Quality and fertility of the frozen-thawed bull semen as affected by the different cryoprotectants and glutathione levels

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Abstract: Five sexually mature Friesian bulls (3-4 years old and 600-650 kg as an average body weight) were used in the present study. Two experiments were carried out. The first experiment aimed to define the effect of different types of cryoprotective agents (7%glycerol: GL and 7% dimethyl sulfoxide: DMSO) and their combinations (3.5% GL plus 3.5% DMSO) on post-thawing sperm motility, freezability and acrosomal damage of spermatozoa. Enzymatic activity (aspartate-aminotransferase: AST, alanine- aminotransferase: ALT, acid phosphatase: ACP, alkaline phosphatase: ALP and lactic dehydrogenase: LDH) in the frozen-thawed semen was also determined. The second experiment was carried out to establish the effects of 3.5% GL plus 3.5% DMSO (the best cryoprotectant in the first experiment) supplemented with different levels of glutathione (0.0, 0.2, 0.4 and 0.8 mM/ 100 ml) on the frozen-thawed semen quality and enzymatic activity during thawing-incubation at 37^{o}C for 2 hours. The conception rates of the cows artificially inseminated with the frozen-thawed semen containing 7% GL, 7% DMSO, 3.5% GL plus 3.5% DMSO with or without 0.4mM glutathione (GSH) were also assessed. The results revealed that the highest (P<0.05) percentage of sperm motility, freezability of spermatozoa and the lowest (P<0.05) percentage of acrosomal damage of spermatozoa were obtained in the post-thawed semen extended with 3.5% GL plus 3.5% DMSO as compared to 7% GL or 7% DMSO alone.Moreover, the frozen-thawed bull semen extended with 3.5% GL plus 3.5% DMSO showed significantly (P<0.05) lower amounts of AST, ALT, ACP, ALP and LDH enzymes released into the extracellular medium than7% GL or 7% DMSO alone the (first experiment). The obtained results in the second experiment showed that the frozen-thawed semen extended with 3.5% GL plus 3.5% DMSO added with GSH at levels of 0.2, 0.4 and 0.8mM showed significantly (P<0.05) increased the percentage of frozen-thawed sperm motility and freezability of spermatozoa and decreased (P<0.05) percentage of acrosomal damage of spermatozoa and amount of AST, ALT, ACP, ALP and LDH enzymes released into the extracellular medium compared to free GSH medium (control). It is of interest to note that, the best level (P<0.05) of GSH which maintained frozen-thawed semen quality and enzymatic activities was 0.4mM/100ml GSH. The advancement of thawing- incubation time at 37^{o}C for up to 2 hours of the frozen-thawed bull semen decreased significantly (P<0.05) the percentage of post- thawing sperm motility and freezability of spermatozoa, while increased significantly (P<0.05) the percentage of the acrosomal damage of spermatozoa and leakage of AST, ALT, ACP, ALP and LDH enzymes into the extracellular medium with the different glutathione levels or free glutathione medium. The highest conception rate (P<0.05) was recorded in the cows artificially inseminated with the frozen-thawed bull semen extended with 3.5% glycerol plus 3.5% DMSO added with 0.4mM GSH, and the lowest (P<0.05) conception rate was observed with the frozen-thawed bull semen extended with 7% GL or 7% DMSO alone.

[M. A. El-Harairy, Laila N. Eid, A. E. B. Zeidan, A.M.Abd El-Salaam and M. A. M. El-Kishk. **Quality and fertility of the frozen-thawed bull semen as affected by the different cryoprotectants and glutathione levels.** Journal of American Science 2011;7(5):791-801]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Bull semen, glycerol, dimethyl sulfoxide, freezing, enzyme, glutathione.

1. Introduction

Success of AI (Artificial Insemination) is dependent on the quality of fresh semen and its capacity for dilution and storage with minimum loss of fertilizing ability. The best preservation technique to date of post-thaw survival is restricted to about 50% of the sperm population (Watson, 1995). The final cryopreservation goal of semen is not only to maintain the initial motility but also to maintain the necessary metabolism to produce energy, plasma proteins to survive in the female reproductive tract at the time of fertilization, acrosomal enzymes for the penetration of the ovum, capacity of progressive movement and to prevent any damage which reduce life span of spermatozoa and its fertilizability (Palacio-Ango, 1994).

The major factor affecting the results of insemination with frozen-thawed semen is the addition of cryoprotectants (Aboagla and Trade, 2004), extension media, extension rate and spermatozoal

damage due to the formation of internal ice crystals, to the increase in solute concentration in the extension media or interaction of both physical factors (Leboeuf *et al*., 2000). The cryoprotectants are added to extenders to protect the sperm from damage during freezing process (Singh *et al*., 1995). The level and type of cryoprotectants in semen diluent influence these events and their effects on the sperm cells during freezing (Jorge *et al*., 2003 and Abdel-Khalek *et al*., 2008).

Most semen preservation protocols still favor glycerol in the cryoprotective media (Zeidan, 1994 and Abdel-Khalek *et al*., 2008). In certain instances other cryoprotectants are possibly better, for example, dimethyl sulphoxide (DMSO) was preferred for bovine (Snedocor and Gaunya, 1970) and buffalo bull (Khalifa, 2001 and Gabr, 2009). Also, ethylene glycol (Rodrigues *et al*., 2004), acetamide and lactamide (Nagase *et al*., 1972) provided good protection to bull spermatozoa during freezing. However, little information is available on their combinations in extenders for bull semen.

On the other hand, mammalian sperm cells are susceptible to lipid peroxidation by O_2 and H_2O_2 due to their high content of polyunsaturated fatty acids in their membranes and the lack of a significant cytoplasmic components containing antioxidants which results in a decreased sperm motility and viability (Von Schantz *et al*., 1999 and Satish and Das, 2005). One of enzymes that are distributed in sperm cells is glutathion (GSH) where one of its functions is to protect cell against the destructive effect of reactive oxygen species (Meister, 1983 and Lewis *et al*., 1997). However, the endogenous antioxidative capacity of semen may be insufficient during storage or dilution (Maxwell and Salamon, 1993) and the addition of antioxidants as GSH, ascorbic acid or catalase (El-Nenaey *et al*., 2006; Ahmed, 2008 and Gabr, 2009) improved the motility and survival of frozen-thawed bull spermatozoa.

Therefore, the present study aimed to study the effect of various cryoprotective agents (glycerol, dimethyl sulfoxide, and their combinations) on post-thawing motility, freezability, acrosomal damage of spermatozoa and enzymatic activity of the frozen-thawed semen (the first experiment). The effect of different levels of glutathion (GSH) addition to the frozen-thawed semen with the best cryoprotectants (based on results of the first experiment) on frozen-thawed semen quality and enzymatic activities during thawing-incubation for 2 hours at 37°C was recorded. Conception rates of the cows artificially inseminated with frozen-thawed semen with the best results in both experiments were also assessed (the second experiment).

2. Material and Methods

The present study was conducted in the Department of Animal Production, Faculty of Agriculture, Mansoura University. The experimental work was carried out at Animal Production Research Station, El-Gemmezah, Gharbiya Governorate, located in the north eastern part of Nile Delta (31°N), belonging to Animal Production Research Institute, Egypt.

Two experiments were carried out. The first experiment was carried out to investigate the effect of various cryoprotective agents (glycerol: GL and dimethyl sulfoxide: DMSO) and their combinations on post-thawed semen quality in terms of sperm motility, freezability of spermatozoa and acrosomal damage of spermatozoa. Enzymatic activity (aspartate-aminotransferase: AST, alanine -aminotransferase: ALT, acid phosphatase: ACP, alkaline phosphatases: ALP and lactic dehydrogenase: LDH) was also determined. The second experiment aimed to examine the effects of 3.5% glycerol plus 3.5% DMSO (the best obtained cryoprotectant in the first experiment) supplemented with different levels of glutathione (0.0, 0.2, 0.4 and 0.8 mM / 100 ml) on post-thawing sperm motility, freezability of spermatozoa, acrosomal damage of spermatozoa and enzymatic activity (AST, ALT, ACP, ALP, LDH). The conception rates of the cows artificially inseminated with the frozen-thawed semen extended with 7% glycerol, 7% DMSO, 3.5% glycerol plus 3.5% DMSO with or without the best level of glutathione (0.4 mM), were also assessed.

Experimental animals

Five sexually mature Friesian bulls aging 3-4 years with an average live body weight of 600-650 kg were used in the present study. They were housed individually under semi-open sheds and partially roofed with asbestos. All bulls were healthy and clinically free of external and internal parasites. Palpation of the external genital tract showed that they were typically normal. The testicular tone was glandular, all epididymal regions were present and both testes were almost in size and moved freely up and down within the scrotal pouches. Coupulatory patterns for all tested bulls at the beginning of the trials were judged to be normal.

Feeding system and management

A concentrate mixture was used in addition to rice straw. The mixture is composed of 25% undecorticated cotton seed cake, 15% corn, 44% coarse wheat bran, 8.5% extracted rice bran, 3% molasses, 3% limestone and 1.5% sodium chloride. The daily ration was offered in a certain mode of feeding starting with concentrate mixture at 8.30a.m followed by the straw at noon and continuing to next morning feeding. Bulls were not allowed to graze or to receive green fodder. The

bulls were allowed to drink clean fresh water *ad libitum*.

Semen collection

Semen was collected from five bulls twice a week for ten weeks by means of an artificial vagina between 08.00 and 09.00 a.m. Two successive ejaculates were obtained from each bull at each day of collection. Only semen with progressive motility of 70% or more was pooled and used for different treatments.

Semen freezing procedure

Semen extension

Semen was evaluated immediately after collection then extended with lactose-yolk – citrate (LYC) at a rate of 1 semen: 20 extender containing different levels of cryoprotectants as presented in Table (1). The final concentration of cryoprotectant was 7% GL (Salisbury *et al*., 1978), 7% DMSO (Hafez, 1993) and 3.5% GL plus 3.5% DMSO in the first, second and third extenders, respectively (the first experiment). In the second experiment ,semen was extended and supplemented with glutathione at levels of 0, 0.2, 0.4, and 0.8 mM/100ml with 3.5% glycerol plus 3.5% DMSO (best cryoprotectant in the first experiment).

Equilibration period

Extended semen with the different cryoprotective agents was kept at 5°C for 4 hours as an equilibration period. Then after, the samples were packaged in straws and frozen in liquid nitrogen (-196°C) as the method described by Salisbury *et al*. (1978).

Evaluation of the frozen-thawed semen

Frozen semen in straws was thawed in a water bath at 37°C for 30 seconds. The frozen-thawed semen was then incubated at 37°C for up to 2 hours. After each incubation time (0, 1, and 2 hours), the percentage of post-thawing sperm motility, freezability of spermatozoa, acrosomal damage of spermatozoa, and enzymatic activity (AST, ALT, ALP, ACP and LDH) of the frozen-thawed semen were recorded.

Post-thawed semen quality

The percentage of post-thaw motile spermatozoa was examined according to Amann and Hammerstedt (1980). The percentage of acrosomal damage was assessed according to Watson (1975).Freezability of the individual samples was examined as a percentage of spermatozoa recovered after freezing and thawing according to Patt and Nath (1969), using the following formula:

$$\textit{Freezability} = \frac{\text{Post-thawing motility (\%)}}{\text{Initial motility (\%)}} \times 100$$

Enzymatic activity (U/10^9 spermatozoa)

In the first and second experiments, frozen-thawed semen samples (5 straws of each experiment) were centrifuged at 600 *g* for 20 minutes. The superratant fluid (diluted seminal plasma) was collected and kept at -20°C till assay of AST, ALT, ACP, ALP and LDH enzymes activity. All enzymatic activities were assayed in post-thawed semen at zero time (immediately after thawing) and in post-thawed semen incubated at 37°C for 1 and 2 hours. Activity of AST and ALT enzymes in the post-thawed semen were determined colourimetrically using QCA kits (Amposta, Spain) according to Reitman and Frankel (1957). While, activity of ACP, ALP and LDH enzymes were determined colourimetrically using commercial kits (Stanbio kits, Texas, USA) according to Graham and Pace (1967). All enzymatic activities were adjusted according to sperm-cell concentration (U/10^9 spermatozoa).

Conception rate

Based on the best results in the first and second experiments, one hundred and twenty normally cyclic Friesian cows (in heat) were divided into six groups. Cows in the first (n=20), second (n=19), third (n=20), fourth (n=21), fifth (n=20) and sixth (n=20) groups were artificially inseminated with the frozen-thawed semen added with 7% glycerol, 7% DMSO, 7% glycerol plus 0.4mM glutathione, 7%DMSO plus 0.4mM GSH, 3.5% glycerol plus 3.5% DMSO and 3.5% glycerol plus 3.5% DMSO with 0.4mM GSH, respectively. For each cow, two inseminations were carried out, one in the morning and the second in the evening. Inseminations were usually carried out on the same day if the cows were notified to be in heat before 10.00 hr. Requests received by the Center after this time were looked to be inseminated on the following day. The straws were thawed by dipping immediately into thermos at 35°C for 30 seconds. The number of motile spermatozoa per insemination was nearly about 20×10^6 for each straw (0.25 ml). Semen was deposited into the uterus by using recto-vaginal insemination technique (Salisbury *et al*., 1978). Conception rate was estimated on the basis of pregnancy diagnosis by the rectal palpation after 60 days from date of insemination.

Statistical analysis

Data were statistically analyzed using Least Square Analysis of Variance according to Snedecor and Cochran (1982). Percentage values were transformed to Arcsine values before being statistically analyzed. The differences between means of individual treatments were evaluated by Duncan's New Multiple Range Test (Duncan, 1955). The conception rate results were analyzed using Chi-Square test.

3. Results

The first experiment:

1. Post- thawed semen quality

The results presented in Table (2) revealed that the highest (P<0.05) percentage of sperm motility and freezability of spermatozoa and the lowest (P<0.05) percentage of damaged acrosome in post-thawed semen were obtained in semen extended with 3.5% GL plus 3.5% DMSO. The highest (P<0.05) percentage of sperm motility (60.20%), freezability of spermatozoa (75.25%) and the lowest(P<0.05) percentage of the acrosomal damage of spermatozoa (10.00%) was recorded with the post-thawed semen extended with 3.5% GL plus 3.5% DMSO as compared to semen extended with 7% GL or 7% DMSO alone. Similar trend was reported by El-Harairy *et al*. (2010) in Friesian bulls. The same author found that the highest percentages of post-thawing sperm motility and freezability of spermatozoa and the lowest acrosomal damage of spermatozoa were recorded in the frozen-thawed bull semen diluted with a combination of glycerol plus DMSO. Gabr (2009) showed that post-thawing and freezability in buffalo semen extended with GL or DMSO were significantly better than ethylene glycol). Moreover, Khalifa (2005) reported that extender containing 3% glycerol had the highest percentage of post-thawing motility as compared to 3% ethylene glycol in goats. On the other hand, Singh *et al*. (1995) showed that both motility and percentage of live goat bucks spermatozoa were improved with a combination of GL plus DMSO**.** Moreover, Abd-El-Salaam (2002) and Zeidan *et al*. (2002) found that 2% glycerol and 4% DMSO maintained higher percentage of acrosomal integrity of frozen rabbit spermatozoa.

It is of interest to note that, the freezing semen at low temperatures causes structural damage as a result of cold shock including damage to the plasma membrane over the acrosome and damage to the plasma membrane of the middle piece. These changes are followed by decrease in the proportion of spermatozoa with intact acrosome. Therefore, the morphological characteristics of sperm acrosome and initial motility, gives the best indication, so far, of initial quality for frozen semen. Penetrating cryoprotectants are used to preserve spermatozoa viability during freezing by reducing or preventing ice crystal formation. The osmotic effects of molar concentrations of cryoprotectants may also result in membrane damage either during the introduction of the cryoprotectant or on its removal depending on the permeability of the cell membrane to the cryoprotectant (Watson, 1995).

2. Enzymatic activity (U/10^9 spermatozoa)

2.1. Activity of transaminase (AST and ALT) enzymes

The results presented in Table (3) revealed that the highest (P<0.05) AST and ALT enzymes activity was significantly (P<0.05) obtained in the post-thawed semen extended with 7% GL or 7% DMSO alone. However, the lowest (P<0.05) activity of AST and ALT was observed with the post-thawed semen extended with 3.5% GL plus 3.5% DMSO. Activity of transaminases (AST and ALT) is a good indicator of semen quality. Good quality of semen was characterized by lower AST and ALT activities (Taha *et al.*, 2000). The present results are in agreement with those obtained by Zeidan *et al*. (2002) and Khalifa (2005), who found that a combination of 2% GL plus 2% DMSO recorded the lowest AST and ALT activities. However, Gabr (2009) found that activity of AST in post-thawed buffalo semen was significantly lower in post-thawed semen extended with ethylene glycol than with glycerol or DMSO, being the highest with DMSO. The activity of AST in post-thawed bull semen was always higher than ALT activity as reported by several authors in semen of Friesian bulls (El-Sherbieny, 2004 and El-Harairy *et al.*, 2010) and in buffalo semen (El-Nagaar, 2008).

2.2. Activity of acid (ACP) and alkaline (ALP) phosphatases and lactic dehydrogenase (LDH)

The results presented in Table (3) revealed that the lowest (P<0.05) ALP, ACP and LDH enzyme activity was recorded in post-thawed semen extended with 3.5% glycerol plus 3.5% DMSO as compared to 7% glycerol or 7% DMSO alone. These results are in agreement with those of Zeidan *et al*. (2002) who found that activity of ACP and ALP was significantly lower in post-thawed semen extended with 4% DMSO and 2% glycerol plus 2% DMSO than with other cryoprotectants. El-Harairy *et al*. (2010) found that the frozen-thawed bull semen extended with 3.5% GL plus 3.5%DMSO had significantly lower amounts of AST, ALT, ACP, ALP and LDH enzymes into the extracellular medium than other cryoprotective agents or their combinations. On the other hand, Gabr (2009) revealed that activity of ACP in post-thawed buffalo semen was significantly lower in post-thawed semen extended with GL than with DMSO or ethylene glycol alone.

Based on the foregoing results, adding a combination of 3.5% glycerol plus 3.5% DMSO as cryoprotectants to lactose-yolk citrate extender during freezing of bull semen showed the best sperm motility, recovery rate of spermatozoa and intact acrosome of spermatozoa and maintained enzymatic activity in post-thawed semen for use in artificial insemination (AI) programmes.

The second experiment:

1. Percentage of post-thawed sperm motility

Data presented in Table (4) showed that GSH supplementation at levels of 0.2, 0.4 and 0.8mM

significantly (P<0.05) increased the percentage of the frozen-thawed sperm motility as compared to free GSH semen (control), during thawing-incubation at 37ºC for up to 2 hours. Among supplementation levels, the highest (P<0.05) percentage of frozen-thawed sperm motility was recorded with the semen supplemented with 0.4mM GSH (57.27%), moderate with 0.2mM GSH (52.94%) and the lowest with 0.8mM GSH (50.08%) versus 46.47% in free GSH semen (Table 4). These results are in agreement with those reported by El-Nenaey *et al.* (2006) and El-Kishk (2010) in Friesian and Gabr (2009) in buffalo bull semen. El-Sherbieny *et al.* (2006) also found that supplementation of bull semen extended with 0.4mM of GSH resulted in spermatozoa maintaining their motility and livability above 50% and abnormality percentage less than 15% at room temperature (25ºC) or at cooling temperature (5ºC) for 48 hours. The observed improvement of post-thawing sperm motility with antioxidant supplementation (GSH) was attributed to maintaining bull spermatozoal motility (Lindemann *et al.*, 1988, Bilodeau *et al.*, 2001 and Foote *et al.*, 2002), protecting spermatozoa against oxidative damage (Alvarez and Storey, 1989) and preserving spermatozoal viability by protecting membrane structure and function (Gadea, 2005).

Due to the effect of thawing-incubation at 37ºC for up to 2 hours, the overall percentage of frozen-thawed sperm motility decreased significantly (P<0.05) in free GSH and all GSH levels with increasing incubation time. The reduction rate in frozen-thawed sperm motility became higher with free GSH semen and with 0.8 m*M* GSH than 0.2 or 0.4mM GSH after 2 hours of thawing - incubation at 37°C (Table 4). Similar trend was reported by Zeidan *et al.* (1998) and El-Harairy *et al.* (2010) in bull semen. Moreover, frozen-thawed motility decreases significantly during incubation because of the gradual decline in ability of spermatozoa to generate ATP through mitochondrial respiration. This mitochondrial impairment might be due to mitochondrial aging and the toxic effect of membrane bound aromatic amino acid oxidase enzyme resulted from dead spermatozoa (Bag *et al.*, 2004). On the other hand, the decreasing motility during incubation may be related to change in spermatozoa morphology, the spermatozoa has usually containing mitochondria in mid-piece and tail after freezing and thawing. In addition, severe dilation or even loss of mitochondria in the mid-piece and tail breakage frequently occurred in the post-thawed spermatozoa. These changes might adversely affect the function of mitochondria and tails, thus reducing the spermatozoa flagellum movement. The loss of mitochondria was probably a result of swelling of mid-piece,the swelling pushed mitochondria away from the mid-piece or caused structural damage within mitochondria that decrease ATP generation(Zhang *etal*.,2003).

2. Freezability of spermatozoa (%)

As shown in Table (5), supplementation of GSH at levels of 0.2, 0.4 and 0.8mM to frozen-thawed semen significantly (P<0.05) increased the mean of freezability of spermatozoa as compared to GSH-free medium, during thawing-incubation at 37ºC for up to 2 hours. The freezability of post-thawed spermatozoa was significantly (P<0.05) highest with 0.4mM GSH (71.58%), moderate with 0.2mM GSH (66.17%) and the lowest with 0.8mM GSH (62.59%) versus 58.09% with free GSH semen. Similar trends were reported by El-Nenaey *et al*. (2006) and El-Kishk (2010) in bovine and Gabr (2009) in buffalo bull semen.

The advancement of thawing-incubation for up to 2 hours at 37ºC, showed significantly (P<0.05) decreased freezability of frozen-thawed semen containing different levels of GSH or free-GSH semen (Table 5). Similar trends were reported by Zeidan *et al*. (1998), Gabr (2009) and El-Kishk (2010).The reduction of post-thawing sperm motility and freezability of spermatozoa with increasing thawing-incubation was discussed by many authors. The production of free radicals during incubation has been suggested as the principal cause of decrease spermatozoa survivability and integrity (Mann *et al*., 1980 and Vishwanath and Shannon, 1997). The leuter authors found that spermatozoa exhibited an aerobic or even a partially aerobic system, the production of reactive oxygen species is inevitable. The superoxide anion (O_2), peroxide (H_2O_2) and hydroxyl free radicals are of the most damaging. The reaction producing these free radicals is more active at higher storage temperatures than in the cryopreserved state. They also found that the latent damage during freezing and thawing is exhibited during incubation and the greatest latent damage will be caused in the female genital tract.

3. Acrosomal damage of spermatozoa (%)

Data presented in Table (6) showed that the supplementation of GSH at levels of 0.2, 0.4 and 0.8 mM to the frozen-thawed semen significantly (P<0.05) decreased the percentage of acrosomal damage of spermatozoa as compared to free GSH medium, during thawing-incubation at 37ºC for up to 2 hours. When comparing the differences between different levels of GSH, extended semen supplemented with 0.4mM GSH showed the lowest(P<0.05) percentage of spermatozoa with acrosomal damage (9.47%), being significantly (P<0.05) lower than 11.80 and 12.60% with 0.2 and 0.8mM GSH levels, respectively (Table 6).However, semen free GSH showed significantly (P<0.05) higher percentage acrosomal damage of spermatozoa (16.73%) than other levels of GSH. These results are in agreement with those recorded by El-Kishk (2010) who found that the addition of 0.4 mM of

GSH to frozen bull semen significantly decreased the percentage of acrosomal damage of spermatozoa than GSH free medium, during thawing-incubation at 37ºC for up to 2 hours.

The advancement of thawing-incubation at 37ºC for up to 2 hours significantly (P<0.05) increased the percentage of acrosomal damage of post-thawed spermatozoa either free or supplemented with different levels of GSH. However, the rate of increase was higher with the control semen as compared to those supplemented with different GSH levels (Table 6). These results are in agreement with those obtained by El-Harairy *et al.* (2010) and Gabr (2009). The observed improvement with all GSH levels may be attributed to the protective mechanism of GSH to the spermatozoa cell against the destructive effect of reactive oxygen species (Meister, 1983 and Lewis *et al*., 1997). Similar trends were reported by El-Nenaey *et al*. (2006) and El-Kishk (2010) in Friesian and Ahmed (2008) and Gabr (2009) in buffalo bull semen. They reported that the addition of antioxidants to extended frozen semen improved spermatozoal quality, being the best with GSH at a level of 0.4mM.

4. Enzymatic activity (U/10^9 spermatozoa):

The results in Tables (7 to 11) confirm that supplementation of GSH at levels of 0.2, 0.4 and 0.8mM to the frozen-thawed semen significantly (P<0.05) reduced the amount of AST, ALT, ACP, ALP and LDH enzymes released into the extracellular medium as compared to free GSH medium. The lowest (P<0.05) amount of these enzymes (AST, ALT, ACP, ALP and LDH) released in the extracellular medium was recorded in post-thawed semen supplemented with 0.4mM, moderate with 0.2mM and the highest with 0.8 mM GSH, being all significantly (P<0.05) lower than free GSH medium. Such findings indicated beneficial effects of GSH addition at a level of 0.4 mM on membrane integrity of spermatozoa compared to other GSH levels. Similar trends were obtained by Gabr (2009) in buffalo and El-Kishk (2010) in Friesian bull semen.

The advancement of thawing-incubation at 37ºC for up to 2 hours significantly (P<0.05) increased the activity of AST, ALT, ACP, ALP and LDH enzymes in post-thawed bull semen supplemented with different levels of GSH or free GSH medium (Table 7 to 11). These results are in agreement with those reported by Zeidan et al . (1998 and 2008). It appears spermatozoal damage during storage may be associated with inccreas sperm membrane permeability and leakage of intracellular enzymes.

It is of interest to note that GSH supplementation at a level of 0.4mM to the extended frozen-thawed bull semen showed that the lowest enzymatic activities (AST, ALT, ACP, ALP and LDH) at different thawing-incubation times. The highest percentage of post-thawing sperm motility and freezability of spermatozoa as well as the lowest percentage of acrosomal damage of spermatozoa, indicating beneficial effects of GSH (0.4mM) on semen quality in terms of less spermatozoal cell injury and less membrane damage of spermatozoa (Rasul *et al*., 2000).

5. Conception rate (%)

The results in Table (12) revealed that the frozen-thawed semen extended with 3.5% glycerol plus 3.5% DMSO added with 0.4mM GSH gave significantly (P<0.05) higher conception rate (CR) than the frozen-thawed semen extended with7% glycerol or 7%DMSO. However, the conception rates of the cows artificially inseminated with the frozen-thawed semen with 7% glycerol, 7%DMSO, 7% glycerol plus 0.4mM GSH, 7%DMSO plus 0.4mM GSH and 3.5% glycerol plus 3.5% DMSO were insignificant (Table 12). Similarly, the conception rate of the cows artificially inseminated with the frozen-thawed semen with 7% GL plus 0.4mM GSH, 7% DMSO plus 0.4mM GSH and 3.5% GL plus 3.5% DMSO with or without 0.4mM GSH were insignificant. In addition, the conception rate of the cows artificially inseminated with the frozen-thawed semen extended with 7% DMSO showed insignificantly higher than the frozen-thawed semen extende with 7%GL. These results revealed that the fertilizing efficiency is nearly equal for the different frozen-thawed bull semen added with the different levels of glycerol. The highest CR of frozen-thawed semen with LYC extender containing a combination of 3.5% glycerol plus 3.5% DMSO supplemented with 0.4mM of GSH was mainly related to the best improvement of post-thawing sperm motility, freezability as well as the lowest of acrosomal damage of spermatozoa**.** Gabr (2009) confirmed that the conception rate of buffalo cows inseminated was recorded with frozen-thawed semen extended with Tris-based extender containing 0.4 or 0.8 mM GSH. In this respect, Foote *et al*. (2002) found that, the 59 days non-return rates for semen containing 0, 0.5 and 1.0 mM of GSH were 71.9, 69.5 and 70.9% respectively. Frozen-thawed semen with 0.5 mM of GSH and 100 U/ml of superoxide dismutase resulted in 74.0 and 73.9% of CR, respectively. El-Nenaey *et al*. (2006) and El-Kishk (2010) suggested that the addition of GSH to the extender during freezing phase improved the quality and fertilizing ability of frozen-thawed spermatozoa. These results are in agreement with those of Nishimura and Morii (1993).

Table (1). Composition of lactose-yolk citrate extender containing different types of cryoprotectants and their combinations .

Ingredients	Type of extender		
	Extender1	Extender2	Extender3
Lactose-yolk citrate (ml)*	73	73	73
Glycerol (ml)	7	-	3.5
Dimethyl sulfoxide (ml)	-	7	3.5
Fresh egg yolk(ml)	20	20	20
Penicillin(IU/ml)	500	500	500
Streptomycin(μg/ml)	500	500	500

* Lactose-yolk citrate (2.9g sodium citrate dihydrate, 0.04g citric acid anhydrate, 1.25 g lactose dissolved in 100 ml distilled water).

Table (2). Effect of cryoprotectants type on the percentages of post-thawing motility, freezability and acrosomal damage of bull spermatozoa.

Type of cryoprotectants	Post-thawing sperm motility(%)	Freezability of spermatozoa (%)	Acrosomal damage of spermatozoa (%)
7% Glycerol (GL)	57.60±0.51^{b}	72.00±0.68^{b}	12.20±0.87^{a}
7% Dimethyl sulfoxide (DMSO)	57.20±0.67^{b}	71.50±1.37^{b}	12.00±0.96^{a}
3.5% GL + 3.5% DMSO	60.20±0.49^{a}	75.25±0.40^{a}	10.00±0.32^{b}

a, b: Means with different superscripts in the same column are significantly different at (P≤0.05).

Table (3). Enzymatic activity (U/10^{9} spermatozoa) of frozen-thawed bull semen as affected by the different types of cryoprotectants.

Type of cryoprotectans	Aspartate aminotransferase	Alanine aminotransferase	Acid phosphatase	Alkaline phosphatase	Lactic dehydrogenase
7% Glycerol (GL)	18.40 ± 0.52^{a}	10.04 ± 0.72^{a}	149.00± 1.87^{a}	59.00 ± 1.14^{a}	278.20 ± 2.08^{a}
7% Dimethyl-sulfoxide(DMSO)	17.60 ± 0.98^{a}	9.40 ± 0.67^{a}	147.02 ± 1.84^{a}	60.01± 1.13^{a}	275.43 ± 2.58^{a}
3.5% GL + 3.5% DMSO	16.02 ± 0.91^{b}	7.01 ± 0.72^{b}	105.02 ± 1.85^{b}	50.02± 1.15^{b}	245.61 ± 1.78^{b}

a, b: Means with different superscripts in the same column are significantly different at (P≤0.05).

Table (4). Mean percentage of motility of frozen-thawed bull spermatozoa supplemented with the different levels of glutathione, during thawing-incubation at 37^{o}C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Overall mean
	0	0.2	0.4	0.8	
0	54.01±0.55	58.62±0.52	62.60±0.28	57.61±0.69	58.21±0.74^{a}
1	48.62±0.52	54.00±0.34	58.81±0.23	51.42±0.99	53.21±0.90^{b}
2	36.80±0.67	46.21±0.82	50.40±0.57	41.21±1.30	43.65±1.24^{c}
Mean	46.47±1.93^{d}	52.94±1.40^{b}	57.27±1.36^{a}	50.08±1.87^{c}	51.69

a, b,c,d: Means with different superscripts in the same row or column are significantly different at (P≤0.05).

Table (5). Mean percentage of freezability of bull spermatozoa supplemented with the different levels of glutathione, during thawing-incubation at 37°C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Overall mean
	0	0.2	0.4	0.8	
0	67.51±0.61	73.27±0.67	78.25±0.28	72.01±0.87	72.76±0.91[a]
1	60.77±0.69	67.50±0.36	73.51±0.23	64.27±1.22	66.51±1.10[b]
2	46.00±0.96	57.76±0.98	63.00±0.76	51.51±1.67	54.56±1.51[c]
Mean	58.09±2.35[d]	66.17±1.72[b]	71.58±1.76[a]	62.59±2.37[c]	64.61

a, b, c, d: Means with different superscripts in the same row or column are significantly different at (P≤0.05).

Table (6). Mean percentage of post-thawed bull spermatozoa with damaged acrosome supplemented with the different levels of glutathione, during thawing-incubation at 37°C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Overall mean
	0	0.2	0.4	0.8	
0	12.40±0.61	9.60±0.60	7.80±0.50	10.40±0.69	10.05±0.47[c]
1	17.00±0.72	11.60±0.52	9.40±0.52	12.40±0.83	12.60±0.70[b]
2	20.80±1.36	14.20±0.55	11.20±0.82	15.00±0.72	15.30±0.89[a]
Mean	16.73±1.03[a]	11.80±0.57[b]	9.47±0.49[c]	12.60±0.63[b]	12.65

a, b, c: Means with different superscripts in the same row or column are significantly different at (P≤0.05).

Table (7). Activity of aspartate-aminotransferase enzyme (U/10^9 spermatozoa) of frozen -thawed bull semen supplemented with the different levels of glutathione, during thawing- incubation at 37°C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Overall mean
	0	0.2	0.4	0.8	
0	17.80±1.04	15.00±0.55	12.80±0.86	15.80±0.8	15.35±0.55[c]
1	30.40±1.75	23.60±0.98	20.40±1.09	25.20±0.98	24.90±1.02[b]
2	42.00±0.85	33.00±1.16	30.20±1.08	36.40±1.04	35.40±1.11[a]
Mean	30.06±2.71[a]	23.86±2.01[c]	21.13±1.96[d]	25.80±2.29[b]	25.21

a, b, c,d: Means with different superscripts in the same row or column are significantly different at (P≤0.05)..

Table (8). Activity of alanine-aminotransferase enzyme (U/10^9 spermatozoa) of frozen- thawed bull semen supplemented with the different levels of glutathione, during thawing-incubation at 37°C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Mean
	0	0.2	0.4	0.8	
0	13.40±0.52	8.60±0.40	7.60±0.39	9.40±0.52	9.75±0.32[c]
1	17.60±0.51	13.20±0.75	11.40±0.76	14.40±0.69	14.15±0.47[b]
2	31.00±0.55	23.20±0.98	21.40±1.23	24.20±1.04	24.95±0.64[a]
Mean	20.66±1.87[a]	15.00±1.66[b]	13.46±1.61[c]	16.00±1.68[b]	16.28

a, b, c: Means with different superscripts in the same row or column are significantly different at(P≤0.05).

Table (9). Activity of acid phosphatase enzyme (U/10^9 spermatozoa) of frozen-thawed bull semen supplemented with the different levels of glutathione, during thawing- incubation at 37°C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Overall mean
	0	0.2	0.4	0.8	
0	144.00±1.48	123.40±1.59	113.60±1.13	131.40±0.51	128.10±2.61^c
1	174.60±1.64	145.40±1.96	125.80±1.77	156.60±3.63	150.60±4.20^b
2	228.40±3.34	172.60±2.30	141.00±3.54	187.00±3.43	182.25±7.36^a
Mean	182.33±9.34^a	147.13±5.44^c	126.80±3.22^d	158.33±6.22^b	153.65

a, b, c, d : Means with different superscripts in the same row or column are significantly different at (P≤0.05).

Table (10). Activity of alkaline phosphatase enzyme (U/10^9 spermatozoa) of frozen-thawed bull semen supplemented with the different levels of glutathione, during thawing-incubation at 37°C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Overall mean
	0	0.2	0.4	0.8	
0	55.00±1.72	44.60±1.18	39.20±0.90	47.20±1.88	46.50±1.45^c
1	86.20±2.86	67.80±1.95	53.60±1.77	74.20±3.88	70.45±2.97^b
2	127.00±5.40	96.00±5.70	80.40±4.95	102.20±6.01	101.40±4.58^a
Mean	89.40±8.05^a	69.46±5.87^b	57.73±4.79^c	74.53±6.35^b	72.78

a, b, c: Means with different superscripts in the same row or column are significantly different at (P≤0.05).

Table (11). Activity of lactic dehydrogenase enzyme (U/10^9 spermatozoa) of frozen-thawed bull semen supplemented with different levels of glutathione, during thawing- incubation at 37°C for 2 hours.

Incubation time (hrs)	Glutathione level (mM)				Overall mean
	0	0.2	0.4	0.8	
0	278.05±2.59	247.41±2.18	222.61±4.01	252.03±1.54	250.02±4.67^c
1	319.02±4.04	276.02±2.45	248.05±5.20	303.42±3.09	286.62±7.96^b
2	352.83±6.36	299.42±4.45	274.80±4.72	312.06±4.13	309.77±6.86^a
Mean	316.63±8.46^a	274.28±5.88^c	248.48±6.15^d	289.17±9.48^b	282.14

a, b, c, d: Means with different superscripts in the same row or column are significantly different at (P≤0.05).

Table (12). The conception rate of Friesian cows artificially inseminated with frozen-thawed semen as affected by the different cryoprotective agents added with 0.4 mM glutathione.

Treatments	No. of cows inseminated	No. of cows conceived	Conception rate (%)
7% Glycerol (GL)	20	14	70.00b
7% Dimethyl sulfoxide (DMSO)	19	14	73.68b
7%GL + 0.4 mM Glutathione (GSH)	20	16	80.00ab
7%DMSO+0.4 mM GSH	21	17	80.95ab
3.5% GL + 3.5% DMSO	20	15	75.00ab
3.5% GL+3.5 %DMSO+0.4 mM GSH	20	17	85.00a

a, b : Means with different superscripts in the column are significantly different (P≤0.05).

4. Conclusion

In conclusion, a high proportion of the bull spermatozoa retained active motility after freezing and thawing from -196°C in a medium containing dimethyl sulfoxide as a cryoprotective agent. The obtained results revealed that dimethyl sulfoxide with glycerol protect bull spermatozoa more effectively than glycerol or dimethyl sulfoxide alone during freezing and thawing. The frozen-thawed bull semen added with 0.4 mM glutathione / 100ml is more efficient in maintaining post-thawing sperm motility, freezability, acrosomal integrity, maintaining enzymatic activities and subsequent fertilizing efficiency of bull spermatozoa than free glutathione medium. Therefore, it can be recommended to extension and freezing of bull semen with 3.5% glycerol plus 3.5% dimethyl sulfoxide supplemented with glutathione at a level of 0.4 mM / 100ml for artificial insemination programmes to enhance conception rates in cows.

References

1. Abd El-Salaam, A.M. (2002). Studies on freezing rabbit semen. M.Sc. Thesis, Fac. Agric., Zagazig Univ., Zagazig, Egypt.
2. Abdel-Khalek, A.E.; M.M. El-Moghazy and Sh.A. Gabr (2008). Influence of some cryoprotectants in comparing with different glycerol levels on sperm viability during freezing process of goat semen. J. Agric. Sci., Mansoura Univ., **33**: 4873-4886.
3. Aboagla, E.M.E. and T. Trade (2004). Effects of egg yolk during the freezing step of cryopreservation on viability of goat spermatozoa. Theriogenology, **62**: 1160-1172
4. Ahmed, I.A. (2008). Effect of adding ascorbic acid to semen of Egyptian buffalo bulls. M.Sc. Thesis, Fac. Agric., Mansoura Univ., Mansoura, Egypt.
5. Alvarez, J.G. and B.T. Storey (1989). Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res., **23**: 77-90.
6. Amman, R.P. and R.H. Hammerstedt (1980). Validation of a system for computerized measurements of spermatozoa velocity and percentage of motile sperm. Biol. Reprod., **23**: 647-656.
7. Bag, S.; A. Joshi; S. M. K. Naqvi and J. P. Mittal (2004). Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenology, **62**: 415-424.
8. Bilodeau, J.F.; S. Blanchette; M.A. Sirard and C. Gagnon (2001). Thiols prevent H_2O_2- mediated loss of sperm motility in cryopreserved bull semen. Theriogenology, **56**: 27
9. Duncan, D.B. (1955). Multiple range and multiple F tests. Biometrics, **11**:1-42.
10. EL-Harairy,M.A.; A.E.B. Zeidan and M.A.M. EL-Kishk (2010). Effect of some cryoprotectants or their combinations on sperm motility and enzymatic activity in post-thawed semen of Friesian bulls. J. Agric. Sci. ,Mansoura Univ**., 1**:71-80.
11. El–Kishk, M.A. (2010). Studies on freezing of bovine bull semen. Ph .D Thesis, Fac. Agric., Mansoura Univ., Egypt.
12. El-Nagaar, H.A. (2008). Studies on frozen buffalo bulls semen. Ph .D Thesis, Fac. Agric., Al-Azhar Univ., Cairo, Egypt.
13. El-Nenaey, M.H.; M.A. El-Sharbieny; A.E. Abdel-Khalek; E.M.E. El-Siefy and M.M. El-Baz (2006). Effect of reduced glutathione supplementation on motility, liveability and abnormality of Holstein spermatozoa in: 2-Frozen semen. J. Agric. Sci., Mansoura Univ., **31**: 5005-5013.
14. El-Sherbieny, M.A. (2004). Physiological study on farm animals. Ph. D. Thesis, Fac. Agric., Mansoura Univ., Egypt.
15. El-Sherbieny, M.A.; M.H. El-Nenaey; E.M.E. El-Siefy and A.E. Abdel-Khalek (2006). Effect of reduced glutathione supplementation on motility, livability and abnormality of Holstein spermatozoa in : 1. Semen stored at room and cool temperature. J. Agric. Sci., Mansoura Univ., **31**: 1913-1931.
16. Foote, R.H.; C.C. Brockette and M.T. Kaproth (2002). Motility and fertility of bull sperm in whole milk extender containing antioxidants. Anim. Reprod. Sci., **71**: 13-23.
17. Gabr, A.A. (2009). Assessment of *In Vitro* and *In Vivo* fertilizing capacity of buffalo sperm treated with various cryoprotectants. Ph.D. Thesis, Fac. Agric., Mansoura Univ., Mansoura, Egypt.
18. Gadea, J. (2005). Sperm factors related to *in vitro* and *in vivo* porcine fertility. Theriogenology**, 63**: 431-444
19. Graham, E.F. and M.M. Pace (1967). Some biochemical changes in spermatozoa due to freezing. Cryobiology**, 4**: 75-84
20. Hafez E.S.E. (1993). Reproduction in Farm Animals. 6th edited by Lea and Febiger, Philadelphia, USA, p.598.
21. Jorge, G.; L. Nils; S. Lennart; R.M. Heriberto (2003). Influence of extender, temperature and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology**, 59**: 1241-1255.
22. Khalifa, T.A. (2001). Effect of some antioxidants on viability of preserved buffalo and ram semen. Ph.D. Thesis, Fac. Vet. Med., Cairo Univ., Cairo, Egypt.
23. Khalifa, E.I. (2005). Effect of some processes of cryopreservation on post-thaw characteristics and fertilizing ability of goat spermatozoa. Ph.D. Thesis, Fac. Agri., Suez Canal Univ., Egypt.
24. Lebouef, B.; B. Restal and S. Salamon (2000). Production and storage of goat semen for artificial insemination. Anim. Reprod. Sci., **62**: 113-141.
25. Lewis, S.E.M.; E.S.L. Sterling and L.S. Young (1997). Comparison of individual antioxidants of semen and seminal plasma in fertile and infertile men. Fertil. Steril., **67**: 142-147.
26. Lindemann, C.B.; J.A. O'Brien and F.J. Giblin (1988). An investigation of the effectiveness of certain antioxidants in preserving the motility of reactivated bull sperm models. Boil. Reprod., **38**: 114-120.
27. Mann, T., R. Jones and R. Sherins (1980). Oxygen damage, lipid peroxidation and motility of spermatozoa. In: Steinberger A., Steinberger E, editors. Testicular

devolpment, structure and function. New York: Raven Press, p. 497.
28. Maxwell, W.M.C. and S. Salamon (1993). Liquid storage of ram semen. Reprod. Fertil. Dev**., 5**: 613-638.
29. Meister, A. (1983). Selective modification of glutathione metabolism. Science, **220** : 472 – 477.
30. Nagase , H .; T. Tomizuka; T. Hosoda and H. Morimoto (1972). Cryoprotection of some amide solutes to spermatozoa of domestic animals. I- Effects of formamide, acetamide and lactamide on the motility of bovine spermatozoa in pellet freezing. Japanese J. Anim. Reprod., **18**: 15-21.
31. Nishimura, K. and H. Morii (1993). Effects of glutathione on the motility of frozen thawed boar spermatozoa. Anim. Sci. Tech., **64**: 433-439.
32. Palacio-Ango, A. (1994). Physiological aspects about semen freezing. Centro Ensenanza Investigacion Extension Production Bovina Caprina Fac. Med. Vet. Zootecnia, Univ. Nacl. Autonoma 4 milpas, Apdo-postal 227, 54600 Tepotzottan, Edo Mexico, Mexico-Veterinaria-Mexico, **25**: 207-210.
33. Patt, J.A. and J. Nath (1969). Effect of diluents, equilibration time and freezing rates on storage of ram semen. Cryobiology, **5**: 385-392.
34. Rasul, Z.; M. Anzar; S. Jalali and N. Ahmad (2000). Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. Anim. Reprod. Sci., **59** : 31 – 41.
35. Reitman, S. and S. Frankel (1957). A colorimetric methods for determination of serum glutamic oxaloacetic transaminases. American J. Clinical and Pathology**, 2**: 56-61.
36. Rodrigues, A.P.R.; C.A. Amorim; S.H.F. Costa; M.H.T. Matos; R.R. Santose; C.M. Lucci; S.N. Bao; O.M. Ohashi and J.R. Figueriedo (2004). Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol. Theriogenology, **61**: 1009-1024.
37. Salisbury. G.W.; N.L. Van Demark and J.R. Lodge (1978). Physiology of Reproduction and Artificial Insemination of Cattle. San Francisco, p. 494.
38. Satish, K. and G.K. Das (2005). Frozen sperm quality with reference to reactive oxygen species: a review. Indian J. Anim. Sci.,**75**: 874-884 .
39. Singh, M.P.; A.K. Sinha and B.K. Singh (1995). Effect of cryoprotectants on certain seminal attributes and on the fertility of goat buck spermatozoa. Theriogenology, **43**: 1047-1053.
40. Snedecor, G. W. and W. G. Cochran (1982). Statistical Methods. 7th Ed., Iowa State Univ. Press, Ames, USA.
41. Snedecor, W.H. and W.S. Gaunya (1970). Dimethyl sulfoxide as acryoprotective agent for freezing bovine semen. J. Anim. Sci., **30** : 953-960.
42. Taha, T.A.; E.I. Abdel-Gawad and M.A. Ayoub (2000). Monthly variations in some reproductive parameters of barki and awassi rams throughout 1 year under subtropical condition.2:Biochemical and enzymatic properties of seminal plasma. Anim. Sci., **71** : 325.
43. Vishwanath, R. and P. Shannon (1997).Do sperm cells age? A review of the physiological change in sperm during storage at ambient temperature. Reprod. Fertil. Dev**., 9**:321-331.
44. Von Schantz, T; S. Bensch; M. Grahn; D. Hasselquist and H. Wittzell (1999). Good genes, oxidative stress and condition-dependent sexual signals. Proc. Royal Soc., London Series, p. 266:1-12.
45. Watson, P. F. (1975). Use of Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. Vet. Record., **97** :12 – 15.
46. Watson, P.F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post thawing function. Reprod. Fertil. Dev., **7**: 871-891.
47. Zeidan, A.E.B. (1994). New aspects in freezing cattle semen. Ph. D. Thesis, Fac. Agric., Zagazig Univ., Zagazig, Egypt.
48. Zeidan, A.E.B.; R.M. Abou-Aiana; E.A. Ahmadi; N.A. Abd El-Aziz; N.S. Sayah; A.H. Ammar and A.H. Abd El-Salaam (2008). Viability and enzymatic leakage of the cooled camel spermatozoa in relation to different extenders. American Eurasian J. Agric. and Envir. Sci**., 4** : 142-149.
49. Zeidan, A.E.B.; A.I. Abulnaga; Z.A. Ibrahim and M.A.M. Hamedi (2002). Quality,enzymatic activity and fertility rate of the cooled rabbit semen supplemented with caffeine. Egyptian J. Rabbit Sci., **12** : 27
50. Zeidan , A.E.B .;M.N.El-Gaafary and F.E.El-Keraby (1998). Effect of new packaging methods for frozen – bull semen in pellets form on some biochemical changes and conception rate. Proc.1st Intern. Conf. Anim.Prod and Health in Semi-Arid Areas,El-Arish,Egypt,pp.223-234.
51. Zhang,Y.Z.; S.C. Zhany; X. Z. Liu; Y. Y. Xu; C. L. Wang; M. S. Sweet and S. L. Chen (2003). Cryopreservation of flounder (*Paralichtys olivacens*) sperm with a practicalmethodology. Theriogenology , **60** :989-996.

4/22/2011