Assiut Vet. Med. J. Vol. 50 No. 100 January 2004

Dept. of Theriogenology
Fac. Vet. Med. Alex. Univ.

FERTILIZING CAPACITY OF FROZEN BUFFALO-BULL SEMEN TREATED BY CALCIUM CHANNEL BLOCKER (VERAPAMIL) AND/OR ANTIOXIDANT (α-OCHOPHEROL)

(With 2 Tables)

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(Received at 29/11/2003)

قدرة السائل المنوى المجمد لطلائق الجاموس على الإخصاب والمعالج بفالق مجرى الكالسيوم (فيراباميل) مع / أو مضاد الأكسدة (الفا- توكوفيرول)

كمال كمال متولى

استهدفت الدراسة تقييم تأثير عالق مجرى الكالسيوم (فيراباميل) مع / او مضاد الاكسدة الفا- توكوفيرول على نوعية السائل المنوى المجمد لطلائق الجاموس وقدرته على الاخصاب. تم تجميع عينات السائل المنوى باستخدام المهبل الصناعى من ثلاثة طلائق جاموسى مرتين اسبوعيا لمدة ثلاثة أسابيع. تم تقييم العينات مباشرة والعينات التى لها حركة فردية للحيوانات المنوية اكثر من ٦٠ % تم استخدامها فى التخفيف باستخدام مخفف صفار البيض وسترات الصوديوم والجليسرول الى ١٠٠ مليون حيوان منوى / مل. تم تقسيم السائل المنوى المخفف إلى أربعة أجزاء. الأول تم استخدامه كضابط أما الثانى فتم إضافة فيراباميل بتركيز ٣٠ ميكروجرام / مل والثالث تم إضافة الفا-توكوفيرول بتركيز ٣مجم / مل أما الرابع فتم إضافة كل من فيراباميل (٣٠ ميكروجرام/ مل) و الفا-توكوفيرول (٣مجم / مل). تم تعبئة كل الأجزاء فى قشات سعة كل واحدة نصف مل وتحتوى على ٥٠ مليون حيوان منوى وتم التجميد فى بخار النيتروجين السائل وتم الحفظ فى النيتروجين السائل لمدة شهر. تم استخدام ٦٠ جاموسة لتقييم قدرة السائل المنوى المجمد على الإخصاب تم تقسيمها إلى أربع مجموعات ١٥ جاموسة لكل مجموعة وتم تلقيح كل مجموعة بنوع من السائل المنوى. أظهرت النتائج أن علاج السائل المنوى المجمد للجاموس بكل من فيراباميل والفا-توكوفيرول أعطت افضل النتائج بعد الإسالة (أعلي حركة للحيوانات المنوية واقل التشوهات واقل نسبة عدم سلامة القلنصوة واقل محتوى فى الوسط من AST,ALT,LDH مقارنة بباقى العلاجات. أيضا أظهرت قياسات الخصوبة فى الجاموس الذى تم تلقيحه بالسائل المنوى المعالج بكل من فيراباميل والفا-توكوفيرول أعلى القياسات (فعدد التلقيحات لكل إخصاب كان الأقل وكانت نسبة الإخصاب لاول تلقيح هى الاعلى).

SUMMARY

This study aimed to evaluate the effects of calcium channel blocker (Verapamil) and /or antioxidant (α-tochopherol) on the quality and fertilizing capacity of frozen thawed buffalo-bull semen. Semen samples were collected from three buffalo-bulls using artificial vagina, twice weekly for three weeks. Semen samples were evaluated directly after collection using standard routine evaluation criteria. The semen samples with motility more than 60% were used for treatment and processing. The semen was diluted to 100 million sperm/ml by egg yolk citrate glycerol diluent and divided into four fractions. 1st fraction used as control. 2nd fraction treated by Calcium channel blocker (Verapamil) in concentration of 30 µg/ml. Third fraction treated by α – tocopherol in concentration of 3mg/ml. Fourth fraction treated by both Verapamil (30 µg/ml) and α – tocopherol (3mg/ml). All samples were packaged in minitubes (0.5ml capacity) containing 50 millions sperm then frozen in liquid nitrogen vapor using instant freezing technique and preserved in liquid nitrogen for one month. 60 buffalo-cows were used for evaluation of the fertilizing capacity of the frozen semen. 15 buffalo-cows were inseminated by one treated semen. The results revealed that, treatment of buffalo-bull semen by both Verapamil and α-tocopherol gave the better quality after thawing (high sperm motility percentages and low abnormalities, acrosomal damage and the content of the medium from AST, ALT and LDH) compared to other treated semen. Also, the reproductive parameters in buffalo-cows inseminated by semen treated by both Verapamil and α-tocopherol were better than that the other treatments (low service/conception "S/C" and high 1st insemination conception rate).

Key words: Calcium channel blocker, antioxidants, semen, buffalo-bull.

INTRODUCTION

Using of the artificial insemination (AI) reduced transmission of the venereal diseases and allowed for the exchange of semen between AI centers (Aurich *et al.*, 1977).

The presence of calcium ions (Ca^{2+}) in diluted semen plasma accelerates the acrosomal and membranal damage or exocytosis during storage (Fraser and McDermott, 1992). As Ca^{2+} is one of the most important factor stimulating indonucleases activities and thereby the

apoptosis (programmed cell death, including cell shrinkage and nuclear fragmentation (Gaido and Cidlowsi, 1991). The concentration and distribution of Ca^{2+} plays an important role in the regulation of contractile and secretory function in many different types of cells, where the intracellular Ca^{2+} had been implicated as a regulatory mediator in spermatozoa (Rasmussen and Goodman, 1977). Extracellular Ca^{2+} strongly affects motility of intact bovine spermatozoa (Babcock *et al.*,1976).

The concentration of intracellular free Ca^{2+} of spermatozoa plays a regulatory role in control of motility (Arver and Sjoberg, 1982). High extracellular calcium inhibits mitochondrial ATP synthesis and motility of spermatozoa (Breitbart *et al.*,1985).

During storage of mammalian spermatozoa, toxic fatty acids peroxides are formed as a result of sperm phospholipids peroxidation (Sinha *et al.*,1996). O'Flaherty *et al.*(1997) reported that, bovine spermatozoa from frozen thawed semen are sensitive to lipid peroxidation which leads to structural damage to the sperm cell accompanied by lowered motility and metabolism. An important reason for the decreased fertility during semen storage is the lipid peroxides formation in the presence of oxygen radicals (Aurich *et al.*,1997). These lipid peroxides act as free radicals initiating an autocatalytic chain reaction, resulting in further damage to the cell membrane (Cotran *et al.*,1989).

The sperm plasma membrane contains a high amount of unsaturated fatty acids and therefore susceptible to peroxidative damage with subsequent loss of membrane integrity, impaired cell function and decreased motility of spermatozoa (Griveau *et al.*,1995). Additions of antioxidant to the semen lead to balance the lipid peroxidation and prevent excessive peroxide formation (Beconi *et al.*,1993 and Griveau *et al.*,1995). Aurich *et al.*(1997) reported that, the endogenous antioxidative capacity of the semen may be insufficient during prolonged storage.

This study aimed to evaluate the effects of calcium channel blocker (Verapamil) and /or antioxidant (α-tochopherol) on the quality and fertilizing capacity of frozen thawed buffalo-bull semen.

MATERIAL and METHODS

I-Collection of the semen samples:

Semen samples were collected from three clinically healthy buffalo-bulls present in the Faculty of Veterinary Medicine Alexandria

University during spring season. The animals were free from any congenital or pathological affections. The semen samples were collected twice weekly using artificial vagina for three weeks, in early morning using a buffalo-cow as a teaser. The semen samples were rapidly transferred to the laboratory.

II- Semen evaluation:

The semen was evaluated to determine:

1- Individual sperm motility (Salisbury *et al.*, 1978).
2- Alive sperm percentage by using Eosin-Nigrosin stain Campbell et al.,1956).
3- Sperm cell concentration using Neubaur hemocytometer (Bearden and Fuquay, 1980).
4- Sperm abnormalities using Alkaline Methyl Violet stain Barth and Oko, 1989).
5- Acrosome integrity using Giemsa stain (Watson, 1975). Semen samples with motility more than 60% were pooled before dilution.

III- Semen dilution and treatments:

The pooled semen was diluted to give sperm cell concentration of 100 millions sperm/ml using egg yolk citrate glycerol diluent that was composed from: Egg yolk: 20ml; Sodium citrate dihydrate 2.9%: 80ml; glycerol: 20ml; Penicillin sodium G: 100,000 IU and streptomycin: 100 mg (Ahmad *et al.*, 1996).

Diluted semen was divided into four parts and treated as follow:

a- First group: no treatment and act as control (cont.).
b- Second group: Calcium channel blocker (Verapamil hydrochloride, Isoptin, Arab Drug Company, Egypt) was added in concentration of 30 µg/ml (Vera.).
c- Third group: α – tocopherol (dl-α–tocopherol acetate, Pharco Pharmaceutical Company, Alexandria, Egypt) was added in concentration of 3mg/ml (α-tocho.).
d- Fourth group: Verapamil (30 µg/ml) and α – tocopherol (3mg/ml) were added (Vera. + α-tocho.).

All samples were packaged in minitubes (0.5ml capacity) containing 50 millions sperm then frozen in liquid nitrogen vapor using instant freezing technique (Gravance *et al.*, 1997) and preserved in liquid nitrogen for one month.

Semen was thawed in a water bath at 37°C for 60 seconds (Gravance *et al.*, 1997) and evaluated for detection of sperm motility percentage, sperm abnormalities and acrosomal integrity.

The contents of every 10 minitubes was pooled and centrifuged. The supernatant was used for determination of the extracellular activity of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) using reagents received from "Biochemical trade Inc., Miami, Florida, USA" (Reitman and Frankel, 1957) and Lactate Dehydrogenase (LDH) using reagents received from "ELITECH diagnostic, Egypt" (Cabaud *et al.*, 1958).

IV- Testing the fertilizing capacity of the frozen semen:

Sixty buffalo-cows (4-6 years old) were used for estimation of the fertilizing capacity of the frozen semen of the buffalo-bulls. Estrus synchronization was carried out using 2 doses of $PGF_2\alpha$ (Estrumate, 3ml per dose, synthetic $PGF_2\alpha$, each ml contain 250 µg cloprostenol) 11 days interval. The animals were divided into four groups, 15 buffalo-cows per each. Each group was inseminated by one treated semen. The buffalo-cows that return to estrus were re-inseminated by the same treated semen of the first time. Pregnancy diagnosis was done 60 days after the last insemination by rectal palpation. Number of Service/conception (S/C), first insemination conception rate, total conception rate and 1st insemination conception interval were recorded.

V- Statistical analysis:

Analysis Of Variance (ANOVA) and Duncan's Multiple Range test were used according to SAS (1988).

RESULTS

The results are presented in tables 1 and 2.

In this study, the results revealed that, addition of calcium channel blocker (Verapamil) or antioxidant (α-tocopherol) to the frozen semen of the buffalo-bulls improved its quality after thawing (high sperm motility percentages and low abnormalities, acrosomal damage and the content of the medium from AST, ALT and LDH) (Table 1). While, addition of both Verapamil and α-tocopherol to the buffalo-bull semen gave better results.

Insemination of buffalo-cows with frozen-semen treated by both Verapamil and α-tocopherol resulted in good reproductive parameters (low number of S/C and high 1st insemination conception rate) compared to that treated by Verapamil, α-tocopherol or control non treated semen (Table 2).

Table 1: Effects of Verapamil and/or α-tocopherol on quality of frozen thawed buffalo-bull semen (mean ± SE)

Evaluation items	Cont.	Vera.	α –tocho.	Vera and α –tocho.
Sperm motility(%)	46.25±1.25^{c}	50.0±2.88^{bc}	55.0±2.88^{ab}	58.75±1.25^{a}
Secondary sperm abnormalities (%)	17.25±1.10^{a}	15.75±1.65^{ab}	14.50±0.95^{ab}	12.50±0.50^{b}
Damaged acrosome(%)	14.0±1.82^{a}	13.25±0.62^{a}	11.0±0.57^{b}	10.75±0.25^{b}
AST (U/L)	69.5±2.10^{a}	54.0±2.21^{b}	39.25±1.65^{c}	30.25±2.09^{d}
ALT(U/L)	330.0±6.48^{a}	285.5±5.85^{b}	247.0±5.80^{c}	195.75±4.04^{d}
LDH(U/L)	1128.75±32.68^{a}	821.0±10.73^{b}	639.5±8.18^{c}	535.0±8.18^{d}

Means in the same row carry different litters are significantly different ($P < 0.05$).

Table 2: Reproductive parameters of buffalo-cows inseminated by different treated semen.

Treated semen	Number of S/C	1st insemin. Conception Rate (%)	Total conception rate (%)	1st insemin. Conception interval (days)
Cont.	3.3^{a}	33.33^{d}	66.66^{b}	16.8^{a}
Vera.	3.1^{a}	40.0^{c}	66.66^{b}	12.6^{b}
α-tocho.	2.16^{b}	53.33^{b}	80.0^{a}	8.75^{c}
Vera.+ α-tocho	1.76^{c}	60.0^{a}	86.66^{a}	6.46^{c}

Means in the same column carry different litters are significantly different ($P < 0.05$).

DISCUSSION

Abnormal spermatozoa must not exceed 15 to 18 % in bovine semen to achieve optimum fertility (Linford *et al.*,1976). As abnormal and dead spermatozoa have adverse effects on companion cells (Lindemann *et al.*,1982) and consequently on fertilization potential of the semen (Saacke and White, 1972).

The results in this study revealed that, the sperm motility percentages were increased significantly ($P < 0.05$) by adding both verapamil and α-tocopherol to the frozen buffalo-bull semen. While, the secondary sperm abnormalities, damaged acrosome and the content of the medium from AST, ALT and LDH were significantly ($P < 0.05$) decreased. Addition of verapamil or α-tocopherol improved the quality of frozen thawed buffalo-bull semen compared to control but the quality was lower than that had both verapamil and α-tocopherol. These results

are in agreement with that of Metwelly and El-Ashmawy (1999) and Metwelly *et al.*(2000). They concluded that, addition of calcium channel blocker or antioxidants to the frozen thawed buffalo-bull semen lead to stabilization of the spermatozoa membrane as indicated by decrease the permeability to AST, ALT and LDH and increased their motility.

The damaging effects of high calcium ion level in incubating media is attributed to the increased influx of calcium ion in the mitochondria by a process which depends up on the pH gradient in the mitochondria cause reduction in ATP generation by the mitochondria leading to decreased motility rate (Breitbart *et al.*,1985). The increase calcium ion influx is considered as apoptotic stimuli, which induce translocation of cytochrome C into the cytoplasm and subsequent activation of endonuclease enzyme. These processes are necessary for DNA cleave between nucleosomes with apoptotic effects (Green, 1997). These reasons might be the cause of obtained results which concluded that, verapamil had a significant ($P < 0.05$) increasing effects on sperm motility and decreasing effects on abnormal spermatozoa and acrosomal damage in frozen thawed buffalo-bull semen.

In this study, the low level of AST, ALT and LDH in the medium of frozen thawed buffalo-bull semen as a result of verapamil addition is in agreement with that recorded by (Bayad, 1995). High level of AST, ALT and LDH in the surrounding medium of spermatozoa was only seen in acute extensive cell damage and leakage (Doxey, 1971). He concluded that, these enzymes are intracellular being located in mitochondria, the cytoplasm or both and consequently circulating levels only increased when cell membranes integrity are damaged.

Gupta and Tripathi (1984) and Slaweta and Laskowska (1987) found that, presence of glutathione as an antioxidant improved the progressive motility of diluted bovine semen. Also, addition of antioxidants to the media of human semen brought beneficial effects in preventing loss of motility and inhibiting lipid peroxidation (Kim and Parthasarathy, 1998). Donoghue and Donoghue (1997) reported that, addition of antioxidants to the extended turkey semen improved sperm survival, membrane integrity and reduce the loss of motility after 48 hrs storage. Also, motility and acrosomal integrity of spermatozoa of liquid stored ram semen were improved by antioxidants addition (Maxwell and Stojanov, 1996). Addition of glutathione during deep freezing preservation of goat semen (Sinha *et al.*,1996) and buffalo-bull semen (Metwelly *et al.*,2000) increased the motility of thawed spermatozoa and

decreased the percentages of acrosomal damage and the content of the medium from AST, ALT and LDH.

Sinha *et al.*(1996) recorded that, the sudden increase in oxygen utilization by spermatozoa during thawing, following the dormant metabolic stage, might be responsible for increased production of free radicals, leading to increased lipid peroxidation and thus spermatozoal membrane damage, so addition of antioxidants to the frozen semen should prevent this phenomenon.

In this study, addition of Verapamil and/or α-tochopherol to the frozen buffalo-bull semen lead to increase the fertilizing capacity of spermatozoa as indicated by improved the reproductive parameters of buffalo-cows inseminated by such semen (table 2) compared to other groups. The best reproductive parameters were obtained in buffalo-cows inseminated by semen contain both verapamil and α-tochopherol.

Acrosomal membrane integrity along with acrosine activity was used as reliable predictor of sperm fertilizing ability (World Health Organization, 1992). So, decreased acrosomal integrity damage, in this study as a results of Verapamil and/or α-tochopherol, is indicative for good fertilizing capacity of the spermatozoa. This also is in agreement with Harrison and Vickers (1990) and Harkema and Boyle (1992) who recorded that, assessment of sperm motility and membrane integrity allows for good estimates of fertilizing capacity.

Conclusion: Calcium channel blocker and/or antioxidants addition during deep freezing preservation of buffalo-bull semen improved the quality of thawing semen and increased the fertilizing capacity of the spermatozoa leading to improve the reproductive parameters of buffalo-cows inseminated by such semen.

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