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The effect of acetyl-cysteine and carnitine on post-thaw sperm characteristics of the sand gazelle (*Gazella subgutturosa marica*)

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Abstract
The current study aimed to estimate the effect of antioxidants **(**N-acetyl-cysteine (NAC) and L-carnitine (LC)) as an antioxidant added on tri lady l semen extender of gazelles. The results showed no significant effect on progressive and mass motility characteristics when compared to the control at (P > 0.05). Assessment of acrosome abnormality and membrane integrity were assessed for (NAC) and (LC), the result was 2.40 ± 0.12% for (NAC) for acrosome abnormality and 49.40 ± 3.06% for membrane integrity, whereas the (LC) was (2.40 ± 0.16% for acrosome abnormality and 42.60 ± 2.94% for membrane integrity respectively with no significant differences among groups. on the other hand, the viability was (3.20 ± 0.17%) for tri lady l with (NAC), and (3.42 ± 0.42%) for (LC) added to tri lady l, furthermore, the (NAC) reduced DNA damage when compared to control group (3.20 ± 0.17%) at (P > 0.05). Among all groups, the pregnancy rates do not show significant differences.

Keywords: antioxidants, Gazelle**,** cysteine, carnitine, motility, acrosome**,** membrane integrity**,** DNA damage

Introduction

Sand gazelle (*Gazella subgutturosa marica*) Population and distribution has significantly affected due to the degradation of natural habitats and excessive hunting during the last decade, so that they are living in a small range and in a few isolated groups as a protected area. Saudi Wildlife Authority has research-breeding centers for specific species, one in all them is that the sand gazelle. A technique of importance in the genetic management of endangered species is the cryopreservation of sperm, it's a regular procedure used in a variety of state inclusive assisted reproduction technologies for human and animals and recently for small ruminants and endangered species such as gazelles (Garde J, *et al*, 2007) [19], nevertheless, sperms are undergoing to effective changes in temperature, the formation of the ice crystal, and various stresses such as (physical, chemical, diffusion, and oxidative) throughout the cryopreservation method, that sorely compromise spermatozoon fertility and quality (Ezzati M *et al* 2020) [17], Previous studies showed that its cause changes in sperm morphology and effect on motility, furthermore the membrane extremely sensitive for freezing and cause cry damage as cold shock and formation of ice crystal, the membranes can be expose to various stresses as osmotic variations, lipid and protein restructurings, oxidative stress (Bailey J, *et al*, 2000) [6]. Oxidative stress has been known in concert various intermediary male physiological state by inflicting sperm dysfunction. At normal condition the reactive oxygen species (ROS) are composed due to reduction of oxygen, therefore as a result sperms loss their motility, integrity and fertility (Gandini L *et al*, 2000) [18]. Due to the richness of cell membranes with polyunsaturated fatty acids this increase the sensibility of sperms to oxidative stress. That produce (ROS) (Bansal A, *et al*, 2010) [7]. Highly concentration of polyunsaturated requires effectual antioxidant to preserve against peroxidation and dysfunction associated with sperm. Sperms and seminal plasma have an antioxidant to avoid oxidative stresses. Nevertheless, the capacity of the cells very limited due to the limitation on antioxidants contents to scavenge Oxidants (Storey B, 1997) [32]. Antioxidant component properties reduce the effect of damage from ROS and cold shocks, therefore to improve sperm quality for human (Alvarez J and Storey B, 1989) [5], ram (Aisen E, *et al* 2002) [2], canine (Michael A, *et al*, 2007) [22], (Bucak M, *et al*, 2008) [9], and goat (Bucak M, *et al*, 2009) [27]. The derivatives of amino acid L-cysteine containing sulfur-containing amino acids named thiol compounds are currently used mainly as an antioxidant and cyto protective in mammalian cells. In addition, daily treatment with NAC results in a significant improvement in sperm motility (Safarinejad M and Safarinejad S, 2008) [26]. Even though (NAC) lowering endogenous oxidant levels and to maintain cells against harmful stress, however, it's a poor scavenger and the molecular mechanisms have remained uncertain (Daria E *et al*, 2018) [15]. Furthermore, (Ciftci H, *et al*, 2009) [14] reported that (NAC) has free radical scavenging activity both *in vivo* and *in vitro* respectively. the post-thaw characteristics for bull (Sarıozkan S, *et al*, 2009) [27], ram (Uysal O and Bucak M, 2007) [38], and goat sperm (Bucak M and Uysal O, 2008) [9] were improved by added of (NAC), moreover, its maintain the viability, chromatin structure and membrane integrity of boar sperm during liquid storage at 5 ^{0}C for goat sperm (Szczesniak B, *et al*, 2003). On the other hand, L-carnitine (LC) highly concentrated in the epididymis of sperms, and has a vital role in the production of energy by transferring fatty acids into mitochondria, its transport long chain fatty acids from cytosol into the mitochondria matrix and play critical role in the regulation bioenergetic metabolism and respiration in most spices including gazelle as published by (Aleissa M, *et al*, 2007) [3], moreover, it is protects the cell, mitochondrial membrane, and DNA integrity against free radicals

(Adewoyin M, *et al*, 2017) [1], also its enhancement of sperm fertility, count, and motility (Tahereh M, *et al*, 2019) [35]. Many studies prove that a close interrelationship among semen carnitine (LC) together with attributes of semen moreover, these studies were confirmed amongst diverse species such as of ram, rats, stallions and human (Brooks D, 1979) [8], stallions (Stradaioli G, *et al*, 2004) [33].

Semen diluent composition is a highly important factor influencing freezing ability. The role of extenders is to protect sperms from cold shock and agglutination; maintain stable pH and osmotic pressure. As far as we know, the antioxidant contributes to protect cells against cryodamage, but due to lack an information and study regarding sand gazelle sperm cryopreservation and influences of (NAC) and (LC). Current study designed to carry out an influence adding N-acetyl-cysteine (NAC) and (LC) into triladyl extender and study the effect of them on characteristics of frozen thawing sperms, viability and fertilizable.

Methodology

Gazelles Preparation:

The commercially available diluent (Triladyl by Minitube, Tiefenbach, Germany), was used in this study for the Cryopreservation of semen. The antioxidants, carnitine, lysine and antibodies were obtained from Sigma–Aldrich Chemical Co, USA. Study was conducted in The King Khalid Wildlife Research Centre (KKWRC), Saudi Arabia. 10 male of the sand gazelles (*Gazella subgutturosa marica*), average weight was 15-18 kg and captured manually by using canvas trap around food point, gazelle's heads were immediately covered after captured to reduce the stress. By using the electro ejaculation method semen was collected according to the procedure described by (Al-Eissa M, *et al*, 2007) [4] based on the method published by (Holt W, *et al*.1996). Prior collection the gazelles were sedative by using xylazine (8.4 ± 1.5 mg/kg) and ketamine hydrochloride (6.9 ± 1.5 mg/kg). 120 ejaculates were collected from the gazelle (12 ejaculates for each gazelle), semen was collected in a conical tube then immediately transfer to water bath at 37C and evaluated within 30 to 60 min of collection. Samples with a sperm concentration of more than 2.8×10^9 /ml, and least 80% motility were used for CASA. Ejaculated is measured by graduated pipette and sperm count by photometer sperma cue (model 12300/0500, Minitube, Germany). 15% of egg yolk and 6% glycerol (v/v) were added to triladyl containing N-acetyl-cysteine (NAC), (LC), then pH was adjusted to 6.8. Each ejaculate was divided into 3 portions then dilute to $60x10^6$/ml sperms. 0.25ml French straws were used to load diluted semen samples then frozen by digital freezing machine (Freeze control, Cl-8000, from Crtologic pty.ltd) afterward, then transferred to vapor nitrogen for 24 hours later on, samples were thawed in water bath for 20s at 38 ^{0}C

Sperm motilities Assessment

Sperm velocity parameters by computer-assisted sperm analyzer (CASA) such as Hamilton-Thorne IVOS 11, Biosciences, Beverly, MA 01915 USA). IVOS CASA system yields precise, objective assessment, and relabels result in different semen parameters based on the estimating of individual sperm (Perumal P, *et al*, 2014) [24]. Recent reports by (Mortimer S, *et al*, 2015) [23], proposed that, CASA does not estimate the proportionality of sperms motile only but also estimates other sperm velocity. The system is equipped with a high-resolution camera connected to a phase-contrast microscope moreover it is needs standardization and validation concerning semen preparation, calibration, and technical settings to provide precise results. For each evaluation 5 µl of diluted sample was placed in the sperm chamber and up to 15 microscopic fields were analyzed to include at least 100 sperms. CASA system is able to measure many sperm velocity include curvilinear velocity (VCL), straight linear velocity (VSL), mean velocity (VAP), linear coefficient (LIN) and amplitude of lateral head displacement (ALH)

Sperm abnormalities Assessment

According to methods published by (Sekoni *et al*. 1988) [29], morphology of sperms was examined by using hancock Stain (P/N HCS-101), the staining mixture consisted of 5% of nigrosin and 1% eosin B in 3% sodium citrate dehydrates solution. Drop of semen was added to drop of the stain, then mixed gently, a fresh smear was applied on slide. At least, 100 sperms, live -dead sperms in an ejaculate were counted and estimated by a microscope at magnification 40x.

Integrity membrane evaluation

Evaluation of membrane integrity carried out by using HOS test according to the method described by (Jeyendran R, 1984) [21], 10 ml of semen with 20 ml of hypo osmolar solution were examined by coumting 200 sperm at magnification 40x. Sperm with swollen tail was consider as positively to HOS test.

DNA viability assessment

According to the method was described by (Yaniz J *et al*, 2013) [40] the DNA viability was estimated by a commercial kit contain acridine orange- propidium iodide, 10 ml of semen with 20 ml of stain applied on glass slide then by using fluorescence microscope the viability was estimated according to the procedure results in selective labelling of live and dead cells visualized in green and red fluorescence colors respectively. A total of 200 sperms were assessed/sample.

Fertility rate

The evaluation of sperm, morphology and quality are highly significant during an evaluation of male fertility due to the strong correlation between them. An accurate morphological assessment for each individual sperm (Varner, D, 2016), 10 female gazelles were artificially inseminated (AI) with frozen-thawed semen. The ability of sperm for fertilizing was estimated based on the non-return rates (proportion %) by the formula given below, the effective non-return rates (NNR) was determined at 80 days post-insemination.

$$NNR = \frac{\text{total no of females inseminated} - \text{no of females returning to estrus}}{\text{total no of females inseminated}} * 100$$

Table 1. Mean (±SE) of post-freeze-thawing sperm motility for Gazelle semen supplemented with different antioxidants.

Groups	Progressive motility (%)	Mass motility (%)
Control	59.50±2.14	68.32±3.55
(LC)	57.21. ±2.4	69.22±2.14
(NAC)	57.44 ±1.3	71.54±6.22

All values are expressed as mean ± SE, Different superscripts in the same column demonstrate significant differences (P> 0.05).

Statistical analysis

Statistical analyses were performed by using the SPSS 17 package program. Results were expressed and as mean ± SEM for all sperm motility, velocity characteristics, abnormalities, and (HOST) tests by analysis of variance significant differences between the groups. The analysis of variance used was compared for DNA damage between groups. Pearson's chi-square test was used to compare the pregnancy rate.

Results

The antioxidant added to diluent in this experiment do not reflect any significant on sperms progressive or, motility characteristics by (CASA) when compare to control group at (P > 0.05) as show in table 1, (57.21. ±2.4%) for (LC) and (57.44 ±1.3) for NAC %) for Progressive motility and furthermore (69.22±2.14), (71.54±6.22) for mass motility. Data of sperm characteristic's by CASA as showed in table 2 are (VAP, VSL and VCL, ALH and LIN) were. The motility pattern parameter for VAP was (114.4±2.1), (118.34±42) for (LC), (NAC) respectively and (119.4±1.2) for control group with no significant difference among them. There were no significant differences in the (VSL) for all treatment compere to control they were (76.26±4.19) for (LC), (77.46±3.6) for (NAC) and (80.12±2.0) for control. None of the (VCL) values were significantly different between groups even (LC) (125.3±6.7), (NAC) (143.6±1.7) or control (134.6±2.4). Furthermore. The (ALH) also shows that, the (LC) and (NAC) values were (10.3±2.8) and (12.4±1.4) respectively and significantly different to the control (22.4±0.24) at (P> 0.05). Finally, linearity (LIN) is the linearity of the curvilinear trajectory calculated as (VSL/VCL) × 100, according to table 2 we don't show and significant between three groups (LC) was (18.4±4.3), (NAC) was (16.62±4.3) and control was (17.34±6.2).

Table 2: Mean (±SE) CASA determination of sperm velocity in post-freeze-thawing sperm of Gazelle semen supplemented with different antioxidants.

Group	VAP	VSL	VCL	ALH	LIN
Control	119.4±1.2	80.12±2.0	134.6±2.4	22.4±0.24	17.34±6.2
(LC)	114.4±2.1	76.26±4.19	125.3±6.7	10.3±2.8[a]	18.4±4.3
(NAC)	118.3±4.2	77.46±3.6	143.6±1.7	12.4±1.4[b]	16.62±4.3

All values are expressed as mean ± SE, values with different superscripts the column demonstrate significant differences at (P> 0.05). VAP=Average value (mm/s), VSL=Rectilinear speed (mm/s), VCL=Curvilinear velocity (mm/s), ALH=, Amplitude of lateral head (mm) and LIN=Linearity index (%).

Semen extender provided with (NAC) and (LC) give a result for acrosomal abnormality as (2.60% ± 0.24) and (2.40% ± 0.16) individually, lower than control and with a significant difference at (P > 0.05) compare to control 8.00% ± 0.44. Regarding the integrity of membrane There were no significant differences between (NAC), (LC) (49.40 ± 3.06%) and (42.60 ± 2.94%), respectively) compare to control (41.40±4.74) but it's clearly that the treatment with (NAC) had more positive effect on the membrane integrity when compare with others. Moreover, the statistical analysis for data collected for viability showed, there are no significant difference between the two groups although the treatment groups were reduced viability compare with control at (P > 0.05) as seen in table 3. Pregnancy rates show no significant difference at (P > 0.05) between the diluent groups (NAC) was 44%, (LC) was 50% and control 47% as showed in table 4.

Table 3: Mean (±SE) for Acrosomal abnormality, integrity of membrane and viability of post-freeze-thawing sperm of Gazelle semen supplemented with different antioxidants.

Group	Acrosomal abnormality (%)	Integrity of membrane (%)	Viability (%)
Control	8.00 ± 0.44	41.40±4.74	4.19 ± 0.24
(LC)	2.40 ± 0.16*	42.60±2.94	3.42±0.42
(NAC)	2.60 ± 0.12*	49.40±3.06	3.20 ± 0.17

All values are expressed as mean ± SE, Different superscripts in the same column demonstrate significant differences (P> 0.05).

Table 4: Pregnancy rates after artificial insemination with freeze-thawing semen of gazelle.

Groups	Pregnancy rate (%)
Control	47%
(LC)	50%
(NAC)	44%

All values are expressed as mean ± SE, Different superscripts in the same column demonstrate significant differences (P> 0.05).

Discussion and conclusion

Sperms Motility reflects the ability of a sperm to transfer through the female genital tracks and acquire ability to penetrate several ovum membranes and fertilizing it. A positive correlation between sperm motility and fertilizing capacity has been shown in many species (Steven P. *et al* 2011) [31]. In this study we examined the influence of (NAC) and (LC) as an antioxidant on sperm characteristics and oxidative stress characteristics post freeze–thawing of gazelle's semen, our results were agreement with the study achieved by (Bucak M, *et al*. 2010a). From the previous result, it can be realized that, motility characteristics do not have sufficient information for the sperms quality and don't showed differences between the effect of (NAC) and (LC) as highlighted in Table 2, 3. Comparison of the differences between (NAC) and (LC) the mean values of the Acrosomal abnormality was lower than control and with a significant difference at (P > 0.05) compare to control, membrane integrity within (NAC) and (LC) revealed that there were no significant changes between them compare to control. Our results show similarities with the previous studies, reported that the sperm parameters do not show significant relationship between antioxidant (LC) therapy and semen parameters (Sedigheh A, *et al*, 2016). This study show that, the triladyl with antioxidants was very efficient in preservation of mass motility and integrity of plasma membrane (Vera.O *et al*, 2009), also the antioxidant, such as (NAC) and (LC) can effectively improve semen parameters, it was similar to studies done by (Sedigheh A, *et al* 2016) for men and bovine (Bucak M *et al*. 2010 a) [13], goat (Bucak M, *et al*, 2010b) [12] against cryo damage. (NAC) and (LC) may equalize free radicals and protects sperm membrane against stress. In addition, structure of the plasma membrane is consisting of high levels of polyunsaturated fatty acids (PUFAs) that improve of membrane flexibility and makes it susceptible to attacked by ROS and eventually loss in fertility (Eskenazi B, *et al*, 2005) [16]. Cryoprotectants are improved recoveries of cells after cryopreservation and can assist in reducing stress effects. Recently many studies focused mainly on the different additives in the diluent in

order to acquire the higher post-thaw motility and fertility rates. Supplementation with (NAC) and (LC) to the cryoprotectants contributes in the protection of DNA materials against cryodamage in the present study, our result was similar with previous study mentioned by (Bucak M, *et al*. 2010a) [13]. Recent studies showed that long-term exposure of sperm to long-term sperm storage caused a theatrical damage in sperm characteristics, this study aims to focus on importance of antioxidative and its role as cryo protectant. During the (HOS) test, sperm with intact plasma membranes go through swelling due to the inflow of water and thus, increase in volume to institute equilibrium between the extra- and intracellular compartments, the test was applied to goat (Ranjan R, *et al*. 2009) [25]. Freezing and long-term storage makes sperm plasma membrane capable to loss its integrity then function, resulting from reactive oxygen species. Viability HOST response showed clearly that, (NAC) and (LC) don't have any protective effect to protect membrane during cryopreservation and remains to be examined its parallel to result for ram by (Aisen E, *et al* 2002) [2]. Incubation of the sperms by using fluoro chromes is considered important prior to the assessment of membrane integrity as reported by (Yaniz J, *et al*, 2012) [39]. Since the AO penetrating live sperm and bound to DNA while the PI penetrating the damaged sperm and replacement of AO. our result showed that, the antioxidants (NAC), (LC) unable to maintain DNA integrity, these results was different than study reported on bovine sperm by (Bucak M, *et al*, 2010A) [13], for the signify role of (NAC) and (LC) in the maintenance of DNA integrity. The diluent contains scavengers that are appropriate to maintain sperms against oxidative stress during cryopreservation, moreover, new findings indicated for no correlation between improvement fertility and supplemented to the diluent, and this finding with our results (Sarıozkan S, 2014) [28]. The following conclusions can be drawn from the present study antioxidants enhanced sperm abnormalities and acrosomal damage for thawed sample. Also, (NAC), (LC) providing an improved DNA integrity. Further research should be done to investigate the effects of different antioxidant on cryopreservation and fertility of endanger Species specially gazelle of the Arabian Peninsula due to Scarcity of information about them.

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