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EFFECT OF PRECOOLING INCUBATION TIME ON THE EFFICIENCY OF ADDED ANTIBIOTICS ON TOTAL BACTERIAL COUNT AND VIABILITY OF FROZEN RAM SEMEN

(With 3 Tables)

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تأثير وقت الحضانة وكفاءة المضادات الحيوية على العد الكلى للبكتيريا وحيوية السائل المنوى المجمد للكباش

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تناولت الدراسة تجميع 16 عينة من السائل المنوى للكباش، حيث تم إختيار هذه العينات المجمعة من كباش ناضجة جنسياً خليط (رحمانى وبرقى)، بحيث لا تقل حركة السائل المنوى لهذه العينات عن 70% وعدد الحيامن عن $10x3000^6$ لكل مللى لتر وعمل تخفيف للسائل المنوى و تقييمه (نسبة الحركة التقدمية، نسبة الحيامن الحية، نسة شواذ الحيامن) عند درجة 35^0 م لمدة 0,30 و45 دقيقة كما تم تجميد السائل المنوى وعمل تقييم لنسبة حركة الحيامن بعد الإسالة ونسبة تشوهات الأكروسوم وكذا معدل الحيوية وتم تقييمه أيضاً بكتريولوجياً من خلال العد البكتيرى، عد عائلة البكتريا المعوية وكذلك المكور العنقودى، وذلك عند زمن الصفر، 30 دقيقة، 45 دقيقة للوصول إلى أنسب وقت للتجميد. وتم تقييم السائل المنوى والعد البكتيري قبل التجميد وبعده كما سبق لإختيار أنسب وقت بما لا يؤثر على حيوية السائل المنوى والحصول على أقل عدد بكتيرى فى نفس الوقت. وأظهرت النتائج أن نسبة الحيامن الحية عند صفر دقيقة أعلى معنويا من 30 و 45 دقيقة وعدم وجود فرق معنوى فى حركة الحيامن عند صفر و 30 دقيقة كما وجد فروق معنوية عند 45 دقيقة. وأظهرت نسبة التشوهات فى الحيامن فروقا معنوية عند زمن صفر، 30 و45 دقيقة. بينما وجد فروقا معنوية فى حركة الحيامن بعد الإسالة وكانت أعلى نسبة عند زمن 30 دقيقة. كما قل معدل الحيوية معنويا عند زمن صفر ،30 و45 دقيقة. وكذلك نسبة تشوهات الأكروسومات بعد الإسالة لم يوجد فروق معنوية عند صفر و 30 دقيقة بينما زادت معنويا عند 45 دقيقة0 تم تقدير العد البكتيرى الكلى وبكتريا العائلة المعوية للسائل المنوى قبل التجميد وبعد الإسالة عند زمن صفر ، 30 و 45 دقيقة. وقد سجل العد البكتيرى الكلى وبكتيريا العائلة المعوية أعلى نسبة عند زمن صفر قبل التجميد وبعد الإسالة ثم قلت هذه النسبة عند زمن 30 دقيقة إلا أن هذا العدد قد زاد عند زمن 45 دقيقة. ووجد ميكروب المكور العنقودى بنسبة قليلة عند زمن صفر قبل التجميد فقط وكانت النتيجة سلبية عند زمن 30 و 45 دقيقة قبل التجميد وبعد الإسالة. وقد أوضحت الدراسة أن حفظ السائل المنوى المخفف عند درجة حرارة 35 ^{5}م لمدة 30 دقيقة قبل التجميد

هو أنسب الأوقات لتأثير المضادات الحيوية المضافة للحصول على سائل منوى يتميز بجودة عالية من حيث حيوية الحيامن والعد البكتيرى.

SUMMARY

A total of 16 pooled raw ram semen samples were collected from healthy rams cross bread (Rahmani & Barki). Semen samples of at least 70% initial sperm motility and $3000x10^6$ sperm/ml were used. Semen samples were tested bacteriologically through determination of total colony count, Enterobacteriaceae count and staphylococci count. Additionally, percentage of sperm motility, percentage of live sperm and total abnormality percentage were performed at 0, 30 and 45 minutes after dilution and holding at 35°C, then freezing process of semen was carried out at the previous time. The post thawing semen samples were evaluated as percentage of post thawing motility, viability index and acrosomal defects. Bacteriological examination of post thawing semen samples was also done at the same time intervals. At 35°C, the percentage of alive sperm increased significantly ($P<0.05$) at 0 minute than at 30 min. and 45 min incubation. The percentage of sperm motility was non significant between 0 min. and 30 min., while it was decreased significantly ($P<0.05$) at 45 min. The percentage of sperm abnormalities decreased significantly ($P<0.05$) at 0, 30 and 45 min. The post thawing motility was highy significant ($P<0.05$) at 30 min. than 0 min. and 45 min. The viability index decreased significantly ($P<0.05$) at 0, 30 and 45 min. respectively, while post thawing acrosomal defects showed non significant difference between 0 min. and 30 min., but increased significantly ($P<0.05$) at 45 min. Before freezing and post thawing, the total bacterial and Enterobacteriacea counts at 0 time were the highest values, which decreased at 30 min. At 45 min. the total bacterial and Enterobacteriacea counts increased than that at 30 min. The staphylococci count was detected with low value at 0 min. and not detected at 30 and 45 min. before freezing, while it wasn't detected in the post thawing samples at all times. The obtained results revealed that the best time for antibiotic action on semen was 30 minutes before freezing process.

Key words: *Semen, sperm, ram, bacterial count*

INTRODUCTION

The main objective of semen freezing from healthy animals having special genetic characters is the preservation of the fertilizing capacity of sperm free from or with the least number of bacterial contaminants. The presence of microorganisms in semen used for A.I. remains a controversial topic with regard to their possible harmful effects on fertilization and/or early embryonic development and their capability to infect the inseminated female animal (Eaglesome *et al.*, 1992).

Bacterial contamination of semen during collection cannot be avoided whatever the hygienic measures were taken; most commonly used media to preserve sperms is that contained egg yolk, milk and sugars, these constituents together with other factors are suitable for growth and multiplication of bacteria (Volk., 1982; Qureshi *et al.*, 1993). The A.I. industry takes steps to prevent transmission of microrganisms in semen by using hygienic measures in collecting and processing semen and by treating semen with antibiotics (Eaglesome *et al.*, 1995).

For the antibiotic to be effective against bacteria, it must have exposure time or chance to act (Hafez, 1987). Many authors used different holding times for semen incubation ranged from 0 time up to 40 minutes (Qureshi *et al.*, 1993; Shisong *et al.*, 1990) respectively.

The objective of this study was directed to determine the proper exposure time chosen for the action of antibiotic in processed semen so as to obtain semen of lowest bacterial contents without adverse effects on sperm vitality.

MATERIALS and METHODS

Semen collection and extension:

Semen samples were collected by artificial vagina twice weekly from five rams cross bread (Rahmani & Barki) aged 3-4 years old for two months. Rams were kept at Animal Reproduction Research Institute (ARRI) Farm, Al – Haram – Giza.

Semen samples of at least 70% initial sperm motility and 3000 x 10^6 sperm/ml were used. The obtained samples were pooled to yield one semen sample in each trial then extended in tris based extender (1 part semen + 19 part extender) which contained antibiotics (Gentamycin 10% 0.5 ml, Lincospectin 0.06 ml and Tylosin 0.5 ml per 100 ml extender) according to Shin *et al.* (1988).

Processing of ram semen:

After semen dilution at 35C° the extended semen was divided into three parts (1, 2 and 3) in clean narrow test tube. Part one, at 0 min. immediately after semen dilution was cooled at 5°C over a period of 45 minutes in cold handling cabinet. Part two was also hold at 35C° for 30 minutes, and part three was hold also at 35°C for 45 minutes; they were cooled at 5C° as previously mentioned. The cooled semen parts were loaded into 0.25 ml French straw at 5°C (Khalifa, 2001). The straws were immersed in liquid nitrogen and stored for 7 days.

Semen evaluation:

After semen dilution of the three parts, percentage of progressively forward sperm motility, percentage of live sperm and percentage of sperm abnormality were determined. Percentage of post thawing motility, viability index (Milovanov, 1962) and acrosomal defect FCF by fast green (Wells and Awa, 1970) were determined for the three parts.

Bacteriological evaluation of semen:

Semen evaluation was carried out bacteriologically through total colony count, total Enterobacteriaceae count and staphylococci count by pouring plate method according to Prescott *et al.* (2005).

The bacterial count and semen evaluation was done at 0, 30 and 45 minutes of incubation at 35^0C. The other portion was frozen at the same times 0, 30 and 45 minutes; then post thawing evaluation was done in order to determine the actual total bacterial number in straw before its use for insemination.

Statistical analysis:

Data were statistically analyzed by using analysis of variance according to Snedecor and Cochran (1982) and the general model program of SAS (1990).

RESULTS

Table 1: Alive sperm. %, sperm motility % and sperm abnormalities % at 35°C of different holding times. (Means ± S.E.).

Holding time	Live %	Motility %	Abnormality %
Part one at 0 min.	84.6 ± 1.5 ^{a}	81 ± 1.5 ^{a}	4.3 ± 0.9 ^{c}
Part two at 30 min.	83 ± 1.8 ^{b}	81.5 ± 1.2 ^{a}	4.7 ± 0.9 ^{b}
Part three at 45min.	79.4 ± 2.0 ^{c}	79 ± 2.1 ^{b}	10.7 ± 1.9 ^{a}

* Means with different superscripts a, b, c , …in the same column, significantly differ at least (P<0.05).

Table 2: Post thawing motility %, viability index and Acrosomal defects % at different holding times (Means ± S.E.).

Holding time	Post thawing motility	Viability index	Post thawing Acrosomal defect
Part one at 0 min.	31.7 ± 1.7 ^{c}	68.3 ± 3.03 ^{a}	22.0 ± 2.1 ^{b}
Part two at 30 min.	36.7 ± 1.7 ^{a}	65.8 ± 3.3 ^{b}	22.0 ± 1.1 ^{b}
Part three at 45min.	33.3 ± 3.3 ^{b}	54.2 ± 0.8 ^{c}	28.0 ± 1.5 ^{a}

* Means with different superscripts a, b, c , …in the same column, significantly differ at least (P<0.05).

Table 3: Mean value of bacterial count for semen before freezing and post thawing/ml.

Bacterial count / Holding time	Total colony count		Enterobacteriaceae count		Staphylococci count	
	Before freezing	Post thawing	Before freezing	Post thawing	Before freezing	Post thawing
Part one at 0 min.	9.6×10^3	8.6×10^3	3.3×10^3	1.8×10^3	0.55×10^2	–
Part two at 30 min.	2.2×10^3	1.6×10^3	0.38×10^2	$0.32\ 10^2$	–	–
Part three at 45 min.	4.1×10^3	3.6×10^3	0.63×10^2	0.52×10^2	–	–

Table (1) showed that the percentage of alive sperm increased significantly (P<0.05) (84.6±1.5) in part one than part two and part three (83± 1.8 and 79 ± 0.2) respectively.

The percentage of sperm motility showed non significant difference between part one and part two while it decreased significantly (P<0.05) in part three (81±1.5, 81.5 ±1.2 versus 79.4 ± 2.0). Moreover the percentage of sperm abnormality decreased significantly (P<0.05) in part one, two and three (4.3 ± 0.9, 4.7 ±0.9 and 10.7 ± 1.9, respectively).

Table (2) revealed that the post thawing motility was high significant (P<0.05) (36.7±1.7) in part two than part one and part three (31.7 ± 1.7 and 33.3 ± 3.3, respectively).

It was also noticed that the viability index decreased significantly (P<0.05) (68.3 ±3.03, 65.8 ±3.3 and 54.2 ± 0.8) in parts one , two and three respectively, while post thawing acrosomal defects showed non significant difference between part one and part two, but increased significantly (P<0.05) in part three (22.0 ± 2.1, 22.0 ± 1.1 and 28.0 ± 1.5, respectively).

Table (3) illustrated the results of bacterial count in the examined semen samples before freezing and post thawing. It was found that, both total bacterial colony count and Enterobacteriaceae count before freezing recorded the highest value ($9.6x10^3$ and $3.3x10^3$,respectively) followed by the post thawing one at 0 min. ($8.6x10^3$ and $1.8\ x10^3$, respectively). At 30 min. before freezing this value decreased reaching $2.2x10^3$ and $0.38\ x10^2$ respectively, whereas the post thawing count was $1.6x10^3$ and $0.32\ x10^2$, respectively. However, at 45 min. the total bacterial colony and Enterobacteriaceae count increased more than at 30 min.; before freezing it was ($4.1x10^3$ and $0.63\ x10^2$, respectively).While the post thawing result was ($3.6x10^3$ and $0.52x10^2$, respectively).

The staphylococci was detected with low count at 0 min. before freezing ($0.55x\ 10^2$) and was not detected at 30 and 45 min. Moreover, it wasn't detected in post thawing samples at all times.

DISCUSSION

In semen ejaculate, usually microorganisms may be found. Most of them are regarded as either commensals or contaminants. Large numbers of such microorganisms may indicate contamination or genital tract infection (Jeyendran, 2000). In the present investigation, the bacterial evaluation of semen was done through total bacterial count, Enterobacteriaceae count and staphylococci count, as the most common types of bacteria found in semen are *E. coli*, Proteus and Citrobacter which are members of the family Enterobacteriaceae and staphylococci (Aleem *et al.*, 1988; Riad, 2000).

The suitable time for effective action of the on bacteria present in semen in this study could be detected through measuring a viable bacterial colony count in semen at intervals of 0 min., 30 and 45 minutes; as Colle *et al.* (1996) who reported that the determination of the suitable time for effective action of antibiotic on bacteria is through measuring total bacterial count.

Prescott *et al.* (2005) enumerated several conditions which influence the effectiveness of antimicrobial agents activity including the duration of exposure and the exposure size of bacterial population. The obtained results as shown in table (3) revealed that; the bactericidal effect of antibiotics on bacteria present in the examined semen was not straight forward with the exposure time. By increasing the exposure time to 30 minutes, there was a decrease in bacterial contents of semen; then there was an increase in bacterial count at 45 minutes. The higher

bacterial count at 0 min. before freezing could be explained by that there was no enough time for antibiotic action on bacteria at 0 min.. On the other hand during 30 minutes the antibiotic is effective against a number of bacteria present in semen, so the bacterial count decreased. As there was no complete elimination of all bacteria in semen after 30 min., the remaining bacteria will multiply after 30 min. because it has a chance and ability for multiplication. This is nearly agree with Hassan (1990) who proved that the antibiotics in diluents required 15 minutes at 35°C to induce its effect on semen contaminants.

It is of importance to put in mind that the generation time during active growth varies with each species of bacterium, although for the majority it will be less than 45 minutes (Volk, 1982).

The Staphylococci count was $0.55x10^2$ at 0 min. and not detected at 30 and 45 min. before freezing, whereas in post thawing it wasn't found at all times. This may be due to the low number of Staphylococci and its sensitivity to Gentamycin in diluents. This result agrees with that reported by Riad (2000).

The addition of antibiotics (Gentamycin, Lincospectin and Tylosin) control different microorganisms without affecting sperm motility or fertility (Ahmed and Foot, 1987; Qureshi *et al.,* 1993).

The present results showed that temperature of 35°C for 30 minutes gave the best results on sperm motility, alive sperm and decrease abnormalities. The post thawing motility gave higher results after held at 35°C for 30 minutes and decrease post thawing acrosomal defects, while viability index showed high results when held at at 0 time; in this respect Hafez *et al.* (1987), Hassan (1990), Shisong *et al.* (1990) and Eaglesome *et al.* (1995) recommended the hold of semen with antibiotics at incubation time for 30 min.; 15 min.; 40 min. and 20 min. respectively, before cooling and these different times of incubation could be safely used without any deleterious effect on spermatozoal viability. In contrast Qureshi *et al.* (1993) processed diluted semen immediately.

The presence of bacteria in semen can affect sperm fertilizing capacity by attaching to spermatozoa (Diemer *et al.,* 1996); decreasing their motility (Kaur *et al.,* 1986); and reducing ability of acrosomal reaction (Kohn *et al.,* 1998). So, these could explain the significant increase of dead sperms and sperm abnormalities, significant decrease in sperm motility as well as significant decrease of post thawing motility viability index and significant increase acrosomal defects. This bad semen quality at incubation time of 45 min. is also due to aging of spermatozoa.

From the present study, it is recommended to keep the diluted semen at 35°C for 30 minutes before processing to obtain semen with good quality from both bacterial view and sperm viability.

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