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Formation of Hydrogen Peroxide by Chilled Goat Spermatozoa and the Effects of Dead Spermatozoa on Motility Characteristics

Research Article

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ABSTRACT

In this study, the formation of hydrogen peroxide (H_2O_2) by chilled goat spermatozoa was measured. Furthermore, the effects of dead spermatozoa on motility characteristics were studied. Fresh collected ejaculates from five Shami bucks were centrifuged and virtually all seminal plasma was removed. A part of the collected spermatozoa was killed by two ways: the first by repeated freezing in liquid nitrogen and thawing at 37 ˚C in water bath and the second by adding the spermatozoa to double distilled water. Two experiments were conducted after two hours of samples incubation in tris-egg yolk (TEY) medium at 5 ˚C. In the first experiment, a fluorometric assay with 10-acetyl-3,7-dihydroxyphenoxazine agent as a probe for H_2O_2 detection was used to measure H_2O_2 formation. In the second experiment, the effects of adding 0 (control), 25, 50 and 75 % (V/V) of dead spermatozoa to live ones on sperm motility were assessed using computer-aided sperm analysis (CASA). Hydrogen peroxide was generated from live and dead chilled spermatozoa and the amounts of this agent increased with time. Moreover, clear significant differences ($P<0.05$) were observed between the generated levels from dead spermatozoa compared to live ones. The dead spermatozoa by repeated freezing-thawing treatment produced the higher H_2O_2 amount ($P<0.05$). The values of percent motile spermatozoa (MOT %), percent of progressively motile spermatozoa (PMOT %) and average path velocity (VAP) were significantly ($P<0.05$) reduced compared to controls when dead spermatozoa were added. The negative effect on the previous CASA parameters was increased when the percentages of dead spermatozoa were increased whatever the way of sperm death was. In conclusion, the high formation of H_2O_2 from dead chilled goat spermatozoa may be responsible for motility decreased. The removal of dead spermatozoa from incubation medium could help to improve the motility characteristics of chilled goat sperm.

KEY WORDS dead spermatozoa, goat, motility, reactive oxygen species.

INTRODUCTION

Reactive oxygen species (ROS) are a generic name given to a variety of molecules and free radicals derived from molecular oxygen. Within anaerobic system, the formation of ROS is unavoidable; the most important agents being the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl-radical ($OH^{\cdot}$). Hydrogen peroxide has been detected as a metabolic product of living rabbit spermatozoa (Holland and Story, 1981).

Moreover, we previously noted the kinetics of H_2O_2 generation from live ram and bull spermatozoa (Alomar and Donnay, 2006; Alomar *et al.* 2016). Mitochondria appear to be the main ROS source, although NADPH-oxidase in the sperm plasma membrane and nitric oxide synthase in the sperm acrosome have been shown to largely contribute in ROS formation (Aitken and Buckingham, 1992; Rodriguez *et al.* 2005). On the other hand, ROS were generated by dead ram and bovine spermatozoa via an aromatic amino acid oxidase (AAAO) system (Shannon and Curson, 1982a;

Shannon and Curson, 1982b; Upreti *et al.* 1998; Alomar and Donnay, 2006; Alomar *et al.* 2016). When reactive oxygen species are found in low concentrations, they act as mediators of normal sperm functions, while when they are produced in excess they are highly toxic to the cells. In this respect, ROS were involved in the sperm capacitation and hyperactivation processes (Aitken and Fisher, 1994; Agarwal *et al.* 2006) by stimulating intracellular cAMP production and tyrosine phosphorylation (Aitken *et al.* 2004; Ford, 2004).

In contrast, ROS could harm spermatozoa during storage and cause lower motility values, DNA fragmentation and membrane damage due to lipid peroxidation (Nair *et al.* 2006; Wagner *et al*. 2018; Bui *et al.* 2018).

Tris-egg yolk (TEY) is one of the most commonly used medium for cooling and cryo-preserving goat spermatozoa. It has been shown to have a beneficial effect on spermatozoa as a protector of the plasma membrane and acrosome against temperature related injury (Purdy, 2006).

However, one of the problems about the media containing egg yolk used for goat semen cryopreservation has been attributed to egg yolk coagulating enzyme (EYCE) which could damage sperm cells (Leboeuf *et al.* 2000).

Moreover, it has been hypothesized that TEY extenders may enhance the formation of hydrogen peroxide, due to the presence of iron and the other transition metals according to Fenton type reactions (Bilodeau *et al.* 2002).

In general, chilled semen suffers from a decrease in motility and structural integrity (Appell *et al.* 1977; Paulenz *et al.* 2002; Slavador *et al.* 2006). Not only temperature degree may cause such effect but also the toxicity resulted from associated dead spermatozoa could play a major role in the decreasing motility of live spermatozoa. In this respect, motility and viability of equine spermatozoa were adversely affected when cooled shocked and dead sperm was added (Trokey and Merilan, 1982). None of literature studies have described hydrogen peroxide formation from chilled goat spermatozoa. Moreover, there are no reported results showing the effect of dead goat spermatozoa on motility characteristics. For that, the present study aimed to analyse the generation of hydrogen peroxide from both live and dead chilled goat spermatozoa and to characterize sperm motility by CASA system with different concentrations of dead sperm in TEY medium.

MATERIALS AND METHODS

Animals and semen processing

This study was carried out at Der Al Hajar Animal Production Research Station, 33 km south-east of Damascus.

Semen was obtained from five sexually-experienced Shami bucks, aged between 3 and 4 years. Semen samples were collected with the aid of an electro-ejaculator (Minitube Electro Ejaculator, Germany) administrating a series of 20 cycles pulses of short electrical stimuli with each cycle (two seconds impulse, then two seconds interval) delivering a slightly higher intensity (from 0 Volt to 20 Volt maximum) until semen production. Upon collection, the semen was immediately evaluated for its general appearance and volume. An initial analysis of sperm motility was performed using CASA system (Hamilton Thorne Biosciences, USA). Sperm samples with a motility score ≥ 75% of motile sperm and a concentration of ≥ 1×10^9 spermatozoa/mL were employed. The semen samples were then diluted with a glycerol free TRIS-based medium (2.44 g tris (hydroxymethyl) aminomethane, 1.36 g citric acid monohydrate and 1 g glucose in 80 mL of distilled water) at a ratio of 1:9 (semen to tris solution, v:v) and centrifuged at 1000 × g for 15 min. The seminal plasma was discarded and the sperm pellet was suspended to a final concentration of 500×10^6 spermatozoa/mL with TRIS medium. It must be noted that the present study was approved by the Local Scientific and Ethical Committee of the Atomic Energy Commission of Syria (AECS), Damascus, Syria (permit number 36/ZM1-2017).

Experimental design

Two experiments were conducted in the present study using 30 ejaculates. To diminish the effect of individual variation between the bucks a mixture of semen from five animals was used in each assay. In the first experiment, hydrogen peroxide formation from chilled live and dead goat spermatozoa was determined. In the second experiment, the effect of dead spermatozoa on motility was assessed. For the two experiments, a part of the collected semen without seminal plasma was immediately killed by two different treatments, the first by repeated freezing in liquid nitrogen and thawing at 37 ˚C in water bath. The second was by adding the spermatozoa to one mL of double distilled water and after 10 minutes of centrifugation at 1000 × g the sperm pellet was restored. Lack of spermatozoa viability was confirmed by viability analysis using fluorescent microscopy after dual staining of propidium iodide (PI) and bisbenzimide (BIS, Hoechst 33342); (Alomar *et al.* 2006).

Sperm concentrations of the three spermatozoa types (live, dead by distilled water and dead by freezing-thawing process) were calculated using a hemocytometer (cell counting chamber; Neubauer Improved Marienfeld, Germany). For the two experiments, the three types were incubated at 5 ˚C in tris-egg yolk (TEY) medium for two hours.

The TEY medium was prepared as a 300 mOsm/Kg solution contained the following: 2.44 g tris (hydroxymethyl) aminomethane, 1.36 g citric acid monohydrate and 1 g glucose in 80 mL of distilled water, plus 20 mL of egg yolk, bringing the total volume to 100 mL.

In the first experiment, and after the incubation of the three spermatozoa types separately at 5 ˚C at 50 × 10^6 in mL, sperm suspensions were diluted to a final concentration of 10 × 10^6 spz/well in TEY medium for H_2O_2 analyses. In the second experiment, spermatozoa samples were divided into aliquots and suspensions of dead spermatozoa were added to achieve final volume of 0.5 mL per aliquot bringing the total spermatozoa concentration to 50 X 10^6 in mL with 0, 25, 50 and 75% levels of dead spz. Each of the two experiments was repeated for three times.

Measurement of hydrogen peroxide

Amplex red (10-acetyl-3, 7-dihydroxyohenoxazine, Molecular Probes) was used to monitor hydrogen peroxide (H_2O_2) production. In the presence of horseradish peroxidase, amplex red reacts with H_2O_2 in a 1:1 stoichiometric reaction to produce resorufin, a highly fluorescent end product. A stock of amplex red was prepared in DMSO (10 m*M*), while horseradish peroxidase was prepared in phosphate buffer (450 unit/mL, pH 7.5). Both stocks were stored at -20 ˚C until the assay. Twenty µL of the sperm suspensions containing 10 × 10^6 of the three spermatozoa types were added to 96-well plates (Nunc, Roskilde, Denmark). Hydrogen peroxide standards (50, 25, 12.5, 6.25 and 3.12 µM) were prepared extemporaneously in TEY medium.

Eighty µL of TEY containing 40 µM amplex red and 1 unit of horseradish peroxidase/mL was added to each well. Sperm samples, standard solutions and blanks were assayed in duplicate. Microplates were incubated at 5 ˚C, and the generation of H_2O_2 was measured using a fluorimeter (excitation wavelength, 530 nm; emission wavelength, 590 nm).

Fluorescence was recorded at 10 minutes intervals for 30 min. The concentration of H_2O_2 was determined from the standard curve based on the blank-corrected fluorescence for each measured time point and expressed in µM.

Motility analyses

The motility characteristics of the sperm were assessed by CASA technique, using the Hamilton Thorne motility analyzer (HTM version 12.3, USA). Five microliters aliquots of diluted spermatozoa were placed in the system lame and loaded into the analyzer. At least three fields were counted for each sample. The motility characteristics included in the analysis were: percent motility (MOT %), average path velocity (VAP, µm/s), percent linearity (LIN %), percent straightness (STR %), and the percent of sperm showing progressive motility (PMOT %).

More details about these parameters are given by Mortimer (2000). The HTM system settings of goat spermatozoa are presented in Table (1).

Statistical analysis

Statistical analysis was conducted with the Minitab program (Minitab Coventry, United Kingdom). The normality of values distribution was first tested with the Shapiro Wilk test. Data were subjected to a factorial analysis of variance general linear model procedure (GLM) followed by multiple pairwise comparisons using a post-hoc (Tukey test). The threshold of signification was set at $P < 0.05$.

RESULTS AND DISCUSSION

During 30 minutes of sperm incubation and at each time point of the analysis (10, 20 and 30 min), H_2O_2 level was significantly different between dead spermatozoa (by double distilled water and also by freezing and thawing) and live ones (Figure 1).

At 30 min point, the dead sperm by freezing and thawing showed the highest formation rate of H_2O_2, while live spermatozoa at 10 min point had the lowest formation rate (36.58 µM *vs*. 0.95 µM). No significant differences ($P>0.05$) were noted between the three time points for live type, while clear and significant differences were observed between these time points for dead spermatozoa by distilled water and also for the dead ones by freezing and thawing treatment.

As indicated by the data in Tables 2 and 3, the CASA parameters (MOT %, PMOT % and VAP) in samples containing the added dead spermatozoa declined ($P<0.05$) more than did in the controls for the two different types of dead spermatozoa.

Moreover, no significant differences for motility parameters (STR % and LIN %; $P>0.05$) were observed between the controls and the samples containing the different dead spermatozoa concentrations.

However, it was obvious from the Table's data that all CASA parameters values did not differ between the dead spermatozoa by distilled water and those dead by freezing and thawing treatment. It is well known that freezing-thawing process impairs the function of spermatozoa and has a negative effect by causing spermatozoa to generate significant amounts of ROS (Salamon and Maxwell, 1995; Ball *et al*. 2001).

Anyhow, only a few researches indicated ROS production from chilled spermatozoa (Chatterjee and Gagnon, 2001).

The present work for the first time provides evidences of hydrogen peroxide formation from chilled goat spermatozoa.

Table 1 The settings for the Hamilton Thorne Biosciences system version 12.3 used to evaluate goat semen

Parameter	Setting
Frame rate (Hz)	60
Frames acquired (no)	45
Minimum contrast	70
Minimum cell size (pixels)	5
Low average path velocity (VAP) cut off	20
Low straight line velocity (VSL) cut off	5
Non-motile head size (pixels)	10
Non-motile head intensity	80
Static size limit (min/max)	0.60/4.32
Static intensity limit (min/max)	0.20/1.92

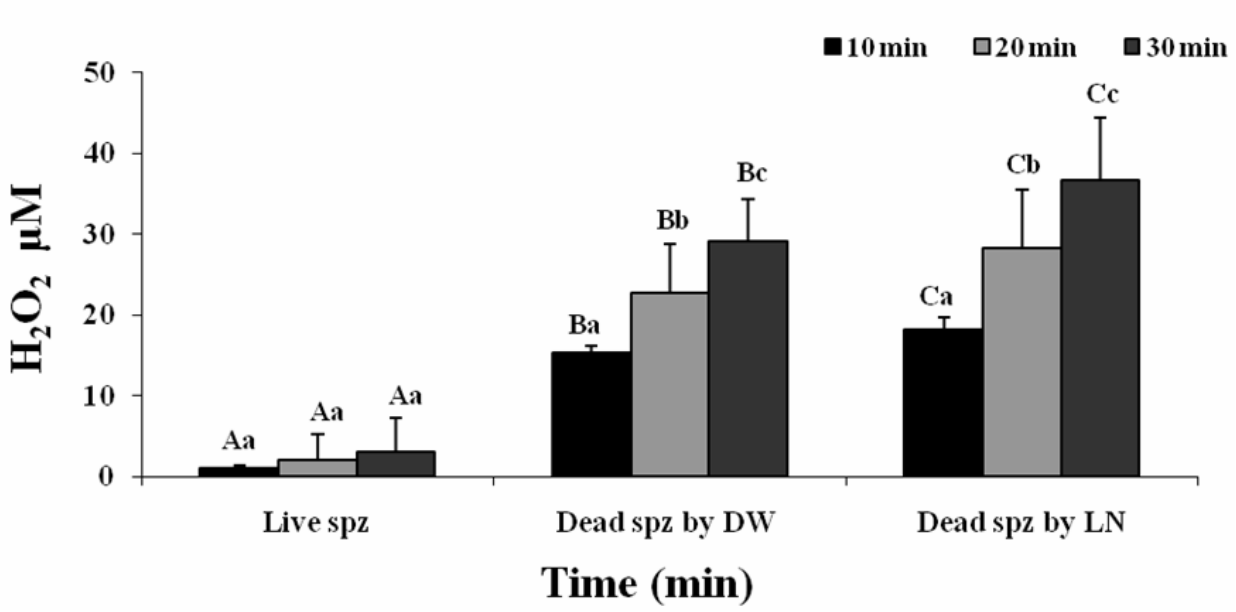

Figure 1 Generation of hydrogen peroxide (H_2O_2; mean±Sd) by live and dead chilled goat spermatozoa during 30 min of incubation in TEY medium

Bucks spermatozoa were incubated in a 96-well micro-plate at concentration of 10×10^6 spermatozoa/well

Different letters (A-C) between spermatozoa types (live, dead by distilled water, dead by liquid nitrogen) at each time point denote significant difference ($P<0.05$)

Different letters (a-c) between time points at each spermatozoa type denote significant difference ($P<0.05$)

Spz: spermatozoa; DW: distilled water and LN: liquid nitrogen

The cooling stage of semen at 5 ˚C which precedes freezing phase is one of the fundamental steps in cryopreservation and it is a very important step to achieve the balance between semen and the surrounding medium.

Any deterious effects during this vital step may have serious consequences on the spermatozoa. The data here confirm our previous results using fresh live and dead goat sperm which were also capable of generating hydrogen peroxide but with different concentrations levels (data not shown). However, not only the status of the spermatozoa (chilled *vs*. fresh) was different between our present and previous results, but also the incubation medium (tris-egg yolk *vs*. tyrode albumin lactate) and the temperature degree (5 ˚C *vs*. 37 ˚C).

It must be noted that ROS formation from spermatozoa may be largely related to the experimental conditions especially the content of semen medium and the incubation degree (Shannon and Curson, 1982a; Shannon and Curson, 1982b). Hydrogen peroxide was generated in higher and significant concentrations from dead spermatozoa compared to live ones whatever the way of sperm death was. In literature, several mechanisms have been proposed for the formation of ROS by spermatozoa (Sanocka and Kurpisz, 2004; Ford, 2004; Wagner *et al*. 2018). The most reported ones are NADPH – oxidase in the sperm plasma membrane and AAAO system in the tail of dead sperm (Shannon and Curson, 1982a; Shannon and Curson, 1982b; O'Flaherty *et al*. 2006; Alomar *et al*. 2016).

Table 2 Mean (±Sd) of three ejaculates from five bucks of CASA parameters for percent motility spermatozoa (MOT %), percent of sperm showing progressively motile (PMOT %), average path velocity (VAP) percent straightness (STR %) as well as percent linearity (LIN %) in spz samples containing various concentration of dead spermatozoa by double distilled water

Dead spz (%)	MOT (%)	PMOT (%)	VAP (µm/s)	STR (%)	LIN (%)
0	84.00±4.97[a]	16.44±4.06[a]	80.78±10.64[a]	55.44±2.88[a]	29.44±3.47[a]
25	70.44±6.87[b]	12.22±3.61[b]	73.89±5.62[b]	57.44±3.11[a]	30.67±3.71[a]
50	61.22±6.86[c]	9.33±3.61[c]	69.89±5.62[c]	58.78±3.11[a]	30.56±3.71[a]
75	41.11±8.87[d]	4.67±2.59[d]	60.33±6.65[d]	56.00±2.45[a]	29.33±2.23[a]

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Table 3 Mean (±Sd) of three ejaculates from the five bucks of CASA parameters for percent motility spermatozoa (MOT %), percent of sperm showing progressively motile (PMOT %), average path velocity (VAP) percent straightness (STR %) as well as percent linearity (LIN %) in spz samples containing various concentration of dead spermatozoa by repeated freezing in liquid nitrogen and thawing in a 37 ˚C water bath

Dead spz (%)	MOT (%)	PMOT (%)	VAP (µm/s)	STR (%)	LIN (%)
0	86.77±3.45[a]	17.33±3.16[a]	79.67±12.68[a]	56.89±3.37[a]	30.00±3.31[a]
25	70.44±6.87[b]	10.78±3.27[b]	70.44±8.47[b]	55.56±3.28[a]	28.22±3.12[a]
50	61.00±3.87[c]	8.33±2.56[b]	65.89±5.16[c]	56.00±3.04[a]	28.56±3.12[a]
75	40.22±10.70[d]	5.89±1.90[d]	61.44±7.25[d]	56.56±4.00[a]	29.11±2.80[a]

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

The exact reason for the higher H_2O_2 formation rate from dead spermatozoa by repeated freezing-thawing treatment is unclear. However, the damage of sperm membrane during the freezing process may be responsible for such a high production level.

In bull, ROS generation from semen diluted in TEY could be located at the surface membrane of spermatozoa because of the presence of transition metals (Bilodeau *et al.* 2002). The presence of transition metals free or bound to membrane lipids can induce lipid peroxidation and the generation of toxic ROS (Halliwell and Gutteridge, 1984).

Anyhow, the content of phenylalanine in egg yolk could also play an important role in hydrogen peroxide formation from goat spermatozoa especially from the dead ones. Phenylalanine is the main AAAO substrate in sperm (Shannon and Curson, 1282a). Dead bull and ram spermatozoa generated a significant amount of hydrogen peroxide when tyrode albumin lactate medium was supplemented with phenylalanine (Alomar and Donnay, 2006; Alomar *et al.* 2016).

In the present study, MOT %, PMOT %, and VAP values showed an important decrease after the incubation of dead goat spermatozoa with live sperm. However, the values of both LIN % and STR % did not differ from controls. VAP is the most important sperm velocity parameter in CASA system, while LIN % and STR % describe sperm trajectory. Thus, dead goat spermatozoa in our experiment have affected the velocity and the percentages of motile sperm but not their trajectory. In contrast to our results, the presence of 75% of dead equine sperm did not adversely affected MOT % or PMOT % of cooled-stored semen (Brinsko *et al.* 2003). The total spermatozoa concentration (25×10^6 spz/mL *vs.* 50×10^6 spz/mL) and the content of seminal plasma in the samples (10% *vs.* 0) were the main differences between Brinsko and co-workers study and ours.

The high concentrated dead spermatozoa in the present study could exert more deleterious effects on live motile sperm. On the other hand, the role of seminal plasma as a supportive medium for spermatozoa is ambiguous if not contradictory (Vishwanath and Shannon, 1997). The presence of 40-50% seminal plasma during storage for 24-48 h was detrimental to sperm motility (Jasko *et al.* 1991; Pruitt *et al.* 1993). Higher progressive sperm motility was found after 24 and 48 h of cooled storage in the samples that contained 1.25% or 2.5% of seminal plasma than in samples containing 10% (Todd *et al.* 2001). Moreover, the non-washed goat spermatozoa which were diluted in 11% egg yolk extender showed significant deterioration in post-thaw cell viability (Memon *et al.* 1985). Thus, removing seminal plasma from our samples could not possibly be the cause of the observed motility decrease. The high formation of hydrogen peroxide from dead spermatozoa may be the most eventual reason for such decrease.

Generally, sperm cell structures such as membranes and chromatin may suffer from oxidative damage resulted from ROS and it remains to be determined whether antioxidant supplementation may protect them. In this respect, the quality of chilled Boer goat spermatozoa was improved when a tris-based extender supplemented with ascorbic acid was used (Memon *et al.* 2013).

Anyhow the removal of dead spermatozoa or the inactivation of toxic products (such as H_2O_2) from the incubation medium could enhance the motility during cooling process.

CONCLUSION

Based on the results of the present study, it can be concluded that chilled goat spermatozoa generate hydrogen peroxide and higher levels of H_2O_2 were produced from dead sperm. Moreover, dead spermatozoa negatively affected

sperm motility and these effects increased when the concentrations of dead sperm were increased. Removal of dead chilled goat spermatozoa from the incubation medium could help to improve motility characteristics whatever the way of sperm death is. The present results may help in developing a deeper understanding of chilled goat spermatozoa storage and could have applications in cooling and freezing process.

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