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UTILIZING KOJIC ACID AND SODIUM ALGINATE AS ANTIBACTERIAL ON CRYOPRESERVED BUFFALO BULL'S SEMEN

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ABSTRACT

This experimental trial was carried out at the Department of Artificial Insemination – Ministry of Agriculture and the laboratories of the College of Agricultural Engineering Sciences/ University of Baghdad from 18 January to 24April 2022. Experiment was conducted to investigate the effect of using sodium alginate and kojic acid in the Tris extender. The goal was to evaluate their effects on the properties of cryopreserved buffalo bull semen and to assess their effectiveness in reducing microbial load. After collecting semen and diluting it with a Tris extender, five experimental groups were designed. T1 (negative control) with no antibiotics, T2 (positive controlled)with conventional antibiotics (Gentamicin 0.4 IU and Tylosin 0.08/100 IU/ml), T3 represents a Kojic acid (0.06 g/L), T4 functioned as Sodium alginate at (0.6 mg/mL), and T5 included the combination of Kojic acid (0.06 g/L) and sodium alginate (0.6 mg/ml). The diluted semen was cryopreserved following recommended procedures, where the cryopreserved semen characteristics were assessed, including the plasma membrane integrity of sperm, individual motility, and bacteriological tests; the total number of bacteria in the treated groups score. In addition, the total count of E. coli bacteria and the total count of Staphylococcus spp. After 2 days, 48 hours, 2 and 3 months of cryopreservation for cooled and frozen sperm. The study findings indicate a significant enhancement (P≤0.05)in individual motility and viability for T3, T4, and T5 treatments compared to the T1 and T2 groups. In addition, a significant decreases (P≤0.05) was observed in the overall bacterial count .

Key words: **Bull semen, bacterial contamination, Tris extender, Bacteriospermia.**

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INTRODUCTION

Since the 1930s, artificial insemination (AI) has been widely utilized by animal producers to enhance livestock population numbers and preserve genetic diversity. However, bacterial contamination remains a critical challenge, potentially occurring at any stage of semen dilution, packaging, or cryopreservation (Labouriau et al., 2003; López-Gatius, 2012). Specific bacterial strains, including *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*, have been well-documented for their detrimental effects on sperm quality and viability (Mahmood et al., 2013).Historically, conventional antibiotics like penicillin and streptomycin were routinely integrated into semen extenders to control these bacterial cycles. Nevertheless, subsequent research has demonstrated that standard antibiotics are increasingly ineffective against highly pathogenic and resilient genera, such as *Pseudomonas* and *Brucella*, due to the rapid evolution of antimicrobial resistance. Alarmingly, recent longitudinal observations indicate that numerous bacterial isolates have developed up to 100% resistance to frontline antibiotics, including penicillin, amoxicillin, and streptomycin (Najee et al., 2012).Consequently, exploring novel, non-conventional alternatives to mitigate bacterial loads without compromising spermatozoa traits has become an urgent priority. Current investigative avenues encompass physical eradication methods, antimicrobial peptides, and bioactive compounds derived from diverse natural origins (Shaoyong et al., 2019). For

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instance, phytogenic compounds, such as the essential oil extract of lemongrass (*Cymbopogon citratus*), have exhibited promising broad-spectrum antibacterial efficacy and significant growth inhibition against various microflora (Kamona & Alzobaay, 2021).Among these innovative alternatives, kojic acid—a natural organic acid synthesized by specific fungi and bacteria—has emerged as a viable candidate for semen extenders owing to its potent antimicrobial, fungicidal, anti-inflammatory, and antioxidant properties (Shaoyong et al., 2019). Concurrently, sodium alginate, a natural polysaccharide extracted from brown algae, has gained traction as a supportive biomaterial (Johnson et al., 1997). Notably, González-Marín et al. (2011) demonstrated that the incorporation of sodium alginates into semen extenders successfully minimized the microbial load during both cooling and freezing preservation processes. Building upon these findings, the current study was designed to evaluate the synergistic or individual efficacy of sodium alginate and kojic acid as biocompatible alternatives to standard synthetic antibiotics, specifically examining their subsequent impacts on the post-thaw quality of diluted buffalo bull (*Bubalus bubalis*) semen.

MATERIALS AND METHODS

Semen Collection

Buffalo bulls (*Bubalus bubalis*) were selected and trained to collect semen using the artificial vaginal method at 3-5 years old.

Substances Used as Antibiotic Alternatives

Chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), which included Sodium alginates (Product Number: 180947/ CAS Number: 9005-38-3/MDL:MFCD00081310) and Kojic acid (Product Number: K3125 /CAS Number: 501-30-4).

Handling the Semen

Semen was diluted using a Tris extender according to Salamon and Maxwell (2000), and then semen was divided into five experimental groups. T1 functioned as a negative control with no antibiotics, while T2 acted as a positive control with conventional Gentamicin 0.4 IU and Tylosin 0.08/100 IU/ml added. Furthermore, T3 represents Kojic acid (0.06 g/L), T4 functioned as Sodium alginate at (0.6 mg/mL), and T5 included the combination of Kojic acid (0.06 g/L) and sodium alginate (0.6 mg/ml).

Semen Cryopreservation

The samples were left for four hours to neutralize the glycerol to lower the temperature of the cooled samples to 5 °C. After that, all semen bacteriological analysis was performed, packed with 0.25 ml of artificial insemination straw, and placed in the liquid nitrogen adware. They were held on a holder for nine minutes at a temperature of -10 °C and five centimeters above the liquid nitrogen surface. The samples were then submerged in liquid nitrogen at -196 °C, and the straw was then taken out of the nitrogen tank at the appropriate times, liquefied for examination, and used for testing according to Mitchell et al. (2004).

Sperm Motility

The individual motility of the sperm after thawing was estimated by placing a diameter of dissolved semen on a warm slice at a temperature of 37 °C and measuring it at a magnification of 400x (Vincent et al., 2012).

Sperm Plasma Membrane Percentage

Sperm's plasma membrane integrity was determined according to the method of Jeyendran et al. (1984) using hypo-osmotic solution, which contained 8.72 gm/L of fructose and 4.74 gm/L sodium citrate, with 100 mOsm/L osmotic pressure and pH 8. Two droplets of semen were overwhelmed on this solution and then incubated in a water bath at 37ºC for 60 min.

Bacteriological Analyses

Aerobic Plate Count: Four straws (4 \times 0.25\ ml) were mixed, and 0.1 ml of it was added in duplicate to the sterile Petri dish to be considered the original sample. Also, from this sample, 0.1 ml of diluted semen was added to 0.9 mL of normal saline step by step to give serial dilutions of 1:10, 1:100, 1:1,000, to 1:1,000,000. The exact original sample from each dilution was poured. 0.1 ml in sterile Petri dishes containing nutrient agar, then mix the sample and the agar medium well and uniformly. The agar was left to solidify, inverted, and incubated aerobically for 24 h at

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37°C. Each dish's growing colonies were then counted manually (El-Tayeb et al., 2007). Bacterial counting was conducted after two hours of cryopreservation, 48 hours, two months and three months of freeze preservation, with five replicates for each treatment.

Bacterial Isolation: Aerobic Bacterial Isolation was conducted following standard procedures described by Tantala et al. (2019). A loopful of semen used for the count was cultured on blood (Oxoid: CM00558) and MacConkey agar (Oxoid: CM0007) simultaneously and incubated at 37°C for 24 hours. Growth characteristics were recorded, including hemolysis on blood, and the types of bacteria that screened on the culture media were diagnosed using Gram stain and biochemical tests and the API-20E system and VITEK 2 Compact. The diagnosis process was carried out for the bacterial genera in the study after two hours of cryopreservation, 48 hours, two months and three months of freeze preservation, with five replicates for each bacterial genera that appeared in each treatment.

Statistical Analysis

The Statistical computations were done using the SAS software program (SAS Institute, 2012) to explore the influence of treatment and time. Duncan's multiple range test for comparison between means (Duncan, 1955) was used ($P < 0.05$).

RESULTS AND DISCUSSION

Sperm Cells' Individual Motility Percentage

The individual motility results showed no statistically significant differences ($P \leq 0.05$) between the five treatments of the experiment in the first preservation period (5 °C/cooling) (Table 1), which amounted to 37.00±4.89, 32.00±2.54, 37.00±3.74, and 40.00±3.16% for T1, T2, T3, T4, and T5, respectively. After two months of cryopreservation, the three treatments T3, T4, and T5, which used kojic acid and sodium alginate, improved the motility of individual sperm. The dense structure of sodium alginate helps preserve sperm by encapsulating the sperm and protecting them as much as possible from the factors they are exposed to during freezing and after thawing (Kumar et al., 2019). A dense medium, because it limits sperm motility during storage, may also reduce the metabolic demands imposed on them according to Yániz et al. (2005) as mentionation (Table 1). Even when buffer solutions are added to the expanders to reduce pH fluctuations, the precipitation that inevitably occurs during preservation may decrease the pH in the sediment cells' region due to the accumulation of toxic metabolites.Because the expander contains alginate, sedimentation is minimized, resulting in a more homogeneous distribution of sperm, which in turn allows the buffer to work more efficiently, according to Nagy et al. (2002), as well as contributing to the preservation of sperm during the storage period by reducing as much as possible the bacterial resistance in the diluted solution to a minimum and thus contributing to maintaining the motility and quality of sperm (Shaoyong et al., 2019), while kojic acid enhances animal quality standards, especially semen. Through its antioxidant effect, it increases total antioxidant capacity (T-AOC) and, as a result, prevents the overproduction of ROS and MDA (Ros-Santaella & Pintus, 2021).

As for the final period of cryopreservation, which lasted up to three months, the additives proved effective in maintaining sperm quality and maintaining the individual motility ratio, as the three parameters T3, T4, and T5 were much better ($P \leq 0.05$). For the third period of cryopreservation, the three treatments (T3, T4, and T5) continued to be significantly superior in the percentage of individual motility of buffalo bull semen.

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Table 1. Effect of using Kojic acid and Sodium alginates to Tris extender on the individual motility percentage of sperm of buffalo bulls after different cryopreservation periods (Mean ± Standard error).

Treatment	Time				Significance
	5C°	48 hrs PC	2months PC	3 months PC	
T1	37.00±4.89 A a	21.00±5.09 B ab	18.00±4.06 B ab	10.00±4.63 AB b	*
T2	32.00±2.54 A a	24.00±4.30 B a	18.00±6.81 B a	10.25±4.90 B a	NS
T3	37.00±4.06 A a	29.00±4.00 A a	26.00±6.78 A a	24.00±6.99 A a	NS
T4	37.00±3.74 A a	30.00±4.18 A ab	28.00±5.61 A ab	22.80±6.09 A b	*
T5	40.00±3.16 A a	30.00±4.74 A a	28.00±6.44 A a	26.00±7,64 A a	NS
Significane	NS	*	*	*	

*(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (negative control-) without adding any antibiotics; T2: (positive control+) adding the conventional antibiotics Gentamicin 0.4 IU and Tylosin 0.08 IU per 100 ml; T3: adding Kojic acid at (0.06 g/L) T4: adding sodium alginate at (0.6 mg/mL) T5: adding a combination of kojic acid (0.06 g/liter) with sodium alginate (0.6 mg/ml).

Plasma Membrane Integrity

Following the two hours of cryopreservation, the study revealed significant differences (P ≤0.05) between the treatments in the percentage of plasma membrane integrity in buffalo bull sperm, with superiority for treatments T3, T4, and T5 (80.60 ± 1.43%, 82.80 ±1.15%, and 84.80 ± 0.86%, respectively) over control T1 and T2 treatments (72.60 ±2.50% and 68.00 ± 0.00%), as listed in Table 2. There were no significant differences (P ≤0.05) between the treatments after three months of freezing compared to the control treatments after one month and two months of cryopreservation. This finding corroborates the results of a previous study in which adding Sodium alginate to boar semen improved post-thaw plasma membrane integrity (Hu et al., 2014). Similarly, sodium alginate effectively maintained the plasma membrane integrity of salmonid fish sperm after short-term storage (10 days) at 4 °C (Merino et al., 2017).

Table 2. Effect of using Kojic acid and Sodium alginates to Tris extender on the Plasma membrane integrity of sperm. of buffalo bulls after different cryopreservation periods (Mean ± Standard error).

Treatment	Time				Significance
	5C°	48 hrs PC	2months PC	3 months PC	
T1	72.60±2.50 B a	66.80±2.08 C ab	59.80±3.12 A bc	54.00±3.67 A c	*
T2	68.00±2.86 B a	60.80±3.05 C ab	43.20±10.98 B b	50.00±2.88 A ab	*
T3	80.60±1.43 Aa	73.40±1.50 Bb	65.00±2.96 A c	59.60±2.89 A c	*
T4	82.80±1.15 Aa	76.00±1.87 AB a	69.80±2.15 A a	51.20±10.56 A b	*
T5	84.80±0.86 Aa	80.00±1.16 A a	72.00±2.60 A b	67.20±2.98 A b	*
Significane	*	*	*	NS	

*(p≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (negative control-) without adding any antibiotics; T2: (positive control+) adding the conventional antibiotics Gentamicin 0.4 IU and Tylosin 0.08 IU per 100 ml; T3: adding Kojic acid at (0.06 g/L) T4: adding sodium alginate at (0.6 mg/mL) T5: adding a combination of kojic acid (0.06 g/liter) with sodium alginate (0.6 mg/ml).

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Total Count of Bacteria

According to the results in Table 3, there were statistically significant differences (P ≤0.05) between the five treatments in the total count of bacterial colonies after two hours of cooling. The T3 (6.05±0.04) treatment was the lowest in the count of bacterial colonies, showing superiority over the rest of the treatments and the two control groups (Table 3).This significant superiority also continued in favor of the treatments to which the antibiotic alternatives were added: T5 (6.04±0.11) and T4 (7.00±0.15), followed by T3 (7.6±6.59), compared to the two control treatments T1 (42.26±22.02) and T2 (43.00±23.0). The decrease in the count of bacterial colonies for these treatments continued even after two and three months of freeze preservation (Table 3).The reduction in bacterial growth is attributed to the addition of sodium alginate and kojic acid, both of which possess anti-microbial properties. Alginates work by coating the sperm, thereby preventing cell leakage and causing the disruption of proton transfer (Ball et al., 2006). This leads to the disappearance of protein bands, ultimately inhibiting the synthesis of proteins and DNA (Peng et al., 2015).Furthermore, given that there was a decline in both the number of *Escherichia coli* bacterial colonies and the total number of expanding bacterial colonies, these results concurred with the findings of Vinodh et al. (2008). The treatments that received the addition of sodium alginate effectively controlled *Escherichia coli* and *Staphylococcus* spp. In addition to its influence on the bacterial cell's metabolic activity and its capacity to deform external microorganisms, this substance also prevents the metabolism of fats in the bacterial cell's outer membrane, which helps explain these outcomes.

Table 3. Effect of using Kojic acid and Sodium alginates to Tris extender on the percentage of the total number of Bacteria of buffalo bulls after different cryopreservation periods (Mean ± Standard error).

Treatment	Time				Significance
	5C°	48 hrs.PC	2 months. PC	3 months PC	
T1	7.91±1.50 B b	20.80±14.79 A a	42.26±22.02 A a	22.0±0.23 A b	*
T2	11.48±1.97 A a	35.31±17.83 A a	43.00±23.0 A a	20.00±0.18 A b	*
T3	6.05±0.04 B b	6.72±0.71 B a	7.6±6.59 B a	6.00±0.01 A c	*
T4	6.14±0.08 B b	6.49±0.47 B a	7.00±0.15 B c	6.00±0.01 B b	*
T5	7.14±1.06 B a	12.86±4.69 A a	6.04±0.11 AB b	0.06±0.01 AB c	*
significance	*	*	*	*	

***(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (negative control-) without adding any antibiotics; T2: (positive control+) adding the conventional antibiotics Gentamicin 0.4 IU and Tylosin 0.08 IU per 100 ml; T3: adding Kojic acid at (0.06 g/L) T4: adding sodium alginate at (0.6 mg/mL) T5: adding a combination of kojic acid (0.06 g/liter) with sodium alginate (0.6 mg/ml).**

Total Count of *Escherichia coli*

The results showed significant difference in the total count of *E. coli* bacterial colonies between all treatments after two hours of cold storage. However, *Escherichia coli* bacteria were less present in treatments T3, T4, and T5, to which antibiotic alternatives were added, compared to the two control treatments, T1 and T2 (Table 4).Also, after 48 hours of freeze preservation, the results were similar, with no significant difference between the five treatments. While the results were different after two months of freeze preservation, there was a significant difference in favor of T3, T4, and T5. This significant decrease in the count of *E. coli* bacterial colonies continued after three months of freeze preservation, where no bacterial growth was recorded in T3. Then, T5 was superior to T4 and the two control treatments (Table 4).Kojic acid affects the integrity and destruction of the bacterial cell membrane and the degradation of genomic DNA (Sone et al., 1982). As for the antibacterial mechanism, it works to block iron

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absorption pathways by bacteria, which may be the reason behind its synergistic antibacterial effect (Wang et al., 2021).

Table 4. Effect of using Kojic acid and Sodium alginates to Tris extender on the percentage of total number of *Escherichia coli* of buffalo bulls after different cryopreservation periods (Mean ± Standard error).

Treatment	Time				Significance
	5C°	48 hrs PC	2months PC	3 months PC	
T1	6.00±0.12 A a	6.26±0.15 A a	8.01±0.01 A a	6.01±0.00 A a	NS
T2	6.14±0.14 A a	6.13±0.15 A a	7.03±0.05 AB a	6.03±0.00 B a	NS
T3	6.02±0.01 A a	6.01±0.01 A a	6.01±0.00 AB a	0.00±0.00 B a	NS
T4	6.08±0.06 A a	6.00±0.01 A a	6.00±0.00 B a	0.00±0.00 B a	NS
T5	6.08±0.06 A a	6.00±0.01 A a	6.00±0.00 B a	0.00±0.00 B a	NS
significance	NS	NS	*	*	

***(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (negative control-) without adding any antibiotics; T2: (positive control+) adding the conventional antibiotics Gentamicin 0.4 IU and Tylosin 0.08 IU per 100 ml; T3: adding Kojic acid at (0.06 g/L) T4: adding sodium alginate at (0.6 mg/mL) T5: adding a combination of kojic acid (0.06 g/liter) with sodium alginate (0.6 mg/ml).**

Total Count of *Staphylococcus* spp.

The results in Table 5 show that there was no significant difference (P ≤0.05) between the five treatments in the total number of *Staphylococcus* spp. bacteria after cryopreservation (5 °C) as well as after freezing for 48 hours. Still, the treatments to which antibiotic alternatives were added recorded the lowest bacterial numbers compared to the control treatments. The results also indicated no significant difference (P ≤0.05) between the treatments for the durations of 48 hours and two months of freeze preservation. However, the number of *Staphylococcus* spp. bacteria continued to decrease in the treatments supplemented with sodium alginate, kojic acid, and their mixture (Table 5).In contrast, a significant difference ($P < 0.05$) was observed between the treatments after three months of freeze preservation. This significant superiority was in favor of the treatments to which antibiotic alternatives were added; the T4 treatment recorded the most negligible growth and lowest number of *Staphylococcus* spp. bacteria. This is due to the effect of adding sodium alginate as an antibacterial agent that acts directly on the composition of the bacterial cell wall, disrupting the transport of essential substances across the membrane and leading to bacterial cell death, which agrees with the findings of Gao et al. (2010).Additionally, Yu et al. (2020) explained the action of sodium alginate against the biofilm formed by bacteria—which normally serves as a defense mechanism against external influences and obstructs antibiotic penetration—demonstrating that sodium alginate works effectively to prevent the formation of these biofilms.

CONCLUSION

In conclusion, supplementing Tris extender with sodium alginate and kojic acid as alternatives to traditional antibiotics enhances the significant role of specific characteristics in buffalo bull semen and effectively reduces microbial load.

JOURNAL DECLARATION

The First author (**Z. A. Mahdi)** serves as an editor for Iraqi Journal of Agricultural Sciences but was not involved in the peer review process of this manuscript beyond their role as an author. The authors declare no other conflict of interest.

CONFLICT OF INTEREST

There is no conflict of interest, according to the authors, when it comes to publicizing the current inquiry.

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during the experimental period to perform the procedure conveniently

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استعمال حامض الكوجيك والجينات الصوديوم كمضاد بكتيري في مخففات السائل المنوي لثيران الجاموس المحفوظ بالتبريد والتجميد

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المستخلص

اجريت هذه الدراسة في مركز التلقيح الاصطناعي / دائرة الثروة الحيوانية / وزارة الزراعة العراقية / في أبي غريب ومختبرات كلية علوم الهندسة الزراعية /جامعة بغداد للفترة من2022/1/18 الى 2022/4/24 لبيان تاثير استخدام كل من الجينات الصوديوم وحامض الكوجيك باستخدام مخفف Tris وكان الهدف هو تقييم تاثير هذه الإضافات في خصائص السائل المنوي لثيران الجاموس المحفوظ بالتبريد والتجميد وتقييم مدئ فعاليتها كمضادات حيوية في تقليل الحمل الميكروبي . بعد اجراء عملية جمع السائل المنوي وتخفيفيه باستخدام مخفف Tris تم تقسيم معاملات التجربة الى خمس معاملات تجريبية حيث كانت المعاملة T1 (Control−) بدون إضافة أي نوع من أنواع المضادات الحيوية والمعاملة T2(Control+) مخفف Tris مع إضافة مضادات حيوية تقليدية (Tylosin 0.08 وحدة دولية /100 مل و Gentamicin 0.4 وحدة دولية / 100مل) والمعاملة الثالثة T3 مخفف Tris مع إضافة حامض الكوجيك 0.06غرام / لتر والمعاملة T4 مخفف Tris مع إضافة الجينات الصوديوم 0.6 ملغم / مل والمعاملة الخامسة T5 توليفة من حامض الكوجيك 0.06 غم /لتر و الجينات الصوديوم 0.6 ملغم /مل .تم حفظ السائل المنوي في التبريد لمدة ساعتين بعدها تم تقييم الحركة الفردية للنطف وسلامة الغشاء البلازمي للنطف وكذلك اجراء الاختبارات البيكترولوجية منها العدد الكلي للبكتيريا والعدد الكلي لبكتيريا *Escherichia coli* و *Staphylococcus* spp لكل معاملة. ومن ثم تم حفظ السائل المنوي بالتجميد واجراء التقيمات السابقة بعد مرور 48 ساعة وشهرين وثلاثة اشهر من الحفظ بالتجميد .اشارت نتائج التجربة الى وجود تفوق معنوي (P<0.05) في الحركة الفردية وكذلك سلامة الغشاء البلازمي للنطف للمعاملات T3وT4 وكذلك T5 مقارنة بمجموعتي السيطرة ، كذلك لوحظ وجود انخفاض معنوي (P<0.05) في العدد الكلي للمستعمرات البكتيرية .

الكلمات المفتاحية: السائل المنوي للثيران ، المضادات الحيوية ،التلوث البكتيري،مخفف Tris،بكتيريا النطف.