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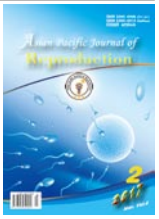

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Impact of silymarin enriched semen extender on bull sperm preservability

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ABSTRACT

Objective: To explore the effect of silymarin on bull spermatozoa during cooling and cryopreservation. **Methods:** Pooled bull semen were diluted by Tris-Citrate-Fructose egg yolk diluents, purified silymarin powder (obtained from the milk thistle silybum marianum), purchased from Unipharma, Al Obour city, Egypt, was soaked in Tris-citric acid-fructose diluent for 48 h at 10 ℃ making a stock solution (70 mg/mL), from this stock solution we obtained concentrations of 0.18 mg/mL, 0.36 mg/mL, 0.54 mg/mL, 0.72 mg/mL, 0.90 mg/mL in addition to the control (0.00 mg/mL) reaching a final volume of 5 mL in each tube. Egg yolk was added to each tube to obtain silymarin enriched semen extender (SEE) with 20% egg yolk, cooled slowly up to 5 ℃ and equilibrated for 4 h. Semen was packed into 0.25 mL polyvinyl French straws (IMV, France). After equilibration periods, the straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen for 10 min and were then dipped in liquid nitrogen. Extended semen was subjected to evaluation (motility, alive%, abnormality%, intact sperm membrane (HOST)% and conception rate) in both chilled and frozen semen. **Results:** Table 1 revealed that Sperm motility of the concentrations 2, 3 and 4 after 8 d of chilling were significantly (P<0.02) higher than control. Sperm motility of the concentration 2 (45.00%±2.89%) after 9 d of chilling was higher than control and the other concentrations. Addition of SEE in concentration 1 and 2 gave post thawing sperm motility as high as the control (47.50±2.81 and 45.00±2.58, respectively) while other concentration have lower effects on motility as compared to the control. Addition of silymarin improved post thawing alive% and was significantly higher (P<0.000 1) than the control. SEE decreased significantly (P<0.000 1) the % of post thawing abnormal sperm in concentration 3 and 4 (11.83±0.65 and 16.00±0.58, respectively). SEE improved significantly (P<0.018) the % of post thawing intact spermatozoa membranes (HOST%) in concentrations 2, 4 and 5 (71.17±0.83, 71.83±0.91 and 75.00±3.42, respectively) (Table 2). **Conclusion:** It could be concluded that silymarin as a natural additive to semen extenders improved preservability in both chilled and frozen bull semen.

1. Introduction

Spermatozoa are the end point of male spermatogenesis and have particular anatomic and metabolic features. Nowadays, sperm cryopreservation and storage are of a great demand for conserving the supergenetic origins of the males, the development of artificial reproductive technologies such as artificial insemination (AI) and *in vitro* fertilization (IVF)[1] are of a great interest. AI with frozen semen is essential in breeding and selection schedules contributing to increase production of domestic species. Nowadays, semen cryopreservation has many biotechnological applications. It can be used to solve infertility problems, life threatening diseases, preservation of semen and DNA from endangered species and conservation of biodiversity. The interaction of several factors

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(cooling rate, storage temperature, chemical ingredients of the extender, cryoprotectant concentration, over accumulation of oxygen free radicals, seminal plasma composition and hygienic control) are the key that affect the life-span of spermatozoa[2]. Cryopreservation of bovine semen often induce an additional source for reactive oxygen species (ROS) attack on sperm due to decreased activities of antioxidant enzymes and the sperm membrane become more susceptible to lipid peroxidation[3] which affect the membrane permeability[4]. Natural antioxidants exert a protective effect preserving the metabolic activity and cellular viability of cryopreserved bovine spermatozoa[5]. Nowadays, the use of herbal natural product has gained interest worldwide. Many of the herbs have been developed into herbal supplement which are claimed to assist in healthy life style. Among these herbs Silymarin is an extract from the seeds and fruits of the milk thistle silybum marianum that contains the flavonolignans silybin which is the major active component. Silymarin is a strong antioxidant used as a remedy for liver protection against oxidative stress and also as a protectant for the testicular tissue and improving semen quality through elevation of blood testosterone level[6,7]. Also, it prevents acetaminophen induced liver injury through restoration of glutathione level[6]. No available literatures were found for interpreting the benefit for using Silymarin.

2. Materials and Methods

2.1. Preparation of silymarin enriched semen extender (SEE)

Tris-citric acid-fructose diluent (TCF) was prepared according to Foote *et al.*[8] purified silymarin powder (obtained from the milk thistle silybum marianum), purchased from Unipharma, Al Obour city, Egypt, was soaked in Tris-citric acid-fructose diluent (TCF), (silymarin: TCF) for 48 h at 10 ℃ making a stock solution (70 mg/mL), from this stock solution we obtained concentrations of 0.18 mg/mL, 0.36 mg/mL, 0.54 mg/mL, 0.72 mg/mL, 0.90 mg/mL in addition to the control (0.0 mg/mL) reaching a final volume of 5 mL in each tube. Egg yolk was added to each tube to obtain SEE with 20% egg yolk (SEEY).

2.2. Semen collection and initial evaluation

Three mature cattle bulls maintained at Semen Freezing Center, General Organization for Veterinary Services Ministry of Agriculture, Abbasia, Egypt, were used as semen donors. Ejaculates were collected using a bovine adapted artificial vagina at weekly intervals for 18 wk. Semen samples were initially evaluated for subjective sperm motility and sperm concentration. Ejaculates fulfilling minimum standard of sperm motility (70%) and sperm morphology were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time for 10 min at 37 ℃ in the water bath before dilution.

2.3. Semen processing

Semen samples were diluted with TCF extender and used as control and other aliquots of pooled semen samples were diluted with TCF extenders containing the different concentrations of silymarin in order to provide concentration of 120 million sperm/mL. Extended semen was slowly cooled (approximately for 2 h) to 5 ℃ and equilibrated for 2 h. Semen was packed into 0.25 mL polyvinyl French straws. After equilibrium periods, the straws were horizontally placed on a rack and frozen in vapor 4 cm above liquid nitrogen for 10 min and were then dipped in liquid nitrogen. A fraction of extended semen from control and each concentration of the additive were kept at 5 ℃ for 7-10 d (chilling) and sperm motility was evaluated daily.

2.4. Assessment of semen quality parameters

The assessment was undertaken on after freeze thawing of bull spermatozoa. Also, sperm motility was evaluated for raw semen, 2 h after cooling and chilled semen daily up to 7-10 d. Frozen straws were thawed at 37 ℃/ 1 min. The parameters studied were subjective semen characteristics (motility, alive, abnormality and hypoosmotic swelling test (HOST) %)[9].

2.5. Statistical analysis

Statistical analysis data were analyzed using the SPSS (2005)[10] computerized program v. 14.0 to calculate the analysis of variance (ANOVA) for the different parameters between control and additives replications. Significant difference between means was calculated using Duncan test at $P<0.05$.

3. Results

3.1. Effect of SEE on cattle bull sperm motility during chilling

Sperm motility of the concentrations 2, 3 and 4 after 8 d of chilling

Table 1

Effect of silymarin enriched extender on cattle bull sperm motility during chilling.

Treatment index	2 h	Days									
		1	2	3	4	5	6	7	8	9	10
Control	90.00±0.00^{a}	88.33±1.67^{a}	88.33±1.67^{a}	86.67±3.33^{a}	68.33±1.67^{a}	60.00±0.00^{c}	40.00±0.00^{d}	40.00±0.00^{a}	36.67±3.33^{bc}	25.00±2.89^{a}	0.00±0.00^{a}
1 (0.18 mg/mL)	90.00±0.00^{a}	90.00±0.00^{a}	88.33±1.67^{a}	86.67±3.33^{a}	76.67±3.33^{a}	76.67±1.67^{a}	46.67±4.41^{cd}	40.00±0.00^{a}	40.00±0.00^{bc}	36.67±1.67^{a}	6.67±6.67^{a}
2 (0.36 mg/mL)	90.00±0.00^{a}	90.00±0.00^{a}	88.33±1.67^{a}	83.33±1.67^{a}	76.67±3.33^{a}	76.67±1.67^{a}	71.67±1.67^{a}	58.33±4.41^{a}	60.00±0.00^{a}	45.00±2.89^{a}	0.00±0.00^{a}
3 (0.54 mg/mL)	86.67±3.33^{a}	88.33±1.67^{a}	86.67±1.67^{a}	80.00±5.00^{a}	75.00±2.89^{a}	73.33±1.67^{ab}	70.00±0.00^{a}	51.67±10.93^{a}	53.33±6.67^{ab}	33.33±8.82^{a}	6.67±6.67^{a}
4 (0.72 mg/mL)	85.00±2.89^{a}	86.67±1.67^{ab}	86.67±1.67^{a}	73.33±3.33^{a}	71.67±1.67^{a}	65.00±5.00^{bc}	63.33±3.33^{b}	55.00±7.64^{a}	51.67±8.33^{ab}	36.67±6.01^{a}	0.00±0.00^{a}
5 (0.90 mg/mL)	86.67±1.67^{a}	83.33±1.67^{b}	76.67±1.67^{b}	73.33±3.33^{a}	73.33±3.33^{a}	63.33±3.33^{c}	53.33±3.33^{c}	46.67±3.33^{a}	33.33±6.67^{c}	25.00±12.58^{a}	6.67±6.67^{a}
F-value	1.33	3.40	7.50	2.25	1.33	7.16	48.64	1.15	4.00	1.22	0.60
P	0.317 9	0.038 2	0.002 1	0.115 5	0.316 3	0.002 5	0.000 1	0.385 7	0.022 8	0.357 5	0.701 3

Different letter superscripts indicate the significant differences between means within column using the multiple range Duncan's test at $P<0.05$.

were significantly ($P<0.02$) higher than control. Sperm motility of the concentration 2 (45.00%±2.89%) after 9 d of chilling was higher than control and the other concentrations (Table 1).

3.2. Effect of SEE on cattle post thawing sperm characteristics

Addition of SEE in concentration 1 and 2 gave post thawing sperm motility as high as the control (47.50±2.81 and 45.00±2.58, respectively) while other concentration have lower effects on motility as compared to the control.

Addition of silymarin improved post thawing alive% and was significantly higher ($P<0.000\ 1$) than the control. SEE decreased significantly ($P<0.000\ 1$) the % of post thawing abnormal sperm in concentration 3 and 4 (11.83±0.65 and 16.00±0.58, respectively). SEE improved significantly ($P<0.018$) the % of post thawing intact spermatozoa membranes (HOST%) in concentrations 2, 4 and 5 (71.17±0.83, 71.83±0.91 and 75.00±3.42, respectively) (Table 2).

Table 2

Effect of silymarin enriched extender on cattle post thawing sperm characteristics.

Treatment Parameters	Motility%	Alive%	Abnormal%	HOST%
Control	45.83 ± 3.52^{a}	61.17 ± 0.98^{d}	18.67 ± 0.33^{bc}	69.50 ± 1.61^{ab}
1 (0.18 mg/ml)	47.50 ± 2.81^{a}	83.83 ± 0.95^{a}	18.67 ± 0.67^{bc}	70.00 ± 1.83^{ab}
2 (0.36 mg/ml)	45.00 ± 2.58^{a}	72.83 ± 1.14^{c}	18.83 ± 1.30^{b}	71.17 ± 0.83^{a}
3 (0.54 mg/ml)	31.67 ± 4.01^{b}	72.83 ± 1.01^{c}	11.83 ± 0.65^{d}	64.83 ± 1.17^{b}
4 (0.72 mg/ml)	25.00 ± 3.42^{b}	78.33 ± 1.67^{b}	16.00 ± 0.58^{c}	71.83 ± 0.91^{a}
5 (0.90 mg/ml)	25.00 ± 2.24^{b}	69.83 ± 0.83^{c}	22.00 ± 1.29^{a}	75.00 ± 3.42^{a}
F-value	11.41	46.40	15.12	3.27
P<	0.000 1	0.000 1	0.000 1	0.017 9

Different letter superscripts indicate the significant differences between means within column using the multiple range Duncan's test at $P<0.05$.

4. Discussion

Cryopreservation of sperm is of a great demand[1]. According to Gadea *et al.*[11], Uysal and Bucak[12] and Bucak *et al.*[13], minimizing the physical and chemical stresses of cooling, freezing and thawing of sperm cells and consequently improving viability and subsequent fertilizing capacity is achieved by including cryoprotectants in the semen extender. Cryopreservation causes wide-ranging physical, chemical and mechanical injures to sperm membranes of all mammalian species[14], which are attributed to temperature changes, alterations in the transition from the lipid phase, production of reactive oxygen species (ROS) and osmotic stress[5,15]. Moreover, the over accumulation of ROS causes a state of oxidative stress that involves structural damage of sperm membranes, fall of intracellular ATP levels causing decreasing in the viability and motility of cryopreserved sperm[16,17]. To decrease the harmful effects of ROS, seminal plasma possesses powerful source of ROS scavengers which offer protection for sperm, including enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and small molecular antioxidants such as ascorbic acid and a-tocopherol[18,19]. The herbal remedies contain antioxidants to counteract the deleterious action of reactive oxygen species (ROS). Sperm motility was kept high in chilled semen at the concentrations of silymarin 0.36, 0.54 and 0.72 mg/mL up to 8 d of chilling. This indicates that chilled extended semen with these concentrations could be used in AI up to 8 d. Sperm motility of the concentration 0.54 mg/mL was kept high up to 9 d of chilling. This means that semen extended with this concentration could be used in AI up to 9 d of chilling. Addition of silymarin improved post thawing alive%, sperm abnormalities, percent of post-

thawing intact spermatozoa membranes and maintained the percent of sperm motility. The improved semen quality in both chilled and frozen semen is due to the strong antioxidant capacity of silymarin [6,7]. Wellington and Jarvis[20] postulated that the mechanism of action of silymarin is through the stimulation of ribosomal RNA protecting the cell membrane from oxidative damage. They stated also that silymarin stimulates the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase. It could be concluded that silymarin as a natural additive to semen extenders improved preservability in both chilled and frozen bull semen.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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