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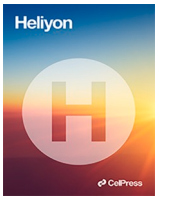

Research article

Hexane fraction of *Prunus japonica* thunb. Seed extract enhances boar sperm motility via CatSper ion channel

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ABSTRACT

Introduction: Mammalian sperm motility is facilitated by flagellar beating, which depends on active ion movement through ion channels and their regulation. *Prunus japonica* Thunb., also known as oriental bush cherry, is a widely used traditional medicinal plant. However, its significance in improving fertility and sperm quality has not been fully elucidated yet. One of our previous reports revealed that *P. japonica* seed extract (PJE) can improve human sperm motility through intracellular pH modulation.

Aim of the study: The present study was designed to investigate the effects of PJE on boar spermatozoa and potential underlying mechanisms.

Materials and methods: Sperm motility changes were examined using a computer-assisted sperm analysis (CASA) system under both capacitated and non-capacitated conditions. Intracellular calcium concentration was measured using either confocal microscopy or a fluorescent microplate reader with Fluo-4AM calcium fluorescent dye. Sperm capacitation-related proteins were analyzed using western blotting.

Results: A significant increase in rapid motility, velocity, and linear displacement of sperm was observed in PJE-treated capacitated boar sperm, whereas the effect was insignificant in the non-capacitated counterparts. Intracellular calcium levels were significantly elevated upon PJE treatment (20–100 μg/L) in a concentration-dependent manner. The increase in intracellular calcium levels was inhibited when the sperm were treated with a CatSper (cation channel of sperm) channel inhibitor, 10 μM Mibefradil, indicating the involvement of the ion channel in the PJE modulatory mechanism. In addition, western blotting revealed an increased level of protein phosphorylation (p-tyrosine and p-PKA), which is a hallmark of sperm capacitation.

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Conclusions: PJE treatment resulted in a combination of increased motility, intracellular calcium concentration, and capacitation, thereby indicating its potential to ameliorate sperm motility parameters and induce capacitation of boar spermatozoa as a result of intracellular calcium elevation via the CatSper channel. Our observations further elaborate ion channel-related underlying mechanisms and show putative implications of the seed extract of traditionally used *P. japonica* Thunb. in ameliorating sperm quality.

1. Introduction

Motility and associated mechanisms are tightly regulated in healthy spermatozoa to facilitate sperm migration from the ejaculation site to the egg for fertilization. Reduced sperm motility and viability are considered proxies of infertility and reproductive pathologies [1]. Over the last few decades, artificial insemination has become one of the most important assisted reproductive technique alternatives to natural fertilization to produce genetic improvement in livestock in the swine industry. However, the beneficial effects of such a process can be hindered by the deterioration of the quality of fresh or preserved spermatozoa. Thus, compounds that can improve the boar sperm quality (especially motility) need to be investigated [2].

Calcium is a secondary messenger molecule that regulates numerous molecular signaling pathways. Therefore, it has a variety of functions in mammalian spermatozoa, including motility, capacitation, and acrosome reaction [3]. The intracellular calcium levels of spermatozoa can be controlled by the internal calcium stores or ion transporters of the sperm plasma membrane. Mature mammalian sperm do not have an endoplasmic reticulum, which is the main internal Ca^{2+} store that can regulate intracellular calcium levels by sequestering or releasing calcium. However, antimonate staining of boar spermatozoa identified two regions with calcium deposits: one in the anterior head region and another in the sperm neck and midpiece region [4]. The presence of the acrosome and mitochondria in these regions with calcium releasing ion channels on the organelle membrane suggests these structures to be possible internal calcium stores of mature spermatozoa [5]. In addition, ion channels, which are responsible for calcium movement across the sperm plasma membrane, are concentrated in these regions [6]. Ca^{2+} transporting ion channels of the sperm plasma membrane mainly include CatSper (cation channel of sperm), voltage-gated calcium channels (VGCCs) [7], transient receptor potential vanilloid (TRPV) [8], and store-operated Ca^{2+} channels (SOCCs) [3,6]. Additionally, Na^{+}/Ca^{2+} ion exchangers can participate in generating calcium oscillations in sperms [9]. Among all calcium ion channels, the CatSper ion channel is vital because of its role in hyperactivated-motility and acrosome exocytosis. Similar to other mammals, boar sperm also comprises CatSper channels [10,11]. Vicente-Carrilo et al. showed that the CatSper ion channel is present and functionally non-redundant in boar spermatozoa [11].

Yeste et al. found that the capacitation induction of boar sperm and increased calcium signals in both the head and the midpiece regions were concomitant [12]. This is further supported by earlier studies showing the dependence on calcium and appearance of p32 tyrosine-phosphorylated sperm protein, which is a hallmark event of mammalian sperm capacitation [13]. Currently accepted molecular signaling pathways of capacitation in boar spermatozoa identify calcium as an essential upstream regulator due to its role in cAMP-activated protein kinase activity pathways and tyrosine phosphorylation of proteins [14–16].

Prunus japonica Thunb., commonly known as Korean bush cherry or Chinese dwarf cherry, is a medicinal and ornamental shrub species belonging to the family Rosaceae. Seeds of the plant are commonly used for antihypertensive treatments [17]. *P. japonica* seeds generally comprise acids (ursolic, vanillic, and protocatechuic), flavonoids (afzelin, kaempferitrin A, multiforin A, and multiforin B), and fatty acids (oleic acid, 11-eicosenoic acid, and linoleic acid) [18,19]. In one of our previous reports, we showed that the PJE can enhance human sperm motility via modulating its intracellular pH levels through the hydrogen voltage gated channel 1 (HVCN1/Hv1) [20]. In human, the proton channel and the CatSper channels are closely associated; and the activation of the CatSper channels is dependent on the increase of intracellular pH that is mainly modulated by the acid extrusion through the voltage-gated proton channels [21,22]. However, the mechanism of action of PJE not fully elucidated yet and its effects on sperm ion channels are relatively unexplored. In this study, we aimed to evaluate the putative pharmacological enhancements of PJE in boar spermatozoa, especially on membrane ion channels, and further elucidate the underlying mechanism of action of PJE. By further elucidating the mechanisms of actions of the PJE on ion channels, this work highlights the potential electrophysiological properties of the PJE extract that can be utilized to improve sperm quality.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Sigma-Aldrich Korea, Yongin, Korea), unless otherwise indicated.

2.2. Preparation and chemical characterization of P. japonica seed extract

Dried seeds of *P. japonica* were purchased from the Kyungdong Oriental herbal market (Seoul, Korea) in June 2018, and identified by one of the authors (Dr. CY Kim). A voucher specimen was deposited in the herbarium of the College of Pharmacy, Hanyang University, Ansan, Korea (HYUP-PJ-001). The *P. japonica* seeds (1 kg) were extracted three times with 75% ethanol. The extract was

filtered and dried under vacuum to obtain the PJE (59.1 g). Subsequently, the PJE (20 g) was suspended in water and fractionated using *n*-hexane [20]. The fractions obtained were 3.27 g of *n*-hexane extract and 16.73 g of aqueous extract.

The PJE, *n*-hexane fraction, and aqueous layer were analyzed using a Waters 2695 Alliance HPLC system (Waters, Medford, MA, USA) connected in parallel with a Waters 2996 Photo Diode Array detector and a Waters Micromass® ZQ™ single-quadrupole mass spectrometer equipped with an Electrospray ionization (ESI) source. Extracts were separated using an Inno C18 column (inner diameter × length: 2.0 × 150 mm, particle size: 5 μm, Young Jin Biochrom, Korea). The mobile phase consisted of acetonitrile and water (solvent A and B), both supplemented with 0.1% formic acid. A linear gradient system was used as follows: 0–60 min, 15–100% A. The sample (10 μL) was injected at 0.3 mL/min flow rate. Mass spectrometry parameters were as follows: ionization mode, negative ESI; capillary voltage, 3.0 kV; extractor voltage, 3 V; source temperature, 120 °C; desolvation temperature, 200 °C; desolvation gas flow, 500 L/h; cone gas flow, 50 L/h; scan range, *m/z* 200–1200; rate, 1 scan/s; and cone voltage, 30 V. The n-hexane fraction increased the intracellular calcium concentration in the initial experiments; therefore, we further purified the extract for chemical characterization. Purification was performed using silica gel open column chromatography with a gradient elution of *n*-hexane:ethyl acetate (4:1 → 0:1) to isolate two fatty acids (oleic acid and linoleic acid) and β-sitosterol.

2.3. Sperm medium

HS medium containing 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM L-(+)-lactic acid, and 20 mM 4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES), was used as the non-capacitating medium (NonCAP). The pH was adjusted to 7.4 with NaOH. For capacitating conditions, 15 mM $NaHCO_3$, 4 mg/mL BSA, and 1 mM caffeine were added to the NonCAP medium.

2.4. Semen sample preparation

This study was performed using commercially available boar seminal doses obtained from a local farm (Bookboo AI Center, South Korea) and the experiments were conducted under the Chungnam Facility Animal Care Committee guidelines (201812A-CNU-01162). Seminal doses were procured after packaging diluted sperm-rich fractions. Packages were then liquid-stored at 17 °C until the experiments. All experiments were performed with the semen within 7 days of collection. All seminal doses used in the experiments had a minimum initial total motility of 70%. The semen was centrifuged for 5 min at 800×*g* at 20 °C, and the pellets were resuspended in HS medium and subsequently washed two times with the respective medium via centrifugation (800×*g* at 20 °C for 5min). Sperm isolated from swim-up were used for the experiments. For capacitation, the sperm samples were diluted to a maximum final concentration of 2 × 10^7 cells/mL in capacitating medium and incubated for 1 h at 37 °C in an atmosphere with a 5% vol/vol CO_2 incubator, whereas the non-capacitating conditions were processed in non-capacitating media (without $NaHCO_3$, BSA, and caffeine). The sperm samples were incubated in HS medium (containing 15 mM $NaHCO_3$ and 4 mg/mL BSA) at 37 °C in the presence of various concentrations of the SA1-17 (hexane fraction of PJE). After 5 min or 1 h of incubation, the intracellular calcium levels and expression of selected proteins were assessed using western blotting. For the viability assessment, and the sperm were incubated for 1, 2, and 4 h with the treatments. Every experiment included a negative control counterpart without any treatment using the sperm of the same seminal dose as its other treatment groups.

2.5. Sperm viability

Boar sperm (2 × 10^7/mL) treated with various concentrations of SA1-17 (10, 50, 100, 200 μg/mL) or 0.1% dimethyl sulfoxide (DMSO) only (time-matched paired controls) were incubated at 37 °C and 5% CO_2. After 1, 2, and 4 h of incubation, the samples were centrifuged at 600×*g* for 10 min at 25 °C and resuspended in 1 mL of HEPES-buffered saline solution containing bovine serum albumin (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7.4). Sperm viability was assessed using the LIVE/DEAD™ Sperm Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Briefly, spermatozoa were incubated with SYBR-14 (final concentration: 100 nM) at 37 °C for 10 min in the dark and with PI (final concentration: 12 μM) for 5 min. The samples were analyzed using flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA 95131–1807, USA). SYBR-14 fluorescence was detected using a 533/30 filter and PI fluorescence was detected using a 585/40 filter. The results are expressed as the percentage of living cells.

2.6. Computer-assisted sperm analysis

Sperm cells were concentrated to 5 × 10^6 cells/mL in Dulbecco's phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4). A computer-assisted sperm analysis (CASA) system (ISASv1®. Proiser S.L., Valencia, Spain) was used to analyze sperm motility and motion kinematics as described previously [23]. Briefly, 20 μL of the sample, after appropriate treatment and incubation, was mounted onto a prewarmed Makler chamber (Makler, Haifa, Israel). The sperm samples were examined under 10 × objective magnification in phase-contrast mode using an Olympus B×41 microscope (Olympus Europe GmbH, Hamburg, Germany). After relaying and digitizing the images, the ISAS program was used to analyze the sperms. A minimum of 100 sperms were recorded per field, and at least five fields were obtained for each condition. Among the motion kinematic parameters of sperm, curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), and mean amplitude of head lateral displacement (ALH, μm) were estimated, where sperm with VCL >5 μm/s were considered motile.

2.7. Intracellular calcium measurements

Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in boar sperm were measured using the fluorescent Ca^{2+} indicator Fluo-4AM [4-(6-Acetoxymethoxy-2,7-difluoro-3-oxo-9-xanthenyl)-4′-methyl-2,2′(ethylenedioxy) dianiline N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl) ester]. Sperm were loaded with 5 μM Fluo-4 AM (Thermo Fisher Scientific) and 0.05% Pluronic™ F-127 (Thermo Fisher Scientific) in HS solution for 30 min at 25 °C in the dark. After incubation, the sperm were washed twice and left for 30 min at room temperature. For single-cell calcium measurements, the sperm were then attached by the head on laminin (1 mg/mL) precoated coverslips, allowing their flagella to move continuously. The coverslip was mounted on a chamber and placed on the stage of an inverted microscope (Eclipse TE 300; Nikon, Seoul, South Korea). The single-cell fluorescence intensities were measured at a 510 nm emission with a 340/380 nm dual excitation using RatoMaster illuminator (Photon Technology International, West Sussex, UK); and time-series images were acquired using a cooled 3 MHz (14 bit) CCD camera (Photometrics CoolSNAP HQ2: ALT, East Lyme, CT, USA) with an exposure time of 100 ms and 1 × 1, 2 × 2, or 3 × 3 binning under the control of the MetaMorph 7.6 software (Molecular Devices, California, USA). Fluorescence microplate reading was employed to measure intracellular calcium changes in the sperm samples. After loading with Ca^{2+} fluorochromes, 200 μL of the samples was transferred into the wells of a 96-well cell culture plate. The wells were then treated with the appropriate amount of PJE to obtain the final concentrations of 0, 5, 10, 20, 50, and 100 μg/mL. The effect of linoleic (LA) and oleic (OA) acids on intracellular calcium levels were measured using the similar method. Final concentration of 20 μM of both OA and LA was used. Additionally, fluorescence was measured in the presence of 10 μM Mibefradil (a specific CatSper channel blocker). Samples treated with ion channel blockers were then treated with different concentrations of PJE (control, 5, 10, 20, 50, 100, 200 μg/mL).

2.8. Western blot

For western blotting experiments, the sperm samples (5 × 10^6 cells/mL) were incubated with various concentrations of SA1-17 (5, 10, 50, 100 μg/mL) or 0.1% DMSO only at 37 °C for 1 h. Sperm, collected after centrifugation at 1,2000×*g* for 5 min, were washed in 1 mL of PBS. Total proteins from sperm were extracted using RIPA lysis and extraction buffer (Thermo Fisher Scientific) supplemented

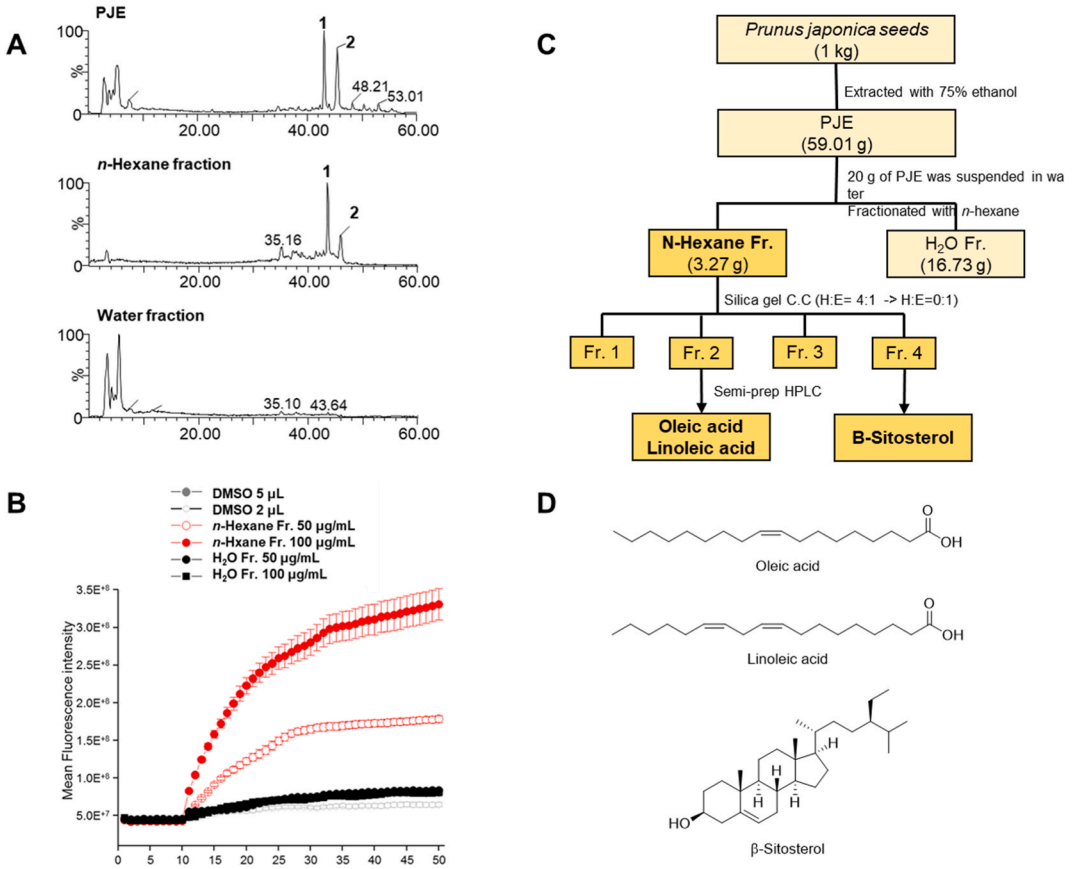

Fig. 1. Chemical characteristics of hexane fraction of PJE (A) Chromatograms of PJE, n-hexane fraction, and water fraction. Peak 1: linoleic acid, peak 2: oleic acid. (B) Intracellular calcium level changes by treatment of n-hexane fraction or water fraction (n = 4–6). (C) Preparation of PJE and purification scheme of n-hexane fraction. (D) Chemical structures of isolated compounds from n-hexane fraction.

with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The homogenates were centrifuged at 12,000×*g* for 15 min at 4 °C, and the protein concentration in each supernatant was determined using a colorimetric assay (Bio-Rad DC protein assay, BIO-RAD, California, USA). The proteins were then separated using 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, CA, USA). Subsequently, the membranes were blocked with 5% skim milk in tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against phospho-tyrosine (1 : 2000; Cell Signaling, catalog no. 9411, RRID: AB_331228, MA, USA), phospho-PKA (1 : 2000; Cell Signaling, catalog no. 9624, RRID: AB_331817), or GSK-3β (1 : 3000; Cell Signaling, catalog no. 9315, RRID: AB_490890); followed by a secondary antibody linked to horseradish peroxidase (Merck Millipore, CA, USA). GSK-3β was used as an internal control to quantify the expression of the target genes. Protein bands were detected using a Bio-Rad Laboratories Chemiluminescence Detection Kit (ECL) (Bio-Rad). Western blot intensity was quantified using the Images J software (NIH, Bethesda, Maryland, USA).

2.9. Statistical analysis

The results are presented as the mean ± S.E.M., and n refers to the number of trials performed. The statistical significance of the data was calculated using one-way ANOVA with Bonferroni correction (Origin Pro 8.1, Northampton, MA, USA) when comparing three or more dataset groups, whereas unpaired or paired Student's t-test was used to compare two groups. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Identification of compounds in the hexane extract of P. japonica seed extract

The n-hexane fraction increased intracellular calcium concentration in boar sperm in a concentration-dependent manner (Fig. 1 B). Therefore, the *n*-hexane fraction was further purified using silica gel open column chromatography and prep-HPLC, which led to the isolation of two fatty acids (linoleic acid and oleic acid) and β-sitosterol (Fig. 1 A). The chemical structures (Fig. 1 D) of the isolated compounds identified using 1H NMR and ESI-MS spectral data confirmed the presence of linoleic and oleic acids, β-sitosterol in PJE (supplementary data). Active ingredients; linoleic acid 7.40 mg and oleic acid 19.30 mg were per 1 g of the extract (Fig. 1C) [20].

3.2. Effect of P. japonica seed extract on boar sperm viability

When treated with 0–200 μg/mL PJE, the viability of boar sperm did not show a significant variation at any tested concentration. The highest viability of all PJE concentrations corresponded to 0 h of incubation, while the lowest was observed after 4 h of incubation. No cytotoxic effect was observed with up to 200 μg/mL PJE in vitro at all incubation times (Fig. 2). Therefore, concentrations of 5–200 μg/mL PJE were used for further experiments.

3.3. P. japonica seed extract enhances motility parameters of boar sperm

Sperm incubated under capacitating conditions with 5–50 μg/mL of PJE hexane fraction showed increased motility parameters compared to the controls (Fig. 3). This effect was evident after both 5 min and 1 h of incubation. Of the capacitated treatment groups

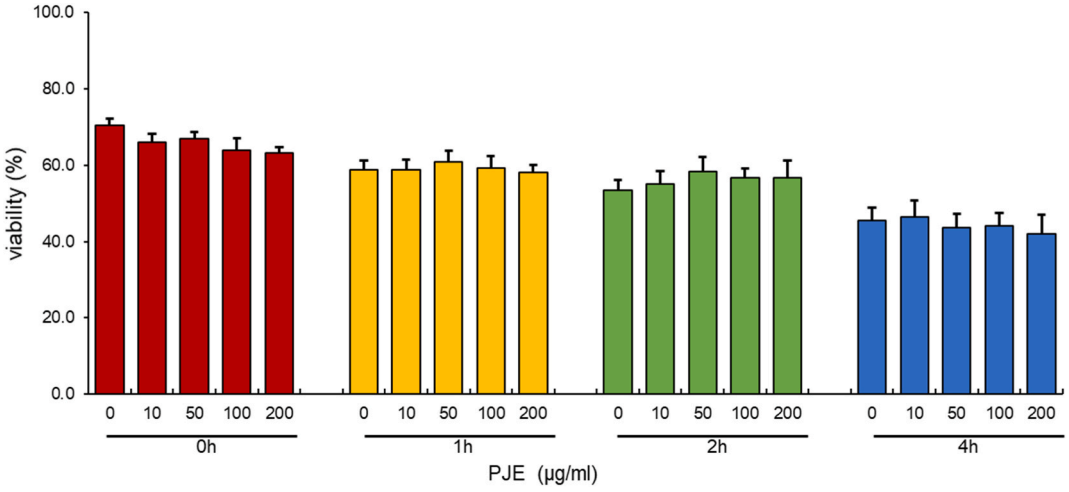

Fig. 2. Cell viability of PJE treated sperms and antioxidant effect of PJE. Percentage of cell viability of sperms after 0–200 μg/ml PJE treatment for the 0–4 h incubation period. Values are expressed as mean ± S.E.M. (n = 5).

incubated for 5 min, rapid motility, VCL, VSL, VAP, and linear displacement at all PJE concentrations were significantly higher than those of the controls (Fig. 3A). Static sperm were the highest in the negative control, whereas a decreasing pattern was observed with increasing concentrations of PJE.

When incubated for 1 h under capacitating conditions (Fig. 3B), rapid motility of the negative control was increased to approximately 65% and the value was higher than that of the PJE-treated counterparts. The static ratio of sperm showed an increasing trend with PJE treatment. Considering the velocity and linear displacement of the 1-h incubated sperm, the 5 and 10 μg/mL PJE treated groups were the highest in all the tested parameters.

The effect of PJE on sperm incubated under non-capacitating conditions (Fig. 4 A, B) was less evident than that on sperm incubated under capacitating conditions. However, rapid motility was significantly higher after 1 h of incubation with PJE (Fig. 4 B) (except for 25 and 50 μg/mL) than that in controls. The static sperm percentages were higher in the non-capacitated sperm samples (Fig. 4 A, B) than those in the capacitated sperm samples (Fig. 3); and the highest tested PJE concentration (50 μg/mL) resulted in the highest static percentages in non-capacitated samples.

3.4. Intracellular calcium levels and CatSper ion channel

According to the single-cell calcium fluorescence measurements, intracellular calcium levels increased upon treatment with 25 μg/mL PJE. The fluorescence further increased with 50 μg/mL PJE (Fig. 5B, D), whereas the internal control containing the corresponding volumes of DMSO treatments did not elevate the fluorescence levels (Fig. 5 A). Calcium levels corresponding to the two PJE concentrations tested were significantly higher than those in the controls (Fig. 5C).

Microplate reader data showed a significant increase in intracellular calcium levels when treated with 20 μg/mL or higher concentrations of PJE, whereas 10 μg/mL or lower concentrations of PJE did not result in a significant increase in fluorescence (Fig. 6 A). The maximum fluorescence values were obtained after 30 s of treatment. A dose-response curve was generated as the fold-changes in mean fluorescence intensities of the sperm against the log values of PJE concentrations tested. According to the calcium fluorescence of sperm, the EC50 of PJE on intracellular calcium levels was 28.12 ± 7.9 μg/mL (Fig. 6B). When sperm were treated with pure fatty acids, a rise in intracellular calcium was observed. The highest calcium signal was corresponding to approximately 10s after the 20 μM LA treatment and there after the fluorescence was slightly decreased and maintained at a constant level (Fig. 6C).

A slight increase in mean fluorescence was recorded in all treatment groups when treated with 10 μM Mibefradil. However, when PJE was added to the Mibefradil-pretreated sperm, intracellular calcium levels did not increase up to 100 μg/mL PJE (Fig. 7 A). However, 200 μg/mL of PJE resulted in an approximately 10-fold increase in fluorescence, which was remarkably different from that of

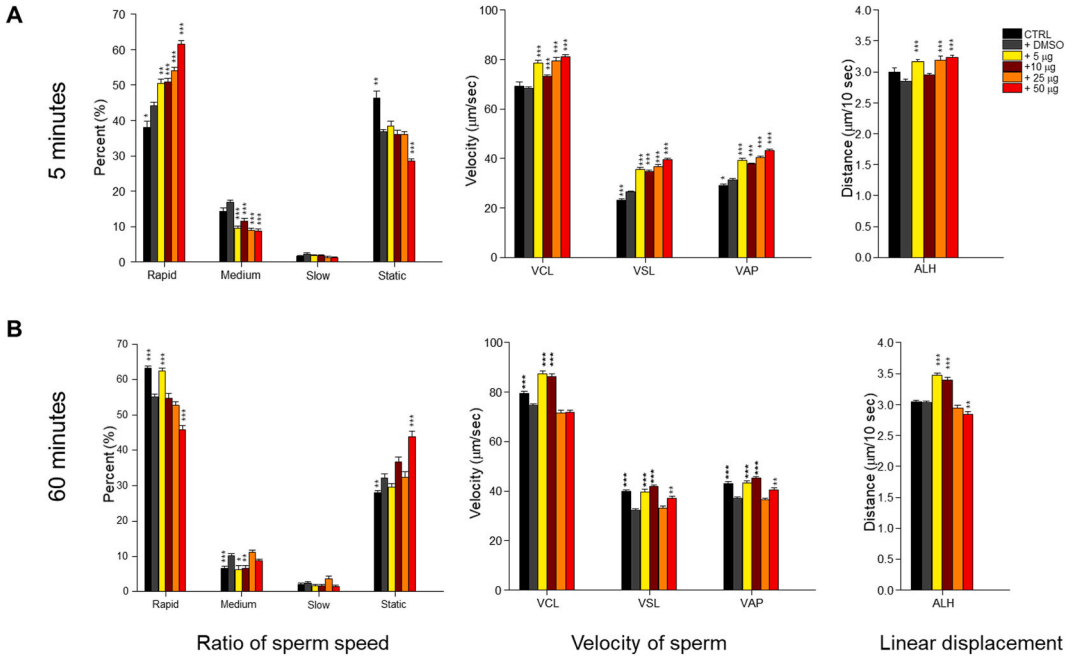

Fig. 3. Motility of PJE treated capacitated sperms (A) Computer assisted semen analysis of capacitated sperms of different PJE treated groups after 5min incubation (Negative control, DMSO, 5–50 μg/ml PJE). Percentage of sperms by different kinematic parameter is categorized differently. Rapid: speed >25 μm/s, Medium: speed 10–25 μm/s, Slow: speed 10-5 μm/s, and Static: speed <5 μm/s. Velocity of sperms: VCL-curvilinear velocity (um/s); VSL straight-line velocity (um/s); VAP average path velocity (um/s), Linear displacement of sperm head: ALH- mean amplitude of head lateral displacement (um)/(10sec). (B) Panel is of capacitated sperms that are corresponding to 60 min incubation with PJE. Asterisk marks indicate significant differences between different treatment groups and the internal control (DMSO). (* $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$) ($n = 10$).

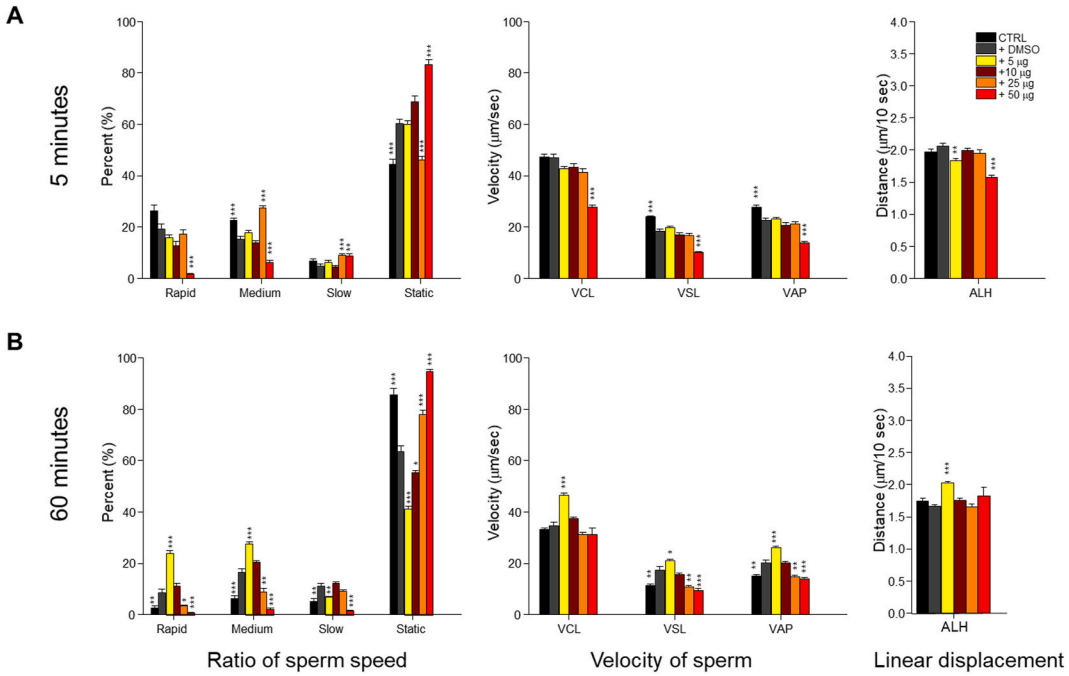

Fig. 4. Motility of PJE treated non-capacitated sperms. Computer assisted semen analysis of non-capacitated sperms with different PJE treatments incubated for (A) 5 mins (B) 60 mins (Negative control, DMSO, 5–50 μg/ml PJE). Percentage of sperms by different kinematic parameter is categorized differently. Rapid: speed >25 μm/s, Medium: speed 10–25 μm/s, Slow: speed 10-5 μm/s, and Static: speed <5 μm/s. Velocity of sperms: VCL-curvilinear velocity (um/s); VSL straight-line velocity (um/s); VAP average path velocity (um/s), Linear displacement of sperm head: ALH-mean amplitude of head lateral displacement (um)/(10sec). Asterisk marks indicate significant differences between different treatment groups and the internal control (DMSO). (* 0.05 > p > 0.01, **0.01 > p > 0.001, ***p < 0.001) (n = 10).

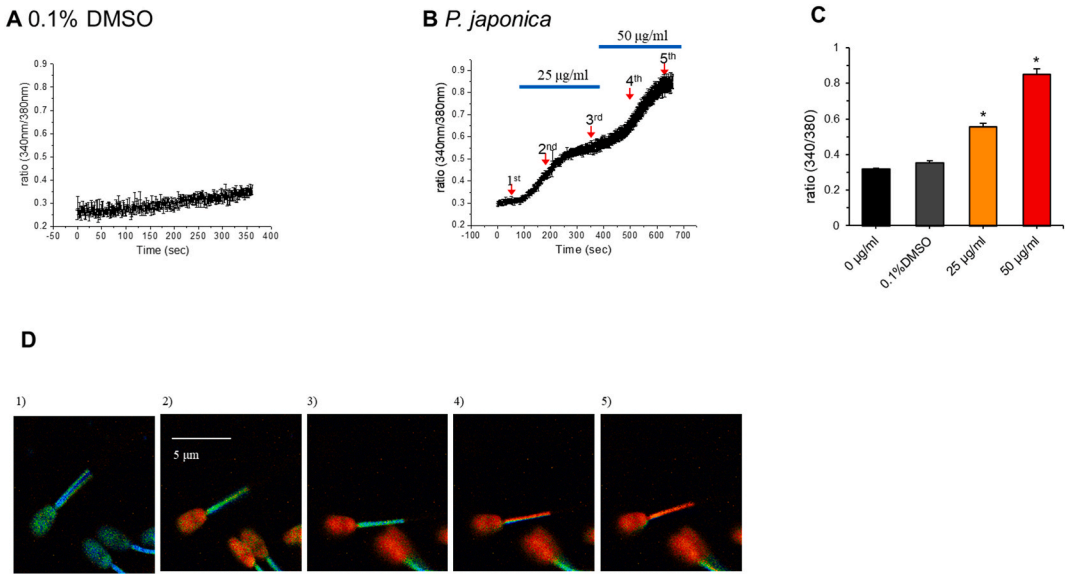

Fig. 5. Effect of PJE on single-cell intracellular calcium levels (A) Single-cell calcium fluorescence measurements with DMSO (internal control) and (B) with PJE, (C) Comparison of mean fluorescence with DMSO, 0,25, and 50 μg/ml PJE, (D) Fluorescence photographs of PJE treated sperms at different time points as indicated in Fig. 5 B (n = 15).

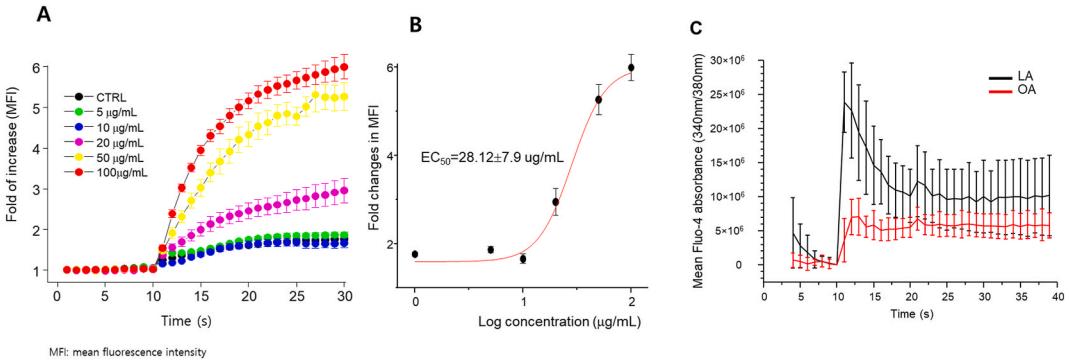

Fig. 6. PJE-dependent intracellular calcium level changes (A) Changes in intracellular calcium levels with different PJE concentrations measured by fluorescence microplate reader (n = 4–6), (B) PJE Dose-intracellular calcium level response curve for boar spermatozoa (n = 4–6) (C) Intracellular calcium level response to 20 μM oleic and linoleic acids (n = 8).

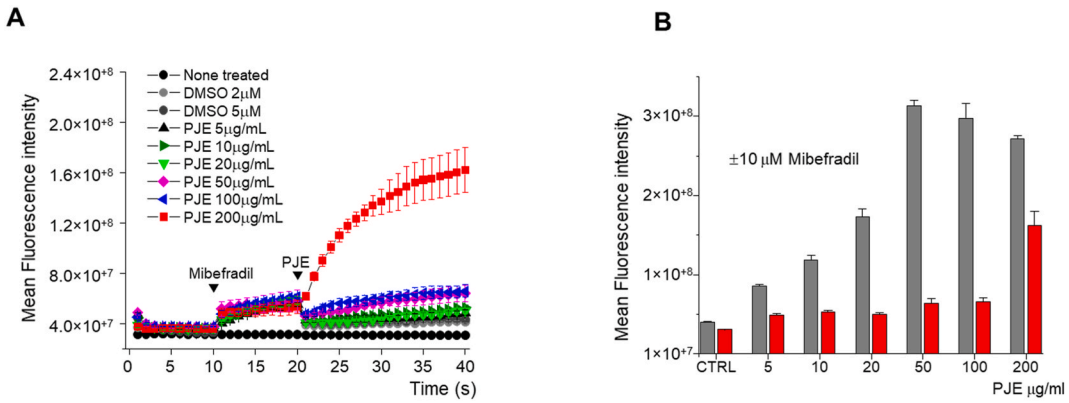

Fig. 7. Effect of PJE on Mibefradil pretreated sperms (A) Intracellular calcium level changes of 10 μM Mibefradil pretreated sperms with different PJE concentrations (B) Mean fluorescence intensity levels Mibefradil treated and untreated sperms with different PJE concentrations. Mibefradil untreated and treated samples are shown in grey and red color, respectively. (n = 4–6)

its counterparts. Compared to that of treatment groups without CatSper blocker pretreatment, intracellular calcium levels of all the treatment concentrations were lower when the sperm were pretreated with the blocker (Fig. 7 B).

3.5. *Effect on protein phosphorylation*

Increased protein phosphorylation was evident in the PJE-treated sperm (Fig. 8 A, B). Compared to the those of PJE-untreated

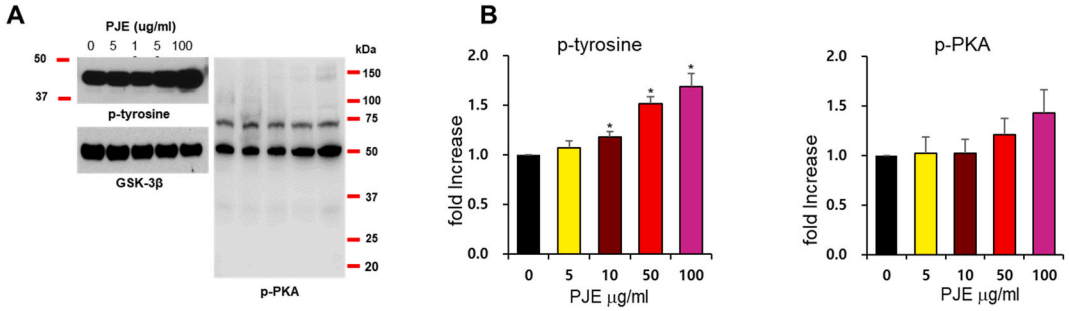

Fig. 8. Effect of PJE on protein phosphorylation of boar spermatozoa. Western blots against tyrosine phosphorylation of P32 (Fig. 8 A) (n = 7) and PKA (n = 4) proteins of boar sperms incubated for 1hr with different PJE treatments. Relative fold increase of each protein expression (Fig. 8 B) corresponding to the condition is shown beside its representative immunoblots. Asterisk (*) mark is indicated where the fold increase is statistically significant to its control groups (p value < 0.05). Uncropped gel images are included in supplementary figures (**S6**).

groups, p-tyrosine levels were significantly elevated at 10 μg/mL PJE. The p-tyrosine expression exhibited a concentration-dependent increase in the range tested, with the highest observed in 100 μg/mL PJE (1.7-fold increase). Phosphorylated PKA levels showed a relatively low increase with PJE treatment, and none of the PJE tested concentrations resulted in a significant increase in phosphorylated PKA. Protein expression levels were given compared to the expression of housekeeping protein: GSK-3β (Fig. 8 B).

4. Discussion

P. japonica seeds are used in the Republic of Korea as a diuretic and laxative in traditional folk medicine. Amygdalin, a cyanogenic glycoside, is a major constituent of *P. japonica* seeds [18]. Only the *n*-hexane fraction increased intracellular calcium levels in boar sperm. Therefore, the active constituents were non-polar compounds, such as free fatty acids or sterols, and not amygdalin. HPLC-ESI/MS analysis of the *n*-hexane fraction confirmed that oleic and linoleic acids were the main active components of PJE (Fig. 1).

β-Sitosterol (5 mg/kg per day per rat subcutaneously) exerted an antifertility effect in male albino rats via decrease in testicular weight and sperm concentrations [24]. However, the highest concentration tested in our experiment was 200 μg/mL of PJE and the study revealed that low concentrations (0.5/kg per day) did not have a pronounced difference among the fertility proxies tested. Hence, we speculated that β-sitosterol in PJE may have minimal to no effect on the results observed in this study.

Boar sperm motility assessment has been widely used as a model system to screen and identify the toxicity of potential drugs because boar spermatozoa can detect cytotoxicity in kinematics-based assays, and it serves as a suitable non-invasive and pain-free cell model [25–27]. In the present study, we used a range of PJE concentrations up to 100 μg/mL. The viability assay revealed that none of the tested concentrations significantly reduced the percentage of viable cells at incubation periods of 0–4 h, thereby indicating that the tested extract concentrations are safe as a therapeutic reagent *in vitro*.

To induce effective capacitation, we applied BSA and caffeine to non-capacitating media, as BSA [28] and caffeine [29] were found to induce capacitation. Among boar sperm incubated under capacitated and non-capacitated conditions, the maximum effect on motility was observed under capacitated conditions. This suggests that the action of the PJE extract is associated with boar sperm capacitation. In addition, changes were observed even after a 5-min incubation with PJE, indicating the involvement of a rapid regulatory mechanism on sperm by the extract. PGE was water-insoluble and was diluted to the desired concentration in DMSO. Therefore, DMSO was used as an internal control to rule out any possible effects of DMSO alone.

We analyzed intracellular molecular signaling to elucidate the possible mechanisms of action of PJE on boar spermatozoa, which may help to better understand our observations regarding motility. Intracellular calcium plays a vital role as an upstream regulator in the capacitation of mammalian spermatozoa and associated hyperactive sperm motility [3]. Consistent with this, we found that PJE-induced boar sperm capacitation and motility were dependent on intracellular calcium levels [13,30]. In our experiment, we used Fluo-4AM, which is a selective calcium-binding fluorescent, dye to observe changes in intracellular calcium levels. We observed a concentration-dependent [Ca^{2+}] increase with PJE. We used single-cell fluorescence analysis to omit the masking effects of a large number of cells in a sample when measured using a microplate reader and to assess the changes at the single-cell level. In both cases, intracellular calcium was found to increase with PJE, confirming PJE's ability to elevate intracellular calcium levels. Agreeing with the results of PJE on $[Ca^{2+}]_{sperm}$, intracellular calcium level was elevated when sperm were treated with the same final concentration of OA and LA separately. Along with our previous work [20] in which LA was found to have a more significant effect on human Hv1, in this study, the highest boar sperm calcium fluorescence increase was corresponding to LA, suggesting its higher potency in modulating sperms ion channels. Furthermore, studies carried out in castrated rats suggests fatty acids including OA to have ameliorative effect and could be used as putative therapy for infertility targeting reproductive tissues [31]. However, there is a paucity of information on their mechanism of action in improving sperm quality and male fertility related parameters.

Mammalian spermatozoa have several ion channels and transporters that may regulate calcium influx across plasma membrane [6] and the CatSper ion channel appears to be the most important one [32]. Further validating this claim, CatSper of boar sperm was found to be crucial in boar sperm capacitation and motility [11]. Upon finding that PJE can increase intracellular calcium levels, we further questioned whether PJE affects CatSper channels. Spermatozoa were treated with PJE in the presence or absence of Mibefradil. The Mibefradil-pretreated PJE group showed significantly reduced calcium levels when compared with their untreated counterparts. As Mibefradil is a selective CatSper channel blocker [11], it was confirmed that PJE could act on the CatSper channel, thereby facilitating a higher calcium influx. However, according to our previous studies, PJE also affects human sperm motility and its intracellular pH by modulating the Hv1 [20]. Since the CatSper channel is regulated by intracellular pH [22], similar possible indirect effects of PJE on CatSper channels should also be considered. Free fatty acids have the potential to directly modify motility-related ion channels by changing the channel conformation or pathways involving fatty acid metabolites and the fatty acids [33]. Hexane fraction of PJE has a relatively high amount of unsaturated fatty acids (oleic acids and linoleic acids). Thus, we construe these fatty acids as putative active compounds of PJE that affect ion channels in boar spermatozoa. Future investigations on effects of free OA and LA on sperm CatSper ion channel in the patch-clamping set up are needed to confirm this claim and it will provide more elaborate explanations regarding the potential electrophysiological properties of PJE.

We measured the protein tyrosine phosphorylation of sperm, which is a hallmark of sperm capacitation [34,35]. Tyrosine phosphorylation is a late event in sperm capacitation which is downstream of the protein kinase A signaling pathway. It is essential for the completion of the sperm capacitation [36,37]. When incubated with PJE, spermatozoa showed increased protein tyrosine phosphorylation. We also measured PKA content in the sperm. The PKA levels in the PJE-treated and untreated groups did not differ significantly. However, with an increase in PJE concentration, a dose-dependent PKA fold increase was observed. Altogether, this confirms that the modulatory mechanism of PJE extract on boar sperm involves the protein tyrosine phosphorylation pathway, which has been found to be essential in boar sperm capacitation in previous studies [12,13,30].

5. Conclusion

In summary, we report that PJE increases intracellular calcium levels in boar spermatozoa by modulating the CatSper channels, leading to the induction of capacitation. Hence, PJE could be used as a putative supplement to increase the quality of boar spermatozoa.

Author contribution statement

Akila Cooray, Mee Ree Chae: Wrote the paper; Performed the experiments; Analyzed and interpreted the data.
Sung Won Lee, Kyu Pil Lee: Wrote the paper; Conceived and designed the experiments.
Chul Young Kim: Wrote the paper; Performed the experiments; Analyzed and interpreted the data; Conceived and designed the experiments.
Tharaka Darshana Wijerathne, Dae Geun Kim, Jeongsook Kim: Performed the experiments; Analyzed and interprited the data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

Appendix B. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e13616.

List of abbreviations

ALH	head lateral displacement
BSA	bovine serum albumin
CASA	computer-assisted sperm analysis
CatSper	cation channel of sperm
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
Fluo-4AM	4-(6-Acetoxymethoxy-2,7-difluoro-3-oxo-9-xanthenyl)-4′-methyl 2,2′(ethylenedioxy) dianiline N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl) ester
HEPES	4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid
HVCN1/Hv1	hydrogen voltage gated channel 1
OA	oleic acid
PI	propidium iodide
PJE	*P. japonica* seed extract
PKA	protein kinase
SOCCs	store-operated Ca^{2+} channels
TRPV	transient receptor potential vanilloid
VAP	average path velocity
VCL:	curvilinear velocity
VGCCs	voltage-gated calcium channels
VSL:	straight-line velocity
LA	linoleic acid

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