

# Phelligrudin D from *Phellinus baumii* Reduces Boar Sperm Viability

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**ABSTRACT :** Phelligrudin D (Phe D) is a compound isolated from *Phellinus baumii*, which is known for various biological activities. In this study, the authors examined the effect of Phe D on boar spermatozoa for its potential application in assisted reproductive technology for mammals. Sperm motility and deubiquitinating activity significantly decreased when boar spermatozoa were incubated with Phe D (>0.5  $\mu$ M). The fluorescence intensities of dead sperm, and reactive oxygen species production increased after sperm incubation in the presence of Phe D. Although Phe D is associated with antioxidant and free radical scavenging activity, sperm viability deteriorated after its addition. This could lead to fertilization failure, including that following artificial insemination or *in vitro* fertilization. Phe D might have other biological functions in spermatozoa, and therefore requires additional studies in the future.

**KEYWORDS :** Boar, Phelligrudin D, *Phellinus baumii*, Spermatozoa, Viability

Many studies have elucidated the biological function of medicinal fungi in the treatment of diseases [1]. Phelligrudin D (Phe D) is a styrylpyrone compound isolated from *Phellinus baumii*, which has been associated with various activities, such as anticancer, anti-inflammatory, antioxidative, and anti-allergic effects [2-4]. Recently, we have reported that fertilization rate of pig oocyte improved in *in vitro* fertilization (IVF) medium supplemented with davallialactone isolated from *P. baumii* [5]. Therefore, the present study was performed to examine the effects of Phe D on boar spermatozoa and to determine whether it could be useful for assisted reproductive technology (ART) in humans and animals.

## Isolation of phelligrudin D

Phe D was isolated from the mushroom *P. baumii*, as previously described [1]. In brief, *P. baumii* was subjected to extraction with MeOH, and the methanolic extract was partitioned between ethyl acetate and H<sub>2</sub>O. The ethyl acetate-soluble fraction was subjected to a column of Sephadex LH-20 eluted with 70% aqueous MeOH, followed by preparative reversed-phase HPLC eluted with 40% aqueous MeOH containing 0.04% trifluoroacetic acid to produce Phe D.

## Sperm motility and proteasomal-proteolytic activity decreased in the presence of Phe D

The present study was performed in accordance with the guidance provided by the Animal Care and Use Committee (ACUC) of Chonbuk National University. Semen was collected from proven fertile adult Duroc boars, 15~22 months of age. The sperm-rich fraction of the ejaculate was collected in an insulated vacuum bottle. Fractions with greater than 85% motile spermatozoa were used, and sperm concentrations were estimated using a hemocytometer. Semen was diluted with Beltsville thawing solution (BTS) [6] to a final concentration of  $1 \times 10^8$  spermatozoa/mL. The diluted semen was maintained in a storage unit at 17°C for a week. Boar spermatozoa ( $1 \times 10^7$  spermatozoa/mL) were incubated in BTS without or with varying con-

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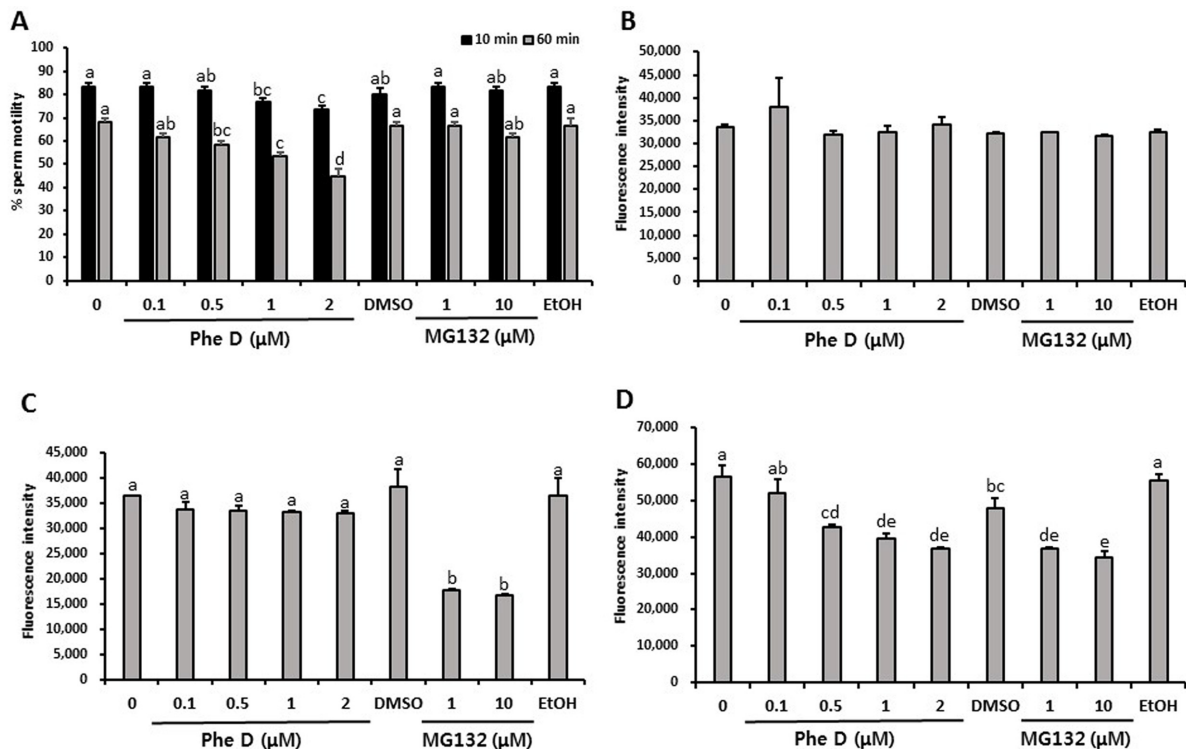
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centrations of Phe D (final concentration [conc.] 0~2  $\mu\text{M}$ ), or in control solutions, (dimethyl sulfoxide [a solvent of Phe D; 2  $\mu\text{M}$  DMSO], MG132 [a potent proteasome inhibitor blocks fertilization without hindering sperm movement; 1 or 10  $\mu\text{M}$ ], or ethanol [a solvent of MG132; 10  $\mu\text{M}$  EtOH]) for 60 min at 37.5°C. After incubation, sperm motility was observed optically under a stereomicroscope at 37.5°C. Sperm proteasomal-proteolytic activity was measured as previously described by Yi et al. [7]. Spermatozoa were loaded into a 96-well black plate (final conc.  $2 \times 10^6$  spermatozoa/mL) and incubated at 37.5°C with Z-LLE-AMC (a specific substrate for 20S chymotrypsin-like peptidyl-glutamylpeptide hydrolyzing [PGPH] activity and not sensitive to MG132; final conc. 100  $\mu\text{M}$ ; Enzo Life Sciences, Plymouth, PA), Suc-LLVY-AMC (a specific substrate for 20S proteasome and other chymotrypsin-like proteases, as well as calpains; final conc. 100  $\mu\text{M}$ ; Enzo), or ubiquitin-AMC (a specific sub-

strate for ubiquitin-C-terminal hydrolase and ubiquitin specific proteases activity; final conc. 1  $\mu\text{M}$ ; Enzo). Fluorescence intensity was measured using a multimode microplate reader (Spark 10M; Tecan, Männedorf, Switzerland) with excitation (ex.) at 380 nm and emission (em.) at 460 nm. Data analyses were conducted with a one-way analysis of variance (ANOVA) in a completely randomized design using SAS package 9.3 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to compare values of individual treatment when the F-value was significant ( $p < 0.05$ ).

As shown in Fig. 1A, significantly lower sperm motility was observed following the addition of 2  $\mu\text{M}$  Phe D (73.3%) after 10 min of incubation than following the addition of 0.1~1  $\mu\text{M}$  Phe D, no additions, and controls (range 76.7~83.3%,  $p < 0.05$ ). After 60 min of incubation, higher spermatozoa motilities were maintained without Phe D, and with DMSO, 1  $\mu\text{M}$  MG132, and EtOH (range



**Fig. 1.** Examination of sperm motility after 10 min or 60 min of incubation in Beltsville thawing solution (BTS) with and without varying concentrations of Phelligrudin D (Phe D) (controls: 1 and 10  $\mu\text{M}$  MG132, 2  $\mu\text{M}$  DMSO and 10  $\mu\text{M}$  EtOH). The percentage of motile spermatozoa was estimated at 37.5°C using a light microscope at 250 $\times$  magnification. Experiments were repeated three times using three different boars (A). Sperm proteasomal-proteolysis activity was assayed using fluorometric substrate AMCs. Fluorescence intensity for 20S chymotrypsin-like peptidyl-glutamylpeptide hydrolyzing (PGPH) activity (B), 20S proteasome and other chymotrypsin-like proteases activity (C), and ubiquitin-C-terminal hydrolase and ubiquitin specific proteases activity (D) were examined after sperm incubation. Experiments were repeated three times with two replicates. Values are expressed as the mean percentages  $\pm$  standard error of the mean. Different superscripts, a~e, in each group of columns denote a significant difference at  $p < 0.05$ .

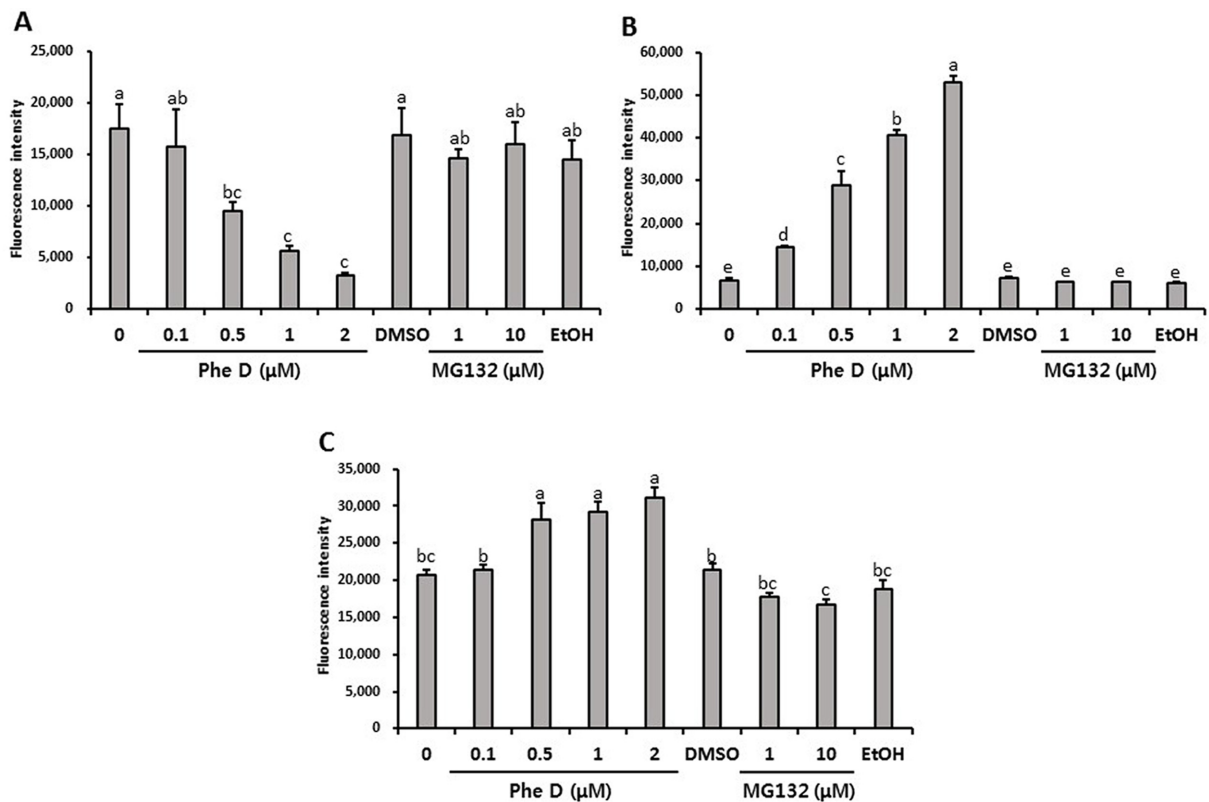
66.7~68.3%,  $p < 0.05$ ; Fig. 1A) than in the presence of 0.1~2  $\mu\text{M}$  Phe D or 10  $\mu\text{M}$  MG132 (range 45.0~61.8%,  $p < 0.05$ ; Fig. 1A). The proteasomal-proteolysis and deubiquitinating activities are known as essential processes in fertilization [8]. Enzymatic activities of sperm borne proteasomes can be used to estimate fertilizing potential [7]. They were assayed using specific fluorometric substrates Z-LLE, Suc-LLVY, and ubiquitin-AMCs in spermatozoa incubated with or without Phe D (Fig. 1B~1D). There were no statistical differences in chymotrypsin-like PGPH activity among spermatozoa incubated in the absence or presence of Phe D, or in the controls (Fig. 1B). No significant differences in 20S proteasome and chymotrypsin-like proteasomal core activity were observed in the absence or presence of Phe D, or DMSO and EtOH controls, but activity significantly decreased with the addition of a proteasome inhibitor (1 or 10  $\mu\text{M}$  MG132,  $p < 0.05$ ; Fig. 1C). Higher fluorescence intensity of deubiquitinating activity was exhibited with no addition of Phe D and with the addition of EtOH, but the intensities gra-

dually decreased in a dose-dependent manner ( $p < 0.05$ ; Fig. 1D) when spermatozoa were incubated in BTS with 0.1~2  $\mu\text{M}$  Phe D or with MG132. As a result, sperm motility and proteasomal-proteolytic activity during incubation decreased in the addition of 0.5~2  $\mu\text{M}$  Phe D, assuming that it could inhibit sperm penetration during fertilization.

**Phe D reduces sperm viability during incubation**

To assess sperm viability, SYBR 14 (final conc. 100 nM; Molecular Probes, Eugene, OR, USA) or propidium iodide (PI; final conc. 5 nM; Molecular Probes) was added to spermatozoa incubated with or without Phe D. The levels of intracellular hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in sperm were assayed using carboxy-DCFDA (final conc. 0.5  $\mu\text{M}$ ; Invitrogen, Eugene, OR, USA). The fluorescence intensity was measured using a multimode microplate reader with ex. at 485 nm and em. at 520 nm for SYBR 14, and carboxy-DCFDA, or with ex. at 540 nm and em. at 620 nm for PI.

Spermatozoa were incubated for 60 min and stained



**Fig. 2.** Assessment of fluorescence intensity for sperm viability, and reactive oxygen species (ROS) level (controls: 1 and 10  $\mu\text{M}$  MG132, 2  $\mu\text{M}$  DMSO and 10  $\mu\text{M}$  EtOH). Incubated spermatozoa were stained with SYBR 14 labels to DNA of live spermatozoa (A), propidium iodide (PI) to detect dead spermatozoa (B), and carboxy-DCFDA to indicate ROS production (C). Experiments were repeated three times with two replicates. Values are expressed as the mean percentages  $\pm$  standard error of the mean. Different superscripts, a~e, in each group of columns denote a significant difference at  $p < 0.05$ .

with SYBR 14 to detect live sperm cells or stained with PI to detect dead cells (Fig. 2A, 2B). Higher intensity of SYBR 14 was shown in the absence of Phe D or DMSO, and the intensity significantly decreased with increasing concentrations of Phe D ( $p < 0.05$ ; Fig. 2A). Conversely, the intensity for dead sperm cells increased with increasing concentration of Phe D compared to no addition or addition of controls ( $p < 0.05$ ; Fig. 2B). Excessive production of reactive oxygen species (ROS) has been implicated in membrane lipid peroxidation, DNA damage of spermatozoa, and fertilization failure [9-11]. However, appropriate levels of ROS play an important role in hyperactivation and capacitation [9, 12]. A previous report suggested that Phe D isolated from *Inonotus xeranticus* exhibited free radical scavenging activity [13], and consequently, we examined ROS levels after sperm incubation with or without Phe D (Fig. 2C). However, significantly higher ROS levels were exhibited in the presence of 0.5~2  $\mu\text{M}$  Phe D than in other concentrations or in controls ( $p < 0.05$ ; Fig. 2C). In our previous study, ROS levels significantly decreased during sperm incubation in the presence of davallialactone, which is a hispidin analogue derived from *P. baumii* [5]. However, Phe D did not suppress ROS production during sperm incubation, suggesting that decreased viability could be associated with ROS production (Fig. 2).

### Conclusion

The present study investigated the effect of Phe D on boar spermatozoa to determine whether it could be applied to ART for humans and animals. Increasing concentration of Phe D ( $> 0.5 \mu\text{M}$ ) decreased sperm motility and deubiquitinating activity, as well as fluorescence intensity of live spermatozoa. Fluorescence intensities of dead sperm, and ROS production increased when spermatozoa were incubated with Phe D. Consequently, boar sperm viability deteriorated in the presence of Phe D. Nonetheless, it has beneficial effects, including antioxidant and free radical scavenging activity in disease, and possibly other biological functions exists in spermatozoa. Therefore, it requires further study in future.

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