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Using Gum Arabic in Place of Egg Yolk as Cryoprotectant for Cryopreservation of the Buck Semen

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ABSTRACT

Background: Egg yolk (EY) is well known to be toxic for buck spermatozoa, which creates restrictions of its use in cryopreservation. Therefore, this study is to compare the effect of different levels of gum Arabic (GA) in an extender on quality and fertility of cryopreserved buck sperm.

Methods: Each ejaculate of six bucks was frozen in Tris with one of concentrations of GA which contained 3, 6, 9 and 12 gm/ 100 ml in place of the EY. Control was Tris extender containing 2.5% of EY.

Result: A percentages of total motile sperm (54.92%; $P<0.05$) and progressively motile sperm (26.22%; $P<0.05$) of semen was frozen in Tris containing 9% of GA. Similar to control group, the pregnancy rate of does inseminated with extender containing 9% (50.0%) were significantly higher than those of does inseminated with extender containing 6% (8.33%), 3% (0.0%) and 12% (0.0%). Semen evaluation and fertility rate were similar when replacing the EY with GA in the Tris cryodiluent, after cryopreservation of buck semen. The present study shows that high motility rate of frozen semen and acceptable pregnancy rate can be obtained when using GA in place of EY for cryopreserving the buck sperm.

Key words: Artificial insemination, Buck semen, Cryopreservation, Egg yolk, Gum Arabic.

INTRODUCTION

Sperm cryopreserved in liquid nitrogen, is a technique to preserve genetic resources and provides some benefits, such as easily shipping of global germ plasma and selective breeding with desirable characteristics (Beir *et al.* 2019).

Frozen semen should be frozen at a very low temperature (-196°C), therefore, it is necessary to extend the semen in appropriate diluents contained cryoprotectants, such as glycerol and EY. However, there were attempts to find alternatives to EY in cryoprotective media, due to a source of bacterial contamination (Ondřej, *et al.* 2019). Therefore, it would be better to use a well-defined and pathogen-free alternative, non-animal origin substitute for EY (Singh *et al.* 2017). In addition, EY of ≥ 3% is well known to be toxic for buck spermatozoa (Ritar and Salamon, 1982), where the egg yolk coagulating enzyme (EYCE) is a harmful to the sperm cells (Purdy, 2006). Egg yolk is considered a cause of the reduction in the respiration and motility of spermatozoa in bulls were caused by the granules (Ugur *et al.* 2019) and caused reduction in the accuracy of computer in order to detection of false sperm, which may actually be EY particles or other debris (García *et al.* 2017). This caused increasing the percentage of immotile spermatozoa. However, in all animal species, the EY is important part of most semen extenders due to protects the sperm from thermal shock during freezing (Anand *et al.* 2014).

Therefore providing a non animal origin, well defined and pathogen free medium for the cryopreservation of semen is highly desired (Muhammet *et al.* 2019). In the present study, GA is used as cryoprotectant to preserve buck sperm at low temperature. According to study by Mohamed Ali *et al.* (2017), a highly quality of post thawed frozen stallion semen was achieved after freezing, when using GA instead of the EY. However, this present study is considered the comparative research related to GA as an alternative to EY for the cryopreservation of buck spermatozoa. Therefore, the aim of the current study is to compare the effect of different levels of GA in an extender on quality and fertility of cryopreserved buck sperm.

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MATERIALS AND METHODS

Animals

The current study was carried out using six fertile bucks and 58 does (Aradi-Damascus Crossbred) with an age range of 2-4 years from the Research Center in Qassim University, Al-Qassim region, Saudi Arabia. All does were clinically healthy at the semiological examination and had given birth at least once before the beginning of the study.

Semen collection

Semen was collected using an artificial vagina and then immediately kept in water bath at 37°C. Semen characterization was achieved, such as color, volume, pH, general motility, percentage motility, concentration and live/dead ratio. Sperm motility and concentration were estimated with the computer assisted semen analysis system (CASA; ISAS® program, Proiser R+D, Valencia, Spain).

Extenders

The samples were frozen using Tris extender. The EY (2.5 mL; as control group) was replaced with GA (Sigma-Aldrich Company, USA) in different concentrations (3, 6, 9 and 12%). Every ejaculate of all bucks was treated the same concentration of GA or EY. The GA was heated at 80°C for 60 minutes to inactive the enzymes. The Tris extender was prepared using sugars (0.625 g glucose), buffering agents (3.786 g Tris and 2.172 g citric acid), 5 mL glycerol and non-pyrogenic water (added to a volume of 100 mL). Measurement of the extender viscosity (cP) was determined by vibroviscometer (model SV-10, Tokyo, Japan) at room temperature. *p*H measurement was adjusted at 7-7.2 (HANNA HI 2211, Italy). Measurement of the extender osmolarity (mOsmol/kgH_2O) was determined by osmometer (model 800 CLG, SLAMED, Germany).

Deep freezing

The semen from each of the six bucks was resuspended with Tris extender containing EY (2.5%) or AG (3, 6, 9, or 12%) and glycerol (5%); the final volume after dilution was one ml semen to 4 mL of extender and then cooled to 4°C for 75 min. After cooling, diluted semen was evaluated using ISAS and then filled into straws (0.5 mL). Freezing processes were performed by putting the straw horizontally on the surface of liquid nitrogen at 3-4 cm for 20 minutes. After seven days kept in liquid nitrogen, straws were thawed in a water bath at 37°C for 60 s immediately and then analyzed by the ISAS system.

Assessment of sperm motility

Cooled diluted semen and frozen semen were examined for a motility pattern using the ISAS® program. A five µL from each diluted semen was placed in a pre-warmed slide. Seven consecutive digitalized images obtained from several fields using a 10X negative-phase contrast objective were examined for sperm motility analysis. At least 300 spermatozoa per sample were analyzed. Subsequently sperm motility parameters were recorded: total motile spermatozoa (% TMS), rapid progressively motile sperm (% PRS), curvilinear velocity (VCL) in µm/s, rectilinear velocity (VSL) in ìm/s, the average path velocity (VAP) in µm/s, straightness index (% STR), linearity coefficient (% LIN), oscillation index (% WOB), amplitude of lateral head displacement (% ALH) and beat cross frequency (Hz, BCF). Spermatozoa with a swimming speed or VAP values below 10 µm/s were considered immotile spermatozoa (IMS).

Cooled and frozen semen evaluation

Cooled semen and frozen-thawed semen were evaluated for the functionality of the plasma membrane by the Hypo-osmotic swelling test (HOST). Twenty µL of semen was added to 2 ml of 125 mOsmol fructose-base solution and then incubated in a water bath at 37°C for 50 minutes. Subsequently, one hindered sperm cells were analyzed for the presence or absence of a coiled tail (Fonseca *et al.* 2005).

The Giemsa staining procedure was used to examine the defected acrosome. The morphologically normal spermatozoa were examined by the nigrosin-eosin stain (Evans and Maxwell, 1987). At least 200 spermatozoa were examined for acrosome defect and defect sperm under a light microscope (1,000x).

Fluorescent stains such as acridine orange (AO) and propidium iodide (PI) were used to assess cell viability (Halotech DNA, S.L., Spain). Fluorescence green of head sperm occurs when AO is retained within intact cells. PI stain can only bind to and stain cellular DNA in non-living or damaged cells, causing them to have red fluorescence. Three hindered sperm per sample was counted.

Artificial insemination protocol

Does were synchronized by using sponge (SYNCRO-PART® sponges contain 45mg of fluorogestone, Ceva Santé Animale, France) that was inserted intra vaginal for 17 days. At Day 17 the sponge was removed and 300 iu of pregnant mare serum gonadotrophine (SYNCRO PART® PMSG, Ceva Santé Animale, France) was injected intramusculary for each doe. After 50-60 hours from sponge removal, the inseminations were performed intracervical using 600×10^6 total spermatozoa/insemination. All does were examined for pregnancy diagnosis using ultrasonography 30 days after insemination.

Statistical analysis

Descriptive analyses were determined for the evaluated variables: total motility (MT), progressive motility (PMS), VCL, VSL, VAP, STR, LIN, WOB, ALH, BCF, acrosome integrity, vitality, HOST and morphological defect. One-way analysis of variance (ANOVA) were performed for statistical comparisons between groups. Analysis of the normal distribution of data was examined with the Kolmogorov-Smirnov test (SPSS, version 16). The data were considered statistically different if $P<0.05$. Data were expressed as the means and SEM.

RESULTS AND DISCUSSION

Sperm parameters, including motility, viability, plasma membrane integrity, normal morphology and acrosome integrity, were noted in samples that were cooled in Tris extender containing GA and evaluated and presented in Table 1. Extenders with 3, 6 and 9% of GA were high efficient in sperm motility than extender with 12%. A similar result of high sperm motility was found in EY extender compared

Table 1: Motility and viability parameters of buck semen were stored at 4°C for 75 min.

Parameter	GA 3	GA 6	GA 9	GA 12	EY
TMS (%)	88.52±4.30^{a}	87.06±2.90^{a}	92.53±2.39^{a}	66.83±8.28^{b}	78.30±5.70^{a}
PRS (%)	69.04±6.97^{a}	68.86±4.90^{a}	72.13±5.76^{a}	35.70±9.06^{b}	61.65±1.85^{a}
VCL (µm/s)	99.04±6.06^{a}	97.62±3.47^{a}	97.68±3.60^{a}	73.38±7.47^{b}	94.20±4.7^{a}
VSL (µm/s)	24.92±1.68^{a}	24.30±0.69^{a}	24.53±0.93^{a}	21.33±1.45^{b}	24.90±0.06^{a}
VAP (µm/s)	48.70±3.05^{a}	48.50±1.55^{a}	50.30±2.22^{a}	40.25±3.21^{b}	44.30±0.01^{a}
LIN (%)	25.26±1.27^{b}	25.03±0.43^{b}	25.21±0.54^{b}	30.01±2.14^{a}	25.50±0.22^{ab}
STR (%)	50.86±2.17^{ab}	50.03±0.96^{ab}	48.35±0.50^{b}	53.43±2.09^{a}	49.20±0.01^{ab}
WOB (%)	49.06±0.99^{b}	50.08±0.73^{b}	51.91±0.65^{b}	55.43±2.04^{a}	55.20±0.00^{a}
ALH (%)	3.80±0.56^{ab}	4.38±0.12^{a}	4.38±0.18^{a}	3.58±0.23^{b}	3.60±0.02^{ab}
BCF (Hz)	4.76±0.28^{b}	6.00±0.19^{ab}	6.18±0.24^{ab}	7.26±0.99^{a}	4.20±0.06^{b}
Vital (%)	72.50±4.51	80.27±1.39	74.87±1.93	67.83±2.77	75.54±2.30
HOST (%)	76.27±2.76	80.00±0.00	65.95±0.95	63.58±9.45	67.87±1.21
Total defect (%)	8.50±0.00	3.10±0.00	6.80±0.30	7.40±0.00	8.76±3.50
Acrosome integrity (%)	78.07±0.00^{b}	79.72±1.08^{b}	85.44±0.39^{b}	79.45±0.41^{b}	91.50±4.50^{a}

^{a,b,ab}Superscript represent significant difference at $P<0.05$ between groups in the same raw.

with 12% GA extender. VCL, VSL and VAP values were significantly higher in GA (3, 6 and 9%) and EY compared to extender containing 12% of GA. Tris extender with 12% of GA was highly significant in LIN, STR, WOB and BCF compared to others. There is no significant different among groups in vitality rate, plasma membrane integrity and morphology defects. The acrosome integrity in extenders containing EY (2.5%) was greater ($P<0.05$) than that of sperm that was cooled in extenders containing GA (3, 6, 9 and 12%).

Extenders should be contain cryoprotectants to protect the sperm against cooled shock such as, egg yolk, egg plasma, skim milk, milk by products, or chemicals for regulation of osmolarity and pH. This research showed that maintaining the sperm motility and progressive motile sperm during cooling and freezing temperature could be achieved more favorably in the diluents containing GA than the diluent containing EY for buck semen. However, this study agrees with the study using GA in cryopreservation of stallion semen (Mohamed Ali *et al.* 2017). Gum Arabic is complex, comprised of polysaccharide and a branch polymer consisting of galactose, rhamnose, arabinose and glucuronic acid (Montenegro *et al.* 2012). Gum Arabic has been better than glycerol, Me_2SO, or lactose in cryoprotecting T2 bacteriophage and it has also been successfully utilized for the cyanobacterium S. platensis (Hubalek, 2003). Gum Arabic is easily soluble in water and forms solutions over a wide range of concentrations. The mechanism of action of polysaccharides in promoting frozen stability is related to the control of the amorphous matrix surrounding the ice crystals (Lopez *et al.* 2005). The GA has a large polysaccharide structure result in used as an extracellular cryoprotectant.

After deep-freezing, parameters of motility and viability of buck sperm are shown in Table 2. Tris extender containing GA of 9% showed a significantly higher in total sperm motile sperm compared to extenders containing GA of 3, 6 and 12% or 2.5% of EY. While progressive motile was significantly higher in 9% of GA and EY compared to others. The probability is due to homogeneity of the extender in pH, viscosity and osmolarity which might be better stability in that extender. In addition this feature provides greater energy support for survival and a sperm cell movement. However, alterations in permeability, functionality and metabolism of sperm cell, resulting in changes of the motility and fertilizing ability, are due to changes in organization of the plasma membrane fluid mosaic (Amann and Graham, 1993). Although the mechanism of sperm protection by using GA is unknown. However, more work is needed to know how the GA protects sperm during freezing. Another factor for the survival of sperm cells and continues positive correlation with ATP concentration during storage is the amount of energy reserves contained in the extender. In the present study, 6% and 9% of GA were the optimal concentrations to keep the average velocity (VCL, VSL and VAP). The viscosity of 12% GA was about 50% and 80% more than that of 9% GA and EY, respectively, being the most effective trait ruling sperm motility (Mohamed Ali *et al.* 2018). However, there was no significantly difference among the extenders after cooling or freezing. Osmolarity of Tris extender contained GA or EY was approximately the same values of pH range between 7.0-7.2 as shown in Table 3. Obviously, > 9% primarily affected fast swimming sperm cells and impede spermatozoa with a velocity slightly above 45 µm/s. Thus, the percentage of immotile spermatozoa (<10 µm/sec) increased with the increase of viscosity. This phenomenon might confirm no fertility resulted of the use of 12% GA extenders in the current study. The ultimate explanation for this impediment of fertility is the high viscosity negatively affected the sperm survival before or during storage in liquid nitrogen. It has been established that the viscosity of the extender affects the pattern of sperm motion by the addition of Ficoll, carboxy methyl cellulose (CMC),

Table 2: Motility and viability parameters and pregnancy rate of buck semen was frozen in liquid nitrogen.

Parameter	GA 3	GA 6	GA 9	GA 12	EY
TMS (%)	15.56±0.85^{b}	33.60±4.09^{b}	54.92±8.50^{a}	17.83±4.00^{b}	40.40±2.60^{ab}
PRS (%)	6.50±1.40^{b}	14.72±2.71^{b}	26.22±5.35^{a}	6.83±1.83^{b}	20.13±2.86^{a}
VCL (µm/s)	50.33±1.33^{b}	62.37±3.44^{a}	52.75±2.35^{b}	49.56±3.64^{b}	52.80±4.20^{b}
VSL (µm/s)	14.53±0.43	14.37±1.30	17.12±1.15	14.26±0.71	14.56±0.53
VAP (µm/s)	27.43±0.73^{ab}	30.50±1.61^{ab}	31.62±1.67^{a}	26.66±1.56^{b}	28.06±1.63^{ab}
LIN (%)	28.86±0.06^{a}	23.20±2.32^{b}	32.40±0.90^{a}	28.86±0.75^{a}	27.90±1.30^{a}
STR (%)	52.96±0.16^{a}	46.95±3.08^{b}	54.05±0.83^{a}	53.40±0.45^{a}	52.23±1.23^{a}
WOB (%)	54.46±0.03^{b}	49.10±1.85^{b}	59.90±0.96^{a}	54.03±1.05^{b}	53.33±1.23^{b}
ALH (%)	3.56±0.06^{a}	3.27±0.32^{ab}	2.85±0.15^{b}	3.40±0.20^{ab}	3.36±0.03^{ab}
BCF (Hz)	2.70±0.10	3.15±0.08	3.80±0.56	3.10±0.41	3.16±0.23
Vital rate (%)	5.00±0.06^{b}	16.75±1.85^{a}	24.86±1.77^{a}	7.90±0.66^{b}	25.00±0.1^{a}
HOST (%)	6.50±0.00^{b}	24.20±4.20^{a}	25.15±3.3^{a}	21.16±4.1^{a}	26.27±3.15^{a}
Total defect (%)	24.41±10.7	20.22±3.08	14.17±4.07	17.25±7.7	9.23±0.10
Acrosome integrity (%)	83.70±1.7	80.67±2.48	85.60±2.70	79.95±3.47	84.76±0.0
Pregnancy rate (%)	0.0% (0/12)^{b}	8.33% (1/12)^{b}	50.0% (6/12)^{a}	0.0% (1/12)^{b}	50.0% (5/10)^{a}

^{a,b,ab}Subscript represent significant difference at P<0.05 among groups in the raw.

Table 3: pH, viscosity and osmolarity of Tris extender supplemented with GA or EY at room temperature.

Extender	pH	Viscosity (cP)	Osmolarity (mOsmol/kgH_2O)
Distiled water	5.6	3.65±0.55	-7
EY (2.5%)	7.2	2.66±0.69	381.07±1.80
GA (3%)	7.1	3.30±0.43	360.50±1.88
GA (6%)	7.1	4.15±0.14	400.50±4.5
GA (9%)	7.0	5.37±0.07	408.33±2.37
GA (12%)	7.0	10.40±0.30	445.67±3.48

methylcellulose, EY or GA (Amann and Hammerstedt, 1980, Hirai *et al.* 1997, Mohamed Ali *et al.* 2017). In the present study, addition of GA could also alter the movement pattern of Buck spermatozoa.

Significantly higher (P<0.05) vitality rate was obtained in extender with GA (6 and 9%) and EY. Although the percentage of sperm defects was higher in GA extenders compared to EY extender, the difference between extenders was insignificant (Table 2). As it is known the cooling and freezing process promoted irreversible cellular injuries resulted in impair the semen fertility. In addition the physical characteristics of the solution like its viscosity and osmolarity might have an impact on the sperm motility and ability for fertilization. Plasma membrane integrity (HOST) was significantly higher (P<0.05) in extender with 6, 9 and 12% of GA or 2.5% of EY extender than with 3% of AG. The plasma membrane allows important molecules pass through to the sperm cytoplasm. As well integrity of plasma membrane is very important for the interaction between the male and female gametes (Tosti and Ménézo, 2016). The superiority of GA and EY by the HOST and biometric testing suggested that it might provide better protection for the plasma membrane during deep freezing.

The pregnancy rate (50%) using semen that was frozen in GA (9%) was similar to EY extender (2.5%). Pregnancy rate derived of AI in small ruminants still beyond the acceptable values. Egg yolk has been extensively used for long time as a cryoprotectant in the semen extenders. Several risk factors are inherited with the use of EY as a biological material. Therefore GA inclusion in Tris extender affords significance on buck's subsequent fertility outcomes. The lesser fertility rate of frozen semen with 3 % of GA resulted in low motility rate, low survival rate and high morphological defect, especially tailless. This may be due to less protection of GA (3%) extender during freezing. There were no pregnant ewes when using 12% GA. The lesser fertility rate following 12% of GA is due to a low survival rate in sperm after deep freezing.

CONCLUSION

Inclusion of GA as a cryoprotectant at 6-9% in the buck semen extender resulted in a relatively a good approach for maintaining buck sperm motility, survival and subsequent fertility. In addition, it surpassed the EY in this trait and subsequent avoids the harmful effect of EYCE enzyme and the infective risk that might arise from using EY in the semen extender.

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