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EFFECT OF ADDING N-ACETYLCYSTIENE AND *AVENA SATIVA* EXTRACT TO TRIS EXTENDER ON POST-CRYOPRESERVATIVE SEMEN CHARACTERISTICS OF HOLSTEIN BULLS

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Abstract

The objective of this study was to evaluate the effect of tris-extender supplemented with various concentrations of NAC and aqueous extract of (*Avena sativa)* seeds (AEASS) on bull semen characteristics. Pooled bull semen were extended with tris-citrate-fructose egg yolk diluents to achieve 60 million motile spermatozoa. Pooled semen was equally divided into nine treatments in the experiment as following : Tris extender only as Control group (C), T_1 contain 2mM NAC, T_2 contain 4mM NAC, T_3 contain 1% AEASS, T_4 contain 1.5% AEASS, T_5 contain 2mM NAC and 1% AEASS , T_6 contain 2Mm NAC and 1.5% AEASS, T7 contain 4Mm NAC and 1% AEASS, T8 contain 4Mm NAC and 1.5% AEASS. Extended semen were subjected to semen freezing protocol. Semen assessment including motility, viability, abnormality, intact sperm membrane (hypo-osmotic swelling test) and acrosome integrity percentage were carried out for both chilled and frozen semen. Results showed that sperm motility after chilling was enhanced in groups treated with various concentrations of NAC and AEASS. In frozen semen after 48 hrs, 1 month and 2month results showed that all the treatments gave the best significance ($pd \leq 0.05$) in sperm motility, viability, intact sperm membrane (hypo-osmotic swelling test) and acrosome integrity percentage in comparison with the control group. All treatments showed numerically decreasing in plasma membrane and acrosome integrity after all the periods of PC compared with the control group. It is concluded that addition of NAC and AEASS to Tris extender had an important role in enhancing some of PC semen characteristics of Holstein bulls.

Key words : N-acetylcystiene, *Avena Sativa*, Semen characteristics, Holstein bulls.

Introduction

Semen cryopreservation is a well-established procedure used in human and veterinary assisted reproduction technology (ART) applications. Over the last 50 years, it was used for genetic improvement in livestock of beef and dairy cattle. It is also used to control venereal diseases and facilitate management of cattle herd fertility. In human, it is usually associated with male fertility preservation which is usually required prior to cancer therapy (Nangia *et al.*, 2013). Spermatozoa are characterized by plasma membrane fluidity and low water content which make it more resistant to cryodamage compared to other cell types (Agca *et al.*, 2002). However, cryopreservation have been shown to induce deleterious changes of sperm structure and function (Watson, 2000). Cryopreservation processes lead to the generation of reactive oxygen species (ROS) that impair sperm motility, membrane integrity, and fertilizing ability (Bilodeau *et al*., 2000; Gsdea *et al.*, 2004; Hammer stedt, 1993). Although bovine semen has natural defense system against the ROS attack, it is insufficient under cryopreservation due to stress (Nichi *et al.,* 2006). In recent years, many cryoprotective agents have been used as a cryoprotectant in bull (Tasdemir *et al.,* 2013) and goat (Turner *et al.,* 2013). Besides, improvement of semen extenders with suitable antioxidant is suggested to reduce oxidative damage during freeze–thawing of bull spermatozoa (Ansari *et al.,* 2011).

N-acetyl-L-cysteine (NAC) is a derivative of L-cystein and the precursor of glutathione is an antioxidant that scavenges free radicals and can be considered as a supplement to alleviate glutathione (GSH) depletion during oxidative stress (Wu *et al.,* 2006; Boothe, 2001; AHFS Drug Information, 2010). Several antioxidant agents, such as vitamin E and C, catalase, dimethylsulfoxide, taurine,

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hypotaurine and N-acetylcysteine have already been tested in *in vitro* or *in vivo* studies concerning human, bovine, boar, rabbit and equine semen (Alvarez and Storey, 1983; Becon i *et al.,* 1993; Kessopoulou *et al.,* 1995; Baker *et al.,* 1996; Aurich *et al.,* 1997; Donnelly *et al.,* 1999; Ball *et al.,* 2001; Bilodeau *et al.,* 2001; Pena *et al.,* 2003) with control versial efficacy and usefulness. In addition, daily treatment with NAC results in a significant improvement in sperm motility in comparison to placebo (Safarinegad, 2009).

Recently many studies involved in using an anti-oxidants from natural origin like plants which produce from its biological metabolism actions more than 4000 phenolic and poly phenol compound which had agood anti-oxidant properties and important benefits in pharmochemicals developments (Youssef *et al.,* 2016 ; Sghair*et al.,* 2016; Seeram, 2005).

Oats (Avena sativa L.) one of the cereal crops which plays an important role in human and animal nutrition via to its high nutritional contents (Reily, 2003) are usually consumed as whole grains and provide the human body with polyphenols, fibres, vitamins, and minerals (Clemens *et al.,* 2014). Their phenolic compounds are mostly located in the bran layer although some are present in groats and hulls (Gangopadhyay *et al.,* 2015). Some phenols present in cereals are ferulic acid, caffeic acid, p-hydroxybenzoic acid, p-hydroxyphenylacetic acid, vanillic acid, protocatechuic acid, syringic acid, p-coumaric acid,sinapic acid, tricin, apigenin, luteolin, kaempferol, and quercetin (Chen *et al.,* 2004). Polyphenols are important food components and are characterized by their anti-oxidative by scavenging both of reactive oxygen and nitrogen species (Chen *et al.,* 2004).

Materials and Methods

NAC with 99.99% purity was purchased from (Sigma Pharmaceuticals, Germeny) and other chemicals were purchased from the local market, oat seed also was obtained from turkey.

Aqueous extract of oat seed (Avena sativa) method

The extraction was made as the method of (Duh and Yen, 1997) by taking 25 gm of grinded seeds and diluted in 500 ml of boiled water on magnetic stirrer for 30 min and then filtered by filter paper and concentrated by using rotary evaporator after that settled in a Petri dishes in 40C for 24 hrs to dried it and put it in dark container in the fridge to use it eventually.

Determination of active compounds in (AEASS)

Total phenolic compounds/total flavonoid concentrations were determined depending on the method of Baba and Malik (2015) and estimation of total terpenoids according to the method of Narayan (2012 (and determination of glycosides concentration according to the method of Tofighi and Ghazi) 2016).

Collection and selection of semen samples

Ejaculates were collected from four healthy, fertile Holsteinbulls 2.5–3 years old, raised at the department of artificial insemination – Baghdad. Semen was collected once a week for 7 weeks. The bulls were kept under standard conditions of feeding and management. Semen was collected using an artificial vagina pre-warmed to 42C. The volume of ejaculates was measured in a conical tube graduated tube and sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). Progressive motility was assessed using a phase-contrast microscope (100X magnification), with a warm stage maintained at 37C. A wet mount was made using a drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score (Bearden and Fuqay, 2000). Pooled semen was made (1ml/ bull) and after calculating the dilution rate the pooled semen was equally divided among the 9 treatments.

Cryopreservation procedures

Semen was cryopreserved using standard production procedures in our AI centers according to Chen *et al.,* (1993), with some modifications. Briefly, semen was gradually diluted at 37C with Tris-yolk fructose (TYF) extender containing 24.2 mg/mL tris aminomethane, 13.4 mg/mL citric acid anhydrous, 10 mg/mL fructose, 6.4% (v/v) glycerol, 20% (v/v) egg yolk, 40 IU/mL Gentamicin and 8 IU/ml tylosin. The extension rate was 1 semen: 20extender. Diluted semen samples were kept at 5^0C in a cooling chamber for 4 h as an equilibration period then automatically filled in 0.25mL french straws (IVM technologies, L' Aigle, France), placed 4cm above liquid nitrogen for 10 min then frozen in liquid nitrogen (-196^0C) as described by Salisbury *et al.,* (1978). Samples were evaluated before dilution, just after dilution (5C), at 48 h and 1 month and 2 month post-cryopreservation during equilibration, and after thawing (37^0C for 30s in water bath).

Assessment of sperm progressive motility

Percentage of progressive sperm motility in each semen sample was determined using phase contrast microscope (Olympus, Tokyo, Japan) supplied with a warm stage adjusted to 37 C.

Assessment of sperm viability

A smear from diluted semen was made on a glass slide and was stained by eosin (1.67%) and nigrosin (10%) stain (Moskovtsev, Librach, 2013), A total of 200 sperm were examined in each sample at 40X under light microscope (Olympus). The number of dead spermatozoa (red stained) and the live spermatozoa (not stain) were counted.

Sperm membrane integrity

Plasma membrane integrity of spermatozoa was assessed using the hypo-osmotic swelling test (HOST) as described by (Jeyendran *et al.,* 1984; Ahmad *et al.,* 2003).

Percentage of acrosome integrity

The dual staining procedure with trypan blue-giemsa stain was performed as described by Kovacs and Foote (1992).

Statistical analysis

Data were analyzed by means of the SAS (2012) computerizedprogram to calculate the analysis of variance (ANOVA) for the different parameters between control and additives with 7 replicate for each treatment, Significant differences between means were calculated using Duncan multiple range test at ($P<0.05$).

Results

Determination of active compounds in (AEASS)

The results showed in table 1 that (AEASS) contain the following ingredients with the values in front of each substance :

Sperm individual motility percentage

Table 1: Concentrations of active compounds in (AEASS).

The compound	Concentration
Phenols	93.4 mg/gm
Flavonoids	67.2 mg/gm
Saponin	5.9%
Glycosides	17.6%
Terpenoid	4.6%
Rutin	179 ppm
Kampferol	513 ppm
Qurcetine	469 ppm
Gallic acid	348 ppm

The values from tablet 2 showed no significant differences between all the treatments at the cooling 5^0C time. While, after 48 hrs PC all the treatments exhibited significant differences ($P\leq0.05$) compared with the control treatment especially T8 with the highest value (53.67%), after 1 and 2 months all the treatments were significantly higher compared with the control treatment which declined and made the lowest values for these two periods (40.42% and 39.28%). There were numerically decreasing in the other treatments and the highest percentage was in T_8 (47.85%) after 2 month PC.

Sperm viability percentage

The data in table 3 showed no significant differences among all the treatments at the (5C cooling). After 48 h PC all the treatments exhibited greater significant differences ($P\leq0.05$) Compared with the control treatment and there were numerically differences among them and their values were (92.93%, 93.09%, 91.94%, 92.17%, 93.10%, 93.12%, 93.26%, 93.44%) for T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8 respectively, After 1 month and 2 month all the treatments were numerically decreased but still exhibiting significant differences ($P\leq0.05$) especially T8 which gave the highest values among all the treatments for these two periods with (92.03%, 91.34) Compared with the control treatment which decreased to the lowest values for the same periods with (89.02%, 87.35%).

Sperm membrane integrity (HOST)

The table 4 showed that there were a numerical differences at the (5C cooling) among all the treatments,After 48 h PC all the treatments exhibited significant differences ($P\leq0.05$) Compared with the control treatment and there were no significant differences among them and their values were (91.42, 91.57, 90.78, 91.56, 90.64, 90.95, 90.57 and 91.67%) for T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8 respectively. After 1 month and 2 month all the treatments were numerically decreased but still exhibiting significant differences ($P\leq0.05$) Compared with the control treatment and there were no significant differences among them especially T8 which gave the highest values among all the treatments for these two periods with (90.50%, 86.92±) Compared with the control treatment which decreased to the lowest values for the same periods with (86.14 %, 83.50%).

Percentage of acrosome integrity

The table 5 showed that there were no significant differences but a numerical differences at the (5C cooling) among all the treatments, After 48 h PC the treatments (T_1, T_2, T_7, T_8) with the values (94.14%, 94.35%, 94.35%, 94.42) respectively exhibited significant differences ($P\leq0.05$) Compared with the control treatment (and there were numerically differences among them at the same time there were no significant differences) Compared with the control , After 1 month and 2 month all the treatments were numerically decreased but still exhibiting significant differences ($P\leq0.05$) Compared with the control treatment especially T_8 which gave the highest

Table 2: Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on post-cryopreservative Sperm individual motility percentage of Holstein bulls (mean ± SE).

Treatments	Periods				Sign.
	At cooling 5C	48 hrs/PC	1month/PC	2month/PC	
C	50.71±2.45Ba	42.17±3.90Cb	40.42±2.60Cb	39.28±1.76Cb	*
T_1	52.57±3.40ABa	48.14±3.19Ba	45.71±2.97Ba	42.28±2.79Bb	N.S
T_2	53.85±3.42ABa	48.57±2.82Bab	46.42±1.79Bb	45.71±1.70ABb	*
T_3	51.57±2.66ABa	46.57±3.24Bab	43.85±3.42Bb	41.85±2.46Bb	*
T_4	51.71±3.84ABa	46.65±3.24Bab	44.00±2.43Bb	42.14±1.84Bb	*
T_5	52.28±2.76ABa	47.18±2.14Ba	44.28±1.70Bb	42.71±0.71Bb	*
T_6	53.34±2.76ABa	47.28±2.40Ba	44.57±2.10Bb	42.85±1.84Bb	N.S
T_7	54.00±3.27ABa	48.85±3.05Ba	45.42±1.79Bb	43.00±2.43Bb	*
T_8	56.42±3.22Aa	53.67±3.40Aa	49.28±3.52Aa	47.85±3.24Aa	N.S
Significance	*	**	**	*	

*(P≤0.05) **(P≤0.01) N.S (no significance) PC/ Post cryopreservation.

Capital letters to compare between columns, Small letters to compare between rows.

Table 3: Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on post-cryopreservative sperm viability percentage of Holstein bulls (mean ± SE).

Treatments	Periods				Sign.
	At cooling 5C	48 hrs/PC	1month/PC	2month/PC	
C	92.97±0.77Aa	90.07±0.61Ba	89.02±0.87Bb	87.35±0.75Bb	*
T_1	93.50±0.77Aa	92.93±0.47Aa	91.35±0.53Ab	90.15±0.52Ab	*
T_2	93.65±0.80Aa	93.09±0.69Aa	91.78±0.63Ab	90.57±0.74Ab	*
T_3	92.56±0.77Aa	91.94±0.61Aa	90.50±0.66Ab	89.55±0.56Ab	*
T_4	92.85±0.28Aa	92.17±0.89Aa	90.68±0.45Ab	89.65±0.41Ab	*
T_5	93.72±0.57Aa	93.10±0.28Aa	91.56±0.57Ab	90.64±0.79Ab	*
T_6	93.74±0.43Aa	93.12±0.92Aa	91.71±0.57Ab	90.59±0.54Ab	*
T_7	93.85±0.40Aa	93.26±0.87Aa	91.90±0.51Ab	90.74±0.72Ab	*
T_8	94.00±0.40Aa	93.44±0.92Aa	92.03±0.68Aab	91.34±0.80Ab	*
Significance	N.S	*	*	*	

*(P≤0.05) **(P≤0.01) N.S (no significance) PC/ Post cryopreservation.

Capital letters to compare between columns, Small letters to compare between rows.

Table 4: Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on post-cryopreservative sperm membrane integrity(HOST) of Holstein bulls (mean ± SE).

Treatments	Periods				Sign.
	At cooling 5C	48 hrs/PC	1month/PC	2month/PC	
C	90.00±0.57ABa	88.06±0.94Bab	86.14±0.98Cb	83.50±1.04Cc	**
T_1	92.57±0.64Aa	91.42±0.97Aa	89.14±1.18ABb	86.78±1.25ABb	**
T^2	92.85±0.70Aa	91.57±0.81Aa	87.71±0.77ABb	85.57±0.61ABb	**
T_3	91.71±0.64ABa	90.78±0.99Aa	87.57±0.99ABb	85.85±0.91ABb	**
T_4	92.42±0.64Aa	91.56±0.83Aa	88.42±1.19ABb	87.00±1.13ABb	*
T_5	91.42±0.86ABa	90.64±0.91Aab	88.92±1.21ABb	86.02±1.48ABb	**
T_6	91.84±0.69ABa	90.95±1.25Aab	88.86±1.24ABb	86.92±1.29ABb	**
T_7	91.42±0.75ABa	90.57±1.11Aab	88.35±0.94ABb	86.57±1.08ABb	**
T_8	92.42±0.81Aa	91.67±0.61Aa	90.50±0.86Aab	89.28±0.86Ab	*
Significance	*	*	*	*	

*(P≤0.05) **(P≤0.01) PC/ Post cryopreservation.

Capital letters to compare between columns, Smallletters to compare between rows.

Table 5: Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on post-cryopreservative Percentage of acrosome integrity of Holstein bulls (mean ± SE).

Treatments	Periods				Sign.
	At cooling 5C	48 hrs/PC	1month/PC	2month/PC	
C	93.42±0.42Aa	92.85±0.48Bab	90.74±0.43Bab	88.86±0.44Bb	*
T_1	94.85±0.55Aa	94.14±0.41Aab	93.71±0.37Aab	93.21±0.37Ab	*
T_2	94.85±0.57Aa	94.35±0.44Aab	93.92±0.38Aab	93.21±0.46Ab	*
T_3	93.80±0.48Aa	93.21±0.40ABab	92.74±0.44Aab	92.00±0.40Ab	*
T_4	93.89±0.51Aa	93.31±0.51ABab	92.78±0.37Aab	92.00±0.28Ab	*
T_5	94.35±0.44Aa	93.85±0.41ABab	93.35±0.38Aab	92.78±0.34Ab	*
T_6	94.57±0.57Aa	93.92±0.41ABab	93.78±0.40Aab	93.00±0.30Ab	*
T_7	94.85±0.55Aa	94.35±0.44Aab	93.78±0.39Aab	93.21±0.34Ab	*
T_8	95.00±0.55Aa	94.42±0.40Aa	94.00±0.44Aa	93.64±0.38Aa	N.S
Sign.	N.S	*	*	*	

*($P \leq 0.05$) N.S (no significance) PC/ Post cryopreservation.
Capital letters to compare between columns, Small letters to compare between rows.

values among all the treatments for these two periods with (94.00%, 93.64%) Compared with the control treatment which decreased to the lowest values for the same periods with (90.74%, 88.86%).

Discussion

The semen cryopreservation process, which includes the decrease in temperature, causes oxidative stress on the sperm. This, respectively, results in irreversible damage to the sperm organelles and changes in enzymatic activity, associated with a reduction in sperm motility, membrane integrity and fertilizing ability (Bucak *et al*., 2009ab), Frozen–thawed bull semen is more easily peroxidized than fresh semen. Besides, intracellular antioxidant capacity in sperm decreases following freeze–thawing (Sarýozkan *et al*., 2009). Seminal plasma has limited antioxidant capacity, thus the use of an extender with strong antioxidant effect is recommended to maintain the viability and subsequent fertilizing capacity of frozen spermatozoa (Gadea *et al*., 2008), In recent years, extensive researches have been conducted to investigate the effect of natural and synthetic antioxidants (herbal origins) on the viability of animal sperm during cooling and cryopreservation (El-Sheshtawy, El-Nattat, 2018).

To the best of my knowledge, this is the first study that deals with the additive effect of (AEASS) and combinations of NAC to Tris extender along on post-cryopreserved semen characteristics of Holstein bulls, The additive effect of both NAC and AEASS certainly enhance the antioxidant influence to protect sperm against ROS and consequently enhances cryopreserved semen quality. In recent years, the combination of antioxidants has been used successfully in the semen extender for human (Rossi *et al.,* 2001), bull (Foote *et al.,* 2002) and boar (Roca *et al.,* 2005) sperm. The use of a single antioxidant, however, was insufficient for enhancing the quality of cryopreserved semen (Rossi *et al.,* 2001; Gadea *et al.,* 2007; Câmara *et al.,* 2011). The protective influence of these combined antioxidants on sperm is associated with the reduction in lipid peroxidation and synergistic effects of the antioxidants in scavenging the ROS generated during the cryopreservation processes (Karaji *et al.,* 2014). The experiment showed that the addition of NAC in T_1, T_2 made a significant differences in sperm individual motility, viability and plasma membrane, acrosome integrity for all the PC periods caused by the anti-oxidant properties of NAC directly by reaction with the ROS and indirectly by its role in glutathione synthesis as precursor of cystiene (Rushworth, Megson, 2013) our results had agreed with (Ciftci *et al*., 2009; Micheal *et al*., 2010) at the same time the adding of AEASS gave the same significant improving in sperm individual motility viability, plasma membrane and acrosome integrity and this because of the good cotenents of the active compounds (phenols, flavonoids)which exhibited an anti-oxidaants properties in scavenging free radicals generated by ROS (Dimberg *et al*., 2005; Matila *et al*., 2005; Diculescu *et al*., 2012).

Adding the combinations of NAC and AEASS in the treatments T_5, T_6, T_7, T_7 to the semen gave a significant improving in the tested parameters for all the PC periods and that thing was related to the synergistic effect of these anti-oxidants and their role in protecting the sperm cell from oxidative stress caused by ROS and support the action of the intracellular anti-oxidants by restricting the metal ions inducing oxidation (Metal-chelating compounds) and reactivate the anti oxidants (Safarinegad, 2009; Greco *et al*., 2005; Owen *et al*., 1976). In concluded that the adding of NAC and AEASS was useful to enhanced some semen parameters during PC and could

be used safely in bull semen extender (Tris).

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