The Haryana Veterinarian (December, 2024) **63(2),** 227-230 ISSN 0033-4359 (P) **Research Article**

EFFECT OF KOJIC ACID AS AN ALTERNATIVE TO ANTIBIOTICS IN BUFFALO SEMEN DILUTOR

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Received: 29.11.2023; Accepted: 15.01.2024

SUMMARY

To reduce the deleterious effects of bacteriospermia, antibiotics are routinely being added to buffalo semen extender. The current study aimed to evaluate Kojic acid (KA) as analternative for antibiotics in buffalo semen dilutor/extender. For this, a total of 4 semen ejaculates were obtained from 4 elite Murrah buffalo bulls. Every ejaculate was dividedinto 6 equal aliquots and diluted into an egg yolk-based semen dilutor having either Strepto-penicillin antibiotics or different concentrations of KA (0.02, 0.04, 0.06, 0.08 and 0.1 g/L) to compose the concluding concentration 80 million spermatozoa/mL and cryopreserved as per the routine procedure. The culture sensitivity test confirmed noantimicrobial effect of KA. Further, the incorporation of KA in semen extender exhibited no significant ($P>0.05$) reduction in Colony Forming Units (CFU/mL) in cryopreserved semen as compared to Strepto-penicillin. The spermatozoal motility assessments revealed that KA did not have a beneficial effect on sperm motility of cryopreserved spermatozoa. Results of the study revealed that KA supplemented dilutor did not differ significantly from the category having antibiotics in the dilutor. Hence, KA cannot be recommended as an alternative to antibiotics in buffalo semen dilutor during cryopreservation.

Keywords: Antibiotics, Buffalo semen, Dilutors, Ejaculates, Kojic acid

How to cite: Yadav,U. Dutt, R., Bansal, K., Jinagal, S., Gupta, A., Bala, R., Bhardwaj, S., Verma, N., Bishnoi, M., Kumar, D. and Kumar, P. (2024). Effect of kojic acid as an alternative to antibiotics in buffalo semen dilutor. *The Haryana Veterinarian* **63(2)**: 227-230.

There is unavoidable presence of microbes in semen as the reproductive tract of male becomes colonized with microbes from environment and from the male itself. Microbes enter into semen through number of routes from diseased animals, preputial sheath, and dilutors, as well as faulty procedures followed during semen collection, extension and packaging into straws. Microorganisms hamper the male reproductive functions directly by altering mechanisms involved in the acrosomal reaction (Morrell, 2016) and agglutination of motile sperm and indirectly, by the formation of reactive oxygen species (ROS) produced by inflammatory response owing to the infection. Bacteria compete with spermatozoa for nutrients and oxygen necessary for growth and normal functioning. The bacterial contamination of semen is a major concern for most of the semen production centres as it adversely deteriorates the semen quality (Diemer *et al.*, 2000). The incorporation of antibiotics in buffalo semen dilutors for artificial insemination is a common practice to ensure the quality and safety of the semen. Contamination of buffalo semen during collection and handling can exert a significant impact on the quality of semen potentially affecting fertility and reproductive outcomes. The addition of antibiotics in semen dilutors is a topic of ongoing research and debate as there are several detrimental effects of the antibiotics can contribute to the genesis of antibiotic-resistant bacteria (Yadav *et al.*, 2023).Furthermore, the production of bacterial endotoxins and leukocytic infiltration along with a massive outburst of ROS, have been repeatedly associated with poor semen quality (Tvrda *et al.*, 2022). Therefore, scientists all over the world are trying to find alternatives to antibiotics in semen dilutor.

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) is an organic acid drawn from several strains of fungi and bacteria (Shaoyong *et al.*, 2019) and shown to have antimicrobial properties, which can help inhibit the growth of microorganisms. It is used as a skin glowing agent (Saghaie *et al.*, 2013), a food additive due to its anti-bacterial and anti-viral properties (Aytemir and Ozcelik, 2010). Therefore, the uses of KA and their derivatives are also relied upon because of their antiplaque, antiparasitic, antiproliferative, anti-inflammatory (Saeedi *et al.*, 2019), anti-neoplastic and anti-fungal properties (Brtko *et al.*, 2004). Previous reports have shown that KA has more anti-bacterial activity against gram-negative bacteriaas compared to Gram-positive bacteria (Bentley, 2006; Song *et al.*, 2019). Kojic acid causes damage to bacterial cells by the potential devastation to the cell membrane integrity, escape of intracellular enzymes, and alterations of the zeta potential of cells, thereby interfering with the localization of some cellular proteins (Wu *et al.*, 2019).In a study, 0.04 g/L KA supplementation in boar semen improved

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spermatozoal quality criteria, spermatozoal capacitation, antioxidant mechanism associated gene expression, spermatocyte binding, embryonic progression and inhibited apoptotic protein expression (Shaoyong *et al.*, 2019). To date, the anti-bacterial effects of KA in cryopreservation of buffalo semen and the mechanism of bacterial growth inhibition by KA have not been elucidated. Therefore, this study was planned to study the effects of KA as an antibacterial agent in buffalo semen dilutor.

MATERIALS AND METHODS

Semen Evaluation: A total of 4 semen ejaculates were obtained from 4 healthy elite Murrah buffalo bulls through artificial vagina method twice a week at Semen Freezing Laboratory, Division ofAnimal Physiology and Reproduction, ICAR- Central Institute for Research on Buffaloes, Hisar. The concentration of spermatozoa was assessed using a photometer (Accucel bovine photometer (IMV), L' Aigla, France). Semen samples were first evaluated for mass and initial motility as per standard procedure and ejaculates with sperm motility above 70% were subjected for further processing. Each ejaculate was divided into six equal parts and extended to a final concentration of 80 million sperm/mL using egg yolk based dilutor (Yadav *et al.*, 2023) either containing strepto-penicillin (SP; Streptomycin 1g/mL and Penicillin 1 lakh units/L) or various concentrations of KA (0.02, 0.04, 0.06, 0.08 and 0.1g/L) as an alternative of Strepto-penicillin. The pH of the dilutor was set at 6.75. The diluted semen was further packed into 0.25 mL capacity plastic straws (IMV, L'Aigle, France), and slowly cooled to 4º C and equilibrated for a span of 4h in a cold cabinet and cryopreserved using an automated biological freezer as described previously by Arjun *et al.* (2022a).

Estimation of bacterial load in fresh and cryopreserved semen: Bacterial load was recorded in undiluted fresh as well as in frozen semen through standard plate count (SPC) method. The SPC was conducted by pour plate method using serial dilution method (Fig. 1). The bacterial load in semen was expressed as the number of colony forming units per milliliter (CFU/mL) of the semen.

Assessment of antibacterial activity of KA: Antibiotic sensitivity testing (AST) is an in vitro method of detecting sensitivity of bacteria to different antibiotics by diffusion techniques on agar media. For this, bacteria obtained from neat semen were subjected to in vitro AST adopting the agar disc diffusion method. The nutrient agar was the test agar. The inoculated plates were permitted to stand for 10 min followed by the placement of paper discs having known concentrations of SP and KA using sterile forceps. The petriplates were upturned and incubated at 37º C for 24 h. Subsequently, zones of inhibition were assessed by calculating the diameters of the zones.

Thermal incubation test: The cryopreserved straws were thawed and deported to 1.5 mL micro-centrifuge tubes at 37º C in a dry bath. The motility of spermatozoa was recorded at 30 min intervals till2 h at 37º C under a phase contrast microscope as described by Arjun *et al.* (2021).

Statistical analysis

The data generated were analysed using IBM SPSS Statistics for Windows, Version 21.0, and expressed as mean ± SE (Mean±Standard error). Shapiro-Wilk test was adopted for a normal distribution of data while Levene's test was opted to assess the data homogeneity of variance. If data were not distributed ordinarily, then arcsine transformation was adopted to evaluate data. The analysis of variance (ANOVA) was opted to analyse the differences among groups, and a significant difference was treated when the least-square mean was $p< 0.05$.

RESULTS AND DISCUSSION

The current study evaluates semen dilutors containing antibiotics (Streptomycin and penicillin solution) and various concentrations of KA. The distinctness was studied in cryopreserved semen through microbial load estimation, CASA for assessing spermatozoal motility, integrity and incubation tests.

Effect of KAas an antibacterial for semen: The antibacterial activity of KA against semen bacteria such as *E. Coli*, Streptococcus, Pseudomonas etc. was assessed through AST, wherein KA did not show a clear zone of inhibition while a clear zone of inhibition was exhibited by SP (Fig. 2A). Further, the bacterial load was calculated in neat semen as well as in frozen-thawed semen. In frozen semen, dilutors incorporated with five concentrations of KA (0.02, 0.04, 0.06, 0.08 and 0.1KA) and SP as an antimicrobial agent were compared. The results were expressed as a number of CFU/mL. The findings of 5 categories of KA were compared with each other and with dilutors containing SP. It was observed that the bacterial load of all the categories was decreased in the frozen-thawed semen as compared to that obtained in fresh semen. However, there was no significant difference in the bacterial load observed in samples frozen in dilutor containing SPas compared to dilutors supplemented with different concentrations of KA (Fig. 2).

Effect on the thermal resistance of spermatozoa assessed through incubation test: The thermal resistance of spermatozoa in all the six categories i.e., dilutor containing SP and dilutor supplemented with 5 different concentrations of KA were assessed for subjective motility under incubation period at different time intervals (0, 30, 60, 90 and 120 min). No significant variation was observed in spermatozoal motility ($p>0.05$) just after thawing (0 min) in all the categories. After incubation for 30, 60, 90 and 120 min, the spermatozoal motility (%) did not exhibit any variation in all the 6 categories ($p>0.05$) (Fig. 3).

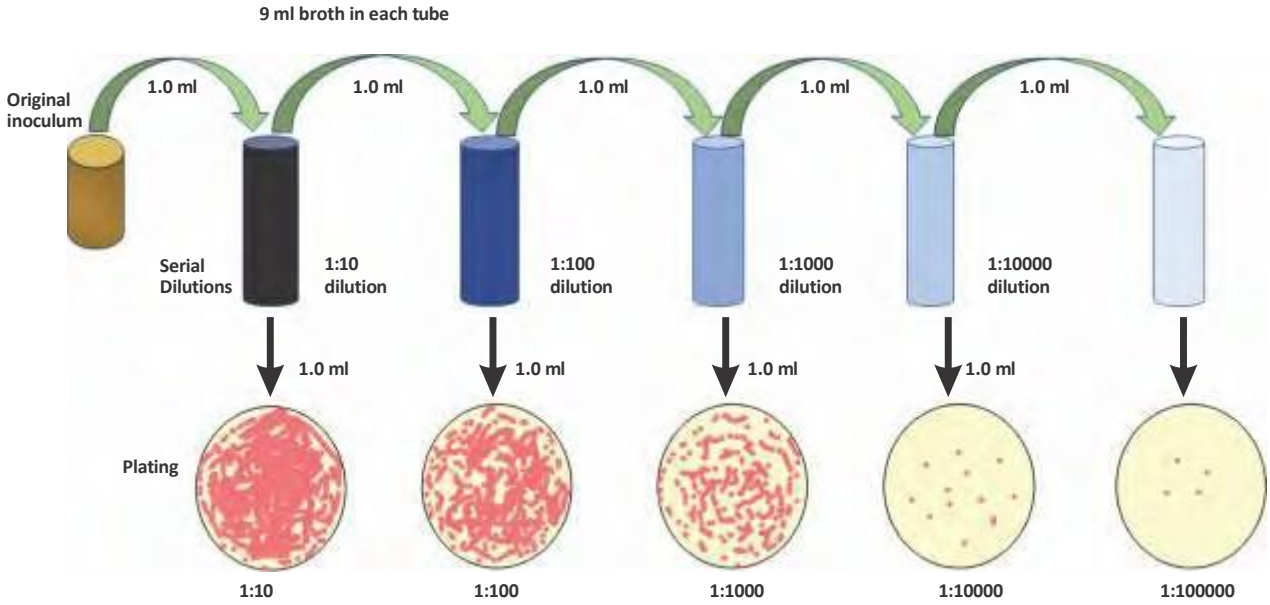

Fig. 1. Diagrammatic representation of estimation of bacterial load using standard plate count method

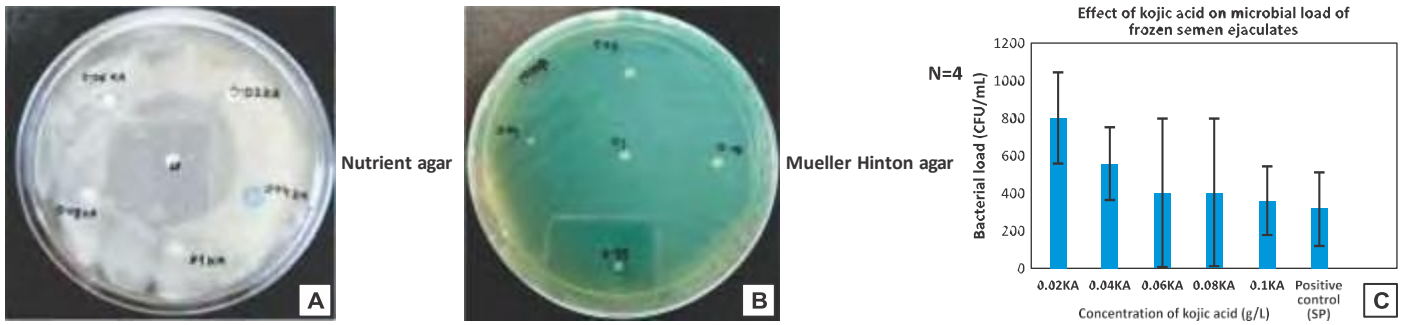

Fig. 2. A. Antibiogram depicting zone of inhibition around discs of antibiotics (streptomycin and penicillin; SP) and 0.02 g/L, 0.04 g/L, 0.06 g/L, 0.08 g/L and 0.1 g/L of Kojic acid on nutrient agar B. Antibiogram depicting zone of inhibition around discs of antibiotics (Streptomycin and penicillin; SP) and 0.02 g/L, 0.04 g/L, 0.06 g/L, 0.08 g/L and 0.1 g/L of Kojic acid on Mueller Hinton agar. C. Bar diagram depicting samples of frozen semen according to level of bacterial load (CFU/mL) in different categories viz 0.02 g/L, 0.04 g/L,0.06 g/L, 0.08 g/L, 0.1 g/L and SP

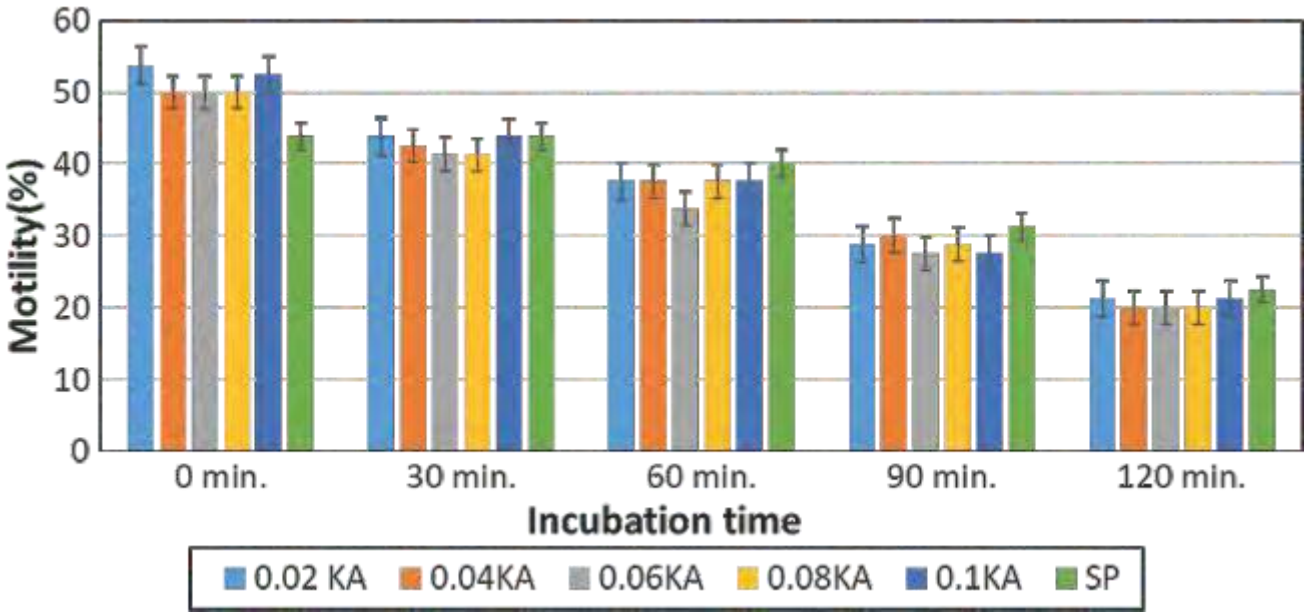

Fig. 3. Incubation test- After 24h of cryopreservation, representative semen straw of each ejaculate was thawed at 37° C for 30s and sperm motility was estimated at 30 min. interval upto 120 min. The mean sperm motility is of 4 samples in each category.

Antibiotics have been used widely in semen dilutors to inhibit bacterial growth during semen cryopreservation. Nevertheless, because of global concern about the negative effects of antibiotics, it is essential to explore effective alternatives for antibiotics. A high bacterial load has detrimental effects on spermatozoal vital parameters, including spermatozoal motility, acrosome integrity, MMP, thermal resistance and plasma membrane integrity. To counteract the problems related to bacterial resistance to the antibiotics used in the dilutors, the emergence of alternative substances is increasingly evident.

After exploring the related literature, it is believed to be the first report on Kojic acid which was used in the dilutor for cryopreservation of buffalo semen as an alternative to conventional antibiotics. In the antibiogram, no clear zone of inhibition was recorded with KA which indicated no antibacterial effect of KA at given concentrations whereas a clear zone of inhibition was shown by SP. This finding with KA contradicts with the observations of Shaoyong *et al.* (2019) in boar semen. However, the decrease in bacterial load was probably due to cryopreservation. The incubation test determines the livability of spermatozoa in the genital tract of the female and thereby reflects the longevity of the spermatozoa. The lowering of motility after post-thaw incubation may be due to an increase in oxidative metabolism, the production of ROS with the elevated number of dead spermatozoa (Sonar *et al.*, 2014).

In this investigation, it was noted that the substitution of antibiotics with KA in semen dilutor did not affect spermatozoal motility parameters. But the findings contradict with Shaoyong *et al.* (2019) who evaluated sperm motility parameters of swine semen stored at 17º C for 5 days and reported that boar spermatozoal motility parameters were significantly increased with the addition of KA as an alternative to antibiotics. However, they could not postulate the reasons for the better semen quality parameters during the cryopreservation as compared to control. The findings on boar semen revealed that the incorporation of antibiotic-free dilutors with 0.04 g/L KA exerts protective effects on liquid swine semen during preservation (Shaoyong *et al.*, 2019). This may be because of inhibition of specific genera of bacteria present in boar semen, differences in seminal plasma composition, different ways of evaluating motility, different composition of diluents, different methods of sample collection and amount of semen contamination.

CONCLUSION

In conclusion, the findings of the current study demonstrated that KA did not have potential anti-bacterial effects and failed to exhibit positive effects on thermal resistance and sperm motility. Further studies are warranted to enable the use of optimal concentrations of these new substances which should be potential alternative of antibiotics without impairing spermatozoal motility and fertilizing capacity.

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