EFFECT OF ADDING PENTOXIFYLLINE AND NITRIC OXIDE TO TRIS EXTENDER ON SOME POST-CRYOPRESERVED SEMEN ATTRIBUTES OF HOLSTEIN BULLS

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ABSTRACT
This study was aimed to explore the effect of adding pentoxifylline (PTX) and nitric oxide (NO) to Tris extender on some post-cryopreserved semen attributes of Holstein bulls for different preservation periods (cooling at 5◦C, 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. PTX (0.192 g / 100 ml extender) and NO (0.018 g / 100 ml extender) were added to Tris extender and comparisons in response were made with the control group (Tris extender, C). The PTX group exhibited greater ($P \leq 0.01$) sperm's cell individual motility percentage as compared with the C group at cooling (5ºc) and 48 hr PC periods, while, PTX and NO groups were superior in these percentages at the remaining PC periods than C group. Excluding data of 2 months PC, greater ($P \leq 0.01$) live sperm percentage was observed in PTX and NO groups in comparison with the C group at all preservation periods. Lesser ($P \leq 0.01$) abnormal sperm percentage were noticed for PTX and NO groups as compared with the C group at all preservation time periods. The PTX and NO groups exhibited greater ($P \leq 0.01$) acrosome and plasma membrane integrity percentages in comparison with the C group at all preservation time periods. In conclusion, adding PTX and NO to Tris extender enhanced some of PC semen characteristics of Holstein bulls at different preservation periods.

Key words: synthetic antioxidants, semen characteristics, acrosome integrity.

تأثير اضافة البنتوكسفايلين واوكسيد النتريك الى مخفف Tris في بعض صفات السائل المنوي لثيران الهولشتاين بعد الحفظ بالتبريد والتجميد

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المستخلص
أجريت هذة الدراسة بهدف بيان تأثير اضافة البنتوكسفايلين واوكسيد النتريك الى مخفف Tris في بعض صفات السائل المنوي لثيران الهولشتاين بعد الحفظ لمدد زمنية مختلفة (التبريد بدرجة حرارة 5ºم والتجميد لمدة 48 ساعة وشهر وشهرين وثلاثة اشهر من الحفظ بالتجميد). تم استخدام سبعة ثيران هولشتاين بعمر 2.5-3 سنوات للمدة من 20 تشرين الثاني/2017 ولغاية اب/2018. تم جمع السائل المنوي وتجميعه ومن ثم تقسيمه الى ثلاث اجزاء باستخدام مخفف Tris. اضيف كل من مركب البنتوكسفايلين (0.192 غم/100مل مخفف) واوكسيد النتريك (0.018 غم/100 مل مخفف) الى مخفف Tris ومقارنتها مع مجموعة السيطرة (مخفف Tris لوحده). اظهرت مجموعة البنتوكسفايلين ارتفاع معنوي ($P \leq 0.01$) في النسبة المئوية لحركة النطف الفردية مقارنة بمجموعة السيطرة عند مدتي الحفظ بالتبريد و48 ساعة من الحفظ بالتجميد، في الوقت الذي تفوقت فيه مجموعتي البنتوكسفايلين واكسيد النتريك على مجموعة السيطرة خلال مدد الحفظ بالتجميد المتبقية. باستثناء نتائج الشهر الثاني من الحفظ بالتجميد، فقد لوحظ اعلى ($P \leq 0.01$) نسبة مئوية للنطف الحية لدى مجموعتي البنتوكسفايلين واوكسيد النتريك مقارنة بمجموعة السيطرة خلال مدد الحفظ الاخرى. حققت مجموعتي البنتوكسفايلين واوكسيد النتريك انخفاض معنوي ($P \leq 0.01$) في النسبة المئوية للنطف المشوهة مقارنة بمجموعة السيطرة خلال مدد الحفظ المختلفة. كما اظهرت مجموعتي البنتوكسفايلين واوكسيد النتريك ارتفاع معنوي ($P \leq 0.01$) في النسبة المئوية لسلامة الاكروسوم والغشاء البلازمي للنطف مقارنة بمجموعة السيطرة خلال مدد الحفظ المختلفة. يمكن الاستنتاج بأن اضافة كل من مركبي البنتوكسفايلين واوكسيد النتريك الى مخفف Tris ادى الى تحسن بعض صفات السائل المنوي لثيران الهولشتاين عند مدد حفظ مختلفة.

كلمات مفتاحية: مضادات اكسدة صناعية، صفات السائل المنوي، سلامة الاكروسوم.

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INTRODUCTION

Lipid peroxidation is a well established mechanism of cellular injury and is used as an indicator of oxidative stress (68). Physiologically, the high concentrations of polyunsaturated fatty acids (PUFA) in sperm are important for maintaining membrane fluidity and flexibility during fertilization process (5). The mechanism by which oxidative stress induced motility loss in mammalian spermatozoa involved the induction of peroxidative damage to the sperm plasma membrane (61). Seminal plasma malondialdehyde, which is the stable lipid peroxidation product, is a simple method to evaluate the effect of lipid peroxidation on sperm (27). Mammalian spermatozoa are extremely sensitive to oxidative damage. Lipid peroxidation plays a leading role in aging of spermatozoa, shortening its life-span *in vivo* and affecting the preservation of semen for artificial insemination (14). The process of peroxidation induces structural alterations; particularly in the acrosomal region of the sperm cell, a fast and irreversible loss of motility, a deep change in metabolism and a high rate of release of intracellular components. Cryopreservation causes a wide range of chemical, physical and mechanical injures to sperm membranes of all mammalian species (65), which are attributed to temperature changes, alterations in the transition from the lipid phase, production of reactive oxygen species (ROS) and osmotic stress (16, 47). Freezing- thawing process causes sperm damage, like loss of lipid membrane integrity, mitochondria activity, and acrosome membrane integrity, leading to a reduction in motility, viability and fertility of sperm (65). Adding of enzymatic antioxidants like GSH and catalase (21) and non enzymatic antioxidants like vitamin A , E and C (12 , 23) amino acids (2, 44), manganese (22) and coenzyme Q10 (58) and their various combinations to Tris extender (1,2,4) have given good results in improving the post-cryopreserved semen quality of Holstein bulls in Iraq. Pentoxifylline (PTX), a methylxanthine derivate, is an inhibitor of phosphodiesterase (28). This substance has also been used to stimulate the motility of fresh and/or frozen–thawed human (33), equine (29, 40), bovine (45), ovine (41) and feline (54) spermatozoa. Pentoxifylline inhibits cAMP phosphod-iesterase, thus increasing the intracellular cAMP concentration (26). A rise in cAMP concentration causes an increase in the cAMP-dependent processes of spermatozoa such as motility, capacitation and acrosome reaction in spermatozoa (8, 11). In addition, pentoxifylline has been proposed too as a cryoprotectant of human spermatozoa due to its protection of spermatozoa membranes from freezing damage, allowing for better post-thaw motility (13) and viability (24). Moreover, it has a protective effect on sperm membranes as it scavenges reactive oxygen radicals and then reduces lipid peroxidation (46). Nitric oxide (NO) as an active non-organic molecule is spreadable and free and non-stable which is considered as endothelium-derived relaxing factor in veins. It is made in body from L-arginine by the enzymatic action of NO synthase (36). After making band with sulfur-iron complexes NO changes the activity of these enzymes. NO is an important transmitter molecule in mammalian cells and plays a main role in physiological and pathological processes (19). The NO has many physiological activities, like sperm motility, acrosome reaction, chemotaxis, ability of sperm to bind to the egg, spermatogenesis and balancing the action of hypothalamic-pituitary-gonadal axis (50). Moreover, incubation of spermatozoa with NO donors increases intracellular cyclic guanosine monophosphate (cGMP) levels of the spermatozoa for bulls (70) and men (49). Sperm hyperactivation motility is promoted by exogenous addition of NO which may increase phosphorylation of flagella proteins (31, 62). Adding of PTX and NO as powerful antioxidants and promotors 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 investigate 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 (51). The Tris-based extender contained 2.42 g Tris, 1.34 g citric acid, 1 g fructose, 19.2% v/v egg yolk, 6.4 ml glycerol (6.4%) and 100 ml distilled water at a pH of 6.8. The extender was mixed with the pooled semen samples and was divided into three groups. PTX (0.192 g / 100 ml extender) and NO (0.018 g / 100 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˚C cooling, 48h , 1,2 and 3 months post-cryopreservation (PC) were estimated according to (63) and (17) 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˚C 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 (60) 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 40x magnification 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 (30) 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 40x magnification microscope. The abnormalities were classified based on (43). Sperm acrosome integrity was determined using the procedure of (38) 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 (100X). 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 (34) 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 37ºC for 60 min.

Statistical analyses

The statistical analysis system (52) was used in the statistical analysis of the data according to the complete random design (CRD) to study the effect of pentoxifylline and nitric oxide on the studied traits. Differences among means were compared using Duncan's multiple range test (20). Chi square test was used to compare different percentages of studies characteristics (55).

RESULTS AND DISCUSSION

Sperm's cells individual motility percentage

PTX group exhibited greater ($P \leq 0.05$) sperm's cells individual motility as compared to the C group at cooling period and 48 hr post-cryopreservation (PC; Table 1). At similar periods, our results didn't show any significant differences between NO group and either C group or PTX group (Table1). Greater ($P \leq 0.01$) sperm's cells individual motility was observed for PTX and NO groups in comparison with the C group at 1,2 and, 3 months PC. (Table 1). Significant ($P \leq 0.01$) differences were noticed among cooling period and all PC periods within PTX and NO groups, however, the differences among PC groups lacked significance (Table 1). Within the C group, 2 and 3 months PC registered lesser ($P \leq 0.01$) sperm's cells individual motility percentage as compared with cooling and 48 hr PC periods (Table 1).

Live sperm percentage

Greater ($P \leq 0.01$) live sperm percentage were observed for PTX and NO groups as compared with the C group at all preservation periods (Table 2). Moreover, the PTX group exhibited

greater (P≤ 0.01) live sperm percentage in comparison with the NO group at 48 hr PC (Table 2). Greater (P≤ 0.01) live sperm percentage was observed at cooling period as compared with the PC periods in all groups. At 2 and 3 months PC, lesser (P≤ 0.01) live sperm percentages were noticed as compared with 48 hr PC in PTX and NO groups and compared with 48 hr and 1 month PC in the C group (Table2).

Abnormal sperm percentage

Lesser (P≤ 0.05) abnormal sperm were observed for PTX and NO groups as compared with C group at cooling period, however, the differences between PTX and NO groups lacked significance at similar period (Table3). At 48hr, 1, 2 and 3 months PC, the lesser (P≤ 0.01) percentages were noticed for PTX and NO Groups in comparison with the C group, however the differences between two former groups lacked significance (Table3). For all groups, the coolong period registered the lesser (P≤ 0.01) abnormal sperm percentages as compared with all PC periods(Table 3). Furthermore, these percentages were consequently increased from 48hr to 3 month PC for all groups.

Table 1. Effect of adding Pentoxifylline (PTX) and Nitric oxide (NO) 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^{b} B	25.00 ± 2.04^{b} BC	21.25 ± 3.14^{b} C	20.00 ± 2.04^{b} C	P≤0.01
PTX	50.00 ± 3.53^{a} A	B 41.25 ± 3.14^{a}	38.75 ± 1.25^{a} B	33.75 ± 2.39^{a} B	32.50 ± 3.22^{a} B	P≤0.01
NO	47.50 ± 4.33^{ab} A	35.00 ± 4.08^{ab} B	35.00 ± 4.08^{a} B	32.50 ± 3.22^{a} B	32.50 ± 3.22^{a} B	P≤0.05
Significance level	P≤0.05	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; PTX=Pentoxifylline *;* NO= Nitric oxide; PC= Post-cryopreservation.

Table 2. Effect of adding Pentoxifylline (PTX) and Nitric oxide (NO) to Tris extender on live sperm cell 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^{b} D	P≤0.01
PTX	77.10 ± 0.78^{a} A	70.10 ± 0.90^{a} B	65.92 ± 1.18^{a} BC	61.55 ± 1.37^{a} CD	58.10 ± 2.41^{a} D	P≤0.01
NO	76.55 ± 1.27^{a} A	66.72 ± 1.16^{b} B	62.17 ± 1.09^{a} BC	58.92 ± 1.60^{ab} CD	56.30 ± 2.20^{a} 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; PTX=Pentoxifylline *;* NO= Nitric oxide; PC= Post-cryopreservation

Table 3. Effect of adding Pentoxifylline (PTX) and Nitric oxide (NO) 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^{a} 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
PTX	11.30 ± 0.63^{b} A	B 15.35 ± 0.47^{b}	18.05 ± 0.35^{b} C	19.92 ± 0.26^{b} D	21.37 ± 0.23^{b} E	P≤0.01
NO	11.15 ± 0.41^{b} A	16.05 ± 0.36^{b} B	18.87 ± 0.42^{b} C	20.50 ± 0.64^{b} D	21.62 ± 0.55^{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; PTX=Pentoxifylline ; NO= Nitric oxide; PC= Post-cryopreservation

Acrosome integrity percentage

PTX and NO groups showed greater significant (P≤0.01) of acrosome integrity percentage as compared with the C group at all preservation periods (Table4). The cooling period exhibited greater (P≤0.01) acrosome integrity percentage as compared with all PC groupds (Table4). For PTX and NO groups, lesser (P≤0.01) acrosome integrity percentage were observed at 1,2 and 3 month PC as compared with 48hr PC (Table4) For C group, 2 and 3 months PC registered lesser (P≤0.01) percentages as compared with 48hr and 1 month PC (Table4).

Plasma membrane integrity percentage

The PTX and NO groups exhibited greater (P≤ 0.01) plasma membrane integrity percentages as compared with the C group at all various preservation periods (cooling at 5ºC, 48h, 1, 2 and 3 months PC) in this study (Table5). 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 PTX and NO to Tris extender on semen characteristics of Holstein bulls. Mammalian spermatozoa are extremely sensitive to oxidative damage (39). Lipid peroxidation plays an important role in spermatozoon ageing, shortening life-span and affecting the preservation of semen for artificial insemination (9). Maintenance of sperm membrane phospholipids and susceptibility to peroxidation depends on adequate antioxidant, which reduces the risk of damage to spermatozoa and increases their survival chances during storage (56,57). Thus, a deficiency of these fractions can affect the overall protection of the spermatozoa from oxidative damage, which can have a negative effect on sperm motility and fertilizing ability (48).

Table 4. Effect of adding Pentoxifylline (PTX) and Nitric oxide (NO) 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^{b} B	55.92 ± 1.99^{b} C	46.92 ± 2.67^{b} D	P≤0.01
PTX	78.97 ± 1.02^{a} A	B 71.05 ± 1.06^{a}	67.75 ± 1.01^{a} C	64.85 ± 1.14^{a} CD	62.85 ± 1.07^{a} D	P≤0.01
NO	78.10 ± 0.98^{a} A	71.12 ± 1.34^{a} B	66.80 ± 1.14^{a} C	62.87 ± 1.39^{a} CD	60.30 ± 0.96^{a} 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; PTX=Pentoxifylline ; NO= Nitric oxide; PC= Post-cryopreservation.

Table 5. Effect of adding Pentoxifylline (PTX) and Nitric oxide (NO) 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^{b} C	51.77 ± 1.66^{b} D	44.72 ± 2.68^{b} E	P≤0.01
PTX	75.35 ± 0.27^{a} A	B 68.15 ± 1.12^{a}	64.47 ± 0.54^{a} C	61.30 ± 0.44^{a} D	58.00 ± 1.13^{a} E	P≤0.01
NO	75.30 ± 0.93^{a} A	67.17 ± 0.46^{a} B	63.05 ± 0.41^{a} C	59.22 ± 0.90^{a} D	56.05 ± 1.20^{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;
PTX=Pentoxifylline ; NO= Nitric oxide; PC= Post-cryopreservation.

The process of peroxidation induces structural changes, particularly in the acrosomal region of the sperm cell, a fast irreversible loss of motility, deep change in metabolism and a high rate of intracellular component release (35). The current results had showed greater ($P \leq 0.01$, $P \leq 0.05$) sperm's cell individual motile, live sperms, acrosome integrity and plasma membrane integrity percentages as well as lesser ($P \leq 0.01$, $P \leq 0.05$) total abnormal sperms for PTX and NO groups all preservation periods as compared with the C group. Among methylxanthines, PTX has been used. Methylxanthine supplementation resulted in better seminal characteristics in fresh and cryopreserved spermatozoa viz., motility and curvilinear velocity (14). A stimulatory effect of PTX on capacitation and acrosome reaction has also been demonstrated (7). Overall, the adding of PTX to sperm suspension seems to improve sperm function leading to better sperm fertilizing ability (25). Antioxidants are used to stop auto-oxidation that causes a chain reaction in the unsaturated fatty acids in oils and lipid, and help in slowing down the oxidation of fats and oils, Oxygen reacts preferentially with antioxidants rather than oxidizing fats or oils, thereby protecting them from spoilage (48). The PTX is a methylxanthine phosphodiesterase inhibitor which reduces superoxide anions responsible for DNA apoptosis (42). PTX is one of the most effective additives for improving the motility and acrosome reactivity of human spermatozoa (28). It has been also used to enhance the motility of fresh and frozen spermatozoa from a number of domestic animals (29, 33, 37, 41, 45, 54). PTX increases cAMP level by a methylxanthine inhibition of phosphodi-esterase and thus improves motility, capacitation and acrosome reaction (53,69). This increase in cAMP causes activation of protein kinase and phosphorylation of endogenous protein (48). Pentoxifylline is thought to act as a cryoprotectant of spermatozoa (13, 24, 64). The current results were in line with those reported by Bhakat et al. (14) who concluded that, the addition of 1.5 mM BHT, 3.6 mM PTX and 1 mg/ml Vit E in the Karan Fries bulls semen extender has more beneficial effect in terms of semen quality and preservability of spermatozoa. The present results confirmed the findings of Pankaj et al. (48) who stated that PTX can be used as a potent antioxidant substance against oxidative stress and subsequent effects to improve sperm motility, abnormal spermatozoa, live-dead count, hypo-osmotic swelling and acrosomal integrity in riverine buffalo. The current results were agreed with Marques et al. (40) who investigated the effects of ascorbic acid, PTX and their combination on the progressive motility, vigor, viability and integrity of the acrosome of cryopreserved stallion spermatozoa. Nitric oxide have a major role in stimulation of lactic dehydrogenize which was a key for metabolic process in sperm motility that may be presumably given as the sperm direct effects responses individual motility, as an end point (18). Nitric oxide as a free radical has actually been shown to be a beneficial antioxidant against reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2) (15, 59, 66), These free radicals are regularly released in mammalian cells during oxygen consumption and cause peroxidative damage to membrane phospholipids (10). This scavenging property gives nitric oxide a major intracellular and extracellular action against oxidative stress (67, 32) that may provide protection against oxidation damage to bimolecular of sperm cell, which in turn elongates sperm life and decreases sperm abnormalities during storage (6). The current results were in agreed with Khodaei et al., (36) who concluded that, the addition of sodium nitroprusside (SNP) as a nitric oxide donor to improved sperm motility, live-dead count, hypo-osmotic swelling and acrosomal integrity in Holstein bulls. The present results confirmed the findings of AL-Ebady et al., (6) who noticed that adding of 0.005 or 0.006 M/ml of arginine as a nitric oxide donor to semen diluents containing poor motile bull sperms improved sperm motility and decreased death, abnormality and defect in acrosoma of sperm.

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