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Research Article

The Effect of Olive Oil Extract Tris-Infusion on Semen Cryopreservability of Cattle Bulls

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Abstract

Sperm cryopreservation is the most widely applied tool for genetic resource conservation of farm animal, livestock production improvement, and spreading of genetic dissemination worldwide. However, semen cryopreservation subjected spermatozoa to the deleterious effects of reactive oxygen species (ROS) overproduction, which are produced by sperm cell and leukocytes resulting in alterations in sperm membrane and acrosome integrities. The objective of the present study was to elucidate the effect of olive oil added to Tris citric fructose extender (TCF) on semen cry preservability of cattle bulls. Semen was collected weekly from three mature bulls for 18 weeks. six tubes were prepared (0 µl/5.0 ml (0%, control), 12.5 µl/5 ml (0.25%), 25 µl/5 ml (0.5%), 37.5 µl/5 ml (0.75%), 50 µl/5 ml (1%) and 62.5 µl/5 ml (1.25%) (v/v) [Olive oil: TCF]). 20% whole egg yolk was added. Semen samples were pooled and added to have a concentration of 60 million sperm/ml and extended semen was exposed to the semen freezing protocol. Semen evaluation was carried out post - cooling and post-freeze thawing including sperm motility, alive sperm, abnormalities, sperm membrane (HOST) and acrosome integrities. Post-cooling results (Table 1), the highest motile sperm percent (MSP) and live sperm percentage (LSP) were at the fourth and fifth concentrations. The abnormal sperm percentage (ASP) showed significant ($P < 0.0001$) low percentages at the first, third and fourth concentrations. The addition of the first concentration showed significant ($P < 0.0001$) high acrosome integrity percentage (AIP). In Post-thawed semen (Table 2), the first concentration revealed the best sperm motility ($P < 0.0004$) and acrosome integrity ($P < 0.0001$), the third concentration gave the superior alive sperm percent ($P < 0.0001$), the second concentration exhibited the lowest sperm abnormalities ($P < 0.0001$) with the highest sperm membrane and acrosome integrities ($P < 0.0001$). It could be concluded that, the first, second and third concentrations of olive oil were beneficial as cryoprotectants during freezing, while the fourth and fifth concentrations possess inferior cryoprotective effect. This improving effect is related to its high content of antioxidant components that scavenge the free radicals and decrease the oxidative damage.

Keywords: *Cattle; Semen; Preservation; Olive Oil*

Abbreviations

ROS: Reactive Oxygen Species; OEE: Olive Oil Enriched Extender; HOST: Hypo-Osmotic Swelling Test/Sperm Membrane Integrity Test; AI: Artificial Insemination; MSP: Motile Sperm Percent; ASP: Abnormal Sperm Percentage; AIP: Acrosome Integrity Percentage; NOS: Nitrogen Reactive Species; PUFA: Polyunsaturated Fatty Acids; LPO: Lipid Peroxidation; TCF: Tris Citrate Fructose Extender; TCFY: Tris Citrate Fructose Egg Yolk Extender

Introduction

Oxygen reactive species (ROS) and nitrogen reactive species (NOS) are strong reactive oxidizing factors secreted in different tissues such as reproductive organs. Normal levels of ROS are greatly useful for sperm maturation, capacitation, acrosome reaction, oocyte penetration, and even its vitality [1]. However, production of excessive ratios of ROS through the stimulation of oxidative stress can have strong hazardous effects on spermatozoa motility, decreasing the mitochondrial function, damage to proteins, lipids, DNA, and enzymatic activities of spermatozoa [2]. Moreover, the spermatozoa plasma membrane is susceptible to peroxidative damage due to its high ratio of polyunsaturated fatty acids (PUFA). Lipid peroxidation (LPO) results in the damage of sperm membrane and affected the sperm flagellum and axonemal structure with subsequent decreased motility [3].

Numerous extracts obtained from the plant leaves, seeds and roots have an antioxidant capacity due to their high levels of polyphenols, flavonoids, carotenes, gallic acid, tannins and essential oils, and it has been recorded that they have superior results than synthetic antioxidants because of their reduced cytotoxicity and residue formation [4]. Olive tree (Olea europaea; Oleaceae family) is a phytoestrogen plant with enormous biological and pharmacological features [5]. Olive oil is markedly related to oxidative stress decrease [6]. In this aspect, the protective effects of olive oil could be related to its high oleic acid level and the antioxidant effect of its polyphenols [7]. D'Angelo., *et al.* [8] recorded that olive oil effectively reduces the hazardous effects of reactive oxygen species in a variety of cellular structures. About 77% of olive oil is mono-unsaturated fatty acids mainly oleic acid [9-11]. Furthermore, olive oil contains saturated fatty acids (~14%), polyunsaturated fatty acids (~9%), alpha-tocopherol, and vegetable mucilage [9,10]. In addition, olive oil contains many biologically active compounds especially antioxidant polyphenols oleuropein [12,13], hydroxytyrosol [14,15] and tyrosol [16,17], antioxidant flavonoids luteolin [18,19] and apigenin [20,21] and other bioactive organic molecules, including squalene [22,23]. These compounds have been linked to the reproductive potential [9,24 25].

Materials and Subject/Methods

The experimental design was approved and certified by the National Research Centre Medical Research Ethics Committee (Egypt) with an ethical certificate no.19146 and dated 2-2-2020.

The different semen extenders

Tris extender

The Tris-citric-fructose diluent (TCF) was prepared as a reference extender (Tris (hydroxy-methyl aminomethane, 3.02 gram/100 ml, Citric acid monohydrate, 1.76 gram/100 ml, Fructose, 1.25 gram/100 ml, Glycerol 6.4 (v/v)) [26]. The extender was distributed into screwed capped tube containing aliquots of 4 ml TCF, and then they were kept in -20°C for further use. While, the addition of 20% Egg yolk (v/v) (TCFY, Tris base extender) was added immediately before use.

Olive oil enriched extender [OEE]

One Tris citric fructose (TCF, control) and 5 tubes of OEE (0 µl/5.0 ml (0%, control), 12.5 µl/5 ml (0.25%), 25 µl/5 ml (0.5%), 37.5 µl/5 ml (0.75%), 50 µl/5 ml (1%) and 62.5 µl/5 ml (1.25%) (v/v) [Olive oil: TCF]). 20% whole egg yolk was added, mixed and the prepared 6 tubes were finally stored at -20°C till used on the next day.

Semen collection and initial evaluation

Three mature bulls kept at Semen Freezing Center, General Organization for Veterinary Services Ministry of Agriculture, Abbasia, Egypt, were used as semen donors. Ejaculates were harvested using a bovine adapted artificial vagina every week for 18 weeks. Semen

samples were primarily assessed for spermatozoal motility and concentration. Ejaculates with minimum standard of sperm motility (70%) and sperm morphological abnormalities were pooled to have enough semen volume for a replicate and to avoid the individual bull variation. The semen was hold for 10 minutes at 37°C in the water bath prior to extension.

Semen processing

Semen samples were extended in TCF diluent and considered as a control and other aliquots of collected semen samples were extended in TCF extenders enclosing the diverse concentrations of olive oil in order to offer a concentration of 60 million sperm/ml. Diluted semen was cooled slowly (approximately for 2 hrs) to 5°C and equilibrated for 2 hrs. Semen was filled in 0.25 ml polyvinyl French straws. After equilibrium interval, the straws were horizontally placed on a special rack and freezed in vapor 4 cm above liquid nitrogen for 10 minutes and were then plunged in liquid nitrogen.

Evaluation of semen quality parameters

The evaluation was implemented on post-cooled and post-thawed bull spermatozoa.

Semen characteristics

Motility percentage

Sperm motility was subjectively assessed using phase contrast microscope set at magnification of ×400 and equipped with a heating plate (37°C). A wet drop of 20 µl was settled on a pre-warmed clean sterile glass slide and then covered with a cover slip. Subjective motility was assessed microscopically with closed circuit television [27].

Alive sperm percentage

The live and abnormal sperms was predicted by staining procedure [28]. To prepare a stain solution of 100 ml, Eosin (0.67 g/100 ml) and Nigrosin (5 g/100 ml) and water were mixed with magnetic stirrer at 30°C. A drop of diluted semen mixed with one drop of stain were mixed on slide and then smeared on pre-warmed slides and was allowed to dry at 30°C. The mounted smear was examined by ×40 objective of the light microscope. Approximately 200 sperms were counted. The dead sperms were stained red and could be easily seen against black background of Nigrosin stain whereas live sperms appeared transparent.

Abnormal sperm percentage

The whole spermatozoon (head, neck, midpiece and tail) was taken into consideration for classification. 200 sperms were counted and the abnormalities classified according to a system developed by Bane [29] and routinely employed in our laboratory. The abnormalities were recorded as a percentage of the total number of the 200 counted sperms.

Sperm membrane integrity (HOST) percentage

The assay was performed by adding and mixing 0.1-ml of the specimen containing the spermatozoa with l-ml of 100 mOsmol/L hyposmotic solutions (equal amount from fructose-sodium citrate). The solution (sperm mixture) was then incubated at 37°C for 1h. Sperm swelling was estimated after incubation of a drop of well-mixed sample on a slide. A cover glass was put on the slide mixture and observed under a phase-contrast microscope at × 400 magnification. A total of 200 spermatozoa were counted in at least 5 different fields of view [30].

Statistical analysis

Control and treatments Replicates were grouped and represented as mean ± standard error of the mean. One way ANOVA test was performed to compare means under the different treatments including the control all over the experimental period for each day (chilling test) and the freeze-thawing test for the different concentrations of MEEY under each semen parameter. The Duncan multiple range test ($P < 0.05$) were used to arrange means in case of significance. All analyses were performed using the SPSS [31] computerized program software version 14.0.

Results

In cooled semen (Table 1), the olive oil addition to the tris extender revealed significant ($P < 0.0001$) differences in the motile sperm percentage (MSP) and live sperm percentage (LSP) between the different treatments. The highest MSP and LSP were at the concentrations of 4 and 5. On the other hand, the abnormal sperm percentage (ASP) showed significant ($P < 0.0001$) low percentages at 1, 3 and 4. The concentrations 1 and 3 are nearly similar to the control. The addition 1 showed significant ($P < 0.0001$) high acrosome integrity percentage (AIP). In Post-thawed semen (Table 2), the concentration 1 revealed the best sperm motility ($P < 0.0004$) and acrosome integrity ($P < 0.0001$), the concentration 3 gave the superior alive sperm percent ($P < 0.0001$), the concentration 2 exhibited the lowest sperm abnormalities ($P < 0.0001$) with the highest sperm membrane and acrosome integrities ($P < 0.0001$).

Treatment	Motile Sperm (MSP)	Live Sperm (LSP)	Abnormal Sperm (ASP)	Plasma Membrane Integrity (SMI)	Acrosome Integrity (AIP)
0% (Control)	$88.33^{b} \pm 0.83$	$92.67^{b} \pm 1.27$	$18.33^{a} \pm 0.83$	$79.67^{a} \pm 0.17$	$78.33^{c} \pm 0.83$
(0.25%)	$81.67^{c} \pm 0.83$	$88.00^{c} \pm 0.58$	$7.00^{c} \pm 0.58$	$77.83^{abc} \pm 0.36$	$84.33^{a} \pm 0.33$
(0.5%)	$91.67^{a} \pm 0.83$	$93.33^{ab} \pm 0.69$	$13.67^{b} \pm 0.44$	$72.33^{d} \pm 2.17$	$78.33^{c} \pm 0.33$
(0.75%)	$91.67^{a} \pm 0.83$	$94.00^{ab} \pm 0.69$	$7.33^{c} \pm 0.17$	$79.00^{ab} \pm 0.76$	$80.33^{b} \pm 1.01$
(1%)	$93.33^{a} \pm 0.83$	$95.56^{a} \pm 0.29$	$7.33^{c} \pm 0.17$	$75.11^{cd} \pm 1.49$	$80.33^{b} \pm 0.44$
(1.25%)	$93.00^{a} \pm 0.76$	$95.56^{a} \pm 0.47$	$12.67^{b} \pm 0.33$	$75.67^{bcd} \pm 0.44$	$81.67^{b} \pm 0.33$
F-cal	28.96	14.65	92.05	5.79	13.59
P<	0.0001	0.0001	0.0001	0.0003	0.0001

Table 1: *Semen parameters in cattle cooled semen with olive oil tris extender (Mean ± SE).*
Different superscript (a, b,...) in the same column are significantly different using Duncan multiple range test at (P < 0.05).

Treatment	Motile Sperm (MSP)	Live Sperm (LSP)	Abnormal Sperm (ASP)	Plasma Membrane Integrity (SMI)	Acrosome Integrity (AIP)
0% (Control)	$33.00^{bc} \pm 0.82$	$54.60^{bc} \pm 1.85$	$13.40^{bc} \pm 0.50$	$21.60^{d} \pm 0.16$	$65.60^{a} \pm 1.28$
(0.25%)	39.00a ± 0.67	$55.80^{b} \pm 1.93$	$12.40^{c} \pm 0.50$	$43.60^{b} \pm 0.27$	68.00a ± 0.92
(0.5%)	$34.00^{b} \pm 1.63$	$58.00^{ab} \pm 0.94$	10.60d ± 0.27	51.60a ± 0.27	68.00a ± 1.41
(0.75%)	$30.00^{bc} \pm 2.11$	61.40a ± 0.91	$14.80^{b} \pm 0.85$	$23.60^{c} \pm 0.65$	$59.20^{b} \pm 1.10$
(1%)	$29.00^{c} \pm 1.94$	$51.60^{c} \pm 0.69$	$14.40^{b} \pm 0.81$	$23.20^{c} \pm 0.88$	$56.00^{c} \pm 1.14$
(1.25%)	$32.00^{bc} \pm 1.33$	$45.00^{d} \pm 1.23$	$17.60^{a} \pm 0.54$	$21.60^{d} \pm 0.45$	$54.60^{c} \pm 0.50$
F-cal	5.47	17.78	15.07	667.63	30.47
P<	0.0004	0.0001	0.0001	0.0001	0.0001

Table 2: *Semen parameters in cattle diluted semen with olive oil tris extender after freeze thawing (Mean ± SE).*
Different superscript (a, b,...) in the same column are significantly different using Duncan multiple range test at (P < 0.05).

Discussion

The results of our study revealed that, the use of olive oil - enriched tris extender improved Post-thawed sperm parameters with the first and second concentrations and deteriorated with the fourth and fifth concentrations.

Olive oil contains numerous biologically active compounds mainly antioxidant polyphenols (oleuropein) [12,13], hydroxytyrosol [14,15], and tyrosol [16,17], antioxidant flavonoids (luteolin) [15,18] and apigenin [20,21], and other bioactive organic molecules, including squalene [22,23]. These compounds are strongly correlated to the reproductive capacity [9,24,25].

The positive influences in spermatozoa motility that obtained with the inclusion of olive oil into semen diluent may account for its very high content of antioxidant components [32]. Previous studies of possible mechanisms of olive oil phenol action indicated that these compounds are able to scavenge free radicals and to break peroxidative chain reactions.

Oleuropein, due to natural antioxidant properties, eliminates the oxygen free radicals, hydroxyl radicals, and other potentially damaging free radicals of metabolism, with inhibition of the oxidative toxicity [3,33]. These materials can reduce lipid peroxidation through metal chelation [34]. Supplementing the extender of roosters' semen with olive oil improved semen quality when semen samples *in vitro* stored at 5°C for up to 72h [35]. The 0.25% concentration of olive oil had already been recorded to be valuable for the mitochondrial function and DNA integrity; olive oil produced the highest number of sperm per oocyte and the greatest penetration rate [6]. Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) revealed a beneficial effect on the frozen-thawed spermatozoa as much as they decreased the LPO [36]. Olive oil has a high percentage of polyphenolic compounds with antioxidant capacity [37] and they have been linked to benefits for human health [38]. The presence of a simple phenol called hydroxytyrosol (3,4 dihydroxyphenylethanol, HT), with a considerable antioxidant potential, has been recorded in the olive fruit (*Olea europea*), with appropriate positive health effects [37]. It has been recorded that HT lowers the oxidation of low-density lipoproteins, protects in opposition to H_2O_2 cytotoxicity and minimizes lactate dehydrogenase activity [39-41]. A further minor simple phenol isolated from olive oil waste is 3,4-dihydroxyphenylglycol (DHPG). This antioxidant has an ortho-diphenolic construction with an additional hydroxyl group in the β position [42], and potent antioxidant and effective anti-inflammatory characteristics [43]. Acrosome integrity enhancement has been recorded when phenolic antioxidants or oleic acid (both obtained from olive oil) were added to rooster [35] and ram sperm [44]. Inclusion of HT and DHPG antioxidants in the freezing semen diluent exerts a beneficial effect on sperm cells; their scavenging activity against oxygen free radicals reduces the produced f ROS [45] and reduces the LPO. The chemical possessions is positive to sperm as it reduces the ratio of the oxygen free radical responsible for oxidation with subsequent damage of the major cellular macromolecules (e.g. proteins, lipids, and nucleic acids) [10]. So, supplementation of olive oil may ameliorate sperm integrity, and accordingly, sperm function, by changing the lipid components of these dynamic cells. This modification is characterized by decreasing the cholesterol content, thus lowering the cholesterol/phospholipids ratio, in the bilayer membrane of sperm [10]. Miersch., *et al*. [46] postulated that, olive oil reduces the cholesterol content in the bilayer membrane and that nitric oxide is vital for sufficient sperm motility, olive oil may improve sperm quality by enhancing nitric oxide signaling. Oleic acid lowers free radicals, which cause damage to the spermatozoal membrane and decreases the lipid peroxidation, thus improving sperm vitality. In this regard [47] recorded that unsaturated fatty acids improved fertility due to its antioxidant effect. Moreover, olive oil contains high levels of many effective antioxidant compounds mainly polyphenols (e.g. oleuropein [12,13], hydroxytyrosol [14,15] tyrosol [16,17], flavonoids (e.g. luteolin [18,19], apigenin [20,21] and vitamin E (roughly 14 mg per 100g of olive oil).

Conclusion

It could be concluded that, tris extender supplemented with olive oil ameliorated the post –freezing sperm characteristics and consequently high fertilizing potential is expected. This improving effect is related to its high content of antioxidant components that scavenge the free radicals and decrease the oxidative damage.

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Conflict of Interest

I am a single author, so there is not any conflict of Interest.

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