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IMPACT OF GELATIN AND α–CHYMOTRYPSIN SUPPLEMENTATION IN RABBIT SEMEN EXTENDER ON KINDLING RATE AND LITTER TRAITS

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The present work was carried out to study the effect of addition gelatin and α-chymotrypsin to semen rabbit extender on semen quality and litter traits during incubation at 37oC for up to 4 hours and storage at 5oC for up to 72 hours of V.line rabbits. Twenty sexually mature bucks and eighty multiparous does of V.Line rabbits in various parities were used. A base extender was prepared (Tris-citric acid-glucose) used a basal buffer. The basal extender was divided into five portions and treated as following: (1) Tris-based extender (control), (2) Tris-based extender + 1gm gelatin/100ml, (3) Tris-based extender + 1gm gelatin/100ml +100mg α-chymotrypsin/100ml (4) Tris-based extender +1gm gelatin/100ml + 200mg α-chymotrypsin/100ml, and (5) Tris-based extender + 1gm gelatin/100ml + 400mg α-chymotrypsin/100ml. The concentrations of total free amino acids were determined in the five portions extenders. Semen was collected by artificial vagina twice weekly for ten consecutive weeks. Semen ejaculates were evaluated microscopically and only the ejaculates with advanced sperm motility ≥ 70% were pooled and used. Semen from different bucks was pooled and divided into five equal portions and each portion was diluted with one of the previous prepared extenders. The final dilution of all groups extender was 1 semen: 4 extender. Thereafter, each extender semen portion was divided into two sub-portions; the first portion was incubated at 37 ◦C for 0, 2 and 4 hours, while the second portion was stored at 5 ◦C for 24, 48, and 72 hours. Fresh semen samples were also collected and treated as described above and used immediately for artificial insemination.

The obtained results indicated that the total free amino acids for treated extender groups were higher ($P<0.05$) compared with those for control group. Extender supplied with the high level of α-chemotrypsin (400mg) showed the highest increase ($P<0.05$) of all free amino acids with the lowest ($P<0.05$) content of ammonia.
Irrespective of cold temperature (5oC) and incubation time (37oC) added with gelatin and α-chemotrypsin to Tris-buffer extender

increased (P<0.05) percentages of advanced sperm motility and decreased abnormality and dead sperm. The percentages of advanced sperm motility decreased (P<0.05), while sperm abnormal and dead spermatozoa increased (P<0.05) by elevated storage temperature or incubation time. Concentrations of ALT and AST in seminal plasma were reduced (P<0.05) in the treated groups. However, the activated of ALT and AST enzymes activities increased (P<0.05) by either the elevated storage temperature or by incubation time. Values of thiobarbituric acid-reactive substances (TBARS) and superoxide dismutase (SOD) decreased (P<0.05) in treated samples compared with the control group. However, the levels of TBARS and SOD increased (P<0.05) and glutathione (GSH) decreased (P<0.05) by advancement of incubation time or by storage temperature. Kindling rate of females artificially inseminated with semen extender supplemented with different levels of α-chemotrypsin and gelatin were improved (P<0.05) compared with the control group. In addition, the total and live litter size at birth, litter size at weaning, bunny weight at birth and bunny weight at 28 days were increased (P<0.05) in treated groups than the control. However, there found no differences between 200 and 400 mg α-chymotrypsin on these trait.

***In conclusion**, rabbit semen extender supplementing with gelatin and α- chemotrypsin not only enhanced the semen quality and extended its storagability but it also improved its subsequent impacts on the reproductive performance.*

Key words: Rabbits, gelatin, α-chymotrypsin**,** semen extender, litter traits

In several mammalian species, it has been suggested that some of the amino acids that are detected in seminal plasma enhanced sperm metabolism and its sperm motility (Gassner and Hopwood, 1952), viability and acrosome reaction (Roth et al., 1988). Ibrahim and Boldizsár (1981) showed that high amounts of total free amino acids in buck semen are important for the semen quality and fertility rate.

The addition of amino acids to extender improved post-thawing sperm motility, viability, acrosome and membrane integrity in goat (Ali Al Ahmad et al., 2008). Numerous studies showed that, supplementation of dipeptides alanin-glutamic and glycine-glutamic can play an important role in decreasing ammonia toxicity for the living cells, which lead to a progressive loss in sperm motility (Kim and Kim, 1998) maturation and fertilization of porcine oocytes (Ka *et al*.,1997). Moreover, it is well known that, mammalian sperm contains a high concentration of polyunsaturated fatty

acids (PUFA) in their membranes and they lack a significant cytoplasmic component containing antioxidants. Therefore, sperm cells are highly susceptible to lipid peroxidation (LPO) by free radicals such as O2 and H2O2 (Gadella *et al*., 2001), which lead to the structural damage of sperm membranes during freezing-thawing process (Sinha *et al*., 1996). Based on this information, the composition of extender and suitable cryoprotectants seem to play an important role for successful semen cryopreservation (Curry *et al*., 1994).

Kutteh (1996) showed that antisperm-antibody (ASA) found systemically in seminal plasma. Antibodies in the blood and lymph belong predominantly to the immunoglobulin G (IgG) isotype, while those found in external secretions are predominantly of the IgA isotype (Mazumdar and Levine, 1998). Bronson *et al*., (1987) showing that treating sperm with autoantibodies by an ASA protease was able to overcome the block to mucus penetration. Bollendorf *et al*., (1994) also found that treating sperm bound with autoantibodies with the protein digestive enzyme α-chymotrypsin followed by an intra uterine injection (IUI) was more effective in achieving pregnancies than IUI with sperm ejaculated with albumin.

Gelatin addition to semen extender was reported by several studies. Lòpez-Gatius *et al.* (2005) mentioned that the use of gelatin may exert beneficial effects through; (1) avoiding sperm cell sedimentation, consequently reducing changes in medium conditions or composition and (2) immobilizing spermatozoa, reducing the metabolic demands of motion, while preserving their fertilization potential. Regarding rabbit, the addition of gelatin (1 g/100 ml extender) had a positive effect on the viability and acrosomal integrity of spermatozoa stored for 72 h (Lòpez-Gatius et al., 2005). Zaghloul (2009) showed that there was a positive effect of adding gelatin on the viability and integrity of rabbit spermatozoa after short-term storage.

Therefore, the present study aimed to define the effects of α-chymotrypsin and gelatin supplementation to the extender on free amino acids in rabbit semen quality, release of antioxidants, kindling rate, fertility and litter traits.

MATERIALS AND METHODS

The present study was carried out at El-Sabahia Poultry Research Station, Alexandria Governorate, belonging to Animal Production Research Institute, Agricultural Research Center, Egypt.

Animals, Semen Collection and Processing

Twenty sexually mature bucks and eighty multiparous does in three sequence parities of V-line (VL) rabbits were used. All rabbits were housed in a

naturally ventilated building and kept in individual wire galvanized cages (60 × 55 × 40 cm) equipped with an internal nest-box. Rabbits were fed ad libitum with a commercial pelleted diet containing 18 % CP, 13.3% CF and 2670 kcal DE/kg diet. Clean tape water was provided as free choice. All the experimental animals were healthy and clinically free from internal and external parasites and kept under the same managerial and hygienic conditions.

A base extender was prepared to use in this experiment consisting of 3.028 g Tris (hydroxymethyl-aminomethane, Germany), 1.5 g citric acid anhydrous, 1.25g glucose, 500 IU penicillin, 500bug streptomycin sulphate in 100 ml distilled water. The extension was carried out by adding the appropriate volume of the semen slowly to the extender. Extended semen (in tubes) was kept below the level of water in a water bath at all times to avoid fluctuations in semen temperatures. The final extension rate was 1 semen: 4 extender.

The base extender was divided into five portions and treated as following:

Extender 1 - base extender (control) (C),

Extender 2 - base extender+1g gelatin/100ml extender (G0),

Extender 3 - base extender+1g gelatin + 100 mg α-chymotrypsin/ 100ml extender (G100),

Extender 4 - base extender+1g gelatin + 200 mg α-chymotrypsin/ 100ml extender (G200),

Extender 5 -base extender +1g gelatin + 400mg α-chymotrypsin/ 100ml extender (G400).

Total free amino acids in all extenders were determined according to the method described by Hamilton (1962), while the individual free amino acids were measured using a method described by Spackman *et al.* (1958) using amino acid analyzer system (Hitachi L-8500, Tokyo, Japan) .Semen was collected by an artificial vagina twice weekly for ten consecutive weeks. Gel plug was removed immediately after collection. Semen ejaculates were individually evaluated microscopically and only ejaculates showing advanced sperm motility ≥70% were pooled and used. Semen from different bucks was pooled and divided into five equal portions and each portion was diluted with one of the above mentioned extenders.

The percentage of motile sperm was estimated by visual examination under low magnification (X100) using a phase-contrast microscope with warm stage. Assessment of abnormal and dead spermatozoa was performed using an eosin–nigrosine blue staining mixture (WHO, 1992).

Each previous extender was divided into two sub-portions, the first portion was incubated at 37◦C and tested at 0, 2 and 4 hours, while the second portion was stored at 5 ◦C and tested at 24, 48, and 72 hours post treatment.

Enzymes and TBARS determinations in diluted seminal plasma

Samples were analyzed biweekly for the Thiobarbituric acid-reactive substances (TBARS) in the diluted seminal plasma using the method of Tappel and Zalkin (1959). Glutathione (GSH) was determined using commercial glutathione reduced kits according to the method of Beutler *et al.* (1963). Superoxide dismutase (SOD) activity was assayed according to Misra and Fridovich (1972). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were detected according to Reitman and Frankel (1957).

Rabbit does insemination

All rabbit does were injected with 20 µg gonadotropin-releasing hormone (Gonadoreline, Fertagyl, Intervet Lab, Holand) to induce ovulation immediately before insemination as described by Lòpez and Alvarino (2000). Rabbit does were divided into five equal experimental groups (16 does /extender) and inseminated artificially by semen diluted with 50 x 106 fertile sperm at zero time. The insemination procedure was done as described by Adams (1981). Kindling rate, total number of born live and weaning kids were recorded according to IRRG (2005).

Statistical analysis

All data were subjected to analysis of variance according to the statistical analysis system described by SAS (2002). The differences among groups means were tested by using Duncan's multiple rang test (Duncan, 1955). Kindling rate results were analyzed by the Chi-square test.

RESULTS AND DISCUSSION

As shown in Table 1 most amino acids significantly ($P<0.05$) increased by the inclusion of gelatin alone or gelatin plus α-chemotrypsin.The predominant amino acids in rabbit seminal plasma were therionine, glutamic and leucine. Total free amino acids were 16.35, 42.44, 64.26, 104.14 and 174.12mg/100 ml extended semen for C, G0, G100, G200 and G400, respectively. Similar results are in agreement with that those of Centenaro *et al.* (2011) who reported that the increase of total free amino acids in extended semen treated with α-chemotrypsin may be due to the digestive enzyme activity that can perform proteolysis and hydrolyzes amid bonds in protein and peptides (gelatin). Protein hydrolysates from various sources have been identified as potential antioxidants (Rossini *et al.*, 2007). Centenaro *et al.* (2011) also noted that the peptide from protein hydrolyses presented free radical scavenging activity. This indicates that the peptides or free amino acids in the hydrolysates have the ability to donate

Table 1. Effect of gelatin and α-chymotrypsin supplementation in rabbit semen extender on free amino acid composition (LSM±SEM)

Amino acid	Gelatin (1 g/100 ml) + α-chymotrypsin level (mg /100ml)				
	Control	0	100	200	400
Aspartic acid	0.60±0.89^{e}	0.74±0.96^{d}	0.98±0.11^{c}	1.16±0.12^{b}	1.29±0.12^{a}
Threonine	8.20±0.11^{d}	16.16±0.14^{c}	16.24±0.13^{c}	23.84±0.18^{b}	34.57±0.25^{a}
Serine	0.14±0.00^{e}	0.47±0.00^{d}	1.11±0.00^{c}	1.58±0.00^{b}	1.87±0.01^{a}
Glutamic acid	3.42±0.14^{d}	6.58±0.16^{c}	8.61±0.18^{b}	8.24±0.17^{b}	9.21±0.19^{a}
Proline	0.66±0.08^{c}	1.86±0.11^{b}	1.96±0.13^{b}	2.11±0.12^{a}	2.15±0.12^{a}
Glycine	0.14±0.00^{e}	0.21±0.00^{d}	0.87±0.00^{c}	1.12±0.00^{b}	1.38±0.00^{a}
Alanine	0.50±0.00^{e}	3.13±0.11^{d}	16.24±0.14^{c}	28.19±0.17^{b}	65.87±2.14^{a}
Cystine	0.54±0.02^{d}	4.43±0.06^{c}	5.53±0.08^{c}	19.54±0.14^{b}	23.15±0.16^{a}
Valine	0.45±0.00^{e}	1.42±0.11^{d}	2.48±0.13^{c}	4.36±0.15^{b}	7.14±0.16^{a}
Methionine	0.61±0.07^{e}	1.21±0.10^{d}	1.81±0.12^{c}	2.02±0.14^{b}	3.10±0.17^{a}
Isoleucine	0.30±0.12^{e}	1.73±0.39^{d}	1.99±0.47^{c}	2.41±0.59^{b}	9.05±0.78^{a}
Leucine	1.60±0.06^{e}	1.85±0.13^{d}	2.38±0.15^{c}	2.96±0.15^{b}	3.22±0.18^{a}
Tyrosine	0.31±0.00^{e}	0.55±0.00^{d}	0.86±0.00^{c}	2.79±0.071^{b}	4.50±0.10^{a}
Phenylalanine	0.94±0.08^{e}	0.96±0.08^{d}	1.89±0.12^{c}	2.07±0.14^{b}	2.43±0.15^{a}
Histidine	0.77±0.07^{d}	0.88±0.07^{c}	0.92±0.09^{c}	1.18±0.10^{b}	4.51±0.16^{a}
Lysine	0.00	0.00	0.00	0.00	0.00
Arginine	0.17±0.00^{e}	0.26±0.00^{d}	0.39±0.00^{c}	0.57±0.00^{b}	0.68±0.00^{a}
Total free amino acids	16.35±1.79^{e}	42.44±2.15^{d}	64.26±2.75^{c}	104.14±3.3^{b}	174.12±5.3^{a}
Amonia	2.56±0.10^{a}	1.88±0.08^{b}	1.56±0.07^{c}	1.38±0.06^{d}	0.26±0.00^{e}

Means with different superscripts in the same row significantly (P<0.05) differ, *n= 25 samples.

hydrogen atoms to the free radicals, slowing the propagation of lipid peroxidation process as reported by Faithong *et al.* (2010).

Ammonia decreased (P<0.05)in semen extended supplied with gelatin and different levels of α-chemotrypsin.This in association with results reported by Bilodeau *et al.*(2009) who demonstrated that the accumulation of ammonia can be reduced in the medium by supplementation with the dipeptides L-alanyl-L-glutamine and L-glycyl-L-glutamine, which can play an important role in motility. The role of α-chemotrypsin plays on protein by its conversion into small peptide and free amino acids might parallel. Similar trend were reported by Bilodeau *et al.* (2009).

Results represented in Tables 2 and 3 that supplementation of gelatin and α-chemotrypsin had a significant (P<0.05) effect on sperm quality. Adding gelatin and α-chemotrypsin to Tris-buffer extender significantly (P<0.05) increased percentages of advanced motility and decreased sperm abnormality (P<0.05) and dead spermatozoa as compared to the control, regardless of storage temperature or incubation time. Percentages of advanced motility significantly (P<0.05) decreased while dead spermatozoa and sperm abnormality (P<0.05) increased by the elevation of storage temperature or incubation time.

Table 2. Effect of gelatin and α-chemotrypsin supplementation in rabbit semen extender and incubation time at 37°C on sperm quality (LSM±SEM)

Items	Advanced Motility %	Abnormal Sperm %	Dead Spermatozoa%
Effect of supplementation			
*C	69.64±2.22 [b]	23.43±.88[a]	18.92±0.96[a]
G0	79.00±1.24 [a]	21.00±0.61[b]	15.01±0.56[b]
G100	83.25±0.80 [a]	20.34b±0.41[b]	13.93±0.46[c]
G200	83.74±0.84 [a]	20.18±0.89[b]	13.21±035[c]
G400	82.92±0.78 [a]	20.24±0.43[b]	13.36±039[c]
Effect of incubation time (h) at 37 °C			
0	85.51±0.70[a]	18.25±0.17[c]	11.40±0.16[c]
2	78.83±1.13[b]	20.88±0.31[b]	15.11±0.41[b]
4	75.37±1.39[b]	23.9±0.41[a]	17.95±0.51[a]

Means in the same column within a category with different superscripts significantly differ (P<0.05). * C= Control, G0= Gelatin, G100= Gelatin + 100 mg α-Chemotrypsin, G200= Gelatin + 200 mg α-Chemotrypsin, G400= Gelatin + 400 mg α-Chemotrypsin (/100 ml extender).

Table 3. Effect of gelatin and α-chemotrypsin supplementation in rabbit semen extender and storage time at 5 ºC on sperm quality (LSM±SEM)

Items	Advanced Motility %	Abnormal Sperm %	Dead Spermatozoa%
Effect of supplementation			
*C	56.81±2.32[c]	37.10±0.86[a]	29.55±1.12[a]
G0	67.59±1.53[b]	27.32±0.56[b]	22.70±0.31[b]
G100	70.72±1.31[a]	26.06±0.47[b]	20.40±0.59[c]
G200	71.64±1.41[a]	25.12±0.47[b]	19.36±0.53[c]
G400	71.99±1.02[a]	26.53±0.48[b]	19.96±0.57[c]
Effect of storage time (h) at 5 °C			
24	76.08±0.87[a]	25.27±0.49[c]	17.82±0.36[c]
48	67.20 ±0.86[b]	28.64±0.75[b]	22.46±0.59[b]
72	59.97±1.50[c]	31.37±0.78[a]	26.91±0.75[a]

Means in the same column within category with different superscripts significantly differ (P≤0.05). * C= Control, G0= Gelatin, G100= Gelatin + 100 mg α-Chemotrypsin, G200= Gelatin + 200 mg α-Chemotrypsin, G400= Gelatin + 400 mg α-Chemotrypsin (/100 ml extender).

The decrease of advanced motility of spermatozoa and the increase of dead spermatozoa abnormality sperm. These results by elevating storage temperature or incubation time are in agreement with those of Matsuoka *et al.*(2006) who reported that cryopreservation induces partially irreversible damage to sperm membranes, which may decrease sperm motility, viability

and the fertilization rate after artificial insemination. Damage during cryopreservation has been attributed to oxidative stress, cryoprotectant toxicity, osmotic changes and lipidprotein reorganizations within the cell membranes (Purdy *et al.,* 2005).

Collectively, increasing total free amino acids due to supplementation with gelatin and α- chemotrypsin was associated with improved semen quality. Several mechanisms have been proposed for the roles of amino acids during cryopreservation. Oltjen *et al*. (1971) showed that high amounts of total free amino acids are important for the semen quality and the fertility of the animals. Ibrahim and Boldizsár (1981) also noted that there is some evidence that the amino acids present in the seminal plasma play an important role in survival of spermatozoa. The function of seminal plasma free amino acids is shown to act as fuels for the spermatozoa, to create favorable conditions for cell survival and to be probably involved in detoxifying function. Kundu *et al*. (2001) suggested that the protective effects of amino acids may stem from their ability to form a layer on the spermatozoa surface, as these positively charged molecules can combine with the phosphate groups of sperm plasma membrane phospholipids. Also, Atessahin *et al*.(2008) showed that addition of amino acids to extender improved sperm motility, viability, acrosomal integrity and membrane integrity in goat and boar. Moreover, cysteine has been shown to improve motility and morphology of ram (Uysal and Bucak, 2007) and goat sperm, (Buck *et al*., 2008) and to maintain the viability, the chromatin structure and membrane integrity of boar sperm (Sariozkan *et al*., 2009).

Likewise, Nagy *et al.* (2003) reported that gelatin addition to extender semen had a positive effect on preserved semen quality. They found a higher percentage of live cells in semen preserved with as compared to free gelatin. On the other hand, some investigators found no differences in goat and sheep semen motility when fresh semen extender was supplemented with gelatin immediately after semen collection (Salvador *et al.,* 2006).

Transaminase enzyme activities

Tables 4 and 5 exhibit alanine - aminotransferase (ALT) and aspatate-aminotransferase (AST) activities in seminal plasma being reduced ($P<0.05$) in treated than controls. As time of preservation progressed at either temperature, there exist significant increases ($P<0.05$) in the ALT and AST activities. However, as the storage temperature increased, there found significant decreases ($P<0.05$) ALT and AST activities. The lower release of these two metabolic enzymes in supplemented extenders could be attributed to the protective effects of free amino acids and peptides on the integrity of

Table 4. Effect of gelatin and α-chemotrypsin supplementation and incubation time at 37◦C on seminal plasma activity of ALT and AST (LSM±SEM)

Items	Seminal plasma enzyme	
	ALT (U/L)	AST (U/L)
Effect of supplementation		
C	62.92±1.40[a]	78.55±1.50[a]
G0	59.25±0.82[b]	72.77±0.85[b]
G100	57.99±0.76[b]	71.84±0.77[b]
G200	57.59±0.67[b]	71.04±0.82[b]
G400	57.66±0.66[b]	71.11±0.79[b]
Effect of incubation time (h) at 37°C		
0	53.05±0.37[c]	67.23±0.22[c]
2	59.04±0.21[b]	72.98±0.59[b]
4	64.45±0.45[a]	78.79±0.68[a]

Means in the same column within category with different superscripts significantly differ (P≤0.05). * C= Control, G0= Gelatin, G100= Gelatin + 100 mg α-Chemotrypsin, G200= Gelatin + 200 mg α-Chemotrypsin, G400= Gelatin + 400 mg α-Chemotrypsin (/100 ml extender). ALT:Aspartate- aminotranseferase AST:Alanine – aminotranseferase.

Table 5. Effect of gelatin and α-chemotrypsin supplementation and storage time at 5◦C on ALT and ASTactivity (LSM±SEM)

Items	Seminal plasma enzyme	
	ALT (U/L)	AST (U/L)
Effect of supplementation		
C	77.62 ± 1.12[a]	104.64 ± 3.44[a]
G0	66.99 ± 0.60[b]	96.52 ± 2.91[b]
G100	65.18 ± 0.59[b]	93.99 ± 2.44[b]
G200	64.33 ± 0.52[b]	90.62 ± 2.17[b]
G400	64.99 ± 0.59[b]	92.41 ± 0.41[b]
Effect of storage time (h) at 5°C		
24	64.11 ± 0.57[c]	76.67± 0.38[c]
48	68.20 ± 0.71[b]	101.93± 0.63[b]
72	72.37 ± 0.96[a]	110.11± 1.33[a]

Means in the same column within a category with different superscripts significantly differ (P≤0.05). * C= Control, G0= Gelatin, G100= Gelatin + 100 mg α-Chemotrypsin, G200= Gelatin + 200 mg α-Chemotrypsin, G400= Gelatin + 400 mg α-Chemotrypsin (/100 ml extender). ALT:Aspartate-aminotranseferase AST:Alanine – aminotranseferase.

the sperm cell membranes. These observations are in agreement with that found in goat semen (Kundu *et al*., 2001). They concluded that addition of glutamine, glycine and cysteine in conventional storage medium improved membrane and acrosomal integrity of spermatozoa. However, Sariozkan *et al*. (2009) demonstrated that amino acid enhanced the defense of

mammalian cell membrane and improved cell membrane integrity during sperm storage. Furthermore, change of biochemical factors have been recognized during cryopreservation, including depletion of amino acids and lipoproteins release of AST (Barbas and Mascarenhas, 2009). Moreover, Numan *et al.* (2010) showed that storage generates sublethal injury to the sperm due to chemical, osmotic, thermal, and mechanical stresses which may result in loss of viability, motility, damage of deoxyribonucleic acid (DNA) and destruction of plasma membrane, while amino acids supplementation have an important roles in preventing damage in animal's spermatozoa during crypreservation stage.

Seminal plasma antioxidants

Inclusion of gelatin and α-chemotrypsin in the rabbit semen extender decreased ($P<0.05$) TBARS concentration and SOD activity, but ($P<0.05$) increased glutathione reduced (Table 6 and 7). Contrariwise, as time of incubation progressed, BARS and SOD in ($P<0.05$). Agarwal *et al.* (2007) mentioned that the sperm are susceptible to reactive oxygen species (ROS) attack. The imbalance between the production of ROS and a biological systems ability to readily detoxify the reactive intermediates or easily repair the resulting damage is known as oxidative stress (Agarwal et al., 2003). Oxidative stress is induced by ROS, or free radicals. However, ROS have been shown to be required for sperm capacitation, hyperactivation and sperm-oocyte fusion. Aitken *et al.* (2004) reported that excessive levels of ROS can negatively impact sperm quality. Since, increased levels of ROS have been correlated with decreased sperm motility; increased sperm DNA damage (Barroso *et al.,* 2000), sperm cellular membrane lipid peroxidation and decreased efficacy of oocyte-sperm fusion (Agarwal *et al.*, 2007).

Antioxidant capability in sperm cells is limited due to deficiency in cytoplasmic components having antioxidant effects to expunction of reactive oxygen. Thus, mammal's sperm have not enough ability to encountering with peroxidation during the storage (Alvarez and Storey, 2005). Moreover, Agrawal et al. (2005) demonstrated that antioxidants are the major defensive mechanism against oxidative stress. Reactive oxygen is responsible to sperm membrane damage and directly damage sperm DNA that cause reduction in the sperm motility, acrosomal integrity and sperm metabolic alterations.

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Table 6. Effect of gelatin and α-chemotrypsin supplementation and incubation time at 37◦C on TBARS, GSH and SOD (LSM±SEM)

Items	Metabolite		
	TBARS (nmol/ml)	GSH (mg/dl)	SOD (IU)
Effect of supplementation			
C	1.53±0.04[a]	422.6±10.34[d]	1.48±0.03[a]
G0	1.48 ±0.03[b]	440.46±7.53[c]	1.44±0.03[b]
G100	1.34 ±0.02[c]	454.52±7.22[b]	1.32±0.02[d]
G200	1.29 ±0.02[c]	453.96±7.82[b]	1.36±0.03[c]
G400	1.33 ±0.02[c]	460.10±6.95[a]	1.37±0.03[c]
Effect of incubation time (h) at 37°C			
0	1.23±0.02[c]	505.14±1.39[a]	1.24±0.01[c]
2	1.51±0.03[a]	429.64±2.32[b]	1.49±0.02[a]
4	1.46±0.02[b]	406.58±3.32[c]	1.45±0.02[b]

Means in the same column within a category with different superscripts significantly differ (P≤0.05). * C= Control, G0= Gelatin, G100= Gelatin + 100 mg α-Chemotrypsin, G200= Gelatin + 200 mg α-Chemotrypsin, G400= Gelatin + 400 mg α-Chemotrypsin (/100 ml extender). TBARS: Thiobarbituric acid-reactive substances GSH: Glutathione SOD: Superoxide dismutase

Table 7. Effect of gelatin and α-chemotrypsin supplementation and storage time at 5°C on TBARS, GSH and SOD (LSM±SEM)

Items	Metabolite		
	TBARS (nmol/ml)	GSH (mg/dl)	SOD(IU)
Effect of supplementation			
C	1.67±0.05[a]	422.10±7.74[d]	1.42±0.03[a]
G0	1.49±0.04[b]	472.75±6.21[b]	1.33±0.03[b]
G100	1.41±0.03[c]	479.32±7.11[b]	1.29±0.02[c]
G200	1.36±0.02[cd]	507.59±3.25[a]	1.27±0.03[c]
G400	1.38±0.03[c]	494.94±5.49[a]	1.28±0.03[c]
Effect of storage time (h) at 5°C			
24	1.24±0.02[c]	506.77±3.61[a]	1.16±0.01[c]
48	1.52±0.02[b]	481.86±3.86[b]	1.40±0.02[a]
72	1.61±0.03[a]	431.38±5.80[c]	1.36±0.02[b]

Means in the same column within category with different superscripts significantly (P<0.05) differ. * C= Control, G0= Gelatin, G100= Gelatin + 100 mg α-Chemotrypsin, G200 = Gelatin + 200 mg α-Chemotrypsin, G400= Gelatin + 400 mg α-Chemotrypsin (/100 ml extender). TBARS: Thiobarbituric acid-reactive substances GSH: Glutathione SOD: Superoxide dismutase

is responsible to sperm membrane damage and directly damage sperm DNA that cause reduction in the sperm motility, acrosomal integrity and sperm metabolic alterations. These findings are similar to results obtained

by Scanchez-Partidata *et al.* (1992) who creased and GSH decreased demonstrated that the addition of low concentration of proline and betaine glycine (a component related to amino acids) to a medium containing egg yolk and glycerol improved the motility of ram spermatozoa. Buck *et al.* (2008) showed a positive effect of cysteine on motility and membrane integrity. Raji *et al.* (2003) reported that low sperm motility and high percentage of abnormal spermatozoa level have been associated with fertility reduction. Sariozkan *et al*. (2009) also demonstrated that cysteine is one of the additives that have been used in freezing extender of human, boar, goat and bull to improve post-thaw sperm parameters. Addition of cysteine and lipoic acid to the semen freezing extender, may prevent cryodamage to spermatozoa metabolism and antioxidant capacities (Rahim Beheshti *et al.*, 2011).

Reproductive Performance

Table 8 indicates that kindling rate of females artificially inseminated with semen extender supplemented with gelatin and different levels of α-chemotrypsin was improved compared with the control group. In addition, the total and live litter size at birth, litter size at weaning, bunny weights at birth and at 28 days were higher for groups of α-chemotrypsin and gelatin

Table 8. Effect of supplementation of the rabbit semen extender with gelatin and α-chemotrypsin on subsequent reproductive performance (LSM±SEM)

Items	Gelatin (1 g/100ml) +α-chemotrypsin (mg/100ml)				
	Control	G0	G 100	G 200	G 400
Kindling rate (%)	55.70±0.75^{c}	66.30±0.69^{b}	79.70^{a}±0.96^{a}	85.80±0.87^{a}	81.30±0.83^{a}
Litter size at birth (n.)	5.38±0.54^{c}	7.13±0.63^{b}	7.03^{b}±0.58^{b}	10.41±0.60^{a}	9.87±0.57a
Live litter size at birth (n.)	4.56±0.53^{c}	6.42±0.65^{b}	6.63^{b}±0.66^{b}	9.00±0.57^{a}	8.61±0.62^{a}
Litter size at weaning (n.)	4.10±0.49^{c}	6.22^{b}±0.58^{b}	6.36^{b}±0.46^{b}	8.07±0.51^{a}	7.87±0.63^{a}
Bunny weight at birth (g)	44.90±0.82^{c}	53.50±0.93^{b}	55.20^{b}±0.81^{b}	68.20±0.73^{a}	65.20±091^{a}
Bunny weight at 28 days (g)	417.90±9.8^{c}	560.20±7.6^{b}	569.20^{b}±8.40^{b}	686.50±9.1^{a}	709.60±8.20^{a}

Means in the same row with different superscripts significantly differ (P<0.05).
G0= Gelatin, G100= Gelatin + 100 mg α-Chemotrypsin, G200 = Gelatin + 200 mg α-Chemotrypsin, G400= Gelatin + 400 mg α-Chemotrypsin (/100 ml extender).

than those of the control group. The levels of 200 and 400 mg/100 ml with gelatin represented the same significant differences with other experimental α-chemotrypsin levels and control. The improvement of the previous mentioned parameters of rabbit reproductive performance is due to the enrichment of semen extender with α-chemotrypsin and gelatin which reflected on semen characteristics enhancement. The positive effect of

amino acid and α- chemotrypsin as an enhancer of reproductive capacity of rabbit bucks could be attributed to its ability to protect mammal cells from oxidation (Alvarez and Storey, 2005). Brun et al. (2002) found that sperm motility significantly ($P<0.05$) influenced the kindling rate. Furthermore, the same authors found that litter size (total born) was significantly influenced by motility and normal sperm. Katsoff et al. (1995) demonstrated that the use of α- chymotrypsin treatment of sperm bound with autoantibody was improve both fertilization and pregnancy rates following conventional insemination of oocytes.

In conclusion, rabbit semen extender supplementing with gelatin and α- chemotrypsin not only enhanced the semen quality and extended its storagability but it also improved its subsequent impacts on the reproductive performance.

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تأثير أضافة الجيلاتين والالفاكيموتربسن في مخفف السائل المنوي للارانب علي نسبة الخصوبة وصفات الخلفة

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أجريت هذه الدراسة لمعرفة تأثير اضافة الجيلاتين و الفا كيموتربسن على مخفف السائل المنوى للأرانب V-Line وذلك على جودة السائل المنوى وصفات الخلفة أثناء التحضين على درجة حرارة ٣٧ درجة مئوسة لمدة ٤ ساعات والتخزين على درجة حرارة ٥ مئوية لمدة ٧٢ ساعة.
تم استخدام عشرون ذكر بالغ وثمانون انثى مرضعة من سلالة الفى لين لبطون مختلفة .يتكون المخفف الاساسى المستخدم من الترس وحمض الستريك و جلوكوز.قسم المخفف الاساسى الى خمس اجزاء وتم اجراء المعاملات الاتية ١- مخفف الترس الاساسى (كونترول) ٢-مخفف الترس الاساسى مضاف الية ١جرام من الجيلاتين ٣-مخفف الترس الاساسى مضاف اليه ١ جرام جيلاتين وكذلك ١٠٠مليجرام من الفا كيموتربسن ٤- مخفف الترس الاساسى مضاف اليه ١ جرام جيلاتين وكذلك ٢٠٠ ملجرام من الفا كيموتربسن ٥-مخفف الترس الاساسى مضاف الية ١ جرام من الجيلاتين وكذلك ٤٠٠ملجرام من الفا كيموتربسن وذلك لكل ١٠٠ مليليتر. يتم تقدير تركيز الاحماض الامينية الحرة فى اجزاء المخفف الخمس . يجمع السائل المنوى مرتين اسبوعيا لمدة عشرة اسابيع متتالية بواسطة المهبل الاصطناعى.تفحص كل قذفة من السائل المنوى على حدة بواسطة الميكروسكوب وتجمع القذفات من السائل المنوى التى تزيد الحركة التقدمية بها عن ٧٠% للاستخدام. السائل المنوى الذى تم تجميعة يتم تقسيمة الى خمس اجزاء على المخففات السابق ذكرها. وتكون نسبة التخفيف النهائى فى كل جزء من المخففات السابق ذكرها ١ من السائل المنوى الى ٤ اجزاء من المخفف .وبعد ذلك يتم تقسيم كل جزء من الاجزاء الخمس الى جزئين الجزء الاول يتم تحضينة على درجة حرارة ٣٧ درجة مئوية لفترة صفر ،٢،٤ ساعة بينما الجزء الثانى يحفظ على درجة حرارة ٥ درجة مئوية لمدة ٢٤،٤٨،٧٢ ساعة ويتم التقيم عند كل زمن. و الجزء الاخر من السائل المنوى المعامل يتم التلقيح بة مباشرة.
اظهرت النتائج ان هناك زيادة فى محتوى الاحماض الامينية الحرة فى الاجزاء المختلفة المعاملة اختلافا معنويا(على مستوى ٠,٠٥) عن المجموعة الغير معاملة الكونترول. اظهرت مجموعة المخفف المعاملة ٤٠٠ ملجرام الفاكيموتربسن (التركيز العالى) زيادة معنوية فى الاحماض الامينية وانخفاض معنوى(على مستوى ٠,٠٥)

فى تركيز الامونيا مقارنة بمجموعة الكونترول.بصرف النظر عن درجة الحرارة وزمن التحضين او التخزين فأن اضافة الجيلاتين و الفاكيموتربسن الى مخفف الترس اظهرزيادة معنوية (على مستوى ٠,٠٥) فى الحركة التقدمية وانخفاض فى كلا من نسبة الشازة والميتة من الحيونات المنوية مقارنة بالكونترول. بينما حدث انخفاض معنوى (على مستوى ٠,٠٥) فى الحركة التقدمية للحيونات المنوية و زيادة معنوية (على مستوى ٠,٠٥) للحيوانات المنوية الشاذة و الميتة مع تقدم فترة التحضين او التبريد. أنخفض معنوى(على مستوى ٠,٠٥) تركيز كلا من AST,ALT فى المخفف (المضاف الية السائل المنوى) فى المخففات المعاملة ، بينما كان هناك زيادة معنوية (على مستوى ٠,٠٥) بتقدم زمن التحضين او التخزين. ينخفض معنويا(على مستوى ٠,٠٥) مستوى كلا من TBRS وSOD فى كافة العينات المعاملة مقارنة بالكونترول. ومع ذلك كان هناك زيادة معنوية (على مستوى ٠,٠٥) فى مستوى كلا منTBARS وSOD وانخفاض معنوى (على مستوى ٠,٠٥) فى مستوى الجلوتثيون مع تقدم الزمن سواء بالتحضين و التبريد .

النسبة المئوية للحمل فى الاناث الملقحة اصطناعيا بالسائل المنوى المضاف اليه الجيلاتين ومستويات مختلفة من الفاكيموتربسن اظهرت تحسن معنوى (على مستوى ٠,٠٥) مقارنة بالكونترول .بالاضافة الى زيادة معنوية (على مستوى ٠,٠٥) فى عدد المواليد وقت الولادة ،عدد الولادات الحية ،عدد الارانب المفطومة ، وزن الخلفات عند الميلاد والفطام فى المجاميع المعاملة بالجيلاتين والالفاكيموتربسن مقارنة بالكونترول بينما لاتوجد فروق معنوية بين المجموعتين المعاملتين ٢٠٠و ٤٠٠ملجرام الفاكيموتربسن على هذه الصفات.