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SOME POST-CRYOPRESERVED SEMEN CHARACTERISTICS OF HOLSTEIN BULLS AS INFLUENCED BY ADDING AQUOEUS EXTRACT OF URTICA DIOICA AND DATE PALM POLLEN POWDER TO TRIS EXTENDER

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Abstract

This study was conducted to investigate the influence of adding aqueous extracts of *Urtica dioica* (AEUD) and date palm pollen powder (AEDPP) to Tris extender on some post-cryopreserved semen characteristics of Holstein bulls for different preservation periods (cooling at 5ºC, 48 hrs., 1, 2 and 3 months post cryopreservation, PC). Seven Holstein bulls of 2.5-3 years old were used in the current study during the period from 20th November, 2017 to 20th August, 2018. Pooled semen was equally divided into three groups within one experiment. AEUD (0.01 g / 50 ml extender) and AEDPP (0.02 g / 50 ml extender) were added to Tris extender and comparisons in response were made with the control group (Tris extender, C). The AEUD and AEDPP groups exhibited greater ($P \leq 0.01$) sperm's cell individual motility percentage as compared with the C group at cooling as well as 1, 2 and 3 months PC periods. Concomitantly, greater ($P \leq 0.01$) live sperm percentage was observed in AEUD and AEDPP groups in comparison with the C group at all preservation periods. Lesser ($Pd \leq 0.01$) abnormal sperm percentage were noticed for AEUD and AEDPP groups as compared with the C group at 48 hr., 1, 2 and, 3 months PC. Greater ($P \leq 0.01$) acrosome integrity percentage was observed for AEUD as compared with the other two groups at 1, 2 and, 3 months PC. The AEUD and AEDPP groups exhibited greater ($P \leq 0.01$) plasma membrane integrity percentage in comparison with the control group at all preservation periods. In conclusion, adding AEUD and AEDPP to Tris extender had a crucial role in improving some PC semen characteristics of Holstein bulls.

Key words: *Urtica dioica* and date palm pollen extracts, semen characteristics, Holstein bulls.

Introduction

The free radicals are generated in normal sperm metabolic function and also can be acquired from the environment (Bhuwan *et al.*, 2015). Free radicals can be oxygen radicals, such as hydroxyl radical, superoxide radical and non free radical species, such as singlet oxygen, hydrogen peroxide and are generated in various redox processes (Gulçin *et al.*, 2002). The endogenous antioxidant like superoxide dismutase, catalase and glutathione peroxidase etc. are the enzymes of antioxidant defense system which trap and destroy these free radicals (Kataki *et al.*, 2012a). The excessive production of free radicals, a decreased level of antioxidant defense enzymes and increased lipid peroxidation are responsible for producing oxidative stress and linked with various pathological conditions (Ellnain-Wojtaszek *et al.*, 2003).

Cryopreservation causes a wide range of chemical, physical and mechanical injures to sperm membranes of all mammalian species (Watson, 2000), which are attributed to temperature changes, alterations in the transition from the lipid phase, production of reactive oxygen species (ROS) and osmotic stress (Ortega Ferrusola *et al.*, 2009 and Câmara *et al.*, 2011a). Moreover, the overproduction of ROS causes oxidative stress that involves structural damage of sperm membranes, fall of intracellular ATP levels causing decrease in the viability and motility of cryopreserved sperm (Baumber *et al.*, 2000 and Agarwal and Said, 2005). Furthermore, mammalian sperm cells are rich in polyunsaturated fatty acids (PUFAs) and have low antioxidant capacity that rendering them highly vulnerable

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to oxidative damage and lipid peroxidation (Lenzi *et al.*, 2002 and Walczak-Jedrzejowska *et al.*, 2013). Adding of enzymatic antioxidants like GSH and catalase (Eidan, 2016) and non-enzymatic antioxidants like vitamin A, E and C (Al-Zaidi, 2014; Eidan *et al.*, 2015b) amino acids (Mohammad *et al.*, 2014; Abdulkareem *et al.*, 2016), manganese (Eidan *et al.*, 2015a) and coenzyme Q10 (Sultan, 2015) and their various combinations to Tris extender (Abdulkareem *et al.*, 2017a,b) have given good results in improving the post-cryopreserved semen quality of Holstein bulls in Iraq.

There is a continuous interest in the utilization of herbal remedies in the developing countries. At present, the use of herbal antioxidants to counteract the deleterious action of reactive oxygen species (ROS) and oxidative stress on body cells and tissues has attracted research attention (El-Sisy *et al.*, 2016; Abdulkareem *et al.*, 2018a,b,c; Abdulkareem and Al-Zaidi, 2018a,b and Eidan and Mohsin, 2019). *Urtica dioica* (UD) an herbaceous perennial plant, belongs to the family *Urticaceae* (Khodaei *et al.*, 2016). In folk medicine, UD seeds used for treatment of cancer (Kaya *et al.*, 2013 and Aktas *et al.*, 2016), urinary tract disorder as well as an anti-inflammatory agent (Di Lorenzo *et al.*, 2013). The UD contains both fat soluble vitamins (A and D) and water-soluble vitamins (C and B), minerals (iron, manganese, potassium and, calcium) and proteins (Toldy *et al.*, 2009 and Upton, 2013). Moreover, UD has salicylic acid, lecithin, sterols, thymol, chlorophyll, carotenoids, flavonoids and antioxidants (Dügenci *et al.*, 2003; Upton, 2013 and Jalili *et al.*, 2014) that promote detoxification, anti-inflammatory and antioxidant capacity (Kataki *et al.*, 2012b).

Date palm pollen (DPP) is used as a traditional medicine for male fertility by improving sperm count, motility, morphology and DNA integrity with increasing testicular and epididymal weights (El-Sheshtawy *et al.*, 2014). These effects are due to the increase in plasma testosterone levels as DPP is rich in flavonoids (Bahmanpour *et al.*, 2006). From ancient times, the DPP was used to improve reproductive performances in men and women as dietary supplement (El-Sheshtawy *et al.*, 2014). The DPP has a potent nutritive importance as it is rich in phytochemicals such as estrone, α-amirin, triterpenoidal, saponins, flavonoids estrone, estradiol, estriol and crude gonadotrophic substance (Abedi *et al.*, 2014), as well as a rich source of natural antioxidants (EL-Sisy *et al.*, 2016). El-Kashlan *et al.*, (2015) observed that DPP extract possessed a powerful free radical scavenging capacity. Hassan, (2011) and Bishr and Desoukey, (2012) attributed the powerful antioxidant capacity of DPP to its high content of phenolic, carotenoid, flavonoid compounds and considerable amount of vitamins A, E and C. Furthermore, DPP has antibacterial, antifungal and antiviral activity (Abedi *et al.*, 2012 and Mallhi *et al.*, 2014).

Adding of AEUD and AEDPP as powerful antioxidants to semen extender may enhance semen quality of Holstein bulls. Moreover, The effect of adding of these extracts to Tris extender on PC semen characteristics of Holstein bulls did not previously investigated. Therefore, this study was conducted to explore theses effects.

Materials and Methods

Animals

Seven Holstein bulls were selected to be the semen source. The bulls were clinically proven to be free from any general or genital diseases and were maintained at the Artificial Insemination Department of the Ministry of Agriculture (Baghdad, Iraq). Ejaculates were collected from the bulls using an artificial vagina twice a week. The ejaculates were ejaculates were pooled to increase the semen volume for replication and to eliminate variability among the samples. This study was replicated four times for each group.

Semen handling and treatment

Tris extender was prepared according to Salamon and Maxwell, (2000). The Tris-based extender contained 24.2 g Tris, 13.4 g citric acid, 10 g fructose, 19.2% v/v egg yolk, 64 ml glycerol (6.4%) and 1000 ml distilled water at a pH of 6.8. The extender was mixed with the pooled semen samples and was divided into three groups. AEUD (0.01 g / 50 ml extender) and AEDPP (0.02 g / 50 ml extender) were added to Tris extender and comparisons in response were made with the control group (Tris extender, C).

The percentages of sperm's cell individual motility for all treatments at 5^0C cooling, 48h., 1, 2 and 3 months post-cryopreservation (PC) were estimated according to Walton, (1933) and Chemineau *et al.*, (1991) by taking a drop of semen and reducing in 3 drops of sodium citrate solution with a concentration of 29% and a dilution of 1:9 semen, sodium citrate solution in a test tube placed in a 37^oC water bath . The droplet was then placed on a warm glass slide with a cover slid and examined under a light microscope .

Live sperm percentage was estimated based on Swanson and Bearden, (1951) method by taking a small drop of fresh semen, placing on 37^oC slide and mixed with a mixture of 5% eosin and 10% nigrosin stains. The smear was examined under 400x magnification

Table 1: Effect of adding aqueous extracts of *Urtica dioica* (AEUD) and date palm pollen powder (AEDPP) to Tris extender on sperm's cells motility percentage of Holstein bulls for different preservation periods (Mean ± SE).

Period / Group	Cooling 5°C	48 hr PC	1 Month PC	2 Months PC	3 Months PC	Significance level
C	38.75±1.25 ^{b}A	30.00±2.04 ^{ab}B	25.00±2.04 ^{b}BC	21.25±3.14 ^{b}C	20.00±2.04 ^{b}C	P≤0.01
AEUD	48.75±2.39 ^{a}A	37.50±4.78^{a} B	35.00±2.88 ^{a}B	35.00±2.88 ^{a}B	35.00±2.88 ^{a}B	P≤0.05
AEDPP	46.25±2.39 ^{a}A	36.25±2.39^{ab}B	32.50±2.50 ^{a}B	32.50±2.50 ^{a}B	32.50±2.50 ^{a}B	P≤0.01
Significance level	P≤0.01	P≤0.05	P≤0.01	P≤0.01	P≤0.01	-

Different small superscripts within the similar column indicate differences; Different large superscripts within the similar row indicate differences. C= Control; AEUD.=Aqueous extract of *Urtica dioica;* AEDPP = Aqueous extract of date palm pollen powder. PC= Post-cryopreservation.

microscope. The dead sperm appear pinkish, while the live sperm is translucent color for non-pigmentation, 200 sperm were counted in different fields of the slide and the percentage of live sperm.

The percentages of sperm's abnormality percentage for all treatments at 5°C cooling, 48h., 1, 2 and 3 months post-cryopreservation (PC) were estimated according to Hancock, (1951) method by taking a small drop of fresh semen, placing on 37°C slide and mixed with a mixture of 5% eosin and 10% nigrosin stains. The smear was examined under 400x magnification microscope. The abnormalities were classified based on Laing and Melrose, (1970).

Sperm acrosome integrity was determined using the procedure of Kovács and Foote, (1992) by using giemsa stain. The fixed smear was immersed in stain solution for 90 min, washed with tap water, dried and examined using a microscope (1000X). The acrosome integrity percentage was calculated by counting 200 sperm at different locations on the slide.

Sperm's plasma membrane integrity was determined according to Jeyendran *et al.*, (1984) method using hypo-osmotic solution which contained 8.72 gm/L of fructose and 4.74 gm/L sodium citrate, with 100 mOsm/L osmotic pressure and pH 8. Two droplets of semen was overwhelmed on this solution than incubated in water bath 37°C for 60 min.

Statistical analyses

The statistical analysis system (SAS, 2012) was used in the statistical analysis of the data according to the complete random design (CRD) to study the effect of *Urtica dioica* and date palm pollen powder on the studied traits. Differences among means were compared using Duncan's multiple range test (Duncan, 1955). Chi square test was used to compare different percentages of sperm's motility and live sperm (Steel and Torrie, 1990).

Results and Discussion

Sperm's cells individual motility percentage

The AEUD and AEDPP groups exhibited greater (P≤0.01) sperm's cells individual motility as compared to the C group at cooling period, while the differences among groups lacked significance at 48 hr post-cryopreservation (PC; Table 1). Greater (P≤0.01) sperm's cells individual motility was observed for AEUD and AEDPP groups in comparison with the C group at 1, 2 and 3 months PC. (Table 1). Significant (P≤0.01) differences were noticed between cooling period and all PC periods within AEUD and AEDPP groups, however, the differences among PC groups lacked significance (Table 1). Within the C group, 2 and 3 months PC registered lesser (P≤0.01) sperm's cells individual motility percentage as compared with cooling and 48 hr PC periods (Table 1).

Live sperm percentage

Greater (P≤0.01, P≤0.05) live sperm percentage was observed for AEUD and AEDPP groups as compared with the C group at all preservation periods (Table 2). Moreover, the AEUD group exhibited greater (P≤0.01)

Table 2: Effect of adding aqueous extracts of *Urtica dioica* (AEUD) and date palm pollen powder (AEDPP) to Tris extender on live sperm percentage of Holstein bulls for different preservation periods (Mean ± SE).

Period / Group	Cooling 5°C	48 hr PC	1 Month PC	2 Months PC	3 Months PC	Significance level
C	71.05±1.92 ^{b}A	61.85±1.20^{c}B	56.47±1.61 ^{b}C	50.47±2.48 ^{b}D	47.72±1.19 ^{c}C	P≤0.01
AEUD	77.02±0.78 ^{a}A	69.17±0.94 ^{a}B	65.92±1.05 ^{a}C	67.17±0.99 ^{a}C	63.02±1.03 ^{a}C	P≤0.05
AEDPP	74.85±2.14 ^{a}A	65.67±1.14^{b}B	61.90±1.57^{a} BC	58.25±2.22^{a} CD	55.55±2.13 ^{b}D	P≤0.01
Significance level	P≤0.05	P≤0.01	P≤0.01	P≤0.01	P≤0.01	-

Different small superscripts within the similar column indicate differences; Different large superscripts within the similar row indicate differences. C= Control; AEUD.=Aqueous extract of *Urtica dioica;* AEDPP = Aqueous extract of date palm pollen powder. PC= Post-cryopreservation.

Table 3: Effect of adding aqueous extracts of *Urtica dioica* (AEUD) and date palm pollen powder (AEDPP) to Tris extender on abnormal sperm percentage of Holstein bulls for different preservation periods (Mean ± SE).

Period / Group	Cooling 5°C	48 hr PC	1 Month PC	2 Months PC	3 Months PC	Significance level
C	13.17±0.80^{ab}A	17.80±0.20^{a}B	21.00±0.67^{a}C	22.37±0.31^{a}C	24.75±0.62^{a}D	P≤0.01
AEUD	10.30±0.57^{b}A	15.67±0.38^{b}B	17.00±0.40^{b}BC	18.30±0.62^{b}CD	19.75±0.47^{b}D	P≤0.05
AEDPP	10.87±0.42^{ab}A	13.75±0.75^{b}B	18.50±0.50^{b}C	20.17±0.68^{b}CD	21.25±0.72^{b}D	P≤0.01
Significance level	NS	P≤0.01	P≤0.01	P≤0.01	P≤0.01	-

Different small superscripts within the similar column indicate differences; Different large superscripts within the similar row indicate differences. C= Control; AEUD.=Aqueous extract of *Urtica dioica;* AEDPP = Aqueous extract of date palm pollen powder. PC= Post-cryopreservation.

live sperm percentage in comparison with the AEDPP group at 48 hr and 3 months PC (Table 2). Greater (P≤0.01) live sperm percentage was observed at cooling period as compared with the PC periods in all groups, however, the percentages were higher (P≤0.01) at 48 hr PC in comparison with the other PC periods for C and AEUD groups and with 2 and 3 moths PC for AEDPP group (Table 2).

Abnormal sperm percentage

Non-significant differences were observed among groups for abnormal sperm percentage at cooling period, however, these percentages were lesser (P≤0.01) for AEUD and AEDPP groups as compared with the C groups at all PC periods (Table 3). The cooling period registered the lesser (P≤0.01) abnormal sperm percentage as compared with the PC groups, however, the percentages were lesser (P≤0.01) at 48 hr PC in comparison with the other PC periods for C and AEDPP groups and with 2 and 3 moths PC for AEUD group (Table 3).

Acrosome integrity percentage

Greater (P≤0.01) acrosome integrity percentage was noticed for AEUD group (78.35±1.01%) as compared with the C group (74.47±0.56%) but not with the AEDPP group (76.67±0.87 %) at cooling period (Table 4). At 48 hr PC, the AEUD and AEDPP groups registered greater (P≤0.01) acrosome integrity percentage in comparison with the C group (Table 4). The AEUD exhibited greater (P≤0.01) percentages as compared with the other remaining groups at 1, 2 and 3 months PC, however, the AEDPP group was superior (P≤0.01) to the C groups at these mentioned periods (Table 4). Concerning the period effect, the acrosome integrity percentages were consequently decreased from cooling to 3 months PC for both AEUD and AEDPP groups. Similar results was observed for C group, except for the non-significance pattern between 48 hr and 1 month PC (Table 4).

Plasma membrane integrity percentage

The AEUD and AEDPP groups exhibited greater (P≤0.01) plasma membrane integrity percentages as compared with the C group at cooling, 48 hr and 3 months PC time periods (Table 5). At 1 and 2 months PC period, AEUD group was superior (P≤0.01) in these percentages than the other two groups, however, the AEDPP group was greater (P≤0.01) than the C groups at these mentioned periods (Table 5). Concerning the period effect, the plasma membrane integrity percentages were consequently decreased from cooling to 3 months PC for all groups (Table 5).

It is worthy to mention that this study is the first study that deals with the adding of AEUD and AEDPP to Tris extender on semen characteristics of Holstein bulls.

An obvious increasing in sperm's cell individual motility, live sperms percentage, acrosome and plasma membrane integrity percentages, as well as decreasing in total abnormal sperms for AEUD and AEDPP groups

Table 4: Effect of adding aqueous extracts of *Urtica dioica* (AEUD) and date palm pollen powder (AEDPP) to Tris extender on acrosome integrity percentage of Holstein bulls for different preservation periods (Mean ± SE).

Period / Group	Cooling 5°C	48 hr PC	1 Month PC	2 Months PC	3 Months PC	Significance level
C	74.47±0.56^{b}A	66.10±0.68^{b}B	61.50±1.54^{c}B	55.92±1.99^{c}C	46.92±2.67^{c}D	P≤0.01
AEUD	78.35±1.01^{a}A	71.42±1.00^{a}B	68.67±0.75^{a}C	66.10±0.60^{a}D	64.30±0.57^{a}D	P≤0.05
AEDPP	76.67±0.87^{ab}A	71.00±1.35^{a}B	65.17±0.11^{b}C	61.05±1.01^{b}D	58.67±1.40^{b}D	P≤0.01
Significance level	P≤0.01	P≤0.01	P≤0.01	P≤0.01	P≤0.01	-

Different small superscripts within the similar column indicate differences; Different large superscripts within the similar row indicate differences. C= Control; AEUD.=Aqueous extract of *Urtica dioica;* AEDPP = Aqueous extract of date palm pollen powder. PC= Post-cryopreservation.

Table 5: Effect of adding aqueous extracts of *Urtica dioica* (AEUD) and date palm pollen powder (AEDPP) to Tris extender on plasma membrane integrity percentage of Holstein bulls for different preservation periods (Mean ± SE).

Period / Group	Cooling 5°C	48 hr PC	1 Month PC	2 Months PC	3 Months PC	Significance level
C	70.42±1.11 ^{b}A	64.35±0.41 ^{b}B	58.92±0.55 ^{c}C	51.77±1.66 ^{c}D	44.72±2.68 ^{b}E	P≤0.01
AEUD	77.55±0.32 ^{a}A	69.80±0.60 ^{a}B	66.77±0.49 ^{a}C	63.97±0.26 ^{a}D	60.55±0.48 ^{a}E	P≤0.05
AEDPP	75.67±0.78 ^{a}A	69.00±0.40 ^{a}B	64.00±0.57 ^{b}C	59.47±0.35 ^{b}D	56.27±1.10 ^{a}E	P≤0.01
Significance level	P≤0.01	P≤0.01	P≤0.01	P≤0.01	P≤0.01	-

Different small superscripts within the similar column indicate differences; Different large superscripts within the similar row indicate differences. C= Control; AEUD.=Aqueous extract of *Urtica dioica;* AEDPP = Aqueous extract of date palm pollen powder. PC= Post-cryopreservation.

as compared with the C group at all preservation periods could attributed to the antioxidant effects of AEUD and AEDPP to scavenged the reactive oxygen species (ROS) through high contents of flavonoids (Hassan, 2011 and Kataki *et al.*, 2012b). Flavonoids are an excellent scavengers of free radicals and the number of hydroxyl group on the phenyl ring seems to enhance the antioxidant capacity of polyphenolic molecule (Wettasinghe and Shahidi, 2000 and LeBlanc *et al.*, 2009). The polyphenols in AEUD is probably responsible for the anti-radical activity of these extracts. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. However, antioxidant activity of plant extracts is not limited to phenolics content but also due to the presence of other antioxidant secondary metabolites (Sidaoui *et al.*, 2015). The antioxidant effect of AEDPP returns to its high concentration of vitamins C, B1, B2, nicotinic acid (Niacin) and vitamin A (Hassan, 2011). Moreover, the AEUD contains both fat soluble vitamins (A and D) and water-soluble vitamins (C and B), minerals (iron, manganese, potassium and calcium) and proteins (Toldy *et al.*, 2009 and Upton, 2013). On the other hand, several factors can be considered to understand the action and the capacity of antioxidants: the capacity for scavenging free radicals, the localization of antioxidant, but also the interaction with other antioxidants and the mobility of antioxidant at the microenvironment (Niki, 2010).

The AEUD have been used in folk medicine and has several pharmacological properties including antioxidant, anti-apoptotic and anti-fibrotic activities (Baninameh *et al.*, 2016). The AEDPP was used to improve reproductive performances in men and woman as dietary supplement (El-Sisy *et al.*, 2016). The AEDPP has a potent nutritive importance as it is rich in phytochemicals such as estrone, α-amirin, triterpenoidal saponins, flavonoids estrone, estradiol, estriol and a crude gonadotrophic substance (Abedi *et al.*, 2014), as well as its rich source of natural antioxidants (El-Sisy *et al.*, 2016) possessed a powerful free radical scavenging capacity (El-Kashlan *et al.*, 2015). Furthermore, AEDPP improved semen preservability through reduction of bacterial growth in the semen extender that used egg yolk as anti-cold shock (Aba Al-Khail *et al.*, 2003).

The current results were in agreement with Jalili *et al.*, (2012) who concluded that *Urtica dioica* hydro-alcoholic extract administration could increase the quality of spermatozoa in mice. The present results confirmed the findings of Inić and Kujundžić, (2012) who observed that *Urtica dioica* can be used as a potent anti-oxidant substance against oxidative stress and subsequent effects. Our results were also in line with El-Sheshtawy *et al.*, (2014) who concluded that the aqueous infusion or extract of the date palm pollen added to the Tris-citrate-fructose extender with or without adding of egg yolk, proved its good preserving and maintaining capacity of chilled (TPGY 150) and thawed (TPGY 250) bull sperms which was expressed mainly by the sperm motility. Similar findings were noticed by El-Sheshtawy *et al.*, (2016) for improving chilled and thawed buffalo bull sperms by adding aqueous extract of date palm pollen to Tris-citrate extender.

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