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KEYNOTE

Herbal Plants improved Boar Reproduction in Thailand

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Abstract. The current experiment was aimed to investigate whether *Extracted Gynostemma Pentaphyllum Makiko* (EGPM) could protect boar sperm from damages due to cryopreservation process. Semen was collected from a mature boar which was fed commercial feed twice daily and had a free access to water. Boar semen was frozen with either 0.25 mg/ml EGPM or 10 mM Cysteine or without any supplementation (control). After 7 days frozen semen was thawed and sperm characteristics were evaluated. It was found that EGPM and Cysteine had similar beneficial effects on semen quality. Supplementation of EGPM or Cysteine in freezing media increased progressive motility, viability and plasma membrane integrity of boar spermatozoa. It can be concluded from this study that EGPM had a great property in preventing spermatozoa from damages due to the cryo-process.

1. Introduction

Thailand is one of the countries where plant biodiversity is so great and herbal plants are abundant. Herbal plants are normally used in humans to cure illness or promote healthiness, but are not frequently used in animals. Livestock, for example, beef cattle, dairy cows, pigs, buffaloes, sheep, goats and chicken is a type of animal providing protein sources for humans. As world population is increased, the numbers of animals required are also increased to make sure that protein sources are sufficient for human consumption. The number of animals produced are primarily depending on breeding techniques. At present, such breeding technique as Artificial Insemination (AI) with frozen-thawed semen are widely adopted among farmers. Making frozen semen, sperm are processed in a very cold environment starting from room temperature to -196^0C in liquid nitrogen. During this cold process some free radicals or oxidants are produced and these will cause oxidative stress, lipid peroxidation (LPO) and damages to sperm cells. To protect sperm from LPO some antioxidants like Superoxide dismutase (SOD), Glutathione, Catalase, Vitamin C or vitamin E are added to freezing media before frozen semen is made. A number of herbal plants in Thailand are rich in antioxidants and can be used in animals. In my research work, *Gynostemma Pentaphyllum Makiko* (GPM), one of the commonly used in humans in Thailand) was used in the semen cryopreservation process. Extracted *Gynostemma Pentaphyllum Makiko* (EGPM) was added to freezing media to investigate whether it can improve frozen-thawed boar semem quality.

2. Materials and Methods

2.2.Animal, herbal extract and chemicals

A 2-yr old mature boar was used in this experiment. It was kept in a 3*2 m2 concrete-floor pen, given commercial pelleted feed twice daily and had a free access to water. The solid/liquid extraction with Soxhlet extractor was employed to obtain the extract of *Gynostemma Pentaphyllum Makiko* (GPM). All chemicals used were from Sigma Chemical Co. (St. Louis, MO, USA). Three media to be used for semen cryopreservation was prepared, namely M1 (BTS containing Glucose, EDTA, $NaHCO_3$ and $Na_3C_6H_5O_7$), M2 (LEY containing Lactose and Egg Yolk), and M3 (LEY+Glycerol+Equex STM).

2.2. Semen cryopreservation

To cryopreserve spermatozoa, boar semen was diluted 1:1 with M1, semen solution was then kept in a cool compartment to allow the temperature to drop down to 15^0C within 2 hours. Subsequently, semen solution was centrifuged for 5 min at 1500g and 15^0C, after which the resulted pellet was mixed with M2 to get the sperm number of $1.5x10^9$/ml. Three test tubes were filled with 10 ml of the solution, and added with either Cysteine or EGPM or none (control). Semen solution in the tubes was then allowed to further drop down from 15^0C to 5^0C within 2 hours. At 5^0C, each tube was added with M3 to get the sperm number of $1.0x10^9$/ml. Finally, 0.5 ml of semen solution was filled in each plastic straw. The straws were put in an automatic freezing machine (Cryobath, CL-3300, Cryologic, Australia). The machine was programmed to decrease the temperature of the solution from 5^0C to -150^0C in 3 steps, namely from 5^0C to -5^0C at 6^0C/min, from -5^0C to -80^0C at 40^0C/min and from -80^0C to -150^0C at 70^0C/min. The straws with frozen semen were then plunged into liquid nitrogen, to be kept at -196^0C.

2.3.Sperm evaluation

After 7 days in liquid nitrogen, straws were taken out of liquid nitrogen, frozen semen was thawed and analyzed for sperm characteristics. Total sperm count and the number of immotile sperm were used for the calculation for progressive motility rates. Sperm were dyed with Eosin-Nigrosin for the evaluation of sperm viability. Sperm with red colour were assumed dead, while those without colour were live. HOST (Hypoosmotic swelling test) was performed to detect plasma membrane integrity of post-thawed sperm.

3. Results

3.1. Sperm progressive motility

After counting the number of spermatozoa using haemacytometer and the calculation was made, it was found that progressive motility rates of fresh spermatozoa were similar in all groups, ranging from 80.4% to 81.3 %. Progressive motility rates of frozen-thawed spermatozoa decreased, when compared to the fresh ones (Table 1). The decline of motility rates were greater in the control group where antioxidant and herbal extract were not added to the freezing media. The percentages of progressive motility rates of frozen-thawed spermatozoa were significantly higher ($p<0.05$) in Cysteine and EGPM supplemented groups (47.3 ± 0.8, 45.2 ± 1.0, respectively), compared to the non-supplemented group (39.6 ± 0.7). However, the motility rates of spermatozoa were not significantly different between Cysteine and EGPM groups.

Table 1. Effect of EGPM and Cysteine in freezing media on Progressive motility of post-thawed spermatozoa (Mean± SD)

	Treatment		
	Control	EGPM	Cysteine
Fresh Semen	80.4 ± 0.4^a	81.3 ± 1.2^a	80.7 ± 0.7^a
Frozen-Thawed Semen	39.6 ± 0.7^a	45.2 ± 1.0^b	47.3 ± 0.8^b

[a, b] Within a row, values with different superscripts differ significantly ($p<0.05$)

3.2.Sperm viability

Cysteine and herbal extract (EGPM) had great effects on viability of frozen-thawed spermatozoa (Table 2). Viability rates of frozen-thawed spermatozoa decreased in all groups when compared to those of fresh semen. The decline rates were more pronounced in the control compared to EGPM and

Cysteine groups. Percentages of live spermatozoa were greater (P<0.05) in Cysteine and EGPM groups when compared to the controls, the figures being 57.3 ± 0.8, 56.2 ± 0.9 and 47.7 ± 0.4, respectively. When compared between the treated groups viability rates of spermatozoa in the EGPM and Cysteine groups were not significantly different.

Table 2. Effect of EGPM and Cysteine in freezing media on viability of post-thawed spermatozoa (Mean± SD).

	Treatment		
	Control	EGPM	Cysteine
Fresh Semen	90.2 ± 0.3[a]	90.5 ± 0.8[a]	88.8 ± 0.6[a]
Frozen-Thawed Semen	47.7 ± 0.4[a]	56.2 ± 0.9[b]	57.3 ± 0.8[b]

[a, b] Within a row, values with different superscripts differ significantly (p<0.05)

3.3.Sperm plasma membrane integrity

Like progressive motility rates and viability rates, plasma membrane integrity rates were greatly affected by Cysteine or EGPM supplementation in freezing media (Table 3). Plasma membrane integrity rates of post thawed spermatozoa declined in all groups, compared to those of fresh semen. It was found that the declining rates were greater in the control group as compared to the Cysteine and EGPM groups. When compared between the Cysteine and the EGPM groups, their declining rates were not different. The percentages of plasma membrane integrity rates were higher (P<0.05) in Cysteine (45.3 ± 0.8) and EGPM (44.5 ± 0.9) groups, compared to those of the controls (40.9 ± 0.7)

Table 3. Effect of EGPM and Cysteine in freezing media on plasma membrane integrity rates of post-thawed spermatozoa (Mean± SD).

	Treatment		
	Control	EGPM	Cysteine
Fresh Semen	80.2 ± 1.0[a]	81.3 ± 0.6[a]	79.7 ± 0.9[a]
Frozen-Thawed Semen	40.9 ± 0.7[a]	44.5 ± 0.9 [b]	45.3 ± 0.8[b]

[a, b] Within a row, values with different superscripts differ significantly (p<0.05)

4. Discussion

Nowadays, Artificial insemination (AI) with frozen-thawed semen is commonly used in pig breeding. This is because of its advantages over fresh semen. It is generally accepted that the process of cryopreservation can cause adversed effects on semen quality [1]. Using this poor quality semen would result in low conception rates and pregnancy rates [2,3]. In the current study, all sperm characteristics measured, namely motility, viability and plasma membrane integrity of frozen-thawed boar semen dropped dramatically when compared to the fresh one. Progressive motility rates, viability

rates and plasma membrane integrity rates dropped from 80.4 ± 0.4 to 39.6 ± 0.7, from 90.2 ± 0.3 to 47.7 ± 0.4 and from 80.2 ± 1.0 to 40.9 ± 0.7, respectively. However, when Cysteine (an antioxidant) and EGPM (herbal extract) were added to the freezing media all sperm characteristics measured were improved.

The decrease of semen quality was believed to be due to the oxidation reaction (lipid peroxidation, LPO) occurred in the sperm plasma membrane, which is known to be rich in unsaturated fatty acids (UFAs). UFAs are known to be highly susceptible to be attacked by free radicals, especially reactive oxygen species (ROS), e.g. superoxide anion (O_2^-), hydroxyl (OH) and hydrogen peroxide (H_2O_2). During semen cryopreservation process, there are some practices that can induce the production of free radicals. These include being centrifuged and exposed to ultra low temperature (-196^{0}C). The increased amounts of ROS will destroy the balanced state between free radicals and antioxidants and causes LPO in plasma membranes of spermatozoa. This will result in the destruction or degradation of plasma membranes, which in turn cause sperm death or abnormality. LPO also causes the decrease in mitochondria activities, resulting in decreased amounts of energy being produced, which subsequently adversely affects sperm motility [4].

To prevent LPO in sperm plasma membranes, antioxidants are commonly supplemented into the freezing media. Antioxidants are substances that can prevent or slow down an oxidation process caused by free radicals [5]. Some examples of antioxidants are Cysteine, Superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx), vitamins A, B, C and E and selenium. Mode of actions of an antioxidants in preventing LPO can be that it scavenges ROS or inhibit ROS production or break or stop chain reaction of LPO [6]. Supplementing Cysteine in freezing media could prevent LPO in sperm plasma membrane [7,8,9]. Extracted *Gynostemma Pentaphyllum Makiko* (EGPM) contains a number of substances possessing antioxidant property, e.g. Gypenoside (triterpine Saponins), Flavonoids, Polysaccharides, amino acids, vitamins B and C, β-Carotene, Calcium, Zinc, Manganese and Magnesium. Gypenosides, vitamin C and β-Carotene are believed to be the main chemicals that counteract free radicals and prevents LPO.

The results of this study were in agreement with Hu et al. [4] who reported that adding EGPM to freezing media at the levels of 0.25 mg/ml and 0.5 mg/ml improved motility rates and plasma membrane integrity rates of frozen-thawed spermatozoa, while at the higher levels (1.0-2.0 mg/ml) the beneficial effects were not found. Sperm viability rates have also been reported to be improved by adding EGPM to freezing media [10]. Vitamin B and C and β-Carotene in EGPM were believed to be the main factors that prevented LPO in sperm plasma membrane. Vitamin C in the form of oxidized ascorbic acid when reacts with α-tocopheroxyl will be transformed to be α-tocopherol which can counteracts with free radicals and prevents LPO and membrane damages. Indeed, Pena et al. [11] reported the increased viability rates of spermatozoa after vitamin C being added to freezing media. Breininger et al. [12] reported that α-tocopherol could improve biochemical and dynamic parameters in cryopreserved boar semen.

Besides EGPM, some other herbal plants have been reported to have positive effects. Zhao et al. [13] reported that adding RSAE (Rhodiola sacra aqueous extract) in freezing media could reduce Malondialdehyde (MDA) production and prevent LPO in plasma membrane of boar spermatozoa. Furthermore, supplementing 10mM Rosemary extract in freezing media was found to have increased progressive motility, viability and plasma membrane integrity of boar spermatozoa [8]. The contents of Rosemary extract are polyphenols and flavonoids [4,8], which are the same types with those found in EGPM.

5. Conclusion

It can be concluded from this study that Solid/Liquid Extraction is a method that can be used to extract substances from *Gynostemma Pentaphyllum Makiko*. The extract of *Gynostemma Pentaphyllum Makiko* (EGPM) had beneficial effects in preventing spermatozoa from damages due to

Lipid peroxidation (LPO) in sperm plasma membrane. It, hence improved motility, viability and plasma membrane integrity of frozen-thawed boar spermatozoa.

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