Effect of Adding Kojic Acid and Sodium Alginate to Semen Extender of Buffalo Bulls on some Biological and Bacteriological Parameters

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Abstract. This experiment examined the effects of adding sodium alginate and Kojic acid as substitutes for conventional antibiotics to Skim milk extender on the characteristics of cryopreserved and frozen buffalo bull semen, as well as the evaluation of their additions as antibiotics that help lower the microbial load. Following the collection and dilution of the Skim milk extender, the experimental treatments were separated into five groups, as follows: T1: (control-) without adding any antibiotics; T2: (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) The diluted semen was cryopreserved in accordance with the recommended procedure, and the characteristics of the cryopreserved semen were then assessed. These characteristics included plasma membrane integrity and individual motility, as well as bacteriological tests that measured the total number of bacteria in the treatments, the total number of E. coli bacteria, and the total number of Staphylococcus bacteria for both cooled and frozen semen after 2 hours, 48 hours, 2 months, and 3 months post cryopreservation. The results of the study revealed There were significant enhancement differences (p 0.05) for the treatments T3, T4, and T5 in the percentages of individual motility and viability in comparison to the two control groups, as well as a significant reduction (p 0.05) in the total number of bacteria and the total number of E. coli and Staphylococcus bacteria for treatments T3, T4, and T5 after all periods of cooling and freezing in comparison with the two control groups. It is concluded from the current experiment that the addition of sodium alginate and kojic acid as an alternative substitute for commonly used antibiotics to the semen extender has a significant role in enhancing some characteristics of the buffalo bulls' semen and helping to reduce the microbial load to a minimum.

Keywords. Sodium alginate, Kojic acid, Antibiotics, Skim milk.

1. Introduction

The management of total quality and pathogenic, environmental contamination are the two key issues facing the bovine Artificial insemination business today. Both variables depend on how the semen cryopreservation extender is prepared and constructed. Whole or skim milk or a TRIS solution with egg yolk added are often the standard extenders used for the cryopreservation of bull spermatozoa [1,2]. The risk of microbiological contamination is increased by the difficulty of standardizing semen extenders that include ingredients like egg yolk and skim milk [3] so antibiotics are added to semen extenders to reduce microbial contamination of the external environment or during semen collection. Different antibiotics, such as penicillin and streptomycin, ceftiofur, apramycin, and aminoglycosides, or linco-spectin + tylosin + gentamycin, have been added to semen extenders [4] Most studies, however, confirmed that most traditional or commonly used antibiotics are ineffective against many bacteria and germs that have a negative effect on sperm, such as Pseudomonas, Brucella, and others, because most bacteria form resistance to these antibiotics. [5], Studies have shown that bacteria become more resistant to antibiotics with time, since most of them have gained 100% resistance to penicillin, amoxicillin, and streptomycin [6]. This was confirmed by [7] in a study on the extent of bacteria's response to antibiotics, using a sensitivity test for *Escherichia coli* and *salmonella*, as well as *staphylococcus*, for some antibiotics: ampicillin, amoxicillin, erythromycin, gentamicin, tetracycline, and streptomycin, where most types were found to be efficacious. Resistance to antibiotics The search for alternatives to bacterial resistance has become an important prerequisite for this Antimicrobial peptides, physical methods to lower the bacterial load, and the use of various materials, whether animal, vegetable, or of other origins, are all alternatives [8]. The antibacterial effect of nanoparticles such as silver nanoparticles, which were bioavailable from microorganism isolates and had an inhibitory effect on bacterial growth, was reviewed [9]. and juniper girls were used in a study to measure the effect of plant extracts as antibacterial agents, as the results showed that the fruits of the juniper plant have a high antibacterial and antiparasitic impact [10]. Semen extenders were given lactoferrin. In addition to removing several bacterial species, it produced positive results in minimizing bacterial contamination [11]. Kojic acid, an organic acid produced by several types of fungi and bacteria, is a recent addition and is classified as one of the alternatives to traditional antibiotics added to sperm extenders. Kojic acid and its derivatives' main applications are rooted in their antimicrobial, fungicidal, and anti-inflammatory effects. And it inhibits the development and growth of pathogens, viruses, and parasites [12]. It was added to sperm extenders and gave positive results [13]. Sodium alginates, a natural polysaccharide extracted from brown algae, are one of the modern additions or alternatives to antibiotics and sperm extenders. [14] As it was added to buffalo semen extender [15] sodium alginates as an antibiotic alternative to antibiotics in semen extenders due to their great effects in reducing the microbial load in semen extenders during semen preservation methods, whether by cooling or freezing. As a side effect, the purpose of the research was to investigate the effect of Kojic acid and sodium alginates as alternative antibiotics to commonly used antibiotics in buffalo bull sperm dilutions. [15] Due to their significant effects on minimizing the microbial load in semen extenders during semen preservation methods, whether by cooling or freezing, sodium alginates were used as an antibiotic alternative to antibiotics in buffalo semen extenders. This study was done to find out how well kojic acid and sodium alginates, two more commonly used antibiotics, worked as semen extenders in buffalo bulls.

2. Materials and Methods

2.1. Semen Collection

Buffalo bulls (*Bubalus bubalis*) were selected and trained to collect semen using the artificial vaginal method at the age of 3-5 years in the department of artificial insemination - ministry of agriculture (Abu Ghraib is /Baghdad Governorate of Iraq)and the laboratories of the college of agricultural engineering sciences / university of Baghdad and operation with laboratories in Mosul university Ethical approval No. um.VET.2021.5. for the period from 7 March to 7 July 2022.

2.2. Handling the Semen
Semen was diluted using skim milk extender which made according to [16] Then semen was divided to five groups as following: T1 (control -) without adding any antibiotics. T2: (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)

2.3. Substances Used as Antibiotic Alternatives
Chemicals were used in this study were purchased from sigma chemical Co. (St. Louis, MO, USA), which including Sodium alginates (Product Number: 180947/ CAS Number: 9005-38-3/MDL: MFCD00081310) and Kojic acid (Product Number: K3125 /CAS Number: 501-30-4).

2.4. 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 measured at a magnification of 400x [17].

2.5. Live Sperm Percentage
The percentage of dead sperm was estimated based on the results [18].

2.6. Bacterial Count
Bacteria were counted using a Miles and Misra plate count method [19] Make a series of tenfold dilutions to semen sample by mixing 0.9ml of phosphate saline solution with 0.1ml of semen sample to get dilution 1:10, and then culture the final three dilutions on MacConky agar and then incubation the petri dishes in incubator at 37°c for 24 hours and then trying to calculate the number of bacteria colonies.

2.7. Bacterial Isolation: Aerobically
Placing the samples were cultured in nutrient broth in incubator at temperature of 37c degree and for a period of 24-48 hours, then were transported the growth from nutrient broth on three kinds of culture media (Blood Agar,MacConky agar, nutrient agar) and cultured by streaking method, then placed in incubator at 37ºc for a period of 24-48 hours, then was diagnosed types of bacteria that appeared on the culture media by using Gram stain and biochemical tests and by using Api-20E system and VITEK 2 Compact.

2.8. Statistical Analysis
The Statistical computations were done using SAS software program [20] to explore the influence of two way analysis of variance test to compassion between means [20].

3. Results and Discussion

3.1. Individual Motility
The study's findings revealed that there were There was no significant difference during cryopreservation, while it was found that there was a significant difference($P \leq 0.05$) in favor of the treatments compared to the two control treatments after 48-hour freeze-preservation in the percentage of individual movement of buffalo bull semen (Table1). This superiority continued in the percentage of individual sperm motility after two and three months of cryopreservation for the treatments to which both kojic acid and sodium alginate were added. This superiority in the percentage of individual sperm motility persisted after two and three months of cryopreservation for treatments to which both kojic acid and sodium alginate were added due to the adhesion property of sodium alginate, which contributed to the preservation of sperm by encapsulating and preserving the sperm. as possible while it is frozen and thawed [13].

Table 1. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to Skim milk 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	2 months. PC	3 months PC	
T1	41.00± 1.87 Aa	34.00± 1.87 Ab	28.00± 2.00 AB bc	23.00± 2.54 Ac	*
T2	41.00 ± 3.67 Aa	23.00 ±1.22 Bb	22.00± 2.00 Bb	19.00± 2.91 Bc	*
T3	45.00± 3.16 Aa	37.00± 3.74 Aab	35.00± 4.18 ABb	32.00± 3.74 Ab	*
T4	42.00± 3.39 aA	36.00± 2.91 Aa	35.00± 3.16 Aa	33.00± 4.35 Aa	NS
T5	42.00± 2.54 Aa	35.00± 3.53 Aa	34.00± 4.30 Aa	31.00± 4.84 Aa	NS
significance	NS	*	*	*	

*(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (control-) without adding any antibiotics; T2: (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).

3.2. Sperm Viability

The results of the current study showed that there was no significant superiority between the treatments in the first preservation period (after two hours of cryopreservation) Table 2, while the results showed a significant superiority(P≤0.05) of treatments T3, T4 and T5 in maintaining sperm vitality compared to the two control treatments after 48 hours. Preservation of freezing compared to the cold period, and this superiority continued throughout the study period, that is, after two and three months of preservation by freezing, in favor of the same treatments compared to the two control treatments, and the reason for this is sodium genes and kojic acid have an effect in preserving sperm from various factors during the period Preservation through several processes, as this material works to encapsulate and preserve the sperm, as well as to get rid of free radicals as much as possible [15] Table 2.

Table 2. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to Skim milk extender on the sperm viability of buffalo bulls after different cryopreservation periods (mean ± standard error).

Treatment	Time				Significance
	5C°	48 hrs.PC	2months.PC	3 months.PC	
T1	81.00± 1.76 Aa	68.60 ±2.44 B Cb	59.60± 2.58 C b	52.00± 3.39 Bb	*
T2	79.00± 3.12 Aa	65.80± 2.28 C b	61.60± 3.54 BC c	59.60± 6.20 AB c	*
T3	83.20± 1.39 Aa	76.00± 1.70 Ab	71.20± 2.15 A bc	67.60± 2.24 Ac	*
T4	81.00± 2.52 Aa	74.60 ±2.03 AB ab	71.20 ±2.08 Ab c	64.20± 4.31 AB c	*
T5	82.20± 1.77 Aa	71.00± 2.38 AB Cb	67.80± 2.45 AB bc	58.00± 5.99 AB c	*
significance	NS	*	*	*	

*(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (control-) without adding any antibiotics; T2: (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/litter) with sodium alginate (0.6 mg/ml).

3.3. Bacterial Number 10 ^{6}CFU /ml

The results of the study showed that there was no significant difference(P≤0.05) in the total number of bacteria in all treatments during cold preservation, while there was a significant superiority in the treatments to which T3, T4, and T5 antibiotic alternatives were added compared to antibiotics. Treatments after 48 hours of cryopreservation (Table 3). This relative decrease in the number of bacteria for these treatments persisted at the expense of the two control treatments after two and three months of cryopreservation. Several mechanisms work together in sodium alginate and kojic acid to destroy the integrity of the cell membrane, allowing vital enzymes to leak into the cells, thus allowing K+ to permeate across the cell membrane to alter the zeta potential and damage sub-cells. Proteins [21] (Table 3) although there were between treatments after two months of freeze-preservation, with regard to the effect of the duration of preservation in one treatment, the results showed a significant superiority (P≤0.05) for all treatments and for all freezing periods, compared to two hours after refrigeration (Table 3). The T3 treatment maintained the lowest number of bacteria among treatments and for all preservation periods.

Table 3. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to Skim milk extender on the percentage of total number of Bacteria 10 ^{6}CFU /ml of buffalo bulls after different cryopreservation periods (mean ± standard error).

Treatment	Timew2				Significance
	5C°	48 hrs.PC	2 months. PC	3 months PC	
T1	0.94±0.76 Aa	5.92±3.003 Aba	9.79±8.822 A abc	3.00±2.00 Ac	*
T2	2.95±1.11 Ab	4.01±0.01 Ab	3.920±1.78 Aa	3.045±0.026 Ab	*
T3	0.73±0.035 Aa	0.82±0.53 Ba	0.89±0.78 Ba	0.011±0.000 Ba	NS
T4	0.61±0.47 Aab	0.0001±0.00004 Bb	1.52±20.71 Aa	0.0057±0.002 Bb	*
T5	3.81±2.18 Aa	1.0002±0.0001 Ab	0.92±1.17 Aa	0.149±0.130 Aa	*
significance	NS	*	*	*	

*(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (control-) without adding any antibiotics; T2: (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/litter) with sodium alginate (0.6 mg/ml).

3.4. Escherichia Coli Bacteria (CFU)

Table (4) showed that there was a decrease in the number of Escherichia coli bacteria in experimental treatments that used T3, T4 and T5 antibiotic alternatives compared to T1, T2 and T2 treatments after 2 hours in cooling and total. Escherichia coli eradication treatments after two and three months. This can be explained because of the addition of sodium alginate and its antibacterial properties, which explain its action according to three different theories to explain the mechanism of its action. It is a polymer with antibacterial properties that works on preventing the exchange of bacterial cells and absorbing nutrition. A chemical chelator demonstrating its ability to remove heavy metals, and as a result, the growth of bacteria and the production of toxins stops.

Table 4. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to Skim milk extender on the percentage of total number of Escherichia coli 10 ^{6}CFU /ml of buffalo bulls after different cryopreservation periods (mean ± standard error).

Treatment	Time				Significan -ce
	5C°	48 hrs.PC	2 months. PC	3 months PC	
T1	0.073±0.0715 Aa	0.0073±0.00644 Aa	1.781±1.7795 Aa	0.000712±0.000 7 Aa	NS
T2	1.108±1.072 Aa	0.0194±0.00893 ABa	0.0090±0.006 Aa	0.00284± 0.00116 Ba	NS

Treatment	Time				Significan-ce
	5C°	48 hrs.PC	2 months. PC	3 months PC	
T3	0.000050±2.5884 Aa	0.000008±4.9091 Bb	0 Ab	0 Ab	*
T4	0.000138±5.8309 Aa	0.000021±0.0000164 Ba	0.0020±0.0013 Aa	0.00027±0.0006 Ba	*
T5	0.000028±0.0000129 Ab	0.00046±0.000180 Bb	0.00292±0.0012 Aa	0.000027±4.130 Bb	*
significance	NS	*	NS	*	

*(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (control-) without adding any antibiotics; T2: (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/litter) with sodium alginate (0.6 mg/ml).

3.5. Staphylococcus Bacteria CFU/ml

The results of the table (Table 5) showed a significant superiority among the experimental treatments during the cooling period, as T3 outperformed the other experimental treatments in the relative decrease in the amount of S*taphylococcus* bacteria. As an antibacterial agent, kojic acid had a direct effect on bacterial cell wall formation, disrupting the process by which vital components of bacteria are transported across the membrane, and ultimately bacterial cell death [22]. While there was no significant difference between the treatments after two and three months of freezing preservation compared to cold preservation, noting that there was a decrease in the number of staphylococcal bacteria in the treatments to which antibiotic alternatives were added (Table 5).

Table 5. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to Skim milk extender on the percentage of total number of Staphylococcus 10 ^{6}CFU /ml of buffalo bulls after different cryopreservation periods (mean ± standard error).

Treatment	Time				Significance
	5C°	48 hrs.PC	2 months. PC	3 months PC	
T1	0.0006±0.00028 Ba	0.00044±0.00039 Aa	0.024±0.021 Aa	0.00002±4.2379 Aa	NS
T2	0.00012±0.00003 Bb	0.00017±0.00004 Ab	0.30±0.18 Aa	0.000014±0.00006 Ab	NS
T3	0.5831±0.0357 Aa	0.000045±0.000034 Aa	0.192±0.189 Aa	0.0002±0.0001 Aa	*
T4	0.00076±0.0003 Bab	0.0000115±9.3434 Ab	0.012±0.005 Aa	0.000004±2.712 Ab	*
T5	0.00043±0.00026 Ba	0.0015±0.0015 Aa	0.020±0.012 Aa	0.0138±0.0125 Ab	*
significance	*	NS	NS	NS	

*(P≤0.05). PC/ Post cryopreservation. Capital letters to compare among treatments. Small letters to compare among periods. T1: (control-) without adding any antibiotics; T2: (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/litter) with sodium alginate (0.6 mg/ml).

Conclusions

The use of both kojic acid and sodium alginate resulted in an improvement in the vital characteristics of semen and a reduction in the microbial load to a minimum.

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