26,53]. Therefore, a potassium channel inhibited by quercetin is most likely to conduct KSper current, which supports the idea that Slo3 rather than Slo1 is the ion channel that performs this task.

Our results suggest that using quercetin as a nutraceutical to boost reproductive health can have varying effects due to its inhibitory effects on Slo3, despite quercetin having been shown to enhance Hv1 as well as CatSper function due to internal alkalization [21,31]. This might explain the reason why some studies have reported quercetin dependent negative effects on fertility, such as reduced numbers of litters in female mice and reduced sperm motility [29,30]. On the other hand, quercetin has also been reported to increase sperm viability and motility, and through this, overall fertility in test subjects [26,53]. In addition to *in vivo* usage, quercetin has been suggested to enhance human sperm function after cryopreservation [54,55]. However, the cryopreservation effect of quercetin is likely to be related to its antioxidant activity rather than its function as an ion channel regulator.

Taken together, our current data suggest that Slo3 is inhibited by OPE and its major active ingredient, quercetin, in a dose dependent manner leading to membrane depolarization. An increase in internal pH during sperm capacitation can hinder the effects of quercetin on Slo3. Entry of sperm into the acidic female reproductive tract from the alkaline male reproductive tract can enhance the effect of quercetin on Slo3 by attenuating minor structural elements of quercetin. In addition to pH, an increase

in internal calcium during sperm capacitation can hinder the inhibitory effect of quercetin. Therefore, it can be concluded that quercetin inhibits Slo3 currents in a pH and calcium dependent manner. The use of quercetin as a pharmaceutical to enhance fertility is therefore likely to have varying effects depending on the method and location of application.

ACKNOWLEDGEMENTS

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI18C2080).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at <http://pdf.medrang.co.kr/paper/pdf/Kjpp/Kjpp2019-23-05-11-s001.pdf>.

REFERENCES

1. Lishko PV, Botchkina IL, Fedorenko A, Kirichok Y. Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. *Cell*. 2010;140:327-337.
2. Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim Biophys Acta*. 2000;1469:197-235.
3. Baldi E, Casano R, Falsetti C, Krausz C, Maggi M, Forti G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J Androl*. 1991;12:323-330.
4. Kirichok Y, Lishko PV. Rediscovering sperm ion channels with the patch-clamp technique. *Mol Hum Reprod*. 2011;17:478-499.
5. Lishko PV, Kirichok Y, Ren D, Navarro B, Chung JJ, Clapham DE. The control of male fertility by spermatozoan ion channels. *Annu Rev Physiol*. 2012;74:453-475.
6. Publicover S, Harper CV, Barratt C. $[Ca^{2+}]_i$ signalling in sperm--making the most of what you've got. *Nat Cell Biol*. 2007;9:235-242.
7. Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. *Proc Natl Acad Sci U S A*. 2011;108:4892-4896.
8. Lishko PV, Miller MR, Mansell SA. The role of sperm Ion channels in reproduction. *Ion Channels in Health and Disease*, Elsevier; 2016. p.223-238.
9. Miller MR, Mansell SA, Meyers SA, Lishko PV. Flagellar ion chan-

- nels of sperm: similarities and differences between species. *Cell Calcium*. 2015;58:105-113.
10. Navarro B, Kirichok Y, Clapham DE. KSper, a pH-sensitive K⁺ current that controls sperm membrane potential. *Proc Natl Acad Sci U S A*. 2007;104:7688-7692.
 11. Zeng XH, Yang C, Kim ST, Lingle CJ, Xia XM. Deletion of the Slo3 gene abolishes alkalization-activated K⁺ current in mouse spermatozoa. *Proc Natl Acad Sci U S A*. 2011;108:5879-5884.
 12. Mannowetz N, Naidoo NM, Choo SA, Smith JF, Lishko PV. Slo1 is the principal potassium channel of human spermatozoa. *Elife*. 2013;2:e01009.
 13. Santi CM, Martínez-López P, de la Vega-Beltrán JL, Butler A, Alisio A, Darszon A, Salkoff L. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. *FEBS Lett*. 2010;584:1041-1046.
 14. Zeng XH, Yang C, Xia XM, Liu M, Lingle CJ. SLO3 auxiliary subunit LRRC52 controls gating of sperm KSPER currents and is critical for normal fertility. *Proc Natl Acad Sci U S A*. 2015;112:2599-2604.
 15. Brenker C, Zhou Y, Müller A, Echeverry FA, Trötschel C, Poetsch A, Xia XM, Bönigk W, Lingle CJ, Kaupp UB, Strünker T. The Ca²⁺-activated K⁺ current of human sperm is mediated by Slo3. *Elife*. 2014;3:e01438.
 16. Chávez JC, Ferreira JJ, Butler A, De La Vega Beltrán JL, Treviño CL, Darszon A, Salkoff L, Santi CM. SLO3 K⁺ channels control calcium entry through CATSPER channels in sperm. *J Biol Chem*. 2014;289:32266-32275.
 17. Abi Nahed R, Martinez G, Hograindleur JP, Le Blévec E, Camugli S, Le Boucher R, Ray PF, Escoffier J, Schmitt E, Arnoult C. Slo3 K⁺ channel blocker clofilium extends bull and mouse sperm-fertilizing competence. *Reproduction*. 2018;156:463-476.
 18. Shafiq S, Shakir M, Ali Q. Role of onion in the fertility issues: a review. *Academ Arena*. 2017;9:40-43.
 19. Musavi H, Tabnak M, Alaei Sheini F, Hasanzadeh Bezvan M, Amidi F, Abbasi M. Effect of garlic (*Allium sativum*) on male fertility: a systematic review. *J Herbmmed Pharmacol*. 2018;7:306-312.
 20. Lanzotti V. The analysis of onion and garlic. *J Chromatogr A*. 2006;1112:3-22.
 21. Chae MR, Kang SJ, Lee KP, Choi BR, Kim HK, Park JK, Kim CY, Lee SW. Onion (*Allium cepa* L.) peel extract (OPE) regulates human sperm motility via protein kinase C-mediated activation of the human voltage-gated proton channel. *Andrology*. 2017;5:979-989.
 22. Seifi-Jamadi A, Kohram H, Shahneh AZ, Ansari M, Macías-García B. Quercetin ameliorate motility in frozen-thawed Turkmen stallions sperm. *J Equine Vet Sci*. 2016;45:73-77.
 23. Al-Roujayee A. Improvement of sexual behavior, sperm quantity and quality by Quercetin in streptozotocin-induced diabetic erectile dysfunction. *Asian Pac J Reprod*. 2017;6:6-12.
 24. Yoshimoto H, Takeo T, Nakagata N. Dimethyl sulfoxide and quercetin prolong the survival, motility, and fertility of cold-stored mouse sperm for 10 days. *Biol Reprod*. 2017;97:883-891.
 25. Moretti E, Mazzi L, Terzuoli G, Bonechi C, Iacoponi F, Martini S, Rossi C, Collodel G. Effect of quercetin, rutin, naringenin and epicatechin on lipid peroxidation induced in human sperm. *Reprod Toxicol*. 2012;34:651-657.
 26. Taepongsorat L, Tangpraprutgul P, Kitana N, Malaivijitnond S. Stimulating effects of quercetin on sperm quality and reproductive organs in adult male rats. *Asian J Androl*. 2008;10:249-258.
 27. Silva LPMC, Araujo EAB, Oliveira SN, Dalanezi FM, Junior LRPA, Carneiro JAM, Rodrigues LT, Hayashi RM, Crespilho AM, Dell'Aqua CPF, Dell'Aqua Junior JA, Papaa FO. Quercetin promotes increase in the fertility rate of frozen semen of Stallions considered sensitive to freezing. *J Equine Vet Sci*. 2018;66:82.
 28. Ardeshirnia R, Zandi M, Sanjabi MR. The effect of quercetin on fertility of frozen-thawed ram epididymal spermatozoa. *South Afr J Anim Sci*. 2017;47:237-244.
 29. Beazley KE, Nurminskaya M. Effects of dietary quercetin on female fertility in mice: implication of transglutaminase 2. *Reprod Fertil Dev*. 2016;28:974-981.
 30. Liang X, Xia Z, Yan J, Wang Y, Xue S, Zhang X. Quercetin inhibits human sperm functions by reducing sperm [Ca²⁺]_i and tyrosine phosphorylation. *Pak J Pharm Sci*. 2016;29(6 Suppl):2391-2396.
 31. Wu SN, Chen BS, Hsu CL, Hsu TI. The large-conductance Ca²⁺-activated K⁺ channels: a target for the modulators of estrogen receptors. *Curr Top Biochem Res*. 2008;10:93-101.
 32. Li PG, Sun L, Han X, Ling S, Gan WT, Xu JW. Quercetin induces rapid eNOS phosphorylation and vasodilation by an Akt-independent and PKA-dependent mechanism. *Pharmacology*. 2012;89:220-228.
 33. Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clin Exp Allergy*. 2000;30:501-508.
 34. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP, Williams RL. Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol Cell*. 2000;6:909-919.
 35. Lindahl M, Tagesson C. Selective inhibition of group II phospholipase A2 by quercetin. *Inflammation*. 1993;17:573-582.
 36. Wijerathne TD, Kim J, Yang D, Lee KP. Intracellular calcium-dependent regulation of the sperm-specific calcium-activated potassium channel, hSlo3, by the BK_{Ca} activator LDD175. *Korean J Physiol Pharmacol*. 2017;21:241-249.
 37. Patton C. MaxChelator [Internet]. 2014. Available from: <https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaEGTA-NIST.htm>
 38. Curtis MJ, Alexander S, Cirino G, Docherty JR, George CH, Gimbycz MA, Hoyer D, Insel PA, Izzo AA, Ji Y, MacEwan DJ, Sobey CG, Stanford SC, Teixeira MM, Wonnacott S, Ahluwalia A. Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *Br J Pharmacol*. 2018;175:987-993.
 39. Leonetti MD, Yuan P, Hsiung Y, Mackinnon R. Functional and structural analysis of the human SLO3 pH- and voltage-gated K⁺ channel. *Proc Natl Acad Sci U S A*. 2012;109:19274-19279.
 40. Jurasekova Z, Domingo C, Garcia-Ramos JV, Sanchez-Cortes S. Effect of pH on the chemical modification of quercetin and structurally related flavonoids characterized by optical (UV-visible and Raman) spectroscopy. *Phys Chem Chem Phys*. 2014;16:12802-12811.
 41. Tsujimoto M, Horie M, Honda H, Takara K, Nishiguchi K. The structure-activity correlation on the inhibitory effects of flavonoids on cytochrome P450 3A activity. *Biol Pharm Bull*. 2009;32:671-676.
 42. Matter WF, Brown RF, Vlahos CJ. The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. *Biochem Biophys Res Commun*. 1992;186:624-631.

43. Wrighton DC, Muench SP, Lippiat JD. Mechanism of inhibition of mouse Slo3 (KCa 5.1) potassium channels by quinine, quinidine and barium. *Br J Pharmacol.* 2015;172:4355-4363.
44. Qi H, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, Kirichok Y, Ramsey IS, Quill TA, Clapham DE. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proc Natl Acad Sci U S A.* 2007;104:1219-1223.
45. Mizuno K, Padma P, Konno A, Satouh Y, Ogawa K, Inaba K. A novel neuronal calcium sensor family protein, calaxin, is a potential Ca^{2+} -dependent regulator for the outer arm dynein of metazoan cilia and flagella. *Biol Cell.* 2009;101:91-103.
46. Shiba K, Baba SA, Inoue T, Yoshida M. Ca^{2+} bursts occur around a local minimal concentration of attractant and trigger sperm chemotactic response. *Proc Natl Acad Sci U S A.* 2008;105:19312-19317.
47. Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S, Gray AJG, Bruce L, Alexander SPH, Anderton S, Bryant C, Davenport AP, Doerig C, Fabbro D, Levi-Schaffer F, Spedding M, Davies JA. The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucleic Acids Res.* 2018;46(D1):D1091-D1106.
48. Chebotarev AN, Snigur DV. Study of the acid-base properties of quercetin in aqueous solutions by color measurements. *J Anal Chem.* 2015;70:55-59.
49. Makler A, David R, Blumenfeld Z, Better OS. Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolarity of semen and urine specimens. *Fertil Steril.* 1981;36:507-511.
50. Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. *In vivo* oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Hum Reprod Update.* 2017;24:15-34.
51. Galantino-Homer HL, Florman HM, Storey BT, Dobrinski I, Kopf GS. Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalinization on capacitation-associated protein tyrosine phosphorylation. *Mol Reprod Dev.* 2004;67:487-500.
52. Zeng Y, Oberdorf JA, Florman HM. pH regulation in mouse sperm: identification of Na^+ , Cl^- , and HCO_3^- -dependent and arylamino-benzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. *Dev Biol.* 1996;173:510-520.
53. Khaki A, Fathiazad F, Nouri M, Khaki A, Maleki NA, Khamnei HJ, Ahmadi P. Beneficial effects of quercetin on sperm parameters in streptozotocin-induced diabetic male rats. *Phytother Res.* 2010;24:1285-1291.
54. Azadi L, Tavalae M, Deemeh MR, Arbabian M, Nasr-Esfahani MH. Effects of tempol and Quercetin on human sperm function after cryopreservation. *Cryo Letters.* 2017;38:29-36.
55. Winn E, Whitaker BD. Quercetin supplementation during boar semen thawing and incubation improves sperm characteristics [abstract]. *J Anim Sci.* 2018;96 Suppl 2:261-262. Abstract no. 489.