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Comparison of Gentamicin and Ciprofloxacin in Dromedary Camels' Semen Extender

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Abstract: Bacterial contamination is an inevitable problem in semen processing and cryopreservation despite taking all hygienic precautions. This is due to the long duration of the camel semen collection and the resemble sitting position during mating for semen collection. The aim of this study was to establish an antibiotic-rich media to eliminate microbial presence in cryopreserved post-thawed camel semen doses. The study was conducted on 3 adult males trained for semen collection. In a primary experiment ejaculates (n=9) were intacted for freezing without the addition of any antibiotics and were analyzed for detection of bacterial contamination. The results showed growth of aerobic gram positive and gram negative bacteria. Several types of antibiotics were tested to eliminate the contaminants; gentamicin and ciprofloxacin were the most effective. The minimum inhibitory concentration (MIC) that showed no growth was 20 μg/ml and 200μg/ml, respectively. Each of gentamicin and ciprofloxacin was added separately in a second experiment to the extender in low and high concentrations, 20μg/ml and 40μg/ml for gentamicin and 200μg/ml and 400 μg/ml for ciprofloxacin. Raw semen samples (n=15) of mean mass motility 64.16 ± 4.3 % were used for cryopresevation with addition of antibiotics. Results showed that addition of ciprofloxacin at high or low doses to the Tris lactose extender had no effect on sperm motility immediately after the addition to the extender. However high dose of gentamicin (40μg/ml), showed sperm motility of 51 ± 4.9% and low dose (20 μg/ml) showed a sperm motility of 59 ± 4.3 %. The same trend was obtained prior to cryopreservation with a decline in sperm motility with high dose of gentamicin. The addition of high dose of ciprofloxacin (400 μg/ml) significantly ($P<0.01$) showed a post-thaw motility of 38.3 ± 5.9, as compared to its low dose (200 μg /ml) with a motility of 28.3 ± 1.1%, while no significant sperm motility differences were observed with different doses (high 20± 1.3, low 21.7± 1.1) of gentamicin. Acrosomal integrity was higher in all antibiotic treatments as compared to control samples. It can be concluded that the addition of ciprofloxacin with a 400 μg /ml concentration for Tris lactose camel semen extender is capable of eliminating bacterial contamination during semen collection and processing with no negative effect on post-thawed semen or intacted acrosomal integrity.

Key words: Dromedary · Camel · Semen · Bacteria · Antibiotics · Cryopreservation

INTRODUCTION

Artificial insemination (AI) has developed over the past 50 years to the extent that it is used in almost every country in the world for almost all animal species [1]. It is described as the most rapid tool used in improving the reproductive efficiency in the camels breeding programs, [2, 3]. Bousseau *et al.* [4] noted that the addition of components of animal origin (egg yolk or milk) to most commercial diluents, used to freeze semen, represents a potential risk of contamination of the doses with bacteria or mycoplasma. The presence of micro-organisms, especially bacteria in the ejaculates can affect fertilization directly, by adhering to spermatozoa [5] impairing their motility [6] and inducing acrosome reaction [7]. Microbial contamination can also have an indirect effect by producing toxins [8].

Indeed, such contamination is a possible source of endotoxins capable of damaging the fertilizing capacity of spermatozoa. Holt [9] pointed out the disease control issue as a basic aspect of frozen storage of semen. Yet, as for dromedary camel, applying AI techniques for breeding

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programs has a long way to go, as still the semen contamination by pathogenic organisms needs more control. Givens and Marley [10] reported that regarding bulls to be used for natural breeding will usually prevent introduction of pathogens that adversely affect reproduction. Barbas and Mascarenhas [11] reported that combinations of storage temperature, cooling rate, chemical composition of the extender, cryoprotectants concentration and hygienic control are the key factors that affect the life-span of spermatozoa. Yet pathogenic contamination is a major problem, as Bielanski [12] noted that semen and embryos generated by assisted reproductive techniques (ARTs) may be contaminated with numerous microorganisms from systemic or local reproductive tract infections in donors or by the inadvertent introduction of microorganisms during semen collection. Bielanski [12] mentioned also that different procedures were used to overcome any contamination including washing procedures, antibiotics, enzymatic treatment, treatment with antibodies or ozone, photo-inactivation, acidification and the use of novel antiviral compounds. Additionally, antibiotics in semen extenders inhibit growth of bacteria in the semen during storage [13]. Researchers saved no effort in studying the most effective antibiotic treatments on semen of different species as bovine [14], porcine [15], rabbits [16], buffalo [17], stallion [18] and human semen [19]. Jasko *et al*. [20] noted that the control of bacteria in semen of stallions has been most effective with the use of seminal extenders containing suitable concentrations of antibiotics. While, Salvetti *et al.* [16] investigated the effects of storage of stallion semen with or without addition of the antibiotic gentamicin and reported that no significant ($p < 0.01$) differences in motility, velocity or viability were observed between treatments, they concluded that addition of antibiotic to samples stored at 5°C had no significant effect on any of the parameters analyzed.

In the present study formulating antibiotic-rich media to eliminate microbial growth in cryopreserved post-thawed camel semen doses by using gentamicin and ciprofloxacin was investigated.

MATERIALS AND METHODS

Animals and Semen Collection: This study was conducted in the artificial insemination laboratory in Maryout Research Station, (Desert Research Center), located 34 Km, North West of Alexandria, Egypt. For this study, 3 adult one humped male camels (*Camelus dromedaries*) with an average weight of 550 kg and aged 12 years were used for semen collection. Semen was collected during the rutting season using an artificial vagina in El Hassanien Dummy [21] for semen collection. Semen was assessed immediately after collection for normal physical characteristics. A phase-contrast microscope (Leica) completed with a warm stage adjusted at a temperature of 37°C was used for assessment of sperm motility in five different fields at a power of 40X magnification. Intacted acrosome and abnormalities were assessed using the methodology reported by Johnson *et al.* [22].

Bacterial Contamination Analysis: Each sample was plated on blood agar and MacConkey agar. The blood agar and MacConkey agar plates were incubated at 37°C (aerobic conditions). The colony characteristics and ability to grow on MacConkey agar were recorded. Each isolate was characterized as the gram reaction, cellular morphology and cellular arrangement of each isolate were determined using gram-stained smears from agar plates. Colonies of gram-positive and gram-negative bacteria were also evaluated with a catalase test or an oxidase test, respectively. The isolates were further identified by several biochemical tests, according to Cowan and Steel`s [23]. All reactions were recorded as positive or negative.

Several colonies from each isolate were emulsified in a small volume of quarter strength Ringer solution. The turbidity was matched against the 0.5 McFarlane turbidity standard tubes. By using a sterile loop of 4mm diameter, the Muller Hinton agar plates were inoculated. Disk diffusion test was performed for the isolates against ampicillin, cephalexin, kanamycin and tetracycline with concentration of 30mcg/disc, erythromycin (15mcg), gentamicin (10mcg) and ciprofloxacin (5mcg) antibiotic disks (Hi-Media, Mumbai, India) were used to screen and interpreted the most effective antibiotic. The zone of inhibition area was read after overnight incubation at 37°C.

MIC: Minimum inhibitory concentration (MIC) was determined for the most sensitive antibiotic tested. A pure culture of the microbial growth was grown in Muller Hinton broth. The culture was standardized to achieve suspension concentration of $1.0X10^8$ CFU/ml as determined by comparison to the MacFarlane index. Final challenge suspensions were prepared in concentration of approximately $1.0X10^6$. By using sterile disposable transparent plastic U-plates, into each well 0.05 ml of broth were dispensed. To the first well in each

row 0.05 ml of the antimicrobial stock solutions was then added. A sterile micro dilutor was used to make doubling dilutions of the antimicrobial agent simultaneously.

The twelfth row of wells contained two sets of controls: wells containing 0.1 ml of broth and wells with 0.05 ml of broth and 0.05 ml of organisms. After inoculation the plates were covered with transparent sealing tapes, carefully agitated to mix the contents of the wells and incubated aerobically at 37°C. MIC of each antimicrobial agent was taken as the least concentration of the agent that shows no growth.

Preparation of Freezing Media: Tris - Lactose extender reported by El-Bhrawi [24] and El-Bahrawy *et al.* [25], was used as the freezing diluent, supplemented with two different antibiotics at two concentrations, ciprofloxacin (as lactate), broad spectrum antibiotic (200mg/100ml) produced by Amriya Pharma, IND., Alexandria, Egypt, with a low dose concentration of 200 μg/ml and a high dose concentration of 400 μg/ml. The another antibiotic was gentamicin (as sulfate) 40 mg, manufactured by: Memphis Co., for Pharma & Chem. IND – Cairo – ARE, under authority of Schering – Plough Corporation/USA, with a low dose concentration of 20 μg/ml and a high dose concentration of 40 μg/ml.

Freezing and Thawing Method: Post thawed semen was analyzed for sperm motility and acrosomal integrity immediately after thawing [22]. Semen assigned to be frozen in straws was packed in 0.5 ml French straws using an automatic mini-tübe filling and sealing machine (Type MPP133). After the completion of the equilibration period, the straws racks were delivered into a programmable freezing unit adjusted at -140°C using liquid nitrogen vapor, kept for 15 minutes at this temperature until frozen and finally were dipped in liquid nitrogen storing tanks. A mini-tübe thawing device was used for slow thawing for the straws. The device was programmed for the desired time and temperature, of 37°C for 40 seconds.

Statistical Analysis: Analysis of variance was detected using GLM procedure by SPSS (SPSS version 11.5 for Windows; SPSS Inc., Chicago, IL, USA).The differences between means were detected using Duncan's Multiple Range Test (DMRT) according to Snedecor and Chocran [26]. The results were quoted as arithmetic mean ± standard error of mean (SEM) and significance was attributed at $p < 0.01$.

RESULTS AND DISCUSSION

Antibiotics are one of the methods used to prevent and treat bacterial infections and contamination. There are two methods for use of antibiotics for semen decontamination. The first method involves treating the male. The second method, sometimes used in conjunction with the first, where the antibiotics are also added to the semen extenders. It is noteworthy to mention that there is no universal disinfectant or procedure to render extenders free from all microbes. Penicillin and streptomycin were used mainly in early experiments where they were effective and safe but now, many bacteria become resistant to them so, the search for a new group of antibiotics is recommended. The parameters used for assessing antibiotics effect in the present investigation are motility of cryopreserved spermatozoa and acrosomal integrity and inhibition of bacteria present in semen under different concentrations of ciprofloxacin and gentamicin which are the most effective antibiotics against the isolates.

The results indicated that, the freezed semen doses without added antibiotics showed growth of gram positive isolates, *Staphylococcus aureus*, *Staphylococcus epidermis* and *bacillus* species and gram-negative ones, as *Escherichia coli and, proteus* species. Several types of antibiotics were tested to eliminate the contaminants. The minimum inhibitory concentration (MIC) was done by using gentamicin and ciprofloxacin where they were the most effective ones at least concentration of 20 and 200 μg/ml respectively, which showed no microbial growths. Accordingly, ciprofloxacin and gentamicin were chosen to eliminate semen contaminants. Gentamicin is an aminoglycoside antibiotic, used to treat many types of bacterial infections, mainly those caused by gram-negative bacteria. Gentamicin is a bactericidal antibiotic that works by binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis [27] while ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class. It is a second generation fluoroquinolone antibacterial. It kills bacteria by interfering with the enzymes that cause DNA to rewind after being copied, which stops DNA and protein synthesis [28, 29] so, both of them are broad spectrum bactericidal antibiotics against many gram-positive and gram-negative bacteria. Each of gentamicin and ciprofloxacin was added separately to the extender at low and high concentrations, being 20 μg/ml and 40 μg/ml for gentamicin and 200 μg/ml and 400 μg/ml for ciprofloxacin.

Table 1: Effect of different concentrations of gentamicin and ciprofloxacin on diluted semen motility, acrosomal integrity pre- and post-freezing

Parameters	Control	Gentamicin 40 µg/ml	Gentamicin 20 µg/ml	Ciprofloxacin 400µg/ml	Ciprofloxacin 200µg/ml
Motility % after dilution (0 min).	59 ± 3.2	51 ± 4.9	59 ± 4.3	59 ± 4.5	60 ± 4.6
Motility % after equilibration (240 min.)	57 ± 3.3	42 ± 6.7	52 ± 6.8	57 ± 5.3	56 ± 6.5
Post-thawing motility %	23.3±1.1^{b}	20 ± 1.3^{b}	21.7 ± 1.1^{b}	38.3 ± 5.9^{a}	28.3 ± 1.1^{ab}
Post-thawing detached acrosome %	7.3 ± 0.7	8.5 ± 1.1	11 ± 2.3	9.6 ± 1.7	9 ± 0.8

Different letters within a line indicates significant difference (P<0.01)

The results presented in Table 1 showed that the addition of antibiotics to the Tris-lactose extender had no significant effect on sperm motility immediately after the addition of the extender except for the high dose of gentamicin (40 µg /ml), which showed a slight non significant decrease (P>0.05) in sperm motility (51±4.9%) as compared with the low dose of the same antibiotic (20 µg/ml) with a sperm motility of 59±4.3 %. The same findings were reported by Jasko *et al.* [20] who mentioned that high levels of gentamicin sulfate should be avoided in seminal extenders used for cooled semen preservation. Gentamicin also resulted in decreased motility and velocity in semen and did not improve motility parameters in semen contaminated with bacteria [30] while no observed effect was detected for high and low dose of ciprofloxacin (400, 200 µg/ml.), respectively. The same trend was obtained after equilibration period of the extended semen after 4 hours. A decline in sperm motility was observed at high dose of gentamicin as compared with the control and the other treatments. The addition of high dose of ciprofloxacin (400µg/ml) significantly (P<0.01) had the highest post-thaw motility 38.3± 5.9, followed by its low dose (200 µg/ml) with a motility of 28.3 ± 1.1%, while no significant differences were observed in sperm motility with different doses (high 20±1.3 %, low 21.7±1.1 %) of gentamicin versus the free antibiotic sample 23.3± 1.1% (the control).

Acrosomal integrity (Table 1) was not significantly affected by the treatment, but mathematically, acrosomal integrity was higher for all treatments and concentrations of antibiotics versus the control sample (antibiotic free). Microorganisms maybe present in semen and maybe transmitted to the females by natural mating, artificial insemination process or any ARTs application causing harmful diseases. These organisms maybe viral, bacterial or any other organisms which maybe either with pathogenic or non-pathogenic effect. These organisms may be transmitted to semen from the animal itself or from lack of attention to the hygienic aspects during semen collection, dilution, processing for cryopreservation or using contaminated equipment and material. However, the detrimental effect of antibiotics on sperm motility may be greater in stored, cooled semen due to the prolonged exposure to the antibiotic. The use of effective semen extenders increased longevity of motility of spermatozoa when compared to the longevity of sperm motility of a raw sample especially in the presence of antibiotics in semen extenders that inhibit growth of bacteria in the semen during storage [13]. However, Griggers *et al.* [31] reported that the addition of semen extender containing an antibiotic restored the motility of urine-contaminated spermatozoa.

CONCLUSION

In conclusion, the addition of ciprofloxacin at a concentration of 400 µg/ml to Tris lactose camel semen extender is capable of eliminating bacterial contamination during semen collection and processing with no negative effect on post-thawed semen or intacted acrosomal integrity. On the other hand gentamicin can negatively affect spermatozoal function in extended cryopreserved semen; therefore, optimal concentrations have to be tested for the respective extender medium.

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