Influence of addition of different antibiotics in semen diluent on viable bacterial count and spermatozoal viability of Awassi ram semen

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Abstract

The objectives of the present study were to determine the effects of six different antibiotics in controlling the growth of semen contaminating bacteria and if these antibiotics have any adverse effect on Awassi ram spermatozoa. Semen samples from six mature Awassi rams were used in this study. A total number of 120 ejaculates were collected from the rams using an artificial vagina once a week. Semen ejaculates were evaluated for volume, sperm concentration, mass motility, individual motility, percentage live sperm, sperm abnormalities, and viable bacterial count. Semen samples were diluted by sodium citrate-fructose-egg yolk. The diluted semen sample was divided into 7 parts. Six types of antibiotics were added to the semen diluent parts including; penicillin G 1000 IU ml^{-1} with streptomycin 1 mg ml^{-1}, gentamicin sulphate 250 mg ml^{-1}, tetracycline 0.5 mg ml^{-1}, lincomycin 1 mg ml^{-1}, cefoperazone sodium 1mg ml^{-1}, cefdinir 1 mg ml^{-1} and the seventh part considered as a control group without antibiotic addition. The diluted semen samples were cooled and preserved at 5 C^{o} for 5 days. Cooled diluted semen samples were examined for individual motility, percent of live sperm, sperm abnormalities, acrosomal defects and bacterial count every 24 h until 5 days. Comparing with the control, all the antibiotics examined were effective in controlling bacterial growth ($P<0.05$) from 24 h to 96 h of preservation at 5 C^{o}. Cefdinir and cefoperazone sodium proved to be significantly ($P<0.05$) effective than other antibiotics in controlling bacterial growth at 96 h of preservation as the bacterial count were $23.3 \pm 3.7 \times 10^{3}$ / ml and $25.4 \pm 6.2 \times 10^{3}$ / ml, respectively. Lincomycin, gentamicin sulphate and tetracycline proved ineffective in controlling bacterial growth at 96 h of preservation as the bacterial count were $57.1 \pm 20.1 \times 10^{3}$ / ml, $52.5 \pm 29.4 \times 10^{3}$ / ml and $46.5 \pm 8.8 \times 10^{3}$ / ml, respectively. The addition of tetracycline to diluted ram semen significantly reduced ($P<0.05$) sperm individual motility and percent live sperm and a significant increase ($P<0.05$) acrosomal defects was observed at 96 h of preservation in comparison to control and other antibiotics. Sperm viability was highly correlated with bacterial count in the control part of diluted semen ($r = 0.794$; $P < 0.01$). It could be concluded from the results of the present study that additions of cephalosporins (cefdinir or Cefoperazone sodium) at the dose of 1 mg ml^{-1} were most effective amongst the antibiotics used in checking the bacterial growth and improving semen quality of Awassi ram.

Key words: Awassi ram; Semen; Dilution; Antibiotics; Bacterial count.

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Introduction

Microorganisms can affect the male reproductive function directly, causing the agglutination of motile sperm, reducing the ability of acrosome reaction and causing alterations in cell morphology and indirectly, through the production of reactive oxygen species generated by the inflammatory response to the infection (Moretti *et al.*, 2009).

However, there is no complete agreement on the detrimental role of the presence of bacteria in the semen. Detection of bacteria in semen does not necessarily indicate infection, because sample contamination and transference of surface genital

colonization can readily occur (Sanocka-Maciejewska *et al.*, 2005). In cases in which bacteria have been detected, sperm morphology was deemed acceptable and few ejaculates contained inflammatory cells. Presence of bacteria in the ejaculates can affect fertilization directly (Morrell 2006), by adhering to spermatozoa (Diemer *et al*. 1996), impairing their motility (Kaur *et al*. 1986), and inducing acrosome reaction (El-Mulla *et al*. 1996). Microbes can also have an indirect effect by producing toxins (Morrell 2006; Fraczek *et al.*, 2007). Thus, in the use of AI, it is important to control efficiently the population of micro-organisms in the semen.

The most available diluents used for storage of spermatozoa are particularly supportive of microbial growth, it is necessary to include antibiotics to prevent massive proliferation of the microorganisms present in ejaculated ram semen. The inclusion of antibiotics is necessary whether the spermatozoa are stored short term in liquid medium. Antibiotics are added to mostly used semen diluents as a prophylactic measures against transmission of pathogenic bacteria as well as to reduce the load of non-pathogenic organisms that contaminate the semen. In ovine artificial insemination, benzyl penicillin and streptomycin are the most widely used antibiotics (Salamon and Maxwell, 2000). Some problems with resistance of bacteria in ram semen have been noted (Ahmad *et al*., 1999). A new combination of antibiotics comprised of cephalosporins and gentamicin has been used successfully to control certain resistant micro-organisms in liquid preserved ram semen (Yániz *et al*., 2010). The objectives of the present study were to determine the effects of six different antibiotics in controlling growth of semen contaminating bacteria and if these antibiotics have any adverse effect on Awassi ram spermatozoa.

Materials and methods

Animals and semen collection: Semen samples from six mature Awassi rams (2-3 years of age) used in this study, and maintained using conventional feeding, housing and lighting conditions. The study was carried out from June 2010 to October 2010 (during the breeding season). Animals were housed at the Animal Research and Practice Farm of the College of Veterinary Medicine, University of Mosul, Mosul at 36°20' N, 43° 8' E.

All these rams were in good health. They were maintained in identical nutritional and managerial condition throughout the period of study. Throughout the experimental period, the animals were kept in open front barrens, were fed individually with concentrated mixture of 1 kg per ram per day, and were given water ad libitum. A total number of 120 ejaculates were collected from the rams using an artificial vagina once a week. For collecting ejaculates, rams were penned with ewes in estrus, in the presence of a handler with an artificial vagina. Ejaculates were evaluated and accepted to include in this study, if the following criteria were met: volume varying between 0.5-2 ml; sperm concentration of 2×10^9 sperm/ml; the motile sperms percentage higher than 70% and less than 10% abnormal sperm in total.

Semen analysis : The volume of each ejaculate was recorded and sperm concentration was determined using semen diluted with 3% NaCl, the diluted semen was placed on a hemocytometer with the sperm counted in five squares of one chamber. Sperm motility was identified as those sperm cells that demonstrated progressive motility. Sperm motility was scored from zero to 100% by a qualified and experienced investigator. Semen was placed on a heated glass slide, and scoring was performed at microscopic magnification of 200X. Each sample was evaluated twice. The mean value was used for data analysis. Assessment of abnormal and normal spermatozoa was performed using an eosin–nigrosin staining method. For the percent of spermatozoa with abnormal acrosomes, fast green stain was used.

Dilution of semen and addition of antibiotics : Semen samples were diluted by sodium citrate-fructose-egg yolk (sodium citrate 2.9 g, fructose 1 g, double distilled water 80 ml and egg yolk 20 ml). Semen quality was re-evaluated to ensure that the dilution has not affected the semen quality. The diluted semen sample was divided

Table-1. Effects of antibiotics (Mean ± SE) on semen parameters and viable bacterial counts of Awassi rams diluted and preserved for 96 hours at 5 °C.

Antibiotics	Motility (%)	Live sperm (%)	Abnormal sperm (%)	Abnormal sperm acrosome (%)	Bacterial count x103/ ml
Penicillin with Streptomycin	57.0 ± 3.1[a]	60.8 ± 2.2[a]	5.0 ± 0.5	24.0 ± 0.6a	25.8 ± 11.2[a]
Gentamicin sulphate	47.9 ± 2.7[b]	53.2 ± 2.8[a]	6.0 ± 0.6	24.1 ± 1.0a	52.5 ± 29.4[b]
Tetracycline	37.5 ± 2.9[b]	42.5 ± 2.8[b]	5.5 ± 0.9	29.8 ± 0.8b	46.5 ± 8.8[b]
Lincomycin	63.6 ± 1.8[a]	68.5 ± 1.5[a]	3.5 ± 0.4	24.5 ± 0.7a	57.1 ± 20.1[b]
Cefoperazone sodium	56.6 ± 2.8[a]	66.7 ± 1.6[a]	3.5 ± 0.7	21.9 ± 0.3a	25.4 ± 6.2[a]
Cefdinir	62.9 ± 2.7[a]	59.9 ± 2.2[a]	2.9 ± 0.4	19.8 ± 0.7a	23.3 ± 3.7[a]
Control	48.3 ± 1.6[b]	52.7 ± 1.6[a]	3.6 ± 0.4	28.3 ± 0.8b	150.9 ± 26.7[b]

Means for each parameter in the same column, with different superscript differ significantly (P < 0.05).

into 7 parts. Six types of antibiotics were added to semen diluent parts including penicillin G 1000 IU ml^{-1} with streptomycin 1 mg ml^{-1}, gentamicin sulphate 250 mg ml^{-1}, tetracycline 0.5 mg ml^{-1}, lincomycin 1 mg ml^{-1}, cefoperazone sodium 1mg ml^{-1}, cefdinir 1 mg ml^{-1} and the seventh part considered as a control group without antibiotic addition. The diluted semen samples were cooled and preserved at 5 C^{o} for 5 days. Cooled diluted semen samples were examined for individual motility, percent of live sperm, sperm abnormalities, acrosomal defects and bacterial count every 24 h until 5 days.

Bacteriological count : For the determination of viable bacterial count, the medium was prepared by dissolving 23 g of dehydrated nutrient agar in 1 L of deionized doubled distilled water and heating it to boiling point. The medium was autoclaved at 121°C under 15 lb/inch pressure for 30 minutes. By cooling down to 50-55°C 10% fresh de-fibrinated sheep blood was added and subsequently poured in sterilized Petri dishes. In order to check the sterility of media, Petri dishes were kept in incubator at 37°C for 24 hours. The viable bacterial count in all diluted semen samples was determined by using spread plate method (Harry and Paul, 1981). As a procedure, 0.1 ml of diluted semen was added into a test tube having 0.9 ml of phosphate buffer solution stepwise 1:10, 1:1000, and 1:10.000 dilutions were obtained. By using double set of Petri dishes for each dilution, 0.5 ml from each diluted samples was spread on nutrient agar plates. The Petri dishes were then incubated at 37°C for a period of 24 hours and a colony counter counted the number of colonies that arose. The number of bacteria present in each Petri dish was calculated by multiplying the number of colonies with the dilution rate at which the colonies developed.

Statistical analysis : Data were expressed as means (±S.E.) and statistical analyses were performed with the software (Sigma Stat, Jandel scientific software V2.0, Richmond, CA, 2004). The differences between means of the same parameter were tested by the analysis of variance (ANOVA) and least significance differences (LSD). For the determination of correlation coefficient between bacterial contamination and sperm viability changes of two variables, the Pearson product moment correlation analysis was used.

Results

Results of the present study showed a decreased viable bacterial count of all samples including control part stored at 5°C for 72 h of preservation. Comparing with the control, all the antibiotics examined were effective in controlling bacterial growth ($P<0.05$) from 24 h to 96 h of preservation at 5°C. Cefdinir and cefoperazone sodium proved to be significantly ($P<0.05$) effective than other antibiotics in controlling bacterial growth at 96 h of preservation as the bacterial cont were $23.3 \pm 3.7 \times 10^3$/ ml and $25.4 \pm 6.2 \times 10^3$/ ml, respectively, as shown in Table-1.

Lincomycin, gentamicin sulphate, and tetracycline proved ineffective in controlling bacterial growth at 96 h of preservation as the bacterial cont were $57.1 \pm 20.1 \times 10^3$, $52.5 \pm 29.4 \times 10^3$ and $46.5 \pm 8.8 \times 10^3$, respectively. The difference in bacterial count between penicillin with streptomycin and cephalosporins (cefdinir

and cefoperazone sodium) used in this study was not significant. The addition of tetracycline to diluted ram semen significantly reduced (P<0.05) sperm individual motility and percent live sperm and a significant increase (P<0.05) acrosomal defects was observed at 96 h of preservation in comparison to control and other antibiotics. Higher sperm individual motility were observed in semen diluted with the addition of lincomycin and cefdinir (63.6 ± 1.8 % and 62.9 ± 2.7 %, respectively) which differ significantly (P<0.05) from tetracycline and gentamicin sulphate (37.5 ± 2.9 % and 47.9 ± 2.7 %, respectively).

Sperm motility, percent live sperm, sperm abnormalities and sperm acrosomal defect were not significantly affected in diluted semen with cefoperazone sodium, cefdinir, lincomycin, penicillin with streptomycin and control at 96 h, but the preservation had lowered semen quality (as measured by sperm motility, percent live sperm and abnormal morphology) more effective than antibiotics. Sperm viability was highly correlated with bacterial count in the control part of diluted semen ($r = 0.794$; $P < 0.01$). Other ram semen diluted parts containing antibiotics showed no correlation coefficient between semen viability and bacterial contamination.

Discussion

Quality of the ejaculate and the diluted semen portion are fundamental for successful artificial insemination. Semen is qualified as good when the there is a minimum contamination of bacteria (plus meeting the standards of motility and morphology). When the seminal dose is contaminated, semen viability decreases within a short period of time sperm and death occurs. As a result, the risk of pathologies in the female reproductive tract increases, resulting in impairment of fertility. There are many efforts in order to substitute penicillin with streptomycin in egg yolk-based diluents by other antibiotics, to minimize the bacterial contamination to dilute sperm during a long-term storage (Maxwell and Salamon, 1993; Salamon and Maxwell, 2000). Yániz *et al*. (2010) demonstrated that 13% of isolated bacteria from ram-diluted semen were simultaneously resistant to penicillin and streptomycin, the most common preservative antibiotic combination used in ovine semen diluents. Results of this study indicated that dilution of ram semen in sodium citrate-fructose-egg yolk diluent with antibiotics has resulted in significant (P<0.05) decrease in the viable bacterial count compared to semen diluted in the same diluent without antibiotic (control). Decreased viable bacterial count of all samples including the control stored at 5 C for 72 h indicate that storage of semen at 5°C affected the viable bacterial count.

These results are in accordance with the findings of Qureshi *et al.* (1993) in diluted bull semen. Tetracycline addition in our study led to lower individual motility and higher acrosomal defects in comparison to the addition of other antibiotics in the same diluent used in the present study. Similarly, Shin *et al*. (1988) and Alavi-Shoushtari *et al*. (2007) demonstrated that the use of tetracycline in the buffalo semen diluent had a harmful effect on spermatozoa. Antibiotics with higher antimicrobial activities in the present study were cefdinir and cefoperazone sodium. Their inclusion in the composition of ram semen diluent have proven an effective inhibition of bacterial growth and no apparent adverse effects on spermatozoa with higher sperm individual motility. These results are in agreement with the findings of Yániz *et al*. (2010) who achieved good antimicrobial activity with addition of ceftiofur (third generation of cephalosporins). Reports of the use of cephalosporins in the extension and cooled storage of ram semen are scarce. Although lincomycin (1 mg ml^{-1}) showed poor ability to inactivate bacterial growth in ram semen. However, higher rate of sperm individual motility was observed in the addition of lincomycin to ram semen diluent. Other concentrations of this antibiotic should be investigated.

High correlation between sperm viability and bacterial contamination in the control group found in this study is in agreement with Moretti *et al.* (2009). Microorganisms can affect the semen viability directly, causing the agglutination of motile sperm, reducing the ability of acrosome

reaction and causing alterations in cell morphology and indirectly, through the production of reactive oxygen species generated by the inflammatory response to the infection.

It could be concluded from the results of the present study that additions of cephalosporins (Cefoperazone sodium or cefdinir) at the dose of 1 mg/ml^{-1} were most effective amongst the antibiotics used in checking the bacterial growth and improving semen quality of Awassi ram.

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Conflict of interest

Authors declare that they have no conflict of interest.

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