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Changes of Enzymes in Epididymal Fluid during Different Steps of Processing (After Dilution, After Cooling and Post Thawing) of Buffalo Bulls

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Abstract

Epididymal fluid (GOT,GPT, and LDH) were assessed in diluted (Tris-egg yolk-citric acid extender 1:5) and Thawing of 0.5 straws was done at 37 °C for 30 seconds using a container contains water at 37°Cepididymal fluid of buffalo bull (control group). Diluted, cooled (4°C/ 2-4 hours) and equilibrated (at 4°C for 2 hrs) epididymal fluid straws were frozen.These frozen straws were stored separately in LN2. Effect of freezing rates on leakage of enzyme was studied by assessing GOT inepididymal fluidof thawed semen indicate that lowest significant (P>0.05) values in T4 (30.64 ± 0.45 mM)and highest significant (P>0.05) value was in T1(57.17 ±0.53mM). While,GPT at post thawing waslowest significant (P> 0.05) Values in T4(0.59 ±0.009 mM), and highest significant (P> 0.05) values in T1 (0.74 ±0.006mM) in comparison with others T2 and T3 (0.70±0.00and 0.65 ±0.008mM).However, values of enzyme (LDH) at post thawing values indicate was lowest significant (P>0.05) values in T4(1433.66±99.512 mM), and highest significant (P> 0.05) values in T1 (3802.50±146.17 mM) in comparison with others T2(2486.92±111.80mM) and T3 (1892.16 ± 113.311 mM) increased in GOT,GPT and LDH activity during different steps of freezing, with highest significant was in post thawing and lowest significant in after dilution.

Key Words: *Epididymal Sperm, Buffalo bull, L-Carnitine,* ***Acrosome integrity***

I. Introduction

Water buffaloes are abundant in southern Iraq, and reproductive biology limits completete buffalo prod uction, as fertility in this population is significantly lower than in animals(Drost, 2007).Enzymes are used as good indicators of semen quality because they measure the stability of sperm in the plasma membrane (Sirat et al. 1996). Changes in biochemical variables were acknowledged during cryopreservation, including depletion of amino acids and lipoproteins, glutamic-oxaloacetic transaminase (GOT), reduce in phosphatase activity, reduce

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in loosely linked cholesterol, protein, inactivation of acrosine enzymes and hyaluronidase, reduce in prostaglandins, increase in sodium, reduce in potassium content Indeed, a primary target for freezing or cold shock injury is the sperm plasma membrane (NumanBucak et al., 2009).

The enzyme leakage reported by Akhtar et al. (1990) is another marker for assessing sperm freezability. There was an adverse correlation between sperm motility and acrosomal integrity with enzyme aspartate amino transferase (AST), while lactic dehydrogenase (LDH) had a positive correlation.Kaker and Anand (1984) revealed that the release of glutamate oxaloacetate transaminase (GOT) and glutamic pyruvic transaminase (GPT) in seminal plasma was affected by glycerol concentration, cold shock, cooling and freezing speed. The adverse correlation of these enzymes with fertility was also reported (Dhami and Kodagali, 1990) after thaw concentrations of GOT and GTP. The enzymes GOT-GPT was estimated in seminal plasma using standard procedures and assay kits procured from Coral Clinical System, Goa, India (Bergmeyer et al., 1986).

In the late maturation events of spermatozoa, particularly in the acrosomal reaction and sperm-egg fusion, this enzyme is believed to play a major role (Yuan et al., 2003). Reproductive seasonality is demonstrated by the presence of such enzyme activities (Mustaffa, 2005) and testes or epididymides are known to be the potential origins of these enzymes in seminal plasma (Kareskoski and Katila, 2008). Due to a lack of information on the enzymatic activities of epididymal buffalo bull fluid, the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were identified in this study. Also, before and after cryopreservation of epididymal fluid in Tris, the activity of these enzymes has been estimated to determine the extent of enzyme leakage from the spermatozoa to the epididymal fluid. L-Carnitine is a water-soluble vitamin such as amino acid that happens naturally in micro-organisms, crops and livestock (Szilagyi, 1998;Vav and Wanders, 2002). L-carnitine also has significant roles in mammalian sperm maturation and metabolism when sperm passes through epididymis (Yakushijiet al., 2006). In mammalian epididymis and spermatozoa, L-carnitine is actually discovered. Epididymal cells and spermatozoa derive power from epididymal liquid carnitine, A commercial research also found that L-carnitine can boost the amount of feasible sperm and AI doses (Yesteet al., 2010).L-carnitine has been alleged to be involved in the development of sperm motility, and the use of carnitine in spermatozoa and its conversion to acetylcarnitine is deemed proof of excellent epididimal function in humans (Stradaioliet al., 2004).

II. Materials and Methods

Experimental Samples

Samples were collected in the current study from Basrah slaughterhouse from December 2018 until the end of August 2019, with an average of 3-4 visits per week for male adult buffalo.

Collect of sperm from tail of epididymis

Thirty-three (33) samples testis of adult male buffalo from a slaughterhouse in Basrah after slaughtering the animal. The testis with attached epididymis were transferred in a cool box at 4-6^{o}C (Lone et al., 2011), to Laboratory at the Research Unit Center of College of Veterinary Medicine/ Basrah University.

Collection of epididymal fluid

Once of collected samples are reached to the laboratory, the organ washed with distilled water, then with normal saline containing antibiotic after that removing of tunica vaginitis using sterile scissor, then theepididymis were separated from the testicle, the tail of epididymis was removed from the entire epididymis and placed in petri dish ,then injected with 3 ml Tyrode Albumin Lactate Pyruvate (TALP) which prepared using sterile needle gauge (no.23), connected to a 5 ml syringe, then sterileblade use for sliced of tail of epididymisfor small pieces according to (Lone et al., 2011).Then the tissue was removed and the remaining medium was centrifugedat 7000 rpm for 2 minute to create a sperm pellet (Baratiet al.,2009). Supernatant was removed from sperm.

Epididymal sperm processing

Tris-citric egg yolk-glycerol (TCEG) extender wasadded theconcentration or density was further adjusted to 451-813 x 10^6 sperm/ml. The plastic tube containing the epididymalsperm, the diluted epididymal sperm will divide into 4 parts 1. T control contains only (Tris-citric egg yolk-glycerol (TCEG). 2. T1 add L-carnitine with (1.0)mmol/ml 3. T2 add L-carnitine with (5.0)mmol/ml 4.T3 add L-carnitine with (10.0)mmol/ml, was placed in a glass beaker containing water and the beaker was placed in a refrigerator for **Cooling 5° C.** All dilutedepididymal sperm containing different concentration of antioxidant were transferred into the cold beaker, allow to reach the stabilize degree 5C° in about 1-1.5 hour to control the time of cooling dilutedepididymal sperm in cold beaker, ice cubes were added to the beaker when temperature of the water in the beaker reached 20C°, so should be bellow 5C° in a controlled manner, this can be done by the aid of sensitive thermometer to determine the degree of the temperature). **Equilibration:** Diluted epididymal sperm containing different concentration of antioxidant at temperature of 5C° was performed for 4 hours at the same temperature (5°C),diluted epididymal sperm was filled in French medium straw (0.5 ml) and stopper was applied using a heat press and preservation at 5°C till equilibration time. Evaluation was performed in the same measured way as after diluted epididymalsperm.Freezing -196°C of straws, straws were kept horizontally, then placed in a container containing liquid nitrogen to be exposed to liquid nitrogen vapor (2-3cm) in the form for 5-10 minutes(Yu et al., 2002). Then collect the straw on each shelf and quickly dip it into special cups containing liquid nitrogen and these cups were transferred to a liquid nitrogen container**.** After 48 hours of storage, thawing is carried out by placing the straw into a water bath at (massage straw by hand) 37°C for 30 second then straws were cut to removes the straw vacuum first drop and put the second one on the slide to start the diluted epididymal sperm was evaluated in the same measured way as after diluted epididymal sperm and after cooling sample**.** Followed by diluted epididymal sperm Evaluation

Biochemical analysis

Activities of Glutamic Oxalo-acetic Tranaminase GOT, Glutamic Pyruvic Transaminase (GPT)and Lactate dehydrogenase (LDH) in epididymal fluid were estimated the samples by using biological kits (Randox England) epididymal fluid was read at a wavelength of 505nm(GOT and GPT) while LDH (absorbance at 340 nm) using the Spectrophotometer (Krefetz and McMillin, 2005).

Statistical analysis

Using the post hoc method from the Statistical Application Program, ANOVA was subjected to data, version 22 (SPSS 22). Differences between after dilution, cooling and post thawingin cauda epididymis, Biochemical (GOT,GPT and LDH) within and between groups were considered to be significant at P<0.05, expressed as the mean ± standard error (SE).

III. Results

Effect of adding different concentrations of L-Carnitine on GOT enzymeof epididymal sperm of buffalo after dilution, after cooling and freezing:After dilution, The result of study regarding GOT activity mili-moleof epididymal sperm of buffalo bull treated with concentrations of L-Carnitine during different steps of processing (after dilution, after cooling and post thawing). The study indicated lowestsignificant (P<0.05) value was in T4 (8.42±0.35mM) and highestsignificant (p<0.05) value was inT1(27.01 ±0.28mM) in comparison with others Table (1).

After cooling, with lowest significant (p<0.05) value of GOT activity mili-Mole was in T4 (21.61±0.23mM), and highest significant (p<0.05) Value was in T1 (32.54 ±0.33mM) in comparison with others Table (1).

At post thawing steps activity of GOT enzyme in epididymal fluid of buffalo bull in different steps (after dilution, after cooling and post thawing), indicate that lowest significant(p<0.05) value was in T4 (30.64 ±0.45mM)and highest significant (p<0.05) value was in T1(57.17 ±0.53mM)in comparison with others Table (1).Regarding the effect of different steps of freezing on GOT activity of epididymalfluid,the result refer that there is gradually significant (p<0.05) increased in GOT activity in epididymalfluid after dilution, after cooling and post thawing in all treatment, and Values was highest significant (p<0.05) in post thawing and lowest significant values in after dilution in all treatment Table (1).

Table (1) Effect of different concentrations of L-Carnitine on GOT enzyme during different steps of processing (after dilution, after cooling and post thawing)(Mean± SE).

Concentration of L-Carnitine	Step of freezing		
	after dilution	After cooling	Post thawing
Control -Tris T1	27.01 ±0.28 Ac	32.54 ±0.33 Ab	57.17 ±0.53 Aa
T2- 1mM/ml	23.81 ±0.28 Bc	27.82 ±0.14 Bb	36.94 ±0.47 Ba
T3 -5mM/ml	21.40 ±0.32 Cc	24.50 ±0.21 Cb	34.03±0.46 Ca

T4-10mM/ml	8.42 ±0.35 Dc	21.61±0.23 Db	30.64 ±0.45 Da

Different capital letters Means significant (p<0.05) different within column.
Different small letters Means significant (p<0.05) different between column.

3.2.Effect of adding different concentrations of L-Carnitine on GPT enzymeof epididymalsperm of buffalo after dilution, after cooling and freezing

The result of GPT enzymatic activity mili-moleof epididymal fluid of bull buffalo treated with different concentrations of L-Carnitine during different steps of freezing (after dilution, after cooling and post thawing), the result indicate in after dilution lowest significant (p< 0.05) Values was in T4 (0.17 ±0.006mM) and highest significant(p<0.05) values in T1(0.34 ±0.00mM) in comparison with others T2(0.28 ±0.009mM) and T3(0.25±0.004mM) Table (2).

After cooling, the GPT activitymili-Mole was lowest significant (p< 0.05) values in T4 (0.23 ±0.014mM), and highest significant (p<0.05) Values was in T1 (0.47 ±0.02mM). Appear non- significant differences between T2 (0.38 ±0.01%), T3(0.30 ±0.017%), andT4 (0.23 ±0.014%),(Table 2).At post thawing the values indicate was lowest significant (p<0.05) values was in T4 (0.59 ±0.009mM), and highest significant(p<0.05) values in was T1 (0.74 ±0.006mM) in comparison with others T2 and T3 (0.70±0.00and 0.65 ±0.008mM)Table (2).Regarding the effect of different steps of freezing(after dilution, after cooling and post thawing), the result in indicate there is gradually significant (p<0.05) increased in GPT activity during different steps of freezing, with highest significant was in post thawing and lowest significant in after dilution, except in T3 there is non- significant differences between the values after dilution and after cooling Table (2).

Table (2): Effect of different concentrations of L-Carnitine on GPT enzyme during different steps of (after dilution, after cooling and post thawing)

Concentration of L-Carnitine	Step of freezing		
	after dilution	**After cooling**	Post thawing
Control -Tris T1	**0.34 ±0.00** **Ac**	**0.47 ±0.02** **Ab**	0.74 ±0.006 Aa
T2- 1mM/ml	**0.28 ±0.009** **Bc**	**0.38 ±0.01** **Bb**	0.70±0.00 Ba
T3 -5mM/ml	**0.25±0.004** **Cb**	**0.30 ±0.017** **Bb**	0.65 ±0.008 Ca

T4-10mM/ml	**0.17 ±0.006** **Dc**	**0.23 ±0.014** **Bb**	0.59 ±0.009 Da

Different capital letters Means significant (p<0.05) different within column.

Different small letters Means significant (p<0.05) different between column.

3.3.Effect of adding different concentrations of L-Carnitine on LDH enzyme of epididymal sperm of buffalo after dilution, after cooling and freezing

The result of LDH enzymatic activity mili-Moleof epididymal fluid of bull buffalo treated with different concentrations of L-Carnitine during different steps of freezing (after dilution, after cooling and post thawing). The result proved in after dilution lowest significant(p< 0.05) values was in T3and T4(925.69±17.128and 874.60±17.0925mM) and highest significant(p<0.05) Values was in T1 (1154.79±52.50mM) in comparison with others Table (3).

After cooling, the LDH activity mili-Mole waslowest non- significant (p<0.05) Values in T2, T3 and T4(1121.56±65.37, 1074.11±52.205and 1059.97±48.18mM) and highest significant (p< 0.05) values in T1 (1293.29±67.63mM) Table (3).At post thawing the values indicate was lowest significant (p<0.05) Value was in T4(1433.66±99.512mM), and highest significant (p<0.05) value was in T1 (3802.50±146.17mM) in comparison with others T2(2486.92±111.80mM) and T3(1892.16±113.311mM) Table (3).

With respect to the effect of different steps of freezing (after dilution, after cooling and post thawing), in LDH, activity of epididymal fluid of bull buffalo, the result proved gradually significant (p<0.05) increased of LDH, activity during different steps of freezing with highest significant(p<0.05) values in post thawing and lowest significant(p< 0.05) values in after dilution Table (3).

Table (3) Effect of different concentrations of L-Carnitine on LDH enzyme during different steps of processing (after dilution, after cooling and post thawing)

Concentration of L-Carnitine	Step of freezing		
	after dilution	**After cooling**	Post thawing
Control -Tris T1	**1154.79±52.50** **Ac**	**1293.29±67.63** **Ab**	3802.50±146.17 Aa
T2- 1mM/ml	**1004.39±30.92** **Bc**	**1121.56±65.37** **Bb**	2486.92±111.80 Ba
T3 -5mM/ml	**925.69±17.128** **Cc**	**1074.11±52.205** **Bb**	1892.16±113.311 Ca

T4-10mM/ml	**874.60±17.0925** **Cc**	**1059.97±48.18** **Bb**	1433.66±99.512 Da

Different capital letters Means significant (p<0.05) different within column.

Different small letters Means significant (p<0.05) different between column.

IV. Discussion

Concerning the investigation of transaminases (GOT, GPT and LDH) values in the epididymal fluid of buffalo bull treated with different concentrations of L-Carnitine during different stage of dilution, cooling and freezing, the result proved that there is gradual significant increase in these parameters during the different steps of freezing of epididymal fluid.This result in an agreement with, **Dhami and Kodagali, (1990)** who reported that freezing –thawing of buffalo spermatozoa lead to leakage of intracellular enzymes .**Al-Daraji (2001)** indicated that GOT activity in seminal plasma was very weak as compared to the GPT activity. Leakage of GOT and GPT enzymes from camel sperm were determined by Duggal et al., (2001). In another study on mice, Elam et al., (2013) stated that L-Carnitine prevent increased the LDH enzyme activity of sperm during post thawing.**Gundogan, (2006)** reported that increased the percentage of abnormal sperm in ejaculate causes high concentration of transaminase enzyme in the extra cellular fluid resulting from sperm membrane damage and leakage of enzymes from spermatozoa. **El-Harairy*et al.,* (2011)** reported that spermatozoa damage during storage may be associated with increase sperm membrane permeability and leakage of intracellular enzymes. Transaminases activity of GOT than GPT in semen is a good indicator of semen quality, good quality semen was exhibited lower GOT than GPT activity **(Taha*et al.,* 2000).**Hence seminal plasma transaminases are evaluated as an index of measurement of injury to spermatozoa during different conditions **(Singh *et al.,* 1996),** whereby membrane become inactive with altered permeability or destroyed resulting into loss of enzymes **(Sidhu et al., 1996).** Sperm damage through oxidative stress results in increased membrane permeability to enzymes and other substances, and therefore, reduced metabolic activity of sperm **(Storey, 1997).** Changes in the activity of enzymes such as GPT or GOT in semen plasma are associated with defects of sperm membranes **(Colebrander*et al.,* 1992).**

Ingale*et al.,* (2000) demonstrated an elevation of GOT in seminal plasma during cryopreservation. **(Gowda*et al.,*2010**) reported changes of GOT activity during different steps of freezing**. Tuli and Holtz (1992)**observed leakage of GPT after freezing in Boer buck semen. In another study on ram, **Upreti*et al.,* (1996)** recorded low level GPT and GOT in semen at various stage of the cryopreservation, whereas LDH leakage was prevalent during cooling and freezing (post thawing) of spermatozoa. **Stefanov*et al.,* (2013)** recorded that in boar semen increase activity of enzymes at cooling due to damage to sperm membrane and subsequent leakage of enzymes into extracellular fluid due to cold shock. While, **Tejaswi*et al.,*(2016)** recorded an increase in the activities of GPT, GOT and LDH after cooling for 2 hrs . **Nasimi*et al.,(*2015)** demonstrated that leakage LDH related to damage of sperm in adult male mice. **Sharma *et al.,* (2013)** demonstrated that increase in the values of GOT and GPT enzyme indicates the damage of perm and abnormal sperm lead to leakage of these enzymes in the extracellular fluid. **Souhayla*et al.,*(2016)**recorded a gradual increase in the value of enzyme GOT and GPT during the different steps of semen processing and highest significant value was observed in frozen semen in

comparison with fresh semen, diluted and cooled semen. **Duan and Goldberg(2003)** demonstrated that GOT,GPT and LDH are proved to be important for various metabolic process which supply energy for motility and fertility of sperm. **Khawaskar*et al.*, (2012)** reported that buffalo bull's enzyme leakage before and after freezing were significantly greater in control sample with dilution only than those containing antioxidant. in another study in ram, the result proved a significant change in LDH activity among which is responsible for driving glycolysis when oxygen is limited by carrying NADH-mediated reduction of pyruvate to lactate, and reduced LDH activity in seminal plasma indicate disturbed spermatozoal function and metabolism **(Jones, 1997) and Salaam, (2008)**suggest that the increase in enzymatic activity at the frozen –thawed epididymal fluid indicates increased cell membrane permeability and damage to the sperm cells at freezing and post thawing incamel .

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