EFFECT OF ADDING ANNATTO *(Bixa orellana)* SEEDS EXTRACT AND TOCOTRIENOL TO SEMEN EXTENDER ON CRYOPRESERVED SEMEN OF HOLSTEIN BULLS

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ABSTRACT

The current study was conducted to investigate the effect of adding various concentrations of annatto alcoholic extract of *(Bixa orellana)* and tocotrienol on characteristics of cryopreserved semen of Holstein bulls. Collected semen were pooled and diluted with tris egg yolk extender, then equally divided into five groups as following: Control group (C) with tris extender only, T1 contain 150 mg annatto seed extract/ 25 ml extender, T2 contain 200 mg annatto seed extract / 25 ml extender, T3 contain 3 Mm tocotreinol /25 ml extender and T4 contain 4 Mm tocotreinol/25 ml extender. All groups were subjected to a cryopreservation protocol. The parameters of semen assessment including: Motility, viability, abnormality, plasma membrane and acrosome integrity, malondialdehyde (MDA), total antioxidant capacity (TAC) and DNA fragmentation Index (DFI). All the previous parameters were done for both cooled and frozen semen after 48 hrs, 3 and 6 months. The results showed that there were a significant differences ($p \leq 0.05$) among the treatments in the percentage of sperm motility, viability, plasma membrane and acrosome integrity compared with the control group, also there were significant differences in sperm total abnormalities, MDA, TAC concentrations and DFI percentage among all the treatments after all cryopreservative periods compared with the control group..

Key words: Alcoholic extract,Tris extender ,sperm quality, bulls.

تأثير إضافة مستخلص بذور الأناتو *(Bixa orellana)* و التوكوترينول إلى مخفف السائل المنوي في خصائص السائل المنوي المحفوظ بالتجميد لثيران الهولشتاين

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المستخلص

أجريت الدراسة الحالية لتقييم تأثير اضافة تراكيز مختلفة من المستخلص الكحولي لبذور الأناتو والتوكوترينول الى مخفف Tris في خصائص السائل المنوي المحفوظ بالتجميد لثيران الهولشتاين. تم تخفيف السائل المنوي المجمّع بمخفف Tris ثم تقسيمه بالتساوي إلى خمسة معاملات في التجربة على النحو التالي: مجموعة سيطرة (C) مخفف Tris فقط, المعاملة T1 تحتوي على 150ملغم مستخلص الأناتو/25مل مخفف, المعاملة T2 تحتوي على 200ملغم مستخلص الأناتو/25مل مخفف, المعاملة T3 تحتوي 3 مليمول توكوترينول/25مل مخفف والمعاملة T4 تحتوي على 4 مليمول توكوترينول /25مل مخفف. تم تجميد السائل المنوي المخفف حسب الطريقة المعتمدة ومن ثم تقييم خصائص السائل المنوي والتي شملت الحركة الفردية للنطف والحيوية والتشوهات وسلامة الغشاء البلازمي وسلامة الاكروسوم وتركيز المالون ثنائي الديهايد (MDA) ومضادات الأكسدة الكلية (TAC) وتضرر المادة الوراثية (DFI) لكل من السائل المنوي المبرد والمجمد بعد 48 ساعة و 3 أشهر و 6 أشهر. وقد اظهرت النتائج وجود فروقات معنوية ($p \leq 0.05$) بين المعاملات في كل من النسبة المئوية للحركة الفردية للنطف والحيوية وسلامة الغشاء البلازمي وسلامة الأكروسوم مقارنة مع مجموعة السيطرة، كما وجدت فروق معنوية في التشوهات الكلية للنطف، تراكيز MDA، TAC ونسبة DFI بين جميع المعاملات بعد كل فترات الحفظ بالتجميد مقارنة مع مجموعة السيطرة.

الكلمات المفتاحية: المستخلص الكحولي، مخفف Tris ،نوعية النطف، الثيران.

Received: 17/4/2023, Accepted:2/8/2023, Published :October 2025

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 extends its storage and physiological value to use it in artificial insemination (15). Spermatozoa are highly susceptible to reactive oxygen species (ROS) due to the specific composition of their plasma membrane which contains poly unsaturated fatty acids which are structurally unstable and highly prone to lipid peroxidation (37). Aldehydes byproducts such as MDA is generated from the peroxidative damage which negatively affects DNA integrity, mitochondrial function, apoptosis, and cellular signaling (11). Improvement of semen extenders with suitable antioxidant is suggested to reduce oxidative damage during freeze–thawing of bull spermatozoa (7), overwhelming results of improved post-cryopreserved semen quality of Holstein bulls were obtained by adding several antioxidant agents such as vitamin e and other antioxidants to tris extender have already been tested in vitro or in vivo studies concerning (6,17,30). 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 (4, 5). Annatto *(Bixa orellana L.*) seed has been used as a natural colorant in many traditional foods found in asia, annatto ranks second place in economic importance worldwide among all natural colorants and its extract shows antimicrobial and antioxidant properties (40), several previous studies have measured the antioxidant activities and color properties of different annatto extract (13). Tocotrienols is a part of vitamin E family members which are natural compounds found in a number of vegetable oils, wheat germ, barley, and certain types of nuts and grains, many of the functions of tocotrienols are related to its antioxidant properties and its varied effects are due to its behaving as a signaling molecule (3). This aim of this study was to investigate the effects of adding various concentrations of alcoholic extract of annatto (*Bixa orellana*) seeds and tocotrienol to Tris - extender on improving cryopreserved Holstein bull semen characteristics**.**

MATERIALS AND METHODS

The study was carried out at the Artificial Insemination Department / Directorate of Animal Resources/ Ministry of Agriculture in Abu Ghraib region during the period from (9). Tocotrienol with 99.16% purity was purchased from (Med Chem Express, USA**)**, while annatto seeds were purchased from (Yamees, Lakewood, USA). The process of Alcoholic extraction of annatto seeds was conducted in the central laboratories of the Department of Food Sciences/ College of Agricultural Engineering Sciences/ University of 50 grams of the dried seeds were powdered by an electric mill and soaked in 60% ethanol for 24 hours and the mixture was filtered and then soxhlet apparatus was used to evaporate organic solvent and the concentrated material was obtained as extract and maintained at 4 ˚C until used according to the method of (28), estimation the cellular toxicity of annatto seeds extract was carried out according to the method of (16) ,total phenolic compounds and total flavonoids concentrations was performed by reversed phase HPLC chromatographic system analysis in the Ministry of Science and Technology laboratories in Al-Jaderiya depending on the method of (9), estimation of total terpenoids according to the method of (32), and determination of glycosides concentration according to the method of (36).

Semen cryopreservation. Ejaculates were collected from four healthy, fertile fresian bulls, 2.5–3 years old, using an artificial vagina pre-warmed to 42 C˚. 1ml of semen was taken from each bull and combining together as pooled semen to remove the individual variations among bulls, and after calculating the dilution rate the pooled semen was equally divided into 5 treatments, semen samples were packaged in 0.25 ml French straws and were finally cryopreserved using standard production procedures in the AI centers according to (14). Semen parameters examined include sperm individual motility as described by (39), viability were assessed according to (34), sperm's plasma membrane integrity according to method of (22), acrosome integrity according to method of (19), as well as DNA damage percentage as

described by (35), assessment of sperm abnormalities were assessed according (31), For the purpose of estimating the total antioxidant activity in seminal plasma, a ready-made laboratory kit prepared from the American company Sigma (1,1-Diphenyl-2-picryl-hydrazyl (DPPH), Sigma Aldrich Co., St.Louis, USA) was used. The concentration of malondialdehyde in seminal plasma was estimated based on the method described by (25).

Statistical analysis

Data were analyzed by means of the computerized program SAS (33) to calculate the analysis of variance (ANOVA) for the different parameters between control and additives with 4 replicate for each treatment. Significant differences between means were calculated using Duncan multiple range test at ($P<0.05$).

RESULTS AND DISCUSSION

Estimation the cellular toxicity showed that alcoholic extract did not make any decomposition of red blood cells and sedimentation in the saline solution for the four tested levels of annatto seeds extract, and did not showed any changes in the shape of the cells and it was similar to the control sample, which indicates that it does not have toxic properties and thus its considered safe for use with semen extenders. the concentration of active compounds in annatto seed extract were summarized in Table (1).

Table 1. Concentrations of active compounds in annatto seed alcoholic extract

No	Compound name	Concentration(mg/gm)
1	Tocotrienol	3.256
2	Tocopherol	00.29
3	Bixin	0.158
4	Squalene	0.059
5	Qurcetine	0.084
6	β-carotene	0.685
7	Rutin	0.090

Sperm individual motility percentage

It is clear from the results at the cooling time in Table 2 that the two tocotrienol treatments T3 (51.25±2.39) and T4 (52.50±2.50) were significantly superior to the control treatment (41.25±2.39) on the other hand, no significant differences were observed among the remaining treatments, after 48 hours and 3 and 6 months of cryopreservation ,the T2 and T3,T4 treatments were significantly superior to the control treatment, while the T1 treatment did not differ with the control treatment for the same preservation period, when comparing the different periods of preservation within the same treatment, it was noted that there was a high significant superiority ($P\leq0.05$) in the percentage of individual sperm motility during the period of cooling preservation (5C˚) compared to the 48hrs, 3 and 6 months periods post- cryopreservation.

Table 2. Effect of adding alcoholic extract of annatto seeds and tocotrienol to Tris extender on sperm motility of Holstein bulls after different cryopreservation periods (Mean ± SE)

Treatment	Time				Significance level
	5C˚	48 hrs/PC	3months/PC	6 months/PC	
C	41.25±2.39 Ba	33.75±1.25 Cb	26.25±1.25 Bc	21.25±1.25 Bd	P≤0.05
T1	46.25±2.39 Aba	38.75±11.25 BCb	27.50±1.44 Bc	24.50±1.44 Bc	P≤0.05
T2	48.75±3.14 Aba	41.25±1.25 ABb	33.75±2.39 Ac	30.00±2.04 Ac	P≤0.05
T3	51.25±2.39 Aa	41.25±2.39 ABb	32.50±1.44 Ac	30.00±2.04 Ad	P≤0.05
T4	52.50±2.50 Aa	45.00±2.04 Ab	37.50±1.44 Ac	33.25±1,25 Ad	P≤0.05
Significance Level	P≤0.05	P≤0.05	P≤0.05	P≤0.05	

PC: Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods .C=Tris only.T1=150mg annatto extract.T2=200mg annatto extract.T3=3mM tocotreinol.T4=4mM tocotreinol

Percentage of sperms viability

The results in Table 3 at the cooling time all the experimental treatments show a highly significant superiority ($P\leq0.05$) compared to the control treatment ,the T4 over the rest of the treatments, including the control treatment, in the same context, the remaining treatments T1,T2 andT3 out performed the control

treatment, as these treatments recorded (81.50±0.61),(84.75±0.51) and(85.37±0.74) respectively while the control treatment recorded the lowest value (73.37±1.39).After 48 hours and 3 and 6 months of post-cryo preservation, all the treatment continued to outperform the control treatment at the same level of significance, when comparing the different preservation periods within the same treatment, it was noted that there was a significant superiority in the percentage of live sperm during the period of cooling compared to the remaining periods post-cryopreservation.

Table 3. Effect of adding alcoholic extract of annatto seeds and tocotrienol to Tris extender on sperms viability percentage of Holstein bulls after different cryopreservation periods (Mean ± SE).

Treatment	Time				Significance level
	5C°	48 hrs/PC	3months/PC	6 months/PC	
C	73.37±1.39 Da	65.12±1.02 Db	59.25±1.49 Dc	54.25±1.49 Dd	P≤0.05
T1	81.50±0.61 Ca	72.25±0.82 Cb	67.25±0.82 Cc	63.12±1.19 Cd	P≤0.05
T2	84.75±0.51 Ba	77.00±0.84 Bb	72.25±0.82 Bc	67.51±1.02 Bd	P≤0.05
T3	85.37±0.74 Ba	77.00±0.45 Bb	72.62±1,02 Bc	70.00±1.02 Bd	P≤0.05
T4	89.50±1.13 Aa	82.50±1.17 Ab	77.50±1.02 Ac	74.35±1.57 Ac	P≤0.05
Significance Level	P≤0.05	P≤0.05	P≤0.05	P≤0.05	

PC: Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods .C=Tris only.T1=150mg annatto extract.T2=200mg annatto extract.T3=3mM tocotreinol.T4=4mM tocotreinol

Sperm plasma membrane integrity

The results upon cooling period, as in Table 4 show that the four experimental were significantly (P≤0.05) superior to the control treatment (78.50±1.19), after 48 hours, and 3,6 months post- cryopreservation the four experimental treatments recorded a significant differences (P≤0.05) compared to the control treatment especially the T4 treatment which recorded the highest value (72.87±2.72) compared to the control treatment, which recorded a significant decrease in this percentage amounting to (55.00±1.76) ,in comparison among the different periods of preservation within one treatment, a significant difference was observed between the cooling period and 48 hours post- cryopreservation for all treatments, and these differences persisted for the remaining periods 3 and 6 months post-cryopreservation.

Table 4. Effect of adding alcoholic extract of annatto seeds and tocotrienol to Tris extender on sperm plasma membrane integrity of Holstein bulls after different cryopreservation periods (Mean ± SE).

Treatment	Time				Significance level
	5C°	48 hrs/PC	3months/PC	6 months/PC	
C	78.50±1.19 Ba	68.87±0.51 Bb	61.25±0.72 Bc	55.00±1.76 Bd	P≤0.05
T1	86.00±0.70 Aa	78.75±1.25 Ab	74.12±1.41 Ac	69.37±1.57 Ad	P≤0.05
T2	86.75±0.60 Aa	79.87±0.92 Ab	75.00±1.02 Ac	70.62±0.62 Ad	P≤0.05
T3	86.37±1.56 Aa	80.62±2.57 Ab	74.25±1.49 Ab	72.50±1.76 Ab	P≤0.05
T4	89.12±0.59 Aa	83.12±1.19 Ab	77.25±2.42 Abc	72.87±2.72 Ac	P≤0.05
Significance Level	P≤0.05	P≤0.05	P≤0.05	P≤0.05	

PC: Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods .C=Tris only.T1=150mg annatto extract.T2=200mg annatto extract.T3=3mM tocotreinol.T4=4mM tocotreinol

Sperm acrosome integrity percentage

The first period (5C˚) there were a significant superiority (P≤0.05) of the experimental treatments over the control treatment, after 48 hours post- cryopreservation the T2, T3, and T4 treatments were significantly superior to the T1 and the control treatment at the same significant level In the 3 and 6 month period post- cryopreservation the same superiority continued among the treatments as the T3,T4 recorded the highest percentages (68.00±1.77) (70.25±2.04) compared to the control treatment (50.00±0.00) as described in Table 5.

Table 5. Effect of adding alcoholic extract of annatto seeds and tocotrienol to Tris extender on sperm acrosome integrity of Holstein bulls after different cryopreservation periods (Mean ± SE).

Treatment	Time				Significance level
	5C˚	48 hrs/PC	3months/PC	6 months/PC	
C	69.75±0.25 Ca	61.25±0.47 Cb	55.00±0.00 Dc	50.00±0.00 Cd	P≤0.05
T1	77.75±1.31 Ba	70.00±2.04 Bb	63.75±1.25 Cc	57.50±1.44 Bd	P≤0.05
T2	81.00±0.57 Aa	74.50±1.56 Ab	67.75±1.31 Bc	61.25±1.25 Bd	P≤0.05
T3	83.50±1.19 Aa	77.50±1.19 Ab	72.50±1.60 Ab	68.00±1.77 Ac	P≤0.05
T4	84.75±1.25 Aa	79.00±1.35 Ab	72.75±1.11144 Ac	70.25±2.04 Ac	P≤0.05
Significance Level	P≤0.05	P≤0.05	P≤0.05	P≤0.05	

PC: Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods .C=Tris only.T1=150mg annatto extract.T2=200mg annatto extract.T3=3mM tocotreinol.T4=4mM tocotreinol

Percentage of sperm total abnormalities

At the cooling time there were a significant differences (P≤0.05) among the four treatments compared with the control treatment which significantly outperformed the rest of the treatments with a rate of (11.75±1.31) compared to the values of the remaining four treatments T1,T2,T3.T4 which amounted to (6.00±0.91), (6.00±1.08) (4.50±0.28),(4.25±0.25) respectively after 48 hours , 3 and 6 months of post-cryopreservation, the control treatment remained with the same significant superiority over the remaining treatments, recording a higher percentage of deformities compared to the rest of the treatments, as for the comparison among the preservation periods within the same treatment, most of the treatments recorded significant differences, especially in the cooling period and after 48 hours post-cryo preservation

Table 6. Effect of adding alcoholic extract of annatto seeds and tocotrienol to Tris extender on sperm total abnormalities of Holstein bulls after different cryopreservation periods (Mean ± SE).

Treatment	Time				Significance level
	5C˚	48 hrs/PC	3months/PC	6 months/PC	
C	11.75±1.31 Ac	17.75±1.60 Ab	22.75±1.10 Aa	24.75±1.31 Aa	P≤0.05
T1	6.00±0.91 Bc	11.75±0.47 Bb	13.50±0.64 Bab	14.50±0.64 Ba	P≤0.05
T2	6.00±1.08 Bb	10.25±0.75 BCa	11.75±1.03 BCa	13.00±0.91 BCa	P≤0.05
T3	4.50±0.28 Bc	8.75±0.62 Cb	10.75±0.62 Ca	12.25±0.47 BCa	P≤0.05
T4	4.25±0.25 Bd	7.50±0.64 Cc	9.75±0.47 Cb	11.25±0.47 Ca	P≤0.05
Significance Level	P≤0.05	P≤0.05	P≤0.05	P≤0.05	

PC:Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods .C=Tris only.T1=150mg annatto extract.T2=200mg annatto extract.T3=3mM tocotreinol.T4=4mM tocotreinol

Concentration of malondialdehyde in seminal plasma (MDA). There were a significant differences (P≤0.05) among the experimental treatments as T4 treatment achieved the lowest value in MDA concentration (77.80±7.38 µmol) While the control treatment recorded the highest value in MDA concentration of (137.98±18.35 µmol) while the remaining three treatmentsT1,T2 and T3 recorded MDA concentrations of (105.13±24.00 µmol) (89.75±10.50µmol) (80.95±10.85 µmol) respectively post cryo preservation as described in table 7.

Seminal plasma total antioxidants capacity. There were significant differences (P≤0.05) among the experimental treatments, as described in table (7). It is noted that the highest levels were in the T4 tocotrienol treatment, as it reached (0.0242±0.002 mg/dcl),The control treatment recorded the lowest levels as it reached (0.0159±0.014 mg/dcl) and the rest of the treatments T1, T2, T3 recorded the values (0.0166±0.002 mg/dcl) (0.0210±0.002 mg/dcl) (0.0217±0.002 mg/dcl) respectively post-cryopreservation..

Sperm's DNA fragmentation percentage

There were significant differences (P≤0.05) among all the experimental treatments, as shown in Table (7). It is noted that the highest percentage were in the control treatment, as it reached (6.91±0.43),The T4treatment recorded the lowest levels as it reached 4.37±0.32) and the rest of the treatments T1, T2, T3 recorded the percentages (5.92±1.10) (5.63±0.33) (4.94±0.99) respectively post-cryopreservation.

Table 7.Effect of adding alcoholic extract of annatto seeds and tocotrienol to Tris extender on MDA,TAC,DNA fragmentation in seminal plasma after 6 months post cryopreservation (Mean±SE)

Treatment	MDA/µmol-PC	TAC/mg/dcl-PC	DNA damage%-PC
C	137.98±18.35 A	0.0159±0.014 B	6.91±0.43 A
T1	105.13±24.00 AB	0.0166±0.002 AB	5.92±1.10 AB
T2	89.75±10.50 AB	0.0210±0.002 AB	5.63±0.33 AB
T3	80.95±10.85 B	0.0217±0.002 AB	4.94±0.99 AB
T4	77.80±7.38 B	0.0242±0.002 A	4.37±0.32 B
Significance Level((P≤0.05)	*	*	*

***(P≤0.05).PC/ Post cryopreservation. Capital letters to compare among treatments .C =Tris only.T1=150mg annatto extract.T2=200mg annatto extract.T3=3mM tocotreinol. T4=4mM**

Several studies investigated the effect of adding plant extracts to semen extenders and reported a beneficial use of it to enhanced the semen characteristics of bulls (5, 1, 24) rams (4,20). The results of the chemical analysis of the crude alcoholic extract of annatto seeds showed that it contained a significant percentage of active compounds (phenols, flavonoids and terpenes) at the concentrations mentioned above which act as antioxidant prevent the harmful effect of free radicals against lipids and protein fractions within cellular compartments (29,38).The content of tocotrienols in the annatto plant is about 140-147 mg / 100 g dry grains when extracted with the Soxhlet device, and there is no type of plant that contains a similar amount as annatto in its content of these active compounds (18).The addition of the alcoholic extract of annatto seeds to Tris extender resulted in a significant improvement of some semen characteristics post-cryopreservation, as the individual motility rate increased in the T2 treatment, as well as the percentage of sperm viability , plasma membrane and acrosome integrity for each of the two treatments T1 and T2 compared to the control treatment after 48 hours, 3 months, and 6 Months post-cryopreservation, on the other hand it decrease in the percentage of damage to sperm genetic material (DNA) among the four tested treatments compared to the control treatment, and the Significant superiority was accompanied by an arithmetic superiority for most of the treatments studied above compared to the control treatment for the rest of the different preservation periods, and the reason may be attributed to the content of the annatto extract of active compounds that have antioxidant properties including ,tocotrienols, bixin, and β-carotene (13). The treatments of alcoholic extract of annatto seeds T1,T2 were significantly (P≤0.05) superior in the percentage of sperm individual

motility after 48 hours, 3 and 6 months post-cryopreservation compared to the control treatment. The addition of tocotrienol to T3 and T4 treatments led to a significant improvement in the percentage of sperm individual motility upon cooling, with an arithmetic improvement of these two treatments for this characteristic at the rest of the preservation periods compared to the control treatment as described in table 2, as this percentage remained within the permissible limits for the purposes of artificial insemination and the reason for the significant superiority may be due to the action of tocotrienols in activating antioxidant enzymes (SOD, NADPH, and GBx) to suppress the formation of free radicals (21,26), what works to protect the sperm from the damage of oxidative stress and thus preserve the sperm motility (8),as the percentage of the plasma membrane integrity and acrosome integrity increased significantly in the two treatments T3 and T4 upon cooling and after 48 hours post- cryopreservation compared to the two treatments of annatto extract T1and T2 and the control treatment at the rest of the preservation periods 3 and 6 months post-cryopreservation. Its known that MDA is a biomarker indicator of the level of oxidative stress (2) and total antioxidant activity in seminal plasma is a vital indicator of the amount of antioxidant protection available (12) T4 treatment recorded the lowest percentage of damage to the genetic material (DNA) (4.37±0.32), followed by the T3 treatment (4.94±0.99) compared to the control treatment (6.91±0.43), it can be said that the addition of the alcoholic extract of annatto seeds and tocotrienols to the Tris extender had an effect mainly in improving the characteristics of the percentage of individual sperm motility, the plasma membrane integrity and the acrosome integrity which is an important factor in the processes of capacitation and acrosome reaction to complete the fertilization process, as it was found that there is a positive relationship between the integrity of the acrosome and the fertility of frozen bulls' sperm (27),generation of reactive oxygen species during the spermatogenesis and during freezing-thawing processes is one of the reasons behind the sperm's DNA damage (41), and the increasing in total antioxidant activity and reducing the concentration of MDA and the genetic material damage would reflected positively in increasing the fertility rate of bulls and achieving a high fertility rate, it was found that bulls which have a lower percentage of the genetic material damage to have a higher fertility rate compared to their counterparts with high genetic material damage percentages (23), with regard to the percentage of total deformities of the sperm previous studies have found a correlation between MDA level and abnormal morphology evaluated by light microscopy (10) the majority of the treatments recorded significant differences compared to the control treatment at cooling period 5C˚ after 48 hours, 3 and 6 months post cryopreservation, and the 4Mm/tocotrienol treatment recorded the less percentage of deformities compared to the rest of the treatments.

Conclusions

Based on the results of this research it was found that the addition of alcoholic extract of annatto *(Bixa orellana)* seeds and tocotrienol to tris extender had beneficial effect in enhancing some of post cryopreserved semen characteristics of holstein bulls for different cryopreservation periods.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received afund.

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