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Research Note

An Antioxidant Davallialactone from *Phellinus baumii* Enhances Sperm Penetration on *In Vitro* Fertilization of Pigs

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Abstract Davallialactone (DAVA) is a hispidin analogue derived from the medicinal fungus *Phellinus baumii*. We examined the effect of DAVA on *in vitro* fertilization (IVF) of pigs. Boar spermatozoa were incubated in fertilization medium with varying concentrations of DAVA, then sperm motility and reactive oxygen species (ROS) level were evaluated. Higher sperm motility was found following the addition of 0.5 or 1 μM DAVA after incubation than addition of other concentrations or controls. ROS level decreased significantly with the addition of DAVA. The rate of normal fertilization was higher in the presence of 1 μM DAVA (65.1%) than were those of other concentrations or controls (45.4~59.4%), and the highest total fertilization rate (mono- and polyspermic oocytes) was observed at 1 μM DAVA (83%). In conclusion, addition of DAVA to fertilization medium improved sperm motility, and reduced ROS level so as to potentially improve sperm-oocyte binding in IVF, suggesting the potential of a compound isolated from mushrooms in assisted reproductive technology for humans and animals.

Keywords Davallialactone, *In vitro* fertilization, *Phellinus baumii*, Pig, Spermatozoa

Mushrooms are a nutritious natural food with unique flavors and properties, such that diverse practical uses of their features have been employed in food and medicine. The various properties of mushroom-derived compounds, including antimicrobial, antiviral, antitumor, antiallergic, immunomodulating, anti-inflammatory, and antioxidant activities, have been proposed for use in treating diseases [1, 2]. *Phellinus baumii* Pilát (Hymenochaetaceae) is a saprophytic fungus that grows wild on deciduous trees such as *Quercus* species [3]. Our previous study discovered biological activities of compounds extracted from this mushroom [4, 5]. One of these compounds, davallialactone (DAVA, Fig. 1), is a hispidin analog, and its multifarious activities have been reported to include antiplatelet-aggregation activity [6], antioxidant activity [7], free radical scavenging properties [4], and inhibition of inflammatory responses induced by lipopolysaccharide [7]. Although many therapeutic properties of mushrooms have been suggested, their applications for fertilization have not been discussed thus far. In the present study, we investigated sperm motility in the presence of DAVA, and pig oocytes matured *in vitro* were inseminated in fertilization medium containing different concentrations of DAVA. We suggest a potential compound from mushrooms for application in artificial insemination and *in vitro* fertilization (IVF) to contribute to assisted reproductive technology (ART) for human and animals.

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Isolation of DAVA. DAVA was isolated from the mushroom *P. baumii* as previously described [4]. In brief, *P. baumii* was extracted with MeOH, and the methanolic extract was partitioned between ethyl acetate and H_2O. The ethyl acetate-soluble fraction was subjected to a column of Sephadex LH-20, and eluted with 70% aqueous MeOH, followed by preparative reversed-phase high-performance liquid chromatography eluted with 40% aqueous MeOH containing 0.04% trifluoroacetic acid, to yield DAVA.

DAVA enhances sperm movement in fertilization medium. All studies were performed in accordance with the guidance of an Animal Care and Use Committee (ACUC) protocol approved by the ACUC of Chonbuk National University. Semen was collected from proven fertile adult Duroc boars, 15~22 months of age. The sperm-rich fraction

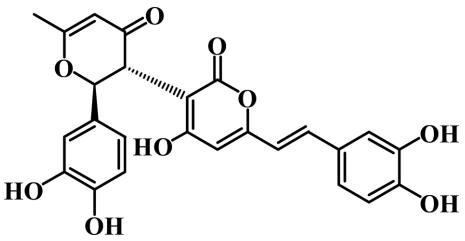

Fig. 1. Structure of davallialactone.

of ejaculate was collected into an insulated vacuum bottle, fractions with greater than 85% motile spermatozoa were used, and sperm concentrations were estimated with a hemocytometer. Semen was diluted with Beltsville thawing solution (BTS) [8] to a final concentration of 1×10^8 spermatozoa/mL. Boar spermatozoa were incubated in modified Tris-buffered medium (mTBM; fertilization medium) containing 2 mM caffeine and 0.2% bovine serum albumin (BSA; A7888; Sigma, St. Louis, MO, USA) in the absence or presence of different concentrations of DAVA (final conc. 0~5 μM; a control dimethyl sulfoxide [the solvent for DAVA]) at 38.5°C, in an atmosphere containing 5% CO_2. After incubation, sperm motility was observed optically under a stereomicroscope at 38.5°C. Higher sperm motility was observed following the addition of 1 μM DAVA after 10 min of incubation (91.7%) than after addition of 1~2 μM DAVA or in controls (no addition or dimethyl sulfoxide [DMSO]; 85.0~86.7%, $p < 0.05$) (Fig. 2A); moreover, addition of 5 μM DAVA resulted in significantly decreased sperm motility with aggregation (80.0%, $p < 0.05$) (Fig. 2A). After 1 hr, these motilities drastically decreased due to sperm hyperactivation that occurred during incubation (Fig. 2A). Significantly higher sperm motility was observed at 0.5 or 1 μM DAVA (56.7~60%) than at 2 μM DAVA or in controls (all 50.0%, $p < 0.05$) (Fig. 2A), and the lowest percentage was observed following the addition of 5 μM DAVA (45.0%, $p < 0.05$) (Fig. 2A), indicating that 0.5~1 μM DAVA may improve sperm movement and penetration into oocytes during fertilization.

DAVA reduces reactive oxygen species (ROS) level during sperm incubation. Boar spermatozoa (1×10^7 spermatozoa/mL) were incubated in mTBM with or without varying concentrations of DAVA or a control of DMSO (the solvent for DAVA) for 1 hr at 38.5°C, in an atmosphere containing 5% CO_2. The level of intracellular hydrogen peroxide (H_2O_2) in sperm was assayed using carboxy-DCFDA (final conc. 1 μM; Invitrogen, Eugene, OR, USA). The fluorescence intensity was measured using a multimode microplate reader (Spark 10M; Tekan, Männedorf, Switzerland) with excitation at 485 and emission at 520 nm. Excessive production of ROS has detrimental effects on sperm functions such as membrane lipid peroxidation, DNA damage, and fertilization [9]. However, an appropriate level of ROS plays an important role in hyperactivation and capacitation of spermatozoa [9, 10]. Recently, DAVA was shown to inhibit ROS production, countering adriamycin-induced ROS accumulation and apoptosis in cardiac muscle cells, and acetaminophen-induced liver injury, via a free radical scavenging activity [4, 5, 11, 12]. Thus, we examined ROS levels during sperm incubation with and without DAVA. As shown in Fig. 2B, ROS levels decreased significantly following the addition of DAVA, and 2~5 μM DAVA in particular significantly decreased ROS production relative to that seen with no addition or DMSO addition ($p < 0.05$). Consequently, 0.5~1 μM DAVA might moderate proper ROS levels through the action of scavengers, whereas higher concentrations of DAVA strongly reduce ROS, but might interfere with the normal functioning of spermatozoa.

DAVA increases fertilization rates on IVF of pig oocytes. IVF is regarded as the most efficient way to verify sperm quality. In order to perform pig IVF, ovaries were collected from prepubertal gilts at a local slaughterhouse. Cumulus oocyte complexes (COCs) were aspirated from antral follicles

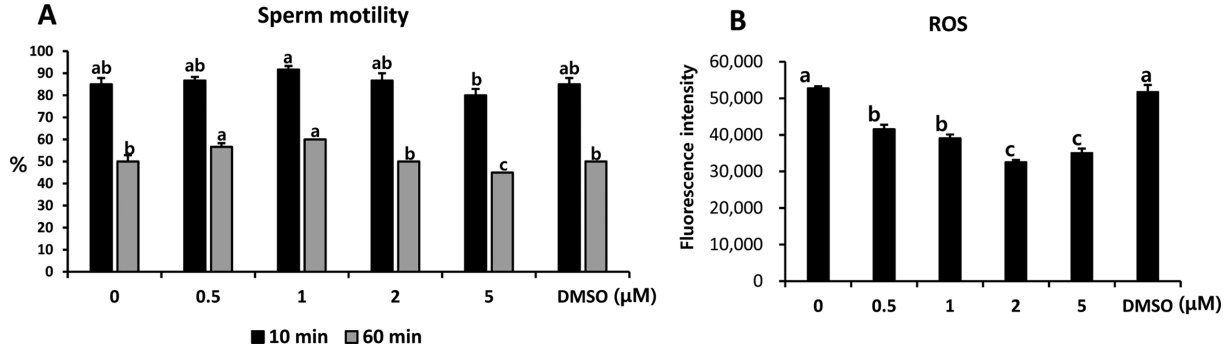

Fig. 2. Examination of sperm motility after incubation (10 min and 1 hr) in fertilization medium with or without varying concentrations of davallialactone (DAVA). A, The percentage of motile spermatozoa at 38.5°C was estimated using a light microscope at 250× magnification; B, Assessment of fluorescence intensity of reactive oxygen species (ROS). Experiments were repeated three times with two replicates. DMSO, dimethyl sulfoxide. Values are expressed as mean percentages ± SEM. The different superscripts (a~c) in each group of columns denote a significant difference at $p < 0.05$.

(3~6 mm in diameter), washed three times in HEPES-buffered Tyrode lactate (TL-HEPES-PVA) medium containing 0.01% (w/v) polyvinyl alcohol (PVA), and then washed three times with oocyte maturation medium [13]. A total of 50 COCs were transferred to 500 μL of maturation medium covered with mineral oil in a 4-well multi-dish equilibrated at 38.5°C in a 5% CO_2 atmosphere. The medium used for oocyte maturation was tissue culture medium 199 (Cat. #50-050-PB; Mediatech, Inc., Manassas, VA, USA) supplemented with 0.5 μg/mL luteinizing hormone (L6420; Sigma), 0.5 μg/mL follicle stimulating hormone (F2293, Sigma), 10 ng/mL epidermal growth factor (E4127; Sigma), 75 μg/mL penicillin G, and 50 μg/mL streptomycin. The oocytes were cultured for 44 hr at 38.5°C in a 5% CO_2 atmosphere. After oocyte maturation, cumulus cells were removed with 0.1% hyaluronidase in TL-HEPES-PVA medium, and oocytes were placed into four 100 μL drops of mTBM medium covered with mineral oil in a 35-mm polystyrene culture dish. One milliliter of liquid semen preserved in BTS was washed, and spermatozoa were resuspended in mTBM medium. After the appropriate dilution, 1 μL of this sperm suspension was added to the medium containing oocytes to give a final sperm concentration of 1×10^5 spermatozoa/mL. Different concentrations of DAVA (final conc. 0~5 μM; DMSO as a control) were added to fertilization drops at the time of sperm addition during IVF. Oocytes were co-incubated with spermatozoa for 2 hr at 38.5°C in a 5% CO_2 atmosphere. After IVF, oocytes were transferred into 500 μL of PZM-3 medium [14] containing 0.4% BSA (A0281; Sigma) for further culture for 20 hr. To evaluate fertilization rate, oocytes/zygotes were fixed with 2% formaldehyde, permeabilized with phosphate buffered saline-Triton X-100, and stained with 2.5 mg/mL 4′,6-diamidino-2-phenylindole, then sperm penetration and the fertilization status of the zygotes (unfertilized, fertilized-monospermic, or fertilized-polyspermic) were assessed under fluorescence microscope (Nikon Eclipse Ci microscope; Nikon Instruments Inc., Seoul, Korea). Data analyses were processed using one-way analysis of variance (ANOVA) using SAS package 9.3 (SAS Institute Inc., Cary, NC, USA) in a completely randomized design. Duncan's multiple range test was used to compare values of individual treatment when the F-value was significant ($p < 0.05$).

A significantly higher rate of normal fertilization (% monospermic oocytes) was observed following the addition of 1 μM DAVA (65.1%) than with other concentrations or controls (45.4~59.4%, $p < 0.05$), although polyspermy rates were also significantly increased at either 0.5 or 1 μM DAVA (12.3~17.8%) relative to those at other concentrations (4.9% at 0 and 2 μM), while no polyspermy was observed at 5 μM DAVA or with DMSO ($p < 0.05$) (Fig. 3). The highest total fertilization was seen at 1 μM DAVA (83%), and the total fertilization rate exceeded 70% with the addition of 0.5 μM DAVA, which are significantly higher rates than those seen in controls (45.4~58.9%, $p < 0.05$) (Fig. 3). It has been reported that a suitable gamete co-

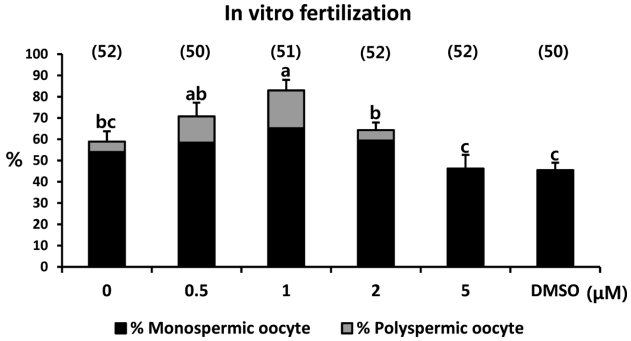

Fig. 3. Effects of davallialactone (DAVA) on sperm penetration and fertilization during pig *in vitro* fertilization. Different concentrations of DAVA were added into fertilization medium, and oocytes were co-incubated with 1×10^5 spermatozoa/mL for 2 hr. DMSO, dimethyl sulfoxide. The diagram indicates % monospermic and % polyspermic fertilization. Numbers of inseminated oocyte are indicated in parentheses. Experiments were repeated three times. Values are expressed as mean percentages ± SEM. The different superscripts (a~c) in each group of columns denote a significant difference at $p < 0.05$.

incubation system could lead to actual capacitation and acrosome reaction, resulted in increasing sperm-oocyte binding [15]. In this study, the incidence of normal fertilization was higher at 1 μM DAVA than with no addition, suggesting that DAVA improved sperm-oocyte binding during IVF.

In conclusion, addition of DAVA to fertilization medium improved sperm motility and reduced ROS level. These benefits led to increased fertilization rates, suggesting the possible use of a novel natural compound isolated from mushrooms to contribute to ART for humans and animals.

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