Effect of addition of N-acetylcystiene and *Avena sativa* Aqueous extract to Tris extender on cooled and post-cryopreservative semen characteristics and some biomarkers of Holstein bulls

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Abstract

Objective: To evaluate effect of tris-extender supplemented with various concentrations of NAC and aqueous extract of (*Avena Sativa)* seeds (AEASS) on bull semen characteristics and some biomarkers. **Methods:** Pooled bull semen were extended with tris-citrate-fructose egg yolk diluter 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) ,T1 contain 2mM NAC,T2 contain 4mM NAC, T3 contain 1% AESASS, T4 contain 1.5% AEASS , T5 contain 2mM NAC and 1% AEASS ,T6 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 , abnormality% , Malondaildehyde (MDA) and (AST & ALT) concentration were carried out for both chilled and frozen semen. **Results:** Results showed that All treatments showed numerically increasing in sperm total abnormalities after all the periods of PC compared with the control treatment ,Furthersome the treatment T8 exhibited numerically lesser Malondaildehyde (MDA) and (AST & ALT) concentrations ,**Conclusions**: 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.*Part of M.Sc. Thesis for the first author.

Introduction

Bull semen cryopreservation had been proceeded for more than 50 years ago for artificial insemination and is being widely used worldwide for genetic improvement and maintained sperms and extend its storage and physiological value to use it in artificial insemination (Calisici, 2010 ;Lemma et al ,2011 ;Crespilho et al,2014).The cryopreservation procedure produces reactive oxy-gen species Ros(Chatterjee and Gagnon, 2001).Two major sources of ROS are leukocytes via the hexose mono phosphate shunt and immature spermatozoa at the level of the plasma membrane or mitochondria(Aprioku ,2013),Spermatozoa are highly susceptible to ROS due to the specific composition of their plasma membrane .They contain polyunsaturated fatty acids, which are structurally un stable and highly prone to lipid peroxidation (Agarwal, et al ,2003) Electrophilic aldehyde byproducts, such as MDA, 4-hydroxynonenal is generated from the peroxidative damage, which negatively affects DNA integrity, mitochondrial function ,apoptosis, and cellular signaling(Łuczaj ,2003 ; Aitken et al ,2012 ; Bisht ,2017).

N-acetyl-cysteine (NAC), a derivative of amino acid L-cysteine, is currently used mainly as an antioxidant (Zafarullah et al ,2003), NAC also contributes to glutathione (GSH) synthesis(Rushworth, Megson,.2013), and may help restore the depleted pool of GSH often caused by oxidative stress and inflammation(Wang et al ,2014; Shimamoto et al ,2011),NAC

has free radical scavenging activity both in-vivo(Ciftci et al ,2009) and in vitro (Oeda et al,1997 ;Erkkila et al ,1998), the development and utilization of more effective antioxidants of natural origin are desired. Hence, the search for natural antioxidant especially plant origin, has greatly increased in recent years (Gu¨ lc¸in et al. 2002; Gu¨ lc¸in et al. 2004).
The bioactive compounds in plant extracts are highly regarded because it is derived from natural sources and compatible with vital systems. Using herbal medicine to increase fertility and improve factors such as hormonal imbalance, oligospermia, low motility of sperm, prostatitis, varicocele, etc has been considered for a long time. Previous studies on laboratory animals indicated that plants such as *Allium cepa*, *Zingiberofficinale*, *Ocimumbasilicum*, *Cinnamomumverum*, *Citrus sinensis*' peel, *Citrulluslanatus* and *Daucuscarota*' seeds as a rich source of vitamins, flavonoids and minerals have significant effects on oligospermia, regulation of blood testosterone levels, increased levels of superoxide dismutase (SOD) and decreased levels of malondialdehyde (MDA) (Khaki et al,2009,2009a,2010,2011; Azab et al,2011 ;Kanzae et al,2009 ;Alasalver et al ,2001 ;Parkash et al,1998 ;Rimmando et al,2005).
Oats have been considered to be a good natural antioxidant sourcefor a long time (Peters ,1937; Ryan et al,2001 ;Guo et al,2012 ;Peterson et al,2001)

2. Materials and methods

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

2.1.Aqueous extract of oat(*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 sterrer for 3o min and then filterd by filter paper and concentrated by using rotary evaporator after that setted in apetri dishes in 40 C for 24 hrs to dried it and but it in dark container in the fridge to use it eventually.

2.2. Determination of active compounds in (AEASS)

Total phenolic compounds / total flavonoids concentrations were determined depending on the method of Baba and Malik,2015 and Estimation of total terpenoids according to the method of Narayan,2015and Determination of glycosides concentration according to the method of Tofighi,Ghazi ,2016.

2.3. Collection and selection of semen samples

Ejaculates were collected from four healthy, fertile Friesian bulls, 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 42_C. The volume of ejaculates was measured in a conical tube graduated tube and sperm concentration was determined by means of an Accu cell photometer (IMV, L'Aigle, France).Progressive motility was assessed using a phase-contrast microscope(100_ magnification), with a warm stage maintained at37 _C. A wet mount was made using a 5-ll 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,Fuqay,2000), Pooled semen was made (1ml per bull)and after calculating the dilution rate the pooled semen was equally divided among the 9 treatments.

2.4. 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 37_C 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 to bring the sperm concentration to 80 _ 106 sperm/mL .Diluted semen samples were kept at 5_C in a cooling chamber for4 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_C) 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_C for 30 s in water bath).

2.5. Assessment of sperm abnormalities

A smear from diluted semen was made on a glass slide and was stained by eosin (1.67%) and nigrosin (10%) stain(Moskovtsev,Librach,2015) . A total of200 sperm were examined in each sample at 400_ under light microscope (Olympus). The number of dead spermatozoa (red stained) were counted. The number of sperm cells bearing head and tail morphological abnormalities were also recorded as previously described(Menon et al,2011).

2.6. Determination of Malondialdehyde MDA concentration in seminal plasma The MDA concentration in seminal plasma had been determined by measuring the values of (TCA,Tri-chloral-acetic acid and TBA ,Thiobarbutiric acid) nmol/ml according to the method of (Muslih et al ,2002).

2.7. Determination of AspartateAminotransferase(AST)concentration in seminal plasma.

By using KITS from (SYRBIO-SWITZERLAND) the) concentration of(AST) in seminal plasma had been determined U/L depending on the instruction of the company as the method described by (Reitman , Frankel,1957) .

2.8. .Determination of Alanine Amino transferase(ALT) concentration in seminal plasma

By using KITS from (RANDOX-ENGLAND) the) concentration of(ALT) in seminal plasma had been determined U/L depending on the instruction of the company as the method described by (Reitman , Frankel,1957)

2.9. Statistical analysis

Data were analyzed by means of the SAS (2012)computerized program 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 $P<0.05$.

3-Results

3.1. Determination of active compounds in (AEASS)

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

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

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

3.2. Assessment of sperm total abnormalities

The values from tablet(2) showed that the control treatment exhibited high significant differences (P≤0.05) compared with the treatment T8 at the same time there was no significant differences among all the treatments compared with the control at the cooling 5C time, after 48 hrs PC the control treatment exhibited high significant differences (P≤0.05) compared with the treatment T8 with the highest value (8.32%) with numerically differences among all the treatments ,after 1 and 2 months all the treatments were numerically lesser compared with the control treatment which made the highest values for these two periods (9.28% and 10.12%).there were numerically increasing in the other treatments and the lowest percentage was in T8 (7.85%%)after 2 month PC**.**

Table(2)Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on cooled and post-cryopreservative percentage of total abnormalities of Holstein bulls (mean ± SE)

treatments	Periods				Sign.
	At cooling 5C	48 hrs/PC	1month/PC	2month/PC	
C	7.30±1.05Aa	8.23±0.77Aa	9.28±0.80Aa	10.12±1.06Aa	N.S
T1	6.75±1.10ABa	7.45±1.14ABa	8.06±1.28ABa	8.91±1.56ABa	N.S
T2	6.14±0.40ABa	7.15±1.05ABb	7.71±0.77ABb	8.72±0.83ABa	*
T3	6.75±0.48ABa	7.71±1.14ABb	8.35±1.05ABb	9.64±0.73ABa	*
T4	6.49±0.36ABa	7.42±0.72ABb	8.00±1.00ABb	8.95±1.61ABa	*
T5	6.57±0.40ABa	7.86±0.36ABb	8.37±0.68ABb	9.67±0.89ABa	*
T6	6.28±0.56ABa	7.63±0.56ABb	8.21±0.61ABb	9.10±0.92ABa	*
T7	6.00±0.48ABa	6.95±0.60ABb	7.55±0.60ABb	8.62±1.16ABa	*
T8	5.42±0.25Ba	6.43±0.21Bb	7.08±0.84Bb	7.85±0.94Ba	*
significance	*	*	*	*	

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

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

3.3. Determination of Malondialdehyde MDA concentration in seminal plasma

The values from tablet(3) showed that the control treatment exhibited high significant differences (P≤0.05) compared with the treatments T1,T2, T8 at the same time there was no significant differences among all the treatments compared with the control at the cooling 5C time, after PC the control treatment exhibited high significant differences (P≤0.05) with the highest value (2.05 nmol/l) compared with the other treatments that made lesser values(1.55 ,1.42, 1.36)nmol/l for T1 ,T2, T8 respectively .

Table(3)Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on cooled and post-cryopreservative Malondialdehyde MDA concentration in seminal plasma (nmol/l) of Holstein bulls (mean ± SE).

treatments	Periods		Sign.
	At cooling 5C	PC	
C	1.87±0.17Aa	2.05±0.19 Aa	**N.S**
T1	1.28±0.11Ba	1.55±0.110Ba	**N.S**
T2	1.19±0.04Ba	1.42±0.15 Ba	**N.S**
T3	1.34±0.17ABa	1.74±0.12ABa	**N.S**
T4	1.36±0.13ABa	1.70±0.18ABa	**N.S**
T5	1.43±0.17ABa	18.30±0.18ABa	**N.S**
T6	1.43±0.09ABa	1.84±0.12ABa	**N.S**
T7	1.37±0.10ABa	1.56±0.12ABa	**N.S**
T8	1.17±0.09Ba	1.36±0.12Ba	**N.S**
Sign.	*	*	

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

3.3. Determination of AST concentration in seminal plasma

The values from tablet(4) showed that the control treatment exhibited high significant differences (P≤0.05) compared with the treatments T1,T2,T4,T7, T8 at the same time there was no significant differences among all the treatments compared with the control at the cooling 5C time, after PC the control treatment exhibited high significant differences (P≤0.05) with the highest value (402.5±61.1 U/L) compared with the other treatments that made lesser values for T1 ,T2,T3,T4,T5,T6,T7, T8 respectively

Table(4) Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on cooled and post-cryopreservative AST concentration in seminal plasma (U/L) of Holstein bulls (mean ± SE)

treatments	Periods		Sign.
	At cooling 5C	PC	
C	267.5±76.6Aa	402.5±61.1Aa	**N.S**
T1	165.0±37.0Ba	285.6±903Ba	**N.S**
T2	170.0±21.2Ba	295.0±1113Ba	**N.S**
T3	212.5±23.9ABa	317.4±47.5Ba	**N.S**
T4	195.0±8.6Ba	287.5±86.0Ba	**N.S**
T5	210.0±11.5ABa	345.0±89.5Ba	**N.S**
T6	230.0±78.8ABa	354.3±60.0Ba	**N.S**
T7	180.0±35.3Ba	300.0±54.4Ba	**N.S**
T8	177.5±21.3Ba	250.0±80.1Ba	**N.S**
Sign.			

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

3.4 . Determination of ALT concentration in seminal plasma

The values from tablet(5) showed that the there were no significant differences among all the treatments compared with the control at the cooling 5C time, after PC the control treatment exhibited high significant differences (P≤0.05) with the highest value (457.6±29 U/L) compared with the other treatments which were numerically than the control treatment with the values for T1 ,T2,T3,T4,T5,T6,T7, T8 respectively.

Table(5) Effect of adding N-acetylcystiene and *Avena sativa* extract to Tris extender on cooled and post-cryopreservative ALT concentration in seminal plasma (U/L) of Holstein bulls (mean ± SE)

treatments	Periods		Sign.
	At cooling 5C	PC	
C	367.5±22.5Aa	457.6±29.0Aa	**N.S**
T1	340.5±15.8Aa	387.5±20.5ABa	**N.S**
T2	335.0±16.5Aa	385.1±29.2ABa	**N.S**
T3	350.0±28.5Aa	385.0±6.4ABa	**N.S**
T4	372.5±36.8Aa	405.4±8.1ABa	**N.S**
T5	367.5±10.3Aa	405.0±20.6ABa	**N.S**
T6	357.5±24.9Aa	407.5±15.4ABa	**N.S**
T7	345.4±23.2Aa	400.0±15.8ABa	**N.S**
T8	337.4±17.9Aa	383.5±25.3ABa	**N.S**
Sign.	N.S	*	

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

4.Discussion

Recently, scientists are interested in the potential health benefits of phyto chemicals and the synergistic effects of their multiple compounds compared to the single purified active fractions(Seeram et al,2005) Semen cryopreservation leads to biochemical and functional damage to spermatozoa, thus reducing its motility and viability**(Graham** et al ,1970) Seminal plasma has limited antioxidant capacity ,so, the use of an extender having strong antioxidant effect is recommended to maintain the viability and fertilizing capacity of frozen spermatozoa (**Gadeaet al 2004)**,NAC was investigated in a randomized placebo controlled study of 120 patients with idiopathic infertility .Patients were randomized to receive a daily dose of600 mg NAC (n = 60) or placebo (n = 60) for 3months. Results revealed a significant improvement in volume, motility, and viscosity of semen with NAC therapy in comparison with placebo(**Ciftci et al ,2009)** The combination of 600 mg NAC and 200 mg selenium resulted in a significant improvement in all semen parameters with a dose dependent positive correlation between the sum of selenium and NAC concentrations, and mean sperm concentration ,motility, and percentage of normal morphology(**Safarinejad and Safarinejad2009).** Administration of nutritional antioxidants as a strategy to prevent or manage health conditions associated with oxidative stress has gained immense interest. Substances isolated from herbal plants such as turmeric are known to exert beneficial effects by scavenging free radicals and modulating the intricate antioxidant defense system (El-Wakf et al., 2011).Oat (*Avena sativa* L.) is a source of

many compounds that exhibit antioxidant activity. Vitamin E(tocols), phytic acid, phenolic compounds, and avenanthramides are the most abundant antioxidants in oat, and flavonoids and sterols are also present. These antioxidants are concentrated in the outer layers of the kernel. Several *in vitro* tests have been used to evaluate antioxidant activity of oat extracts(**Peterson, 2001)**.

Malondialdehyde level in seminal plasma has been reported to negatively correlate with, sperm morphology (Shamsi et al., 2010; Atig et al., 2012; Benedetti et al., 2012) previous studies have found correlation between MDA level and abnormal morphology evaluated by light microscopy (Ben Abdallah et al., 2009; Atig et al.,2012; Benedetti et al.,2012)our results showed numerical increasing differences in MDA in frozen-thawed semen compared with fresh semen , A great number of enzymes in seminal plasma have been investigated. Guerin et al,1979reported that the existence mode of enzymes in seminal plasma was similar to that in sperm, indicating that the enzymes in seminal plasma might play a role in ensuring the normal metabolism of sperm Like serum, seminal plasma consists of rich biochemical components. It has been shown recently that seminal plasma proteins could serve as important biomarkers for male infertility(Macanovic, et al ,2015) The concentration of transaminase enzymes (AST and ALT) in semen is a good indicator of semen quality because it measures sperm membrane stability and acrosomal damage (Corteel 1980). the high concentration of transaminase enzyme e.g. AST in seminal plasma indicate increasing percentage of abnormal spermatozoa in ejaculate occurring due to sperm membrane damage leading to leakage of enzymes from spermatozoa (Gundogan, 2006) high activity of ALT and AST in seminal plasma might have some adverse effects on sperm function which may be used as a marker to evaluate sperm quality. However ,further studies are needed to analyze the origins of the high levels of ALT and AST in seminal plasma, and we might speculate that some of damaged sperm or epithelial cells in the reproductive tract contribute to such elevated ALT and AST levels in seminal plasma. (Dhami and Kodagali,1990; Khan et al 2012Forejtek and Avratil,1984) our results showed numerical increasing differences in both of AST and ALT values and agreed with the outcomes of (**Gafer et al,2015)**,

Adding the combinations of NAC and AEASS in all the treatments and especially in T5 ,T6 ,T7 ,T8to the semen made a significant improving in the tested parameters for PC period 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 (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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