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COMPARISON OF CULTURE MEDIA (SMART AND RPMI-1640) AND TRIS EXTENDER FOR COOLING RAM SPERM

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Abstract
The object of the study was to investigate the impact of using various types of culture media from Rodwell park memorial institute (RPMI-1640), SMART (simple medium for assisted reproduction medium). After three days of cryopreservation, the rams' semen parameters were compared with those of Tris diluted. Three Awassi rams' semen was collected using an artificial vagina, and the samples were examined before being diluted with Tris diluent and culture media. The samples were placed at 4℃ for 1 hour, then they were examined, as The motility, plasma membrane integrity, dead, and abnormality of sperm were evaluated. The results of this study showed that the use of SMATR, RPMI-1640, and Tris extender on parameters of the sperm of rams stored in cooling for one hour showed nonsignificant differences (P>0.05) in all sperm parameters. Except for the percentage of sperm abnormalities, which showed a significant increase (P>0.05) when using the Tris reducer. For a period of 1,2 and 3 days, there was a significant increase (P>0.05) using SMART culture media, RPMI-1640, in comparison with the use of Tris reducer of the result of mass activity, individual and active motility, and the HOST test. Whereas the percentage of abnormality and mortality of sperm showed a significant decrease (P>0.05) compared to Tris reducer in the results of each of the percentages of the abnormality and mortality of sperm. On the other hand, there were no significant differences (P>0.05) between the treatments in the percentage of inactive motility. Finally, the results indicate the possibility of using SMART, RPMI-1640 culture media as semen diluents for ram's semen during chilling storage.
Keywords: culture media, SMART, RPMI-1640, ram sperm

Introduction

For the purpose of obtaining the best results from the use of the artificial insemination process in farm animals, especially in small ruminants. The males with excellent genetic characteristics to spreading these characteristics to the rest of the animals and thus ensure obtaining the best results(1). The importance of using methods that maintain quality semen contributes to protecting the fertilizing capacity of the sperm without causing damage to it. This is one of the important conditions that must be met when conducting the process of collecting and preserving semen for artificial insemination(2). The viability of sperm during cooling periods can be preserved by adding some substance to the semen extender.

The preservation of semen involves the storage of the sperm also in fluid or under refirgeration (3). This process involves slowing down the biochemical role by cooling the sperm at temperatures between 0-15℃. However, The fertility of chilled or fresh sperm after fluid

preservation in a ram is limited to 4 hours, with an average of 10-35% reduction in fertility every day using cervical arterial insemination(3).

The sperm of rams are very sensitive to temperature variations due to their containing less cholesterol to phospholipids within the cell membrane compared to other species. Diluents are accountable for protecting and providing adequate resources for the viability of spermatozoa during the different stages of the preservation of semen. Diuretics protect the sperm in several ways, including stabilizing and maintaining the plasma membrane. Besides, the concentration of ions inside the cells and the membrane reduces the damage of cold shock and maintains the osmotic pressure. Moreover, In order to protect the sperm from the cold shock, certain additions to the semen diluent were used (3, 4, 5, 28, 29, and 30). Thinners include diverse bioactive components, making them meet the special requirements of the species and routes of preservation used.

Because of these biological differences, each species' sperm requires different safeguards. The optimal pH for respiration in sperm differs between species (6, 7), with the optimal pH range for rams being 7.3–7.5, and for bulls being 6.7–7.5. (9).

Each storage method has its own unique set of pressures that must be considered to maximize sperm length in light of the aforementioned biological variations. As the main components of the diluents vary depending on the type of storage, each diluent has its unique properties. For instance, glucose or fructose are used as an energy source, and egg yolk is included in the refrigerant to protect the sperm from the cold shock (3, 33).

Diluents like Tris-fructose (Tris), Salamon's egg yolk-based expander (EY), and phosphate-buffered saline (PBS) are commonly used in ram fluids to protect the sperm from heat shock, cold shock, and osmotic shock (10). Phosphate-buffered saline (hydroxymethyl aminomethane (tris), citric acid, fructose, egg yolk, bovine serum albumin, and various antibiotics are some of the main ingredients. These include BSA and tris, which serve as a protein source and pH buffer, respectively, to aid in spermatogenesis (3), and fructose, which supplies sugar that supplies essential substrates for metabolism (8,11,34, 12, and 13).

Egg yolk's low-density lipoproteins (LDL) and cholesterol provide membrane support, and citric acid acts as a pH buffer to maintain peak metabolic rates during rest and repair (1, 8). (12).

The objective of this study is to investigate using culture media (SMART and PRMI-1640) to dilute ram semen during cooling storage.

Materials and methods:

Aim of the study:

This study aims to investigate using culture media (SMART and RPMI-1640) to dilute ram semen during cooling storage.

Experiment design:

This study was conducted at the college of agricultural engineering sciences/university of Baghdad, in 2022. Three rams were used in this experiment, and the samples were mixed to eliminate individual differences, and the samples were divided into three parts.

A- Part diluted 1:10 with Tris extender with egg yolk 5%

B- Part diluted 1:10 in SMART culture medium, egg yolk 5%

C- Part diluted 1:10 in culture medium RPMI-1640 egg yolk 5%

Preparation of culture media and tris diluent:

Preparation of SMART medium:

The Simple Medium for Assisted Reproduction Technologies (SMART) culture medium was prepared by dissolving the chemicals and the quantities shown in Table (1) at a pH of 7.2. Then passing the resulting solution through a millipore filter of 0.22 µm size, then kept in a refrigerator until use (14).

Table (1) SMART culture medium extender components

materials	Quantity
Bicarbonate	29mM
$CaCl_2$	0.27g
Distilled water	500mL
(BSA) Bovine Serum Albumin	5%
KCl	0.4g
NaCl	6.0g
Na-lactate	3.2g
Na-pyruvate	0.01g
Penicillin	100IU/mL
Phenol red	0.5g
Streptomycin	100µg/mL

Ref **(Fakhrildin** and **Flayyih, 2099)**

Preparation of RPMI-1640 medium:

Roswell park memorial institute (RPMI-1640) medium was prepared by dissolving the chemicals and the quantities in 100 distilled water RPMI-1640 powder (Sigma Co: Germany) 1.039g, Human serum albumin (HSA) 5%. Plus, Na-pyruvate 0.1mg, Penicillin 100IU/mL, and Streptomycin100µg/mL at a pH of 7.2, then, the resulting solution was passed through a millipore filter of 0.22 µm size, then kept in a refrigerator until use

Preparation of Tris extender:

Tris diluent was prepared according to the Salamon and Maxwell (3) method:

Table (2) Tris extender components

materials	Quantity
Tris	3.63g\100ml
Citric acid	1.99g\100ml
Glucose	0.50 g\ 100ml

Distilled water	500mL
Egg yolk (Fresh unfertilized eggs)	5ml\100ml
Penicillin	100IU/mL
Streptomycin	100µg/mL

Ref : (Maxwell and Salamon, 2000)

Semen collection:

The semen collection process begins at 8:00 in the morning, with one ejaculate/ram/week, using the artificial vagina of sheep and goats. Rams are primed to perform a false mount to increase their sexual desire (15)

Dilution and cooling of semen:

The semen sample was diluted using SMART or RPMI-1640 culture media, or Tris diluent, according to the study parameters, and then the diluted semen was cooled by gradually lowering the temperature to 4 °C for 3 hours using a water bath and placed in the refrigerator.

Semen assessment:

Sperm motility:

A drop of thawed semen was placed on a warm slide at 37 degrees Celsius, and the sperm's motility was estimated using a magnification of (400x) following the protocol proposed by (16).

Dead and abnormal sperm percentage:

The percentage of dead sperm was evaluated according to what was stated by Swanson and Beardon (17). the percentage of abnormal sperm was estimated according to what was stated by Hancock (18),), in the same slide for estimating the percentage of sperm mortality

Membrane integrity test:

This test is called the HOST- test. The percentage of the integrity of the plasma membrane was estimated based on what it came with (19).

Statistical analysis:

The SPSS statistical program was used to analyze the data, the complete random design (CRD) was calculated, and the significant differences between the means were compared by the multinomial test (20).

Results and Discussion:

The deterioration of semen quality is often due to exposure to cold shock when cooling to 5 °C, which affects the metabolic ability of sperm due to the release of enzymes and ions (21,22) and (22,30). The stages of the freezing process, including the cooling process, lead to mechanical stress on the plasma membrane of the sperm and loss of the osmotic system, which leads to a change in the amount of water inside the sperm (23, 24, 26, and 27).

Using a 1-hour cooling period, Table (3) compares the effects of the culture media SMART, RPMI-1640, and Tris extender on the characteristics of sheep sperm. However, there were no statistically significant differences between treatments for any of the sperm parameters. In comparison, the use of the tris extender was associated with a statistically significant increase ($P>0.05$) in the percentage of abnormal sperm. Each type of sperm storage involves its own unique set of pressures that must be considered to achieve the desired elongation, and the extenders

themselves are technology-specific in terms of their main components due to these biological differences. To prevent sperm damage from cold shock, some refrigerators use egg yolk as a cooling agent (3,4 and 35).

Table (4) shows the effect of using SMART, RPMI-1640 culture media, and Tris extender diluent on sperm characteristics of sheep that were chilled for one day. The percent of sperm abnormality and sperm mortality showed a significant decrease (P>0.05) compared with the tris extender in the results of each of the percent of abnormality of sperm. As well as, sperm mortality for the results of each group ratio, individual motility, active motility, and the HOST test. In contrast there was no significant difference among all groups of study.

The results showed that the use of SMART, RPMI-1640 culture media and Tris extender on sperm characteristics of sheep stored in cooling for two days showed significant improvement (P<0.05). Whereas the percentages of sperm abnormality and sperm mortality showed a significant decrease (P>0.05) compared to the Tris extender in the results of each of the percentages of sperm abnormality. Likewise, sperm mortality for the results of each group ratio, individual motility, active motility, and the HOST test.On the other hand, there was no significant difference here (P>0.05) among all groups of this work (Table 5).

The use of SMART media, RPMI-1640, and Tris extender resulted in a significant increase (P<0.05) in the characteristics of spermatozoa of sheep stored in cooling for 3 days. In the same role, the percent of sperm abnormality and mortality showed a significant decrease (P>0.05) compared to the Tris extender in the results of each of the percent of sperm abnormality and sperm mortality. Moreover, there were no significant differences here among all groups of this experiment (table 6).

Tris-citrate-fructose (TRIS), salmon egg yolk-based expander (EY), and phosphate-buffered saline (PBS) are common diluents used in ram fluids to protect the sperm from temperature change, cold shock, and osmotic shock (10). Ingredients such as phosphate-buffered saline, tris (hydroxymethyl aminomethane), fructose, citric acid, egg yolk, bovine serum albumin (BSA), and various antibiotics are used. As protein sources and a pH buffer, respectively, BSA and tris extender are useful for spermatogenesis (3, 25). Fructose, on the other hand, is the sugar that provides the substrate necessary for the metabolic process (11, 12 and 13). Egg yolk provides membrane support via low-density lipoproteins (LDL) and cholesterol, and citric acid acts as a pH buffer, keeping the metabolic rate for respiration at its highest possible levels (1, 8, and 24). (12). The outcomes demonstrated the potential for using SMART and RPMI-1640 culture media as semen diluents for rams semen while chilling store.

Table 3: Effect of using SMART medium, RPMI- 1640 medium, and tris diluent on the characteristics of ram sheep sperm after one hour of cooling.

Parameters	tris	SMART medium	RPMI-1640 medium	P-value
Individual Motility%	84.233±0.25^{A}	85.000±0.71^{A}	86.766±0. 57^{A}	0.1124 NS
Progressive motility%	48.700±0.67^{A}	49.750±1.03^{A}	48.750±0.88^{A}	0.0772 NS
Non-progressive motility%	37.300±0.22^{A}	34.250±1.65^{A}	38.000±1.58^{A}	0.7222 NS
Abnormality %	13.785±0.63^{A}	8.750±0.75^{B}	9.500±0.29^{B}	0.0401*
Mortality %	15.522±0.33^{A}	15.000±0.61^{A}	14.500±0.50^{A}	0.7156NS
Host test %	85.760±1.23^{A}	88.020±1.81^{A}	86.750±1.62^{A}	0.0886NS

Different letters for each mean show significant differences (P<0.05).
Like letters for each mean show non-significant differences NS.
NS There are non-significant differences.
* Significant difference (P<0.05).
** Significant difference (P<0.01).

Table 4: Effect of using SMART medium, RPMI- 1640 medium, and tris diluent on the characteristics of ram sheep sperm after one day of cooling.

parameters	tris	SMART medium	RPMI-1640 medium	P-value
Individual Motility%	61.500±0.86B	71.290±0.99A	69.350±1.11A	0.0449*
Progressive motility%	24.600±0.21C	37.250±1.28A	32.733±0.48B	0.0013**
Non-progressive motility%	36.410±0.87A	34.022±1.18A	37.050±1.78A	0.7572NS
Abnormality %	32.300±0.86A	22.760±0.95C	24.580±0.75B	0.0012**
Mortality %	35.250±1.43A	25.800±0.25B	27.500±1.87B	0.0317*
Host test %	66.010±1.91B	76.250±2.11A	75.799±1.22A	0.0328*

Different letters for each mean show significant differences (P<0.05).
Like letters for each mean show non-significant differences NS.
NS:- There are non-significant differences.
* Significant difference (P<0.05).
** Significant difference (P<0.01).

Table 5: Effect of using SMART medium, RPMI- 1640 medium, and tris diluent on the characteristics of ram sheep sperm after two days of cooling.

parameters	tris	SMART medium	RPMI-1640 medium	P-value
Individual Motility%	41.722±1.33 B	62.250±1.21A	61.250±1.31A	0.0422*
Progressive motility%	9.777±1.32B	26.250±1.52A	25.250±1.55A	0.0307*
Non-progressive motility%	32.33±2.66A	36.066±1.01A	36.000±1.08A	0.6589NS
Abnormality %	52.288±1.83 A	32.750±1.19C	41.560±1.34B	0.0008**
Mortality %	55.000±1.77 A	36.730±0.65C	44.500±1.56B	0.0021**
Host test %	46.278±1.87 B	67.250±0.85A	66.550±1.49A	0.0143**

Different letters for each mean show significant differences (P<0.05).
Like letters for each mean show non-significant differences NS.
NS There are non-significant differences.
* Significant difference (P<0.05).
** Significant difference (P<0.01).

Table 6: Effect of using SMART medium, RPMI- 1640 medium, and tris diluent on the characteristics of ram sheep sperm after three days of cooling.

Parameters	tris	SMART medium	RPMI-1640 medium	P-value
Individual Motility%	25.043 ± 1.87^{C}	44.266 ± 1.70^{A}	39.766 ± 1.65^{B}	0.0012**
Progressive motility%	5.540 ± 0.95^{B}	20.088 ± 0.41^{A}	8.723 ± 1.75^{B}	0.0076**
Non-progressive motility%	19.570 ± 2.71^{C}	23.250 ± 1.22^{B}	30.232 ± 1.58^{A}	0.0046**
Abnormality %	72.077 ± 1.89^{A}	42.750 ± 1.55^{C}	54.580 ± 1.45^{B}	0.0081**
Mortality %	71.711 ± 1.95^{A}	45.750 ± 1.85^{C}	57.520 ± 1.87^{B}	0.0099**
Host test %	30.598 ± 2.86^{c}	54.250 ± 1.93^{A}	43.728 ± 1.61^{B}	0.0091**

Different letters for each mean show significant differences (P<0.05).
Like letters for each mean show non-significant differences NS.
NS:- There are non-significant differences.
* Significant difference (P<0.05).
** Significant difference (P<0.01).

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