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Effect of clarified Tris egg yolk extender supplemented with strawberry juice (*Fragaria* Spp.) on some characteristics of chilled and frozen cattle semen

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Abstract: Recently, investigations on semen extenders, natural antioxidants which present substantially in fruits are used for semen preservation either in chilled or frozen semen. The aim of this study is to explore the effect of clarified tris egg yolk extender to which added different concentrations of strawberry juice (1-6%) as a cryoprotective agent for cattle semen preservation. Tubes containing 5 ml tris and 20 % (V/V) egg yolk (except the control) were centrifuged to separate LDL fraction. Tris with LDL fraction were transferred to other tubes to which the different concentrations (1- 6%) of strawberry juice from 10 % stock solution. Semen was collected from 5 mature cattle bulls; weekly / 5 weeks. Evaluation of pooled ejaculates has been done for dilution processing. Semen samples were diluted in TCYF as control (0% SB) and in the different concentrations from the 10% stock SB juice (1 to 6%). Diluted semen were equilibrated for 4 h. at 5 ºC; then packed into 0.25 ml straws. The straws were frozen in a vapor 4 cm above liquid nitrogen (LN_2) for 10 minutes and then dipped in LN_2. Sperm motility of chilled cattle semen was evaluated 2 hours after cooling and chilling up to 6 days. Frozen straws were thawed at 37˚C/ 60 s. The motility, alive, abnormality and membrane integrity (HOST) percentages were studied. In chilled semen, the concentrations from (1-5%) strawberry in tris extender showed higher sperm motility at 6 days post chilling. In frozen semen, the concentrations 1, 2, 4, 5 and 6% exhibited higher sperm motility. The best motility was at concentration 4 %.While 3 % gave the best alive and sperm abnormalities percentages. HOST % was maintained in all concentrations as compared to control.
In conclusion, we propose that clarified tris extender containing different concentrations of strawberry as cryoprotective improved semen characteristics is both chilled and frozen semen.
Keyboards: Tris egg yolk extender, strawberry juice (*Fragaria* Spp.), chilled cattle semen, frozen cattle semen.

Introduction

As semen is considered of the high value products of male; its preservation, either by chilling or freezing, is very important to be used in artificial insemination[1]. To conserve the super genetic constitution of the male, so cryopreservation of sperm is of a great demand[2]. According to[3,4,5] 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 cryoprotectant in the semen extender. [6] mentioned that, preserving cattle and caprine semen with natural extenders, extracted and infused from fruits, have strong protective natural property.

Since fruits are containing natural antioxidants which are more acceptable than synthetic antioxidants, [7] found that Strawberry fruit is an important source of natural antioxidants, phytochemicals (anthocyanins, flavonoids, phenolic compounds) and ellagic acid which have strong antioxidant activity. [8]stated that strawberry fresh juice detoxifies living cells from oxidative agents due to its high antioxidant capacity. Moreover, [9] recorded that the strawberry fruit is rich in potassium, vit. C, E, folic acid, carotenoids as well as phenolic compounds, so it has strong antioxidant capacity.

Regarding the semen extenders from animal sources, hen egg yolk is widely used as a cryoprotective agent in semen freezing extenders in order to protect the spermatozoa against cold shock. The protective action of yolk is largely presumed to be due to low density lipoproteins (LDL). Recently, the use only of the LDL extracted from egg yolk in the extender is a new trend applied by many authors[10]. Several concentrations of LDL (between 2.5 and 20 % W/V) were tested in freezing extenders for bull semen.

A new generation of semen extenders free from animal origins based on the presence of natural products is the target of our study to minimize the risk of contamination and improve the potential of cryopreservation.

Material and Methods

Egg yolk clarification: The tubes containing 5 ml. tris and 20 % (V/V) egg yolk (except the control) were centrifuged at 4000 r.p.m. for 20 minutes to separate LDL fraction with tris; leaving the egg yolk deposit. Tris with LDL fraction were transferred to other tubes to which the different concentration of strawberry juice were added.

Fruit juice preparation: Fresh mature strawberry (*Fragaria spp.*) fruits (SB) were purchased from local market. They were well cleaned and cut to be squeezed in a blender machine with filter mesh. Stock solution of the SB juice (10%) in TCYF was prepared. Then, the 10% juice was added to tris extender (TSB) at concentrations of 1 to 6%.

Semen processing: A basic control extender (Tris-citric acid-egg yolk-fructose [TCYF]) was prepared according to Foote [11]. Semen samples were diluted in TCYF (control, 0 % SB) and in the former concentrations of TSB (1-6%) to ensure 60 million motile spermatozoa /mL, cooled slowly up to 5 °C 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 (LN_2) for 10 minutes and were then dipped in liquid LN_2.

Semen quality assessment: The assessment was under taken post freeze-thawing of cattle bull spermatozoa. Also, sperm motility was evaluated for raw semen, 2 hours after cooling and chilled semen daily up to 10 days. Frozen straws were thawed at 37°C/ 60 s. The parameters studied were subjective semen characteristics (motility%, alive%, abnormality% and membrane integrity (hypo-osmotic swelling test HOST) %).

Subjective motility was assessed using a phase-contrast microscope (100x magnification), with a warm stage maintained at 37 °C. A wet mount was made using a drop of semen placed directly on a pre-warmed slide and covered by a pre-warmed cover slip under the same temperature conditions. Sperm motility estimations were performed in three different microscopic fields for each semen sample. Visual motility was assessed microscopically with closed circuit television system[12].

Live and abnormal spermatozoa (%): This was evaluated using eosin-nigrosin stained smear as described by [13]. Two hundred spermatozoa were assessed.

Sperm membrane integrity (%): Sperm membrane integrity was assessed using the hypo-osmotic swelling test (HOST) [14]. Two hundred spermatozoa were assessed and the percentage of spermatozoa with curled tails (swollen/ intact plasma membrane) was calculated.

Statistical analysis: Statistical analysis data were analyzed using the SPSS, 2005, computerized program v. 14.0 to calculate the analysis of variance (ANOVA)[15] for the different parameters between control and additives

replications. A significant difference between means was calculated using Duncan multiple range test at P<0.05.

Results

In chilled semen the concentrations from (1-5%) strawberry in tris extender showed significant (P<0.0001) higher sperm motility at 6 days post chilling 56.67 ± 3.33, 51.67 ± 1.67, 70.00 ± 5.77, 75.00 ± 2.89 and 46.67 ± 3.33 as compared to the control (41.67 ± 1.67). In frozen semen, all the concentrations (1, 2, 4, 5 and 6%) exhibited higher sperm motility (30.00 ± 5.77, 31.67 ± 4.41, 43.33 ± 1.67, 35.00 ± 2.89 and 38.33 ± 1.67) as compared to the control (16.67 ± 3.33). The best motility was at concentration 4 %. The concentration 3 % gave the best alive sperm percentage as compared to the control (81.33 ± 0.67 and 68.67 ± 3.67 respectively). The lowest sperm abnormalities was observed in concentration 3 % as compared to the control (23.67 ±1.86 and 27.67 ±2.33) respectively. HOST % was maintained in all concentrations as compared to control.

Table 1. Sperm motility of Chilled cattle semen using different concentrations of clarified TSB.

periods / treatment	2 hours	Days					
		1	2	3	4	5	6
Control	88.33^{a} ± 1.67	85.00^{ab} ± 2.89	81.67^{a} ± 1.67	78.33^{a} ± 1.67	73.33^{ab} ± 3.33	70.00^{a} ± 5.77	41.67^{c} ± 1.67
TSB 1%	88.33^{a} ± 1.67	90.00^{a} ± 2.89	83.33^{a} ± 1.67	81.67^{a} ± 1.67	80.00^{a} ± 2.89	78.33^{a} ± 3.33	56.67^{b} ± 3.33
TSB 2%	88.33^{a} ± 1.67	91.67^{a} ± 1.67	83.33^{a} ± 1.67	81.67^{a} ± 1.67	80.00^{a} ± 2.89	78.33^{a} ± 3.33	51.67^{bc} ± 1.67
TSB 3%	90.00^{a} ± 0.00	91.67^{a} ± 1.67	83.33^{a} ± 1.67	81.67^{a} ± 1.67	80.00^{a} ± 2.89	78.33^{a} ± 3.33	70.00^{a} ± 5.77
TSB 4%	90.00^{a} ± 0.00	91.67^{a} ± 1.67	81.67^{a} ± 1.67	81.67^{a} ± 1.67	80.00^{a} ± 2.89	78.33^{a} ± 4.41	75.00^{a} ±2.89
TSB 5%	88.33^{a} ± 1.67	86.67^{ab} ± 3.33	75.00^{ab} ± 2.89	73.33^{ab} ± 3.33	68.33^{ab} ± 6.01	66.67^{a} ± 6.67	46.67^{bc} ± 3.33
TSB 6%	86.67^{a}± 1.67	81.67^{b} ± 1.67	66.67^{b} ± 8.82	63.33^{b} ± 8.82	60.00^{b} ± 7.64	58.33^{a} ± 8.33	45.00^{c} ± 5.00
f-cal	0.67	2.83	2.78	3.27	3.11	2.20	12.18
Sig.	0.6781	0.0508	0.0540	0.0317	0.0375	0.1053	0.0001

Different superscripts (a, b…etc.) indicate significant difference between means using Duncan multiple range test (P<0.05).

Table 2. Sperm parameters of post-thawed cattle semen using different concentrations of clarified TSB.

periods / treatment	Motility	Alive	HOST	Abnormality
Control	16.67^{d} ± 3.33	68.67^{b} ± 3.67	76.67^{a} ± 3.33	27.67^{abc} ± 2.33
TSB 1%	30.00^{bc} ± 5.77	72.67^{b} ± 1.45	80.00^{a} ± 0.00	31.33^{a}± 1.33
TSB 2%	31.67^{bc} ± 4.41	74.33^{b} ± 0.67	77.67^{a} ± 3.93	28.33^{abc} ± 1.67
TSB 3%	23.33^{cd} ± 3.33	81.33^{a} ± 0.67	80.67^{a} ± 2.96	23.67^{c} ± 1.86
TSB 4%	43.33^{a} ± 1.67	73.67^{b} ± 1.33	86.33^{a} ± 1.33	25.67^{bc} ± 0.67
TSB 5%	35.00^{ab} ± 2.89	73.67^{b} ± 1.67	71.00^{a} ± 1.00	31.00^{a} ± 1.00
TSB 6%	38.33^{ab}± 1.67	74.00^{b}± 2.08	78.33^{a}± 4.41	30.33^{ab}± 0.33
f-cal	6.40	3.88	2.60	3.84
Sig.	0.0021	0.0171	0.0657	0.0178

Different superscripts (a, b…etc.) indicate significant difference between means using Duncan multiple range test (P<0.05).

Discussion

The results of the present study in chilled semen revealed significant (P<0.0001) maintenance of sperm motility in concentrations 2 and 3% up to the third day of chilling. This indicates that it could be used in field insemination up to the third day of chilling. Frozen semen explored improvement in sperm motility post-thawing at concentrations 1 and 2%. These results are in agreement with [10] who stated that sperm motility and semen characteristics improved with LDL in the tris extender as compared to the tris extender containing egg yolk with optimum LDL 8 % concentration in the extender.

The improved results in semen preservability by using strawberry juice as a cryoprotectant in tris semen extender is mainly due to its strong antioxidant properties. This strong antioxidant capacity is due to its high contents of vitamins, flavonoids and phenolic compounds as these components are strong antioxidants [16, 17]. Strawberry is rich in anthocyanins that have strong antioxidant property as radical scavenger and alleviating oxidative stress and cellular damage[18]. The main polyphenolic compounds in strawberry responsible for antioxidant effect are anthocyanins[19]. The mechanisms to prevent oxidation are associated with the defense system, including antioxidant enzymes and antioxidants which play an important role in preventing oxidative injury through their abilities to scavenge free radicals that cause cellular damage[20].

In conclusion we propose that extender containing LDL extracted from egg yolk could be used as cryoprotective media with better efficacy in preserving both chilled and frozen semen in cattle bulls.

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