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GELATIN AS A HIGH VISCOSITY PROTEIN IN TRIS EXTENDER AND ITS EFFECT ON POST-THAWED RAM SEMEN QUALITY

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ABSTRACT

Gelatin as a high viscosity protein can reduce sperm metabolism by reducing sperm movement. So, this study was set out to examine the impact of adding gelatin to Tris-citric-egg yolk extender (0, 0.5, 1.0 and 1.5%) on visual sperm characteristics, CASA analysis parameters, total antioxidants capacity, and enzyme activity in in post-thawed ram semen. Total of 5 rams have been used for collecting semen once weekly for 5 weeks. Pooled semen was diluted with the different levels of gelatin, equilibrated at 5^{o}C for 4h and stored in liquid nitrogen. Semen had been evaluated post thawing. Results showed a positive effect of gelatin supplementation in extender on sperm characteristics, including progressive motility, livability, abnormality, and membrane integrity. Gelatin addition improved CASA -motility parameters and normality, while decreasing sperm velocity of sperm cells as well as increasing total antioxidant capacity and glutathione peroxidase, and decreasing enzyme activity (LDH) of post-thawed sperm medium. In conclusion, gelatin supplementation to Tris semen extender at a level of 1.5% is considered as a good enhancement on sperm function in post-thawed ram semen.

Key Words: Gelatin, Ram semen, Computer assisted sperm analysis (CASA).

INTROD0UCTION

In sheep, increment of spermatozoa's motility and metabolic activity is necessary to be successfully stored in liquid, thereby prolonging their fertile life and artificial insemination (AI) **(Allai *et al.*, 2015)**. Fresh semen's capacity to fertilize may be sustained at 15 ºC through 48 or 72 h **(Roca *et al.*, 2000; López-Gatius *et al.*, 2005)**. It would be intriguing to employ frozen semen to enhance genetic improvement and storage time. Nonetheless, despite the fact that numerous research has been conducted to increase the effectiveness of reproduction when frozen semen is utilized, a decrease in motility, viability, and fertility or prolificacy following insemination is typically observed **(Mocé *et al.*, 2003; Castellini *et al.*, 2006; Kashiwazaki *et al.*, 2006; Si *et al.*, 2006)**. Frozen semen is needed to extend storage period, but the freezing process is linked to a decrease in sperm characteristics

and fertility following artificial insemination **(Castellini *et al.*, 2006; Kashiwazaki *et al.*, 2006)**.

The gelatin is a high viscosity protein may exert a beneficial effect through preventing sperm cell precipitation, which can limit changes in medium conditions or composition. It can also immobilize spermatozoa, which lowers the metabolic needs of mobility and preserves their fertilizing ability **(López-Gatius *et al.*, 2005; Cortell and Viudes-De-Castro, 2008)**. *In vitro* preservation of sperm motility, viability metrics, and fertility have been enhanced by the use of more viscous extenders, such as those enhanced with gelatin in rabbits **(Nagy *et al.*, 2002; López-Gatius *et al.*, 2005; El-Sherbieny *et al.*, 2012)**, sheep **(Yániz et al., 2005)**, goats **(Salvador et al., 2006),** and boars **(Corcini *et al.*, 2011)**. Gelatin solution stays liquid at normal temperature, but at low temperatures (5ºC) it becomes very viscous and the medium solidifies **(dos Santos *et al.*, 2015)**.

Gelatin increases the viscosity of the extender which affects motility characteristics, in terms of decreasing sperm metabolism and movement in post-diluted and equilibrated semen and minimizing lactic acid formation in prolonged semen **(Hirai *et al.*, 1997; Echegaray-Torres *et al.*, 2004)**. Furthermore, it should be mentioned that gelatin is a big molecule that cannot enter spermatozoa and harm them internally or dramatically change the osmolarity of diluents, leading to plasmolysis or dehydration **(Resseguie, 1979)**.

This study was purposed to find out the impact of adding varying levels of gelatin to a semen extender (0.5, 1.0, and 1.5%) on the ram semen's physical parameters, computer assisted sperm analysis **(**CASA), antioxidant status and enzyme activity in post-thawed ram semen.

MATERIALS AND METHOD

The current study was conducted in Karada Station, Kafr el-Sheikh Governorate, APRI, ARC, Egypt from May to August of 2024.

Extender preparation and experimental design:

Tris base extender containing 3.025 g Tris, 1.66 g citric acid monohydrate, 1.25 g glucose, 15 mL egg yolk, 7% glycerol, 100 µg/mL lincomycin, and 100 µg/mL streptomycin which have been mixed and made up to 100 mL distilled water. The osmolarity level and pH value of the base extender was 280-300 mOsmol/L and 6.8-7.0, respectively.

In this experiment, semen was diluted with Tris-base extender supplemented with gelatin at levels of 0, 0.5, 1.0, 1.5% .Gelatin was mixed with Tris-extender during the preparation of the diluent.

Semen collection:

Total of 5 sexually mature Ossimi rams, as semen donors, having 70-80 kg body weight and 2-4 years old kept on the same environmental conditions with feeding system. Semen was collected early morning by

an artificial vagina once/week for 5 weeks, as semen collection period. Semen ejaculates (≥70% initial sperm motility) were kept at 37 °C in water bath and promptly taken to the lab, then, diluted at 1:20 (semen/extender) by different types of Tris-extender, equilibrated for 4 hours at 5 °C, stored in liquid nitrogen (-196 °C) for at least one week, and thawed at 37 °C for 30 seconds.

Semen evaluation:

Physical parameters of semen:

Progressive motility, livability **(Moskovtsev and Librach (2013)**, abnormalities **(Menon *et al.*, 2011)** and membrane integrity by hypo-osmotic swelling test (HOS-t) **(Neild *et al.*, 1999)** have been evaluated manually in post-dilution, equilibrating, and thawing semen**.**

Computer assisted sperm analysis (CASA)*:*

A drop of post-thawed semen (5µL) was put into a slide that had been preheated from each extender. Before the CASA analysis, the sample was allowed to settle on the mini-thermal heating stage (37-38°C). Two to three drops of each sample included about 200 spermatozoa were evaluated for each specimen.

Percentages of progressive (rapid + slow), non-progressive, and total motility (progressive + nonprogressive) as well as immotile spermatozoa were included in the final analysis of each sample.

Sperm kinetic parameters as curve linear velocity (VCL), straight linear velocity (VSL), and average path velocity (VAP) were determined. However, rates of linearity (LIN%) =VSL/VCL ×100, straightness (STR%) =VSL/VAP ×100 and wobble (WOB%) =VAP/VCL ×100 were calculated.

Total antioxidants capacity and enzyme activity*:*

Total antioxidant capacity (TAC) level **(Ohkawa *et al.*, 1979)**, and activity of glutathione peroxidase (GPx) and lactate dehydrogenase (LDH) **(Bais and Philcox, 1994)** were measured in sperm medium of post-thawed semen. Sperm medium was obtained by centrifugation of post-thawed semen at 3000 rpm for 10 min.

Statistical analysis:

Data were statistically analyzed by one-way ANOVA design using computer SAS program **(SAS, 2007)** and Duncan's multiple range test **(Duncan, 1955)** was used to check the significant differences between means at $P<0.05$.

RESULTS

Physical parameters:

Results of physical characteristics (Table 1) revealed that visual progressive motility, livability, and membrane integrity percentages significantly ($P<0.05$) improved, in E3 after dilution, equilibration, and

thawing. However, sperm abnormality percentage (P<0.05) was reduced significantly (P<0.05) by E3 only in post-thawed ram semen as compared to the control extender (E0). On the other hand, motility, livability, and membrane integrity percentages in post-equilibration and thawed semen were significantly (P<0.05) improved by E2 compared with E0, in lease extend than in T3. However, E2 did not affect significantly sperm characteristics. Also, there was insignificant effect of extender type on sperm abnormality percentage by gelatin supplementation in post diluted and equilibrated ram semen.

Table 1. Effect of gelatin supplementation on sperm characteristics (%) in ram semen

Item	Progressive motility	Livability	Abnormality	Membrane integrity
Diluted semen				
E0 (Control)	**83.23±0.58^{b}**	**85.20±1.30^{b}**	**11.51±0.68**	**83.70±0.30^{b}**
E1 (0.5% gelatin)	**84.57±0.28^{b}**	**85.30±1.28^{b}**	**11.21±0.18**	**83.38±0.44^{b}**
E2 (1.0% gelatin)	**84.21 ±0.67^{b}**	**85.42±1.30^{b}**	**11.58±0.34**	**84.67 ±0.67^{b}**
E3 (1.5% gelatin)	**86.61±0.29^{a}**	**87.28±1.10^{a}**	**11.90±0.47**	**86.97±0.29^{a}**
P-value	**0.0405**	**0.0358**	**0.3357**	**0.0311**
Equilibrated semen				
E0 (Control)	**74.19±1.04^{c}**	**75.42±1.20^{c}**	**24.12±0.63**	**75.45±1.12^{c}**
E1 (0.5% gelatin)	**75.26±0.40^{c}**	**76.85±1.35^{c}**	**23.50±0.67**	**76.69±0.44^{c}**
E2 (1.0% gelatin)	**78.37±1.41^{b}**	**80.42±1.14^{b}**	**23.44±0.48**	**79.57±1.46^{b}**
E3 (1.5% gelatin)	**84.94±0.35^{a}**	**84.85±1.40^{a}**	**22.51±0.96**	**84.94±0.58^{a}**
P-value	**0.0111**	**0.0125**	**0.2281**	**0.0252**
Thawed semen				
E0 (Control)	**44.36±0.55^{c}**	**45.42±1.10^{c}**	**40.90±0.63^{a}**	**45.89±0.55^{c}**
E1 (0.5% gelatin)	**44.48±0.40^{c}**	**46.63±1.48^{c}**	**40.64±0.25^{a}**	**46.39±0.40^{c}**
E2 (1.0% gelatin)	**46.67±0.41^{b}**	**48.27±1.34^{b}**	**37.24±0.64^{b}**	**48.32±0.41^{b}**
E3 (1.5% gelatin)	**50.75±0.35^{a}**	**51.39±1.18^{a}**	**33.14±0.28^{c}**	**50.68±0.35^{a}**
P-value	**0.0222**	**0.0248**	**0.0047**	**0.0143**

$^{a, b, c}$: Means denoted within the same column for each parameter at each stage with different superscripts are significant at P<0.05.

Semen motility (CASA analysis):

All levels of gelatin significantly (P<0.05) increased percentages of slow progressive, and total motility while significantly (P<0.05) decreased rapid progressive and immotility percentages in post thawed semen compared to control (Table 2). However, E2 and E3 significantly (P<0.05) increased total progressive motility compared with E0 and E1. Also, non-progressive motility was not affected by gelatin treatment in post –thawed semen.

Table 2. Effect of gelatin supplementation on sperm motility parameters (%) in post-thawed ram semen by CASA analysis

Item	Rapid progressive	Slow progressive	Total progressive	Non progressive	Total motility	Immotility
E0 (Control)	29.25±0.56[a]	13.9±0.25[b]	43.15±0.56[c]	5.11±0.10	48.26±0.89[c]	51.74±0.89[a]
E1 (0.5% gelatin	20.69±0.35[b]	24.26±0.12[a]	44.95±0.78[c]	5.28±0.14	50.23±0.64[b]	49.77±0.64[b]
E2 (1.0% gelatin	21.46±0.79[b]	25.32±0.36[a]	46.78±0.65[b]	6.25±0.20	53.03±0.54[a]	46.97±0.54[c]
E3 (1.5% gelatin	23.35±0.84[b]	25.88±0.14[a]	49.23±0.51[a]	5.65±0.43	54.88±0.61[a]	45.12±0.61[c]
P-value	0.0389	0.0325	0.0228	0.1258	0.0163	0.0364

[a, b, c]: Means denoted within the same column with different superscripts are significant at P<0.05.

Kinetic parameters (CASA analysis):

All sperm velocity parameters and sperm kinetic indexes were significantly (P<0.05) decreased by all levels of gelatin, except WOB as compared to control (Table 3). Results revealed that VCL, VSL, VAP, LIN, and STR were the lowest with the highest level of gelatin and WOB showed an opposite trend.

Table 3. Effect of gelatin supplementation on sperm velocity parameters and sperm kinetic indexes in post-thawed ram semen.

Item	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)
E0 (Control)	84.36±0.36[a]	43.26±0.64[a]	60.86±0.28[a]	51.28±0.51[a]	71.08±0.35[a]	72.14±0.44[b]
E1 (0.5% gelatin)	81.4±0.35[b]	40.46±0.38[b]	59.8±0.29[a]	49.70±0.41[a]	67.65±0.27[b]	73.46±0.36[b]
E2 (1.0% gelatin)	70.86±0.39[c]	34.96±0.25[c]	54.4±0.21[b]	49.33±0.39[a]	64.26±0.24[c]	76.77±0.41[a]
E3 (1.5% gelatin)	66.93±0.26[d]	31.26±0.37[d]	50.86±0.26[c]	46.70±0.48[b]	61.46±0.29[d]	75.98±0.40[a]
P-value	0.0001	0.0001	0.0014	0.0112	0.0001	0.0231

[a, b, c, d]: Means denoted within the same column with different superscripts are significant at P<0.05.

Abnormalities (CASA analysis):

Data of sperm normality percentage and abnormality forms by CASA in post-thawed ram semen have been showed in Table 4. Results revealed that the highest normality percentage and the lowest neck and tail abnormality of spermatozoa were achieved significantly (P<0.05) by E3 as compared to E0. However, E2 significantly (P<0.05) increased normal forms and decreased tail abnormality percentage. While E1 had no effect on normality and abnormality percentages. Insignificant effect of different gelatin supplementations on head abnormality of sperm have been investigated.

Table 4. Effect of gelatin supplementation on sperm normality abnormality percentage in post-thawed ram semen.

Item	Normality (%)	Abnormality (%)		
		Neck	Head	Tail
E0 (Control)	58.1±1.25[a]	22.1±0.45[a]	11.33±0.35	8.47±0.54[a]
E1 (0.5% gelatin)	59.36±1.12[a]	22.24±0.36[a]	11.25±0.25	7.15±0.24[a]
E2 (1.0% gelatin)	63.76±1.02[b]	21.73±0.64[a]	12.36±0.61	2.15±0.62[b]
E3 (1.5% gelatin)	64.86±1.22[b]	19.36±0.61[b]	12.91±0.34	2.87±0.35[b]
P-value	0.0413	0.0258	0.5481	0.0369

[a, b]: Means denoted within the same column with different superscripts are significant at P<0.05.

Total antioxidant capacity and enzyme activity:

The effect of gelatin supplementation on TAC level and activity of GPx and LDH in post-thawed ram semen had been showed in Table 5. Results revealed insignificant effect of gelatin supplementation on TAC. E3 showed increment (P<0.05) in the activity of GPx and decrement in the activity of LDH, while E2 significantly decreased LDH activity in sperm medium as compared to E0.

Table 5. TAC, GPx and LDH in post-thawed ram seminal plasma supplemented with gelatin

Item	TAC (mM/L)	GPx (mU/mL)	LDH (U/mL)
E0 (Control)	2.71±0.50	4.25±0.25[b]	70.16±6.35[a]
E1 (0.5% gelatin)	2.20±0.64	4.36±0.10[b]	66.35±4.93[a]
E2 (1.0% gelatin)	2.63±0.38	4.97±0.12[ab]	55.29±6.06[b]
E3 (1.5% gelatin)	2.80±0.54	5.12±0.16[a]	57.49±5.19[b]
P-Value	0.5368	0.0356	0.0328

[a, b]: Means denoted within the same column with different superscripts are significant at P<0.05.

DISCUSSION

The spatial orientation of cryopreserved spermatozoa inside the medium determines their survival and integrity. Because gelatin-supplemented extenders expose sperm to consistent solute concentrations and the medium's viscosity, which lowers the metabolic needs of spermatozoa, sperm quality may increase **(Holt, 2000; Nagy *et al.*, 2002; López-Gatius *et al.*, 2005)**.

Aim of the current study was to evaluate different gelatin levels on sperm quality of cryopreserved ram semen. In this context, we found that gelatin addition at a level of 1.5% (E3) showed a significant improvement (P<0.05) in the percentage of motility, livability, and membrane integrity after all stages of cryopreservation. Additionally, gelatin addition (1.5%) improved motility and abnormality parameters as measured by CASA, In the same way of the current work, **Bandeira et al. (2018)** indicated that 1.5% gelatin was the most effective for enhancing ram sperm quality during storage. Several authors reported that adding gelatin in the extender improved freezing ability,

effectiveness, and fertility in post-thawed rabbit semen **(El-Sherbieny et al., 2012)**, sheep semen **(Yániz *et al.*, 2005)**, and boar semen **(Corcini et al., 2011)**. Also, gelatin addition to semen extender improved semen quality and provided a high proportion of living cells in preserved semen by increasing acrosome integrity and vitality 72 hours following semen collection compared to gelatin free extender **(Nagy *et al.*, 2002).** On the other hand, several researchers did not observe any differences in the motility of goat and sheep semen when gelatin was added to fresh semen extender after semen collection **(Yániz *et al.*, 2005; Salvador *et al.*, 2006; Elspeiy and Elhanoun, 2015)**. Adding 1.5% gelatin showed little sperm agglutination, whereas those containing 3.0% gelatin showed no agglutination **(Corcini *et al.*, 2011)**.

Improving sperm membrane integrity and normal forms in our study indicated maintenance of sperm normal structure. This intact structure was reported to be preserved by gelatin, because its physical form provided protection in the cells that was comparable to encapsulation, avoiding a cold shock throughout the cooling curve and assisting in keeping this structure stable as it transitions to room temperature until it reaches 5ºC **(Gheller *et al.*, 2018)**. Since the acrosome is an essential structure in fertilization, enabling chemical and physical interactions between sperm and egg that result in conception, gelatin has a favorable effect on the cooling process of sperm cells. Cooling may cause the acrosome to break. The acrosome reaction can be triggered in sperm cells with unstable membranes with a temperature drop of 0.5°C/min **(Gadella *et al.*, 2001; Yániz *et al.*, 2011)**.

AS such, we found that decreasing all sperm velocities and LDH activity in post-thawed sperm medium. Also, gelatin addition resulted in increasing slow progressive motility and decreasing rapid progressive motility. However, total progressive motility improved by gelatin addition These findings were due to increasing viscosity of sperm medium which reduce the motility of sperm cells, subsequently reducing sperm metabolism and reducing pH value of sperm medium. For ram sperm to remain viable, the pH level of the semen should typically be kept between 6.0 and 6.5. However, this study also found that the natural lactic acid synthesis during the chilling and dilution phases of semen lowers intracellular and extracellular pH levels **(Bartoov *et al.*, 1980; Gadea, 2003; Dziekońska *et al.*, 2009; Yániz *et al.*, 2011)**. Sediment region's pH increases acidic when sperm agglutinate because of a higher concentration of several harmful spermatozoa metabolic products **(Nagy *et al.*, 2002)**. Gelatin kept the cooled semen's pH constant for 48 and 72 hours while reducing sperm sedimentation **(Bandeira *et al.*, 2018)**. The increased homogeneity may provide a more favorable environment by lessening the amplitude of the pH fluctuations that naturally occur

because the buffers and sperm are uniformly distributed **(Salvador *et al.*, 2006; Rahman, 2011; dos Santos *et al.*, 2015; Elspeiy and Elhanoun, 2015)**.

Additionally, the cryoprotectant systems could better shield the sperm from cold shocks due to the increased homogeneity of the fluid **(Holt, 2000; Corcini *et al.*, 2011)**. Because gelatin-supplemented samples don't need to be homogenized, the extender's buffers work better to minimize pH fluctuation and lessen exposure to cold shock. However, despite the viscosity of the medium preventing movement, suspended swine spermatozoa still burn energy while attempting to migrate inside it **(Esbenshade and Nebel, 1990)**. Thus, the protein content of the gelatin may be the reason for increases in sperm quality in extenders rather than the viscosity. A gelatin-supplemented extender might be a low-cost substitute for storing and transporting semen for extended periods of time **(Corcini *et al.*, 2011)**.

Adding gelatin to the extender helped to maintain a consistent environment and altered certain of the sperm kinematic properties. In similarity with **(Bandeira et al., 2018),** the sperm in the gelatinized medium exhibited more irregular or curvilinear motions by decreased VSL, VAP, and STR values following 48 hours of storage,. The current research showed reduction in sperm kinetic parameters as the level of gelatin in the diluent increases. (G3) 1.5% showed the lowest sperm kinetic parameters in post-thawed semen. These findings are also in line with those of **Mortimer (1997)**, who found that the amplitude of flagellar movement decreases with increasing diluent viscosity. Additionally, **Hirai et al. (1997)** noted that in very viscous medium, the percentage of increasingly motile bull sperm and their velocity decreased. An apparent benefit would have been shown if assessments of these traits had been carried out right away upon collection and dilution as well as after a 24-hour holding period at 5°C **(Bandeira *et al.*, 2018)**. On the other side, **Cortell and Viudes-De-Castro (2008)** reported that the inclusion of gelatin did not enhance semen characteristics, fertility and prolificacy after thawing.

After a brief period of storage, the percentage of viability and intact acrosome in extender with gelatin were greater than in the control extender. The sperm cells sediment during preservation, even if buffers are added to the extenders to reduce pH variations caused by the spermatozoa's metabolic products. As a result, the area of sedimented cells may have a lower pH value and a greater concentration of some harmful metabolic products. Sperm cells are more evenly dispersed because gelatin inhibits sedimentation, and buffers are better able to stop pH fluctuations. Another benefit of gelatin is that prolonged semen

samples are solid, which facilitates and secures sample handling and postal delivery **(Levis, 2000; Bandeira *et al.*, 2018)**.

CONCLUSION

So, it can be concluded that gelatin supplementation in extender led to improvements of manual sperm characteristics, motility parameters, sperm velocity, and normal forms as well as superiority of antioxidant status and enzyme activity of post-thawed sperm medium of cryopreserved ram semen. Gelatin at a level of 1.5% showed the highest substantial effects on sperm quality in post-thawed ram semen.

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الجيلاتين كبروتين عالي اللزوجة في مخفف التريس وتأثيره على جودة السائل المنوي للكباش بعد التجميد و الإسالة

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الجيلاتين بروتين عالي اللزوجة يؤثر على حركة الحيوانات المنوية مما يؤدي إلى تقليل عملية التمثيل الغذائي. لذا الغرض من هذه الدراسة هو تقييم تأثير إضافة مستويات مختلفة من الجيلاتين (% 1.5 –1 –0.5 –0) الى مخفف السائل المنوي تريس–سيتريك– صفار البيض على خصائص الحيوانات المنوية ومقاييس تحليل السائل المنوى باستخدام الكمبيوتر وقدرة مضادات الأكسدة الكلية والنشاط الإنزيمى في السائل المنوي للكباش بعد الاسالة. تم استخدام 5 كباش لجمع السائل المنوي مرة واحدة أسبوعيًا لمدة 5 أسابيع. تم خلط السائل المنوي و تخفيفه بمستويات مختلفة من الجيلاتين وموازنته عند 5 درجات مئوية لمدة 4 ساعات وحفظه في النيتروجين السائل. تم تقييم السائل المنوي بعد التخفيف و الموازنة والاسالة بالعين و الكمبيوتر و كذلك النشاط المضاد للأكسده و النشاط الانزيمى فى بيئة السائل المنوى. أظهرت النتائج تأثيرًا إيجابيًا لإضافة الجيلاتين في المخفف على خصائص الحيوانات المنوية ومقاييس تحليل السائل المنوى باستخدام الكمبيوتر وقدرة مضادات الأكسدة الكلية والنشاط الإنزيم في السائل المنوي للكباش بعد الاسالة. كان لأعلى مستوى من الجيلاتين (1.5%) التحسين الافضل للحركة والحيوية والشذوذ وسلامة الغشاء البلازمى والحركة التقدمية والحركة الكلية ومقاييس الحركة باستخدام الكمبيوتر والشكل الطبيعي في السائل المنوي للكباش بعد الإسالة مما يعتبر تعزيزًا جيدًا لوظيفة الحيوانات المنوية في السائل المنوي للكباش بعد التجميد والاسالة.