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Abstract. This experiment examined the effects of adding sodium alginate and KOJIC acid as substitutes of Conventional antibiotics to soybean lecithin extender on the characteristics of cryopreserved and frozen buffalo bull semen, as well as evaluation of their additions as antibiotics that to help lowering the microbial load. Following the collection and dilution of in the soybean lecithin 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 characteristics of the Cryopreserved semen were then assessed. These characteristics included sperm viability 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 total number of Staphylococcus bacteria for both cooled and frozen semen after 2 hours, 48 hours, 2 months, and 3 months post cryopreservation Results of the study revealed A significant enhancement differences (p 0.05) for the treatments T3, T4 and T5in the percentages of Individual motility and viability in comparison to the two control groups, as well as 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 T5after all periods at cooling and freezing in compared with the two control groups. it is concluded from the current experiment that 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 helped to reduce the microbial load to a minimum.

Keywords. Kojic Acid, Sodium Alginate, Buffalo Bull, Semen Cryopreserved.

1. Introduction

Since the (1930s) of the previous century and up to the present, animal producers have utilized the artificial insemination technique to increase the number of animals and maintain genetically different species. This bacterial contamination process can occur during any stage of dilution, packing, or preservation [1-3]. Bacillus subtilis, E. coli, and Staphylococcus aureus were identified as bacteria that have a negative effect on sperm quality [4]. As a result, antibiotics such as penicillin and streptomycin, were added to end the cycle of bacterial contamination. 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] used 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 look into the effect of Kojic acid and sodium alginates as alternative antibiotics to commonly used antibiotics in buffalo bull sperm dilutions. [15] Due to its 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. In order to investigate the functionality of Kojic acid and sodium alginates as more commonly used antibiotics in buffalo bull semen extenders, this study was conducted.

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 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 April to 21 Juan 2022.

2.2. Handling the Semen

Semen was diluted using soybean lecithin 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 treatment and time. Duncan's multiple range test [1955] to compassion between means [20].

3. Results and Discussion

3.1. Individual Motility

The study's findings revealed that there were substantial differences between the five treatments of the experiment, as shown in Table Table1, with treatment T5 outperforming the other treatments in the percentage of individual movement after two hours of cryopreservation over a length of 5 C. Table Table(1), while treatment T3 continued to outperform the other treatments in the second period of preservation, which is the freezing period after 48 hours of collection and extender, due to the addition of Kojic acid, which contributed to the preservation of sperm during the storage period by preserving as much of the bacterial load in the diluent as possible. Sperm motility and quality improved after T4 and T5 treatments compared to T1 and T2 control treatments, as well as in the third period of 2-month freeze preservation due to sodium alginate's adhesive property, which contributed to sperm preservation by encapsulating and preserving the sperm as much as possible while it was frozen and subjected to thawing [13].

Table 1. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to soybean lecithin extenderon 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	26.00±2.91 B a	19.00±2.44 AB b	13.00±2.54 B bc	10.00±02.37 BC c	*
T2	25.00± 2.73 B a	17.00±2.54 B ab	13.00±2.00 B bc	8.0±1.22 C c	*
T3	37.00±3.74 A a	29.00±4.58 A ab	27.00±5.38 A ab	21.00±4.00 AB b	*
T4	35.00±3.53 AB a	27.00±4.35 AB a	23.00±5.14 AB a	21.00±6.00 AB a	NS
T5	37.00±4.06 A a	27.00±3.39 AB b	24.00±3.67 AB b	23.00±4.06 A b	*
significance	*	*	*	*	

*(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 Table Table2 showed that there were no significant differences between the treatments in the percentage of sperm viability during the period of Preservation at cooling at (5 C) for two hours, whereas the results recorded significant differences between the treatments in the second period of preservation. Additionally, sodium alginate has an effect in the preservation of the sperm during the preservation period through several processes [15]. While the T2 treatment had the lowest percentage of sperm viability (Table 2), the three experimental treatments, T3, T4, and T5, showed substantial differences from the other treatments, T1 and T2, after freezing for two months (Table 2).

Table 2. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to soybean lecithin 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	82.00±0.70 A a	65.80±3.15 BC b	55.00±1.84 C c	46.00±3.22 C c	*
T2	82.40±1.46 A a	70.80±2.47 AB b	61.60±0.50 AB c	56.60±2.71 AB d	*
T3	71.40±3.45 B a	74.40±1.96 A b	65.00±2.09 A c	59.60±2.71 A c	*
T4	78.40±2.65 A a	63.00±2.00 C ab	58.60±2.54 BC b	49.00±4.3- BC c	*
T5	82.00±0.70 A a	64.60±0.58 BC b	58.80±2.26 BC bc	54.00±2.44 ABC c	*
significance	*	*	*	*	

*(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).

3.3. Bacterial Number (CFU)

The study's findings revealed that the five experimental treatments had significantly different total bacterial counts after two hours of cryopreservation. This superiority was seen in the experimental treatments that included antibiotic alternatives as opposed to the two control treatments, with the T5

treatment having the lowest total bacterial counts (Table 3).Several mechanisms work together in the Gram-negative Gram stain to destroy the integrity of the cell membrane, which allows vital enzymes to leak inside the cells and, in turn, allows K+ to permeate through the cell membrane to alter the zeta potential and harm subcellular proteins [22] (Table 3) Though there between the treatments after two months of freeze-preservation, as for the effect of the duration of preservation in one treatment, the results showed There was a significant superiority ($P \leq 0.05$) for all treatments and for all periods of freezing, compared to two hours after refrigeration (Table 3). Also, treatment T3 maintained the lowest number of bacteria among treatments and for all periods of preservation (Table 3).

Table 3. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to soybean lecithin extenderon the percentage of total number of Bacteria (10^6) of buffalo bulls after different cryopreservation periods (mean ± standard error).

Treatment	Time				Significance
	5C°	48 hrs.PC	2 months. PC	3 months PC	
T1	15.74±7.33 A b	23.06±10.89 A a	2.012±1.09 A c	0.23±0.13 A d	*
T2	3.18±0.28 B ab	8.26±0.29 B a	0.86±0.38 A b	0.0018±0.001 B b	*
T3	1.13±0.50 B a	0.13±0.00 B b	0.031±0.017 A b	0.004±0.0027 B b	*
T4	0.48±0.02 B a	1.09±0.55 B a	1.92±1.67 A a	0.073±0.064 AB a	*
T5	0.25±0.05 B b	2.06±0.72 B a	0.34±0.138 A b	0.025±0.14 B b	*
significance	*	NS	*	*	

*($P \leq 0.05$). 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).

3.4. Escherichia Coli Bacteria (CFU)

Table (4) displayed that after two hours in the refrigerator and total elimination transactions on bacteria of the type *Escherichia Coli* after two months and three months, there is a decrease in the numbers of *Escherichia COLI* bacteria in the experimental transactions in which the alternatives to antibiotics T3, T4, and T5 were used compared to the T1, T2, and T2 transactions. The inclusion of sodium alginate and its antibacterial properties are the main reasons for its fame. Three alternative theories can be used to explain how the polypolymers, particularly sodium alginate, possess antibacterial properties: a) It works to cover the surface of the sperm, preventing cell exchange and the absorption of nutrients by bacteria; b) It expresses sodium alginate from chelating substances, which means that it has the ability to remove heavy metals, and as a result, the growth of bacteria and the production of toxins end and c) It works to cover
the surface of the sperm [23,24].

Table 4. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to soybean lecithin extenderon the percentage of total number of Escherichia coli(10^6) of buffalo bulls after different cryopreservation periods (mean ± standard error).

Treatment	Time				Significance
	5C°	48 hrs PC	2months PC	3 months PC	
T1	0.010±0.0053 B b	0.0018±0.00023 B b	0.021±0.0085 A a	0.000017±5.537 A b	*
T2	0.0024±0.0015 A ab	0.0028±0.00033 A b	0.033±0.0142 AB a	0.000047±0.000043 A b	*
T3	0.00027±0.000029 B b	0.000038±0.000014 C b	0.0028±0.00122 B a	0 A b	*
T4	0.00045±0.000027 B b	0.00025±0.000013 C b	0.0025±0.0011 B a	0.000019±0.000011 A b	*

Treatment	Time				Significance
	5C°	48 hrs PC	2months PC	3 months PC	
T5	0.00012±0.000075 B b	0.000064±0.000041 C a	0 B b	0 A b	*
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).

3.5. Staphylococcus Bacteria (CFU)

The results of Table (Table5) demonstrated a moral superiority between experience transactions during the refrigeration period, with the T3 outperforming other experiment transactions in the proportionate decrease in the quantity of Staphylococcus bacteria. As an antibacterial agent, sodium alginate has a direct effect on the formation of the bacterial cell wall, the disruption of the process by which vital components for bacteria are transported through the membrane, and ultimately the death of the bacterial cell [22]. The antibiotics (Table 5) significantly reduced pain as compared to stretching therapy, and the outcomes were similar for all transactions made during the refrigerated period.

Table 5. Effect of adding different concentrations of Kojic acid and sodium alginate and their mixture to soybean lecithin extenderon the percentage of total number of Staphylococcus (10^6) of buffalo bulls after different cryopreservation periods (mean ± standard error).

Treatment	Time				Significance
	5C°	48 hrs PC	2months PC	3 months PC	
T1	47.000±3.741 A a	0.0034±0.00120 B b	0.00025±7.028 A b	0.000174±1.536 A b	*
T2	42.400±3.124 A a	0.0005±0.000026 B b	1.000±0.999 A b	0.000022±3.839 A b	*
T3	4.2000±0.3741 BC a	0.0000±2.154 B b	0.000014±0.00004 A b	0.000021±0.0009 A b	*
T4	8.8000±1.157 B a	7.600±0.6000 A a	0.00001±0.00001 A b	0.000037±0.00001 A b	*
T5	1.600±0.5099 C a	0 B b	0.0000196±0.000088 A b	0.0000153±0.0000063 A b	*
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/liter) with sodium alginate (0.6 mg/ml).

Conclusions

The addition of standard antibiotic alternatives to semen extenders resulted in an increase in semen biomarkers and a minimum reduction in bacteria load.

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