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Control of sperm penetration using stereumamide A derived from *Trichaptum fuscoviolaceum* in the *in vitro* fertilization of pig oocytes

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Research Article

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Summary

Fungal metabolites are known to have potent and diverse properties such as antiviral, antidiabetic, antitumour, antioxidant, free radical scavenging, and antibacterial effects which can be utilized to treat diseases. In this study, we investigated the functional activity of stereumamide A (StA) derived from a culture broth of *Trichaptum fuscoviolaceum* during the *in vitro* fertilization (IVF) of pig oocytes, to determine its effects on sperm penetration. Oocytes matured *in vitro* were fertilized in the absence or presence of varying concentrations of StA (0-50 μg/ml StA). When StA was directly added into the IVF medium, significantly lower fertilization rates were seen with the 20 or 50 μg/ml StA (2.0–17.5%) treatments compared with those of 10 μg/ml StA or the controls (60.9–62.3%), whereas StA had no influence on the survival of oocytes and spermatozoa throughout the IVF process. For evaluating the control of sperm entry, mature oocytes were pre-incubated in a medium containing 20 μg/ml StA for 1 h, and then IVF was subsequently performed. The incidence of polyspermy was significantly reduced when oocytes were pre-incubated with StA (15.0% *vs.* 50.4–57.5% in controls). In conclusion, sperm penetration was inhibited in the medium in the presence of StA during IVF, while StA did not affect sperm motility and fertility competence. Fertilization was controlled when mature oocytes were incubated with StA prior to IVF, suggesting the possible use of the fungal metabolite in assisted reproductive technology for humans and animals.

Introduction

Subfertility is a leading global health issue affecting individuals of reproductive age worldwide. Available data over the past few years suggests that the percentage of infertile couples is steadily increasing in the world. Infertility may occur due to female or male factors or complications involving both (Malina and Pooley, 2017). It is a very sensitive issue of social concern, and many pharmacological, surgical, and assisted reproductive techniques (ART) have been developed to enable couples to have children. *In vitro* fertilization (IVF) is a type of ART commonly used in this field (Klemetti et al, 2004; Malina et al., 2016). The use of porcine models in research is more popular due to their physiological similarity to humans. Many factors affect the success of the *in vitro* fertilization technique, including the condition of the sperm and the eggs. When conducting IVF with boar spermatozoa, the occurrence of polyspermy is of major concern (Alcântara-Neto et al., 2020). The term polyspermy is simply defined as the interaction of more than one male sperm nucleus with the female nucleus (Markert, 1979). The incidence of polyspermy can reach up to 30–40% with boar sperm compared to other mammalian species and under IVF conditions it can reach up to 65% (Xia et al., 2001). The reasons for polyspermy are still unclear and several articles have suggested that it could occur due to factors such as the quality of mature oocytes, IVF conditions, and the semen quality at fertilization (Sirard et al., 1993; Wang et al., 1994; Marchal et al., 2001), as well as variations within the same species and the influence of the ejaculate on the sperm penetration and the polyspermy (Almiñana et al., 2005; Gil et al., 2007; Alcântara-Neto et al., 2020). Polyspermy results in nuclear chromosomal abnormalities, a reduced number of cells in the blastocyst, and an overall reduction in the success rate of IVF (Nguyen et al., 2020). Therefore, researchers are paying attention to the use of artificially derived or naturally derived compounds to enhance the success rate of IVF.

Plant-derived compounds have been in use for many years and there is considerable ongoing research with regard to the medicinal uses of different plant extracts (Lee et al., 2019). A wide variety of mushrooms are commonly considered nutritious foods with unique properties including their medicinal value (Wasser, 2011; Ma et al., 2013). Secondary metabolites derived

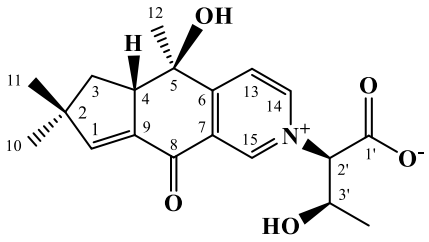

Figure 1. Structure of the isolated compound.

from the fungal strains of mushroom origin have antibacterial, antitumour, antiviral, anti-inflammatory, immunomodulatory, and antioxidant activities (Lindequist et al., 2005; Lee and Yun, 2006; Wasser, 2011; Yi et al., 2016). The fungal species *Trichaptum fuscoviolaceum,* commonly known as the violet tooth is of the order *Hymenochaetales* and is widely disseminated on coniferous trees in the temperate regions of the world (Seierstad et al., 2021). However, little is known about its functional activity. In this study, while exploring the effects of fungal metabolites on pig oocytes, we found that stereumamide A (StA) derived from the culture broth of *T. fuscoviolaceum* exhibited activity in regulating sperm penetration during IVF.

Materials and methods

Isolation and purification of StA from the culture broth of T. fuscoviolaceum

The fungal strain *T. fuscoviolaceum* was cultured on potato dextrose agar at 27°C for two weeks. Small pieces of fresh mycelium were inoculated into 20 one-litre flasks containing 400 ml of potato dextrose broth and cultured on a rotary shaker at 120 rpm and 27°C for four weeks. The culture broth was filtered to separate mycelium and the broth filtrate. The broth filtrate was subjected to Diaion® HP-20 column chromatography eluted with 50% aqueous methanol (MeOH), followed by silica gel column chromatography with stepwise chloroform ($CHCl_3$)-MeOH (30:1→0:100, v/v) to form fractions labelled A-C. Fraction B was chromatographed on a column of Sephadex® LH-20 eluted with MeOH. The fraction containing StA was separated using medium pressure liquid chromatography eluted with a gradient of an increasing amount of methanol in water. Finally, StA (142 mg) was obtained using a Sep-Pak C_{18} cartridge eluted with 10% aq. MeOH (Figure 1).

Structure determination of StA

The chemical structure of the isolated compound was determined by electrospray ionization (ESI)-mass spectrometry and nuclear magnetic resonance (NMR) measurements. The molecular weight was established as 345 by the ESI-mass spectrometry, which showed a quasi-molecular ion peak at m/z 245.9 $[M+H]^+$ in the positive mode. The ^1H NMR spectrum in dimethyl sulfoxide (DMSO)-d_6 exhibited signals due to three aromatic methines at δ 9.31, 9.12 (d, $J = 5.4$ Hz), and 8.27 (d, $J = 5.4$ Hz), one olefinic methine at δ 6.90, three methines at δ 5.00 (d, $J = 4.2$ Hz), 4.51 (m), and 3.69 (m), one methylene at ***δ*** 1.97 (dd, $J = 13.2$, 8.4 Hz)/1.88 (dd, $J = 13.2$, 7.8 Hz), and four methyls at ***δ*** 1.21, 1.19, 1.10, and 0.98 (d, $J = 6.6$ Hz) (Figure 2). In the ^{13}C NMR spectrum, 19 carbon peaks including two carbonyl carbons at ***δ*** 180.7 and 165.0, four sp^2 methine carbons at δ 153.4, 148.5, 144.7, and 122.6, three sp^2 quaternary carbons at *δ* 168.4, 136.3, and 128.8, two

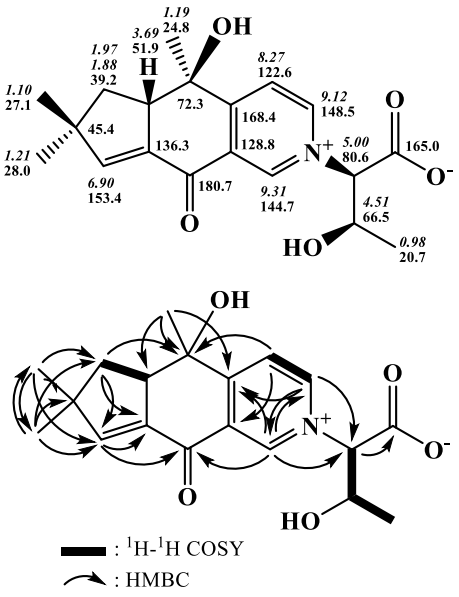

Figure 2. ^1H (italic) and ^{13}C peaks assignments and two-dimensional nuclear magnetic resonance (NMR) data.

oxygen- or nitrogen-conjugated methines at δ 80.6 and 66.5, one oxygenated quaternary carbon at δ 72.3, one methine at δ 51.9, one quaternary carbon at δ 45.4, one methylene at δ 39.2, and four methyls at δ 28.0, 27.1, 24.8, and 20.7 were evident (Figure 2A). All the proton-bearing carbons were assigned by the heteronuclear multiple quantum correlation spectrum, and the ^1H-^1H homonuclear correlation spectroscopy spectrum revealed three partial structures. Further structural elucidation was performed with the aid of the Heteronuclear Multiple Bond Correlation spectrum, which showed long-range correlations from H-1 to C-8 and C-9, from H-3 to C-1, C-5 and C-9, from H-10 and H-11 to C-1, C-2, and C-3, from H-12 to C-4, C-5, and C-6, from H-13 to C-5 and C-7, from H-14 to C-6 and C-15, and from H-15 to C-6, C-8, and C-14, establishing the presence of sterostrein Q moiety. Finally, the long-range correlations from H-14 and H-15 to C-2′ and H-2′ to C-1′ indicated that the threonine moiety was connected to sterostrein Q via a carbon-nitrogen bond (Figure 2B). Therefore, the structure of the isolated compound was identified as a zwitterionic alkaloid StA (Duan et al., 2018).

Assessment of boar sperm motility

Liquid boar semen was purchased from a local artificial insemination (AI) centre. Sperm motility was examined using a computer-assisted sperm analysis system (CASA; Sperm Class Analyzer®, Microptic, Barcelona, Spain). Spermatozoa were incubated for 2 h at 37.5°C with or without StA (controls; without addition of StA [w/o] or a solvent control with DMSO), and a 1 µl aliquot of sperm sample was then placed on a pre-warmed (38°C) Leja counting slide (Leja products B.V., Nieuw-Vennep, The Netherlands). Ten fields were analyzed at 37.5°C, assessing a minimum of 500 spermatozoa per sample. The proportion of total motile spermatozoa (%), progressive motile spermatozoa (%), and hyperactive spermatozoa (%) was determined. The kinetic parameters measured for each spermatozoon included: curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), the percentage of linearity (LIN, %), the percentage of straightness (STR, %), and the wobble percentage (WOB, %).

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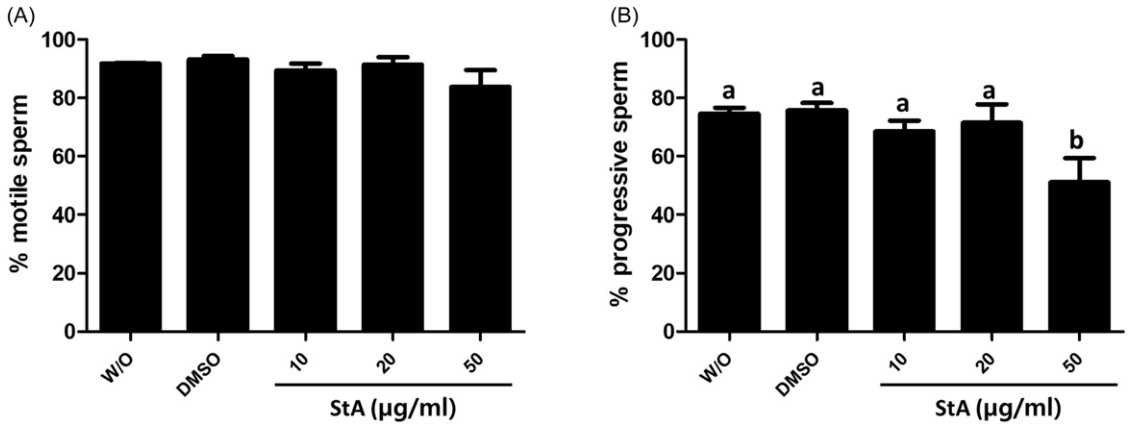

Figure 3. Incubation of boar spermatozoa in the absence (W/O) or presence of StA or DMSO (a solvent control). Motile (A) And progressive (B) Spermatozoa were assessed after 2 h of incubation. Values are expressed as the mean ± SEM. The different superscripts (a&b) in each group of columns denote a significant difference at $p < 0.05$. StA: stereumamide A; DMSO: dimethyl sulfoxide.

Collection and in vitro maturation (IVM) of pig oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from the antral follicles (3-6 mm in diameter), washed three times in N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Tyrode lactate (TL-HEPES) medium supplemented with 0.01% (w/v) polyvinyl alcohol (PVA), (TL-HEPES-PVA), followed by three washes with the oocyte maturation medium (Abeydeera et al., 1998). A total of 50 COCs were transferred to 500 μl of the maturation medium and layered with mineral oil in a 4-well multi-dish equilibrated at 38.5°C in a 5% CO_2 atmosphere. The oocyte maturation medium used was the tissue culture medium 199 supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml luteinizing hormone (Sigma-Aldrich, Seoul, Korea), 0.5 μg/ml follicle-stimulating hormone (Sigma), 10 ng/ml epidermal growth factor (Sigma), 75 μg/ml penicillin G, and 50 μg/ml streptomycin. The oocytes were cultured in TCM199 for 44 h at 38.5°C and 5% CO_2 in air.

In vitro fertilization (IVF) and culture (IVC) of pig oocytes

After IVM, cumulus cells were removed by treating with 0.1% hyaluronidase in the TL-HEPES-PVA medium (Abeydeera et al., 1998), and metaphase II oocytes were selected by observation under a stereomicroscope. Thereafter, in a 35-mm polystyrene culture dish, oocytes were placed into four 100 μl drops of modified Tris-buffered medium (mTBM) and covered with mineral oil. One millilitre of liquid semen preserved in Beltsville Thawing Solution was washed twice with phosphate-buffered saline (PBS) containing 0.1% PVA (PBS-PVA), at 800 x *g* for 5 min. The washed spermatozoa were resuspended in mTBM, appropriately diluted, and 1 μl of the sperm suspension was added to the medium containing the oocytes to give a final sperm concentration of 1×10^5 spermatozoa/ml. Oocytes were co-incubated with spermatozoa for 5 h at 38.5°C in an atmosphere containing 5% CO_2. To observe the effects of StA on IVF, the following experiments were performed: i) Oocytes were inseminated in the absence or presence of StA (0–50 μg/ml), ii) Spermatozoa were incubated with 20 μg/ml StA for 30 min, and were then used for IVF, and iii) Oocytes were incubated with 20 μg/ml StA for 1 h, and then were used for IVF. After IVF, oocytes were transferred to 500 μl porcine zygote medium (PZM-3; Yoshioka et al., 2002) supplemented with 0.4% bovine serum albumin (BSA, A0281, Sigma), and cultured for an additional 20, 48, or 144 h. The IVM, IVF, and IVC studies were repeated five times for each treatment regimen.

Evaluation of fertilization and embryonic development

Oocytes/embryos were fixed with 2% formaldehyde for 40 min at room temperature, washed twice with PBS, permeabilized with PBS-Triton X-100 for 30 min, and stained with 2.5 mg/ml 4′,6-diamidino-2-phenylindole (DAPI; DNA staining; Molecular Probes, Eugene, OR, USA) for 40 min. The fertilization status of the zygotes (unfertilized, fertilized-monospermic, or fertilized-polyspermic), cleaved embryo number, blastocyst formation, and the cell number per blastocyst were assessed under a fluorescence microscope (Nikon Eclipse Ci microscope; Nikon Instruments Inc., Tokyo, Japan).

Statistical analysis

Values are exhibit as the mean ± standard error of the mean (SEM). Data analyses were conducted using one-way analysis of variance with SAS package 9.4 (SAS Institute Inc., Cary, NC, USA). The completely randomized design was used, and Duncan's multiple range test was performed to compare values of individual treatments when the F-value was significant ($p < 0.05$).

Results

StA did not interfere with sperm movement during incubation

The boar spermatozoa were incubated in the presence of StA at concentrations of 10, 20 and 50 μg/ml for 2 h, or controls (W/O addition of StA or a solvent control with DMSO), and then sperm motility and progression were examined using a CASA system (Figure 3). As shown in Figure 3A, there was no significant difference in the percentage of motile spermatozoa between the treatment groups, and the controls. On the other hand, a significantly lower rate of progression was observed in spermatozoa incubated with 50 μg/ml StA, compared to those of spermatozoa incubated with 10 and 20 μg/ml StA or the controls ($p < 0.05$, Figure 3B).

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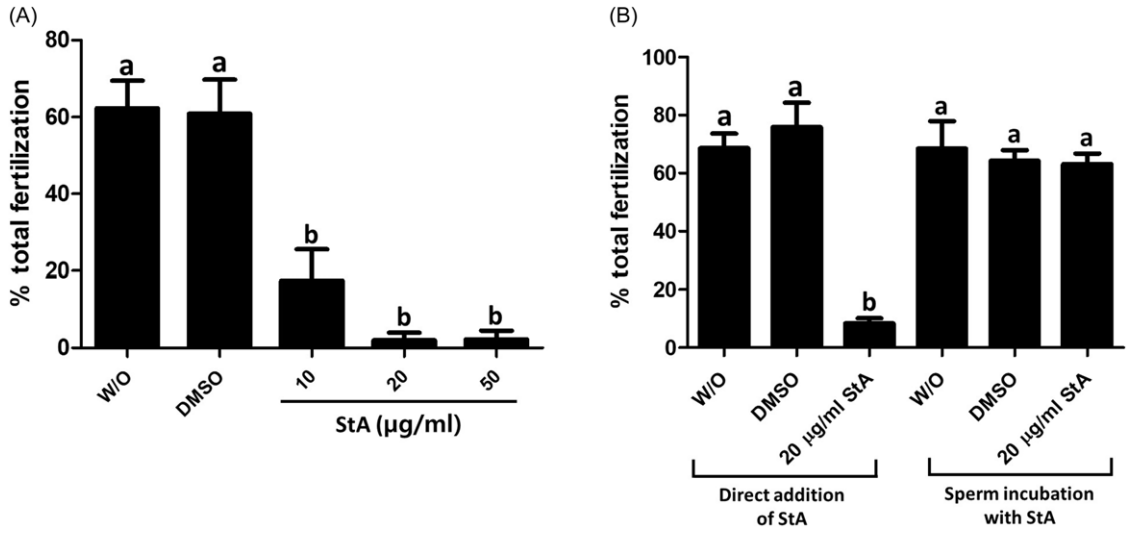

Figure 4. Oocytes were inseminated in the IVF medium with different concentrations of StA or controls (without addition of StA [w/o] or a solvent control with DMSO) (A). StA (20 μg/ml) was directly added into the IVF medium or boar spermatozoa were incubated with 20 μg/ml StA, and then IVF was subsequently performed (B). Values are expressed as the mean ± SEM. The different superscripts (a&b) in each group of columns denote a significant difference at $p < 0.05$. StA: stereumamide A; DMSO: dimethyl sulfoxide; IVF: *In vitro* fertilization.

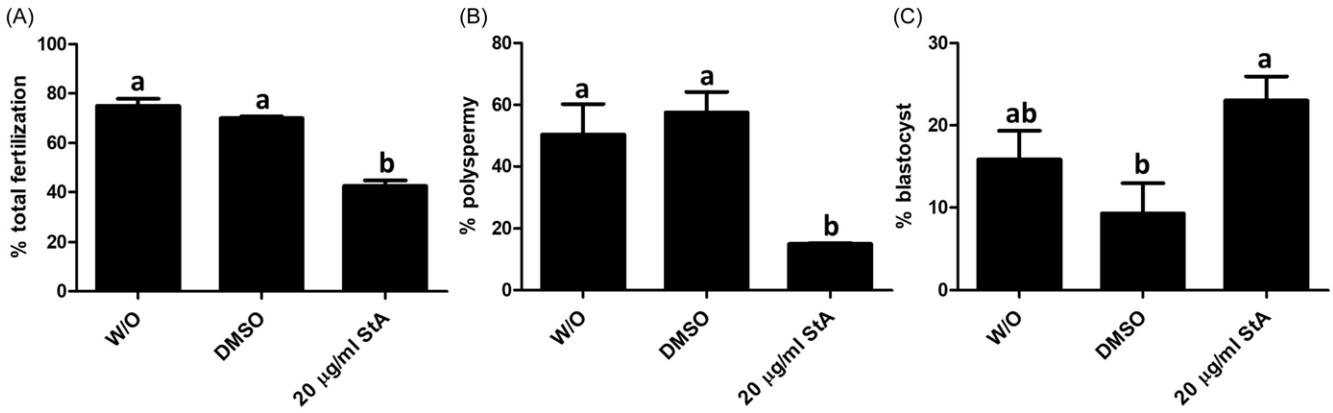

Figure 5. Oocytes were incubated with 20 μg/ml StA or DMSO (a solvent control), and then IVF was performed subsequently. The rate of total fertilization (monospermic+ polyspermic oocytes; (A), Polyspermy rate (B), And blastocyst formation (C) were examined after embryo culture for 16, and 144 h, respectively. Values are expressed as the mean ± SEM. The different superscripts (a&b) in each group of columns denote a significant difference at $p < 0.05$. StA: stereumamide A; DMSO: dimethyl sulfoxide; IVF: *In vitro* fertilization.

StA inhibited sperm penetration during IVF, affected oocytes but not spermatozoa

To observe the effects of StA on IVF, oocytes were inseminated in the IVF medium in the absence or presence of StA for 5 h. Significantly lower rates of fertilization were observed in oocytes inseminated in the presence of 10–50 μg/ml StA compared with the fertilization rate of the controls ($p < 0.05$, Figure 4A). In addition, spermatozoa were pre-incubated with 20 μg/ml StA for 30 min and were then used for IVF (Figure 4B). When compared with the groups wherein StA was present in the IVF medium during insemination, the direct addition of StA inhibited sperm penetration significantly ($p < 0.05$, Figure 4B), while there were no differences in the fertilization rate of spermatozoa pre-incubated with or without StA (Figure 4B). Therefore, it is believed that StA affects oocytes rather than sperm during IVF.

Incidence of polyspermy was reduced when IVF was performed with oocytes pre-incubated with StA

Based on the results, oocytes pre-incubated with 20 μg/ml StA for 1 h prior to the IVF process exhibited a significantly lower total fertilization rate, where controls showed above 60% of total fertilization ($p < 0.05$, Figure 5A). However, the incidence of polyspermy was significantly decreased in the oocytes pre-incubated with StA, and a higher percentage of monospermic oocytes was observed, compared with the controls ($p < 0.05$, Figure 5B). Further, in the fertilized oocytes cultured for 144 h, a higher rate of blastocyst formation was observed in oocytes pre-incubated with StA ($p < 0.05$, Figure 5C). Thus, StA at various concentrations and periods of incubation could reduce the frequency of polyspermy in IVF using pig oocytes.

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Discussion

IVF is a type of ART that involves retrieving oocytes after ovarian stimulation and combining them with sperm to accomplish fertilization in a well-established laboratory, and implanting one or more fertilized embryos in the uterine cavity. Among the factors that result in the success of IVF, the use of spermatozoa with good motility is one of the most important (Eskandar, 2002; Xie et al, 2010). Similarly, the survival of the oocyte also directly affects the success of the IVF process (Catalá et al., 2012; Ingilizova et al., 2014; Lonergan and Fair, 2016). Factors that reduce the success of IVF include impairments in the cumulus cell penetration, sperm/oocyte binding, sperm /oocyte fusion oocyte activation, sperm processing, and pronuclear formation (Swain and Pool, 2008). Even though sperm penetration is a positive factor, polyspermy is a persistent obstacle to the success of IVF. Polyspermy could depend on the quality of oocyte and sperm, and their sperm number at the fertilization site (Hunter, 1991). In general, polyspermy is higher in IVF than in the IVM system and it is necessary to avoid polyspermy for successful fertilization and embryo development (Coy and Avilés, 2010). Polyspermy leads to abnormal embryo development, early embryonic death, or spontaneous abortions (Jacobs et al., 1978). Within the body, several mechanisms prevent polyspermy such as oviduct-based mechanisms which prevent the entrance of a large number of spermatozoa into the proximity of the oocytes, the egg-based mechanisms that alter the membrane, and the zona pellucida (ZP) reaction to the fertilizing sperm (Coy and Avilés, 2010). However, in *in vitro* systems, it is somewhat difficult to prevent polyspermy (Funahashi, 2003; Li et al., 2003). Alcântara-Neto et al. (2020) stated that supplementation of extracellular vesicles (EVs) can decrease polyspermy and improve embryo production because EVs are the key components in the oviductal fluid. Chemical compounds that have the ability to inhibit sperm hyaluronidase activity and increase ZP hardening can reduce polyspermy (Tatemoto et al., 2006; Coy et al., 2008). Similar findings in the literature have shown that the resistance of ZP to proteolysis reduces the incidence of polyspermy (Coy et al., 2008; Mondéjar et al., 2013a; Mondéjar et al., 2013b). Also, the pre-incubation of the oocytes or sperm with oviductal epithelial cells prior to IVF can reduce penetration by multiple sperm (Wang et al., 2003). Several studies have shown that polyspermy is very commonly seen in pigs rather than in other mammals (Li et al., 2003; Hao et al., 2006; Kitaji et al., 2015). In the current study, we have observed that the polyspermy was significantly reduced in the StA incubated group (Figure 5B). This result indicates that StA has the ability to control polyspermy, which is commonly seen during IVF. Moreover, we observed that varying concentrations of StA did not affect sperm motility or the overall survival of the sperm and the oocytes and the percentage of the blastocysts was higher in the StA-treated group (Figure 3–5). Many studies have shown that a higher number of quality blastocysts help in carrying out the IVF process successfully (Gardner et al., 2000; Xiong et al., 2020). Currently, researchers are focusing on the use of different compounds extracted from plant materials to improve ART. Medicinal mushrooms have beneficial compounds like polysaccharides, triterpenoids, steroids, phenolic components, cordycepin, etc. that have anticancer, anti-inflammatory, antioxidant, antihypertensive, and immunomodulatory effects (Wasser, 2011; Johnson et al., 2019). Lin et al. (2019) stated that the nutritive and essential trace elements, and/or concentrated compounds of medicinal plant extracts have the ability to improve fertility, but the underlying scientific mechanism is unclear. We can conclude from our results that the extracts of *T. fuscoviolaceum* have the ability to improve some aspects of the IVF process and we need more studies to identify the compounds responsible and their scientific mechanism during IVF. Also, these results indirectly indicate the possible benefits of the compounds derived from this medicinal mushroom in ART programmes.

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Competing interests. Authors have no conflict of interest with this study.

Ethical standards. The ovaries were collected at a commercial slaughterhouse, and liquid boar semen was purchase from local AI centre, therefore it was not necessary to obtain approval of the Ethics Committee.

References

Abeydeera L.R., Wang W.H., Prather R.S. and Day B.N. (1998). Maturation in vitro of pig oocytes in protein-free culture media: fertilization and subsequent embryo development in vitro. *Biology of Reproduction* **58**(5), 1316–1320.

Alcântara-Neto A.S., Fernandez-Rufete M., Corbin E., Tsikis G., Uzbekov R., Garanina A.S., Coy P., Almiñana C., and Mermillod P. (2020). Oviduct fluid extracellular vesicles regulate polyspermy during porcine in vitro fertilisation. *Reproduction, Fertility and Development*, **32**(4), 409–418.

Almiñana C., Gil M.A., Cuell C., Roca J., Vazquez J.M., Rodriguez-Martinez H. and Martinez E.A. (2005). Adjustments in IVF system for individual boars: value of additives and time of sperm-oocyte co-incubation. *Theriogenology*, **64**(8), 1783–1796.

Catalá M.G., Izquierdo D., Rodríguez-Prado M., Hammami S. and Paramio M.T. (2012). Effect of oocyte quality on blastocyst development after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in a sheep model.*Fertility and Sterility*, **97**(4), 1004–1008.

Coy P. and Avilés M. (2010). What controls polyspermy in mammals, the oviduct or the oocyte? *Biological Reviews Cambridge Philosophical Society*, **85**(3), 593–605.

Coy P., Grullon L., Canovas S., Romar R., Matas C. and Aviles M. (2008). Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction*, **135**(1), 19–27.

Duan Y.C., Feng J., Bai N., Li G.H., Zhang K.Q. and Zhao P.J. (2018). Four novel antibacterial sesquiterpene-α-amino acid quaternary ammonium hybrids from the mycelium of mushroom Stereum hirsutum. *Fitoterapia*, **128**, 213–217.

Eskandar M. (2002). Is 24-h sperm motility a useful IVF measure when male infertility is not apparent?*Acta Obstetricia et Gynecologica Scandinavica*, **81**(4), 328–330.

Funahashi H. (2003). Polyspermic penetration in porcine IVM–IVF systems.*Reproduction, Fertility and Development*, **15**(3), 167–177.

Gardner D.K., Lane M., Stevens J., Schlenker T. and Schoolcraft W.B. (2000). Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer.*Fertility and Sterility*, **73**(6), 1155–1158.

Gil M.A., Almiñana C., Cuello C., Parrilla I., Roca J., Vazquez J.M. and Martinez E.A. (2007). Brief coincubation of gametes in porcine in vitro fertilization: role of sperm: oocyte ratio and post-coincubation medium. *Theriogenology*, **67**(3), 620–626.

Hao Y., Mathialagan N., Walters E., Mao J., Lai L., Becker D., Li W., Critser J. and Prather R.S. (2006). Osteopontin reduces polyspermy during in vitro fertilization of porcine oocytes. *Biology of Reproduction* **75**(5), 726–733.

Hunter R.H. (1991). Oviduct function in pigs, with particular reference to the pathological condition of polyspermy *Molecular Reproduction and Development* **29**(4), 385–391.

Ingilizova G., Ivanov D., Kovachev E., Evrev M., Kostov I. and Necheva V. (2014). Oocyte quality as a predictive marker for assessment of IVF/ICSI procedure outcome. *Akusherstvo i Ginekologiia*, **53**(6), 41–46.

https://doi.org/10.1017/S0967199424000327 Published online by Cambridge University Press

Jacobs P.A., Angell R.R., Buchanan I.M., Hassold T.J., Matsuyama A.M. and Manuel B. (1978). The origin of human triploids. *Annals of Human Genetics*, **42**(1), 49–57.

Johnson A., Cheng S.C., Tsou D. and Kong Z.L. (2019). Attenuation of reproductive dysfunction in diabetic male rats with timber cultured Antrodia cinnamomea ethanol extract. *Biomedicine & Pharmacotherapy* **112**, 108684.

Kitaji H., Ookutsu S., Sato M. and Miyoshi K. (2015). A new rolling culture-based in vitro fertilization system capable of reducing polyspermy in porcine oocytes. *Animal Science Journal*, **86**(5), 494–498.

Klemetti R., Gissler M. and Hemminki E. (2004). Equity in the use of IVF in Finland in the late 1990s.*Scandinavian Journal of Public Health*, **32**(3), 203–209.

Lee F.K., Lee W.L. and Wang P.H. (2019). Medicinal plants and reproduction. *Journal of the Chinese Medical Association*, **82**(7), 529–530.

Lee I.K. and Yun B.S. (2006). Hispidin analogs from the mushroom Inonotus xeranticus and their free radical scavenging activity. *Bioorganic and Medicinal Chemistry Letters* **16**(9), 2376–2379.

Li Y.H., Ma W., Li M., Hou Y., Jiao L.H. and Wang W.H. (2003). Reduced polyspermic penetration in porcine oocytes inseminated in a new in vitro fertilization (IVF) system: straw IVF. *Biology of Reproduction* **69**(5), 1580–1585.

Lin C.Y., Chen Y.J., Lee S.H., Kuo C.P., Lee M.S. and Lee M.C. (2019). Uses of dietary supplements and herbal medicines during pregnancy in women undergoing assisted reproductive technologies - a study of taiwan birth cohort. *Taiwan Journal of Obstetrics and Gynecology*, **58**(1), 77–81.

Lindequist U., Niedermeyer T.H. and Jülich W.D. (2005). The pharmacological potential of mushrooms. *Evidence-based Complementary and Alternative Medicine : eCAM* **2**(3), 285–299.

Lonergan P. and Fair T. (2016). Maturation of oocytes in vitro. *Annual Review of Animal Biosciences* **4**, 255–268.

Ma Z., Zhang W., Wang L., Zhu M., Wang H., Feng W.H. and Ng T.B. (2013). A novel compound from the mushroom Cryptoporus volvatus inhibits porcine reproductive and respiratory syndrome virus (PRRSV) in vitro. *PLoS One* **8**(11), e79333.

Malina A., Błaszkiewicz A. and Owczarz U. (2016). Psychosocial aspects of infertility and its treatment. *Ginekologia Polska*, **87**(7), 527–531.

Malina A. and Pooley J.A. (2017). Psychological consequences of IVF fertilization–Review of research. *Annals of Agricultural and Environmental Medicine*, **24**(4), 554–558.

Marchal R., Feugang J.M., Perreau C., Venturi E., Terqui M. and Mermillod P. (2001). Meiotic and developmental competence of prepubertal and adult swine oocytes. *Theriogenology*, **56**(1), 17–29.

Markert C.L. (1979). Gametogenesis, fertilization and early development. *Mead Johnson Symposium on Perinatal Developmental Medicine*, **16**, 3–6.

Mondéjar I, Avilés M and Coy P (2013a). The human is an exception to the evolutionarily-conserved phenomenon of pre-fertilization zona pellucida resistance to proteolysis induced by oviductal fluid. *Human Reproduction* **28**(3), 718–728.

Mondéjar I., Martínez-Martínez I., Avilés M. and Coy P. (2013b). Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. *Biology of Reproduction* **89**(3), 67.

Nguyen H.T., Dang-Nguyen T.Q., Somfai T., Men N.T., Linh N.V., Nguyen B.X., Noguchi J., Kaneko H. and Kikuchi K. (2020). Selection based on morphological features of porcine embryos produced by in vitro fertilization: Timing of early cleavages and the effect of polyspermy. *Animal Science Journal*, **91**, e13401.

Seierstad K.S., Fossdal R., Miettinen O., Calsen T., Skrede I. and Kauserud H. (2021). Contrasting genetic structuring in the closely related basidiomycetes *Trichaptum abietinum* and *Trichaptum fuscoviolaceum* (Hymenochaetales). *Fungal Biology*, **125**(4), 269–275.

Sirard M.A., Dubuc A., Bolamba D., Zheng Y. and Coenen K. (1993). Follicle-oocyte-sperm interactions in vivo and in vitro in pigs. *Journal of Reproduction Fertility Supplment*, **48**, 3–16.

Swain JE and Pool TB (2008) ART failure: oocyte contributions to unsuccessful fertilization. *Human Reproduction Update* **14**(5), 431–446.

Tatemoto H., Tokeshi I., Nakamura S., Muto N. and Nakada T. (2006). Inhibition of boar sperm hyaluronidase activity by tannic acid reduces polyspermy during in vitro fertilization of porcine oocytes. *Zygote*, **14**(4), 275–285.

Wang W.H., Abeydeera L.R., Okuda K. and Niwa K. (1994). Penetration of porcine oocytes during maturation in vitro by cryopreserved, ejaculated spermatozoa. *Biology of Reproduction* **50**(3), 510–515.

Wang W.H., Day B.N. and Wu G.M. (2003). How does polyspermy happen in mammalian oocytes? *Microscopy Research and Technique*, **61**(4), 335–341.

Wasser S.P. (2011) Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. *Applied Microbiology and Biotechnology* **89**(5), 1323–1332.

Xia P., Wang Z., Yang Z., Tan J. and Qin P. (2001). Ultrastructural study of polyspermy during early embryo development in pigs, observed by scanning electron microscope and transmission electron microscope. *Cell and Tissue Research*, **303**(2), 271–275.

Xie L., Ma R., Han C., Su K., Zhang Q., Qiu T., Wang L., Huang G., Qiao J., Wang J. and Cheng J. (2010). Integration of sperm motility and chemotaxis screening with a microchannel-based device. *Clinical Chemistry*, **56**(8), 1270–1278.

Xiong F., Sun Q., Li G., Yao Z., Chen P., Wan C., Zhong H. and Zeng Y. (2020). Association between the number of top-quality blastocysts and live births after single blastocyst transfer in the first fresh or vitrified–warmed IVF/ICSI cycle. *Reproductive Biomedicine Online*, **40**(4), 530–537.

Yi Y.J., Lee I.K., Lee S.M. and Yun B.S. (2016). An antioxidant davallialactone from Phellinus baumii enhances sperm penetration on in vitro fertilization of pigs. *Mycobiology*, **44**(1), 54–57.

Yoshioka K., Suzuki C., Tanaka A., Anas I.M. and Iwamura S. (2002). Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biology of Reproduction* **66**(1), 112–119.

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