NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

Slovak J. Anim. Sci., 52, 2019 (1): 1–8

ISSN 1337-9984
E-ISSN 1338-0095

EFFECT OF L-GLYCINE AND L-CARNITINE ON POST-THAW SEMEN PARAMETERS AND FERTILITY IN CHICKEN

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ABSTRACT

The effect of supplementing L-glycine and L-carnitine in cryopreservation mixture on post-thaw semen and fertility parameters was studied. Semen from adult PD6 line roosters was collected, pooled and used. The samples after equilibration at 5°C for 30 minutes were mixed with the diluent with the final concentration of supplements L-glycine of 5 or 15 mM and L-carnitine of 1mM along with a cryoprotectant (4 % dimethylsulfoxide; DMSO), and the semen was cryopreserved in 0.5 ml French straws. Different *in vitro* semen quality parameters, fertility and hatchability were assessed in post-thaw samples. Post-thaw sperm motility, live sperm, MTT dye reduction test and seminal plasma lipid peroxidation were significantly lower ($P < 0.05$) in the cryoprotectant only (4 % DMSO) as well as in the L-glycine- and L-carnitine- supplemented treatments. Fertility was significantly ($P < 0.05$) lower the L-glycine- and L-carnitine-supplemented treatments compared to fresh semen, however, hatchability on fertile egg set was similar in all the treatments. The post-thaw semen parameters, fertility and hatchability in L-glycine- and L-carnitine- supplemented treatments were similar to that of the cryoprotectant only treatment. In conclusion, L-glycine and L-carnitine supplementation did not improve post-thaw semen parameters or fertility and hatchability. Thus, inclusion of these compounds in the chicken semen cryopreservation mixture may not provide advantage during the cryopreservation process.

Key words: carnitine; chicken; cryopreservation; fertility; glycine; semen

INTRODUCTION

The sperm survival and fertilizing ability are reduced during semen cryopreservation process due to the effect of freeze-thawing steps on membrane integrity and other functional parameters (Holt, 2000). In the cryopreservation process, high levels of reactive oxygen species (ROS) are formed (Chatterjee and Gagnon, 2001). The avian sperm membrane has higher polyunsaturated fatty acid concentration in comparison to mammalian sperm (Cerolini *et al*., 1997) and is, therefore, highly susceptible to the deleterious effects of lipid peroxidation (LPO). There may be a compromise in the antioxidant system of semen during the cryopreservation process, which further increases the intensity of LPO and potentiates the damaging effects (Li *et al*., 2010; Partyka *et al*., 2012).

L-Carnitine is a quaternary ammonium compound (Bieber, 1988) and water-soluble amino acid naturally biosynthesized in the kidney and liver of animal body from lysine and methionine (Bremer, 1983). It plays a key role in reducing the availability of lipids for peroxidation by facilitating transport of fatty acids into mitochondria for β-oxidation to generate ATP energy (Hinton *et al*.,

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Received: July 12, 2018
Accepted: August 15, 2018

1979; Neuman *et al*., 2002; Zhai *et al*., 2007). Antioxidant characteristics and anti-apoptotic activities of L-carnitine have a protective role against damaging effects of ROS and may stabilize mitochondrial membrane and DNA structure (Qi *et al*., 2006). L-Carnitine also increases the activity and levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Neuman *et al*., 2002). Supplementation of L-carnitine in sperm freezing medium prior to freezing has been found to increase post-thaw motility (Banihani *et al*., 2014), acrosomal integrity (Aliabadi *et al*., 2018) and morphology (Bucak *et al*., 2010) of spermatozoa. Supplementation of L-carnitine in extender has resulted in significant improvement in post-thaw sperm motility in rainbow trout (Kutluyer *et al*., 2014) and bull (Sarıözkan *et al*., 2014).

Supplementation of Beltsville extender with 1 and 2 mM L-carnitine significantly improved post-thaw rooster sperm quality parameters resulting in higher total motility, progressive motility, membrane functionality, viability and lower lipid peroxidation (Fattah *et al*., 2017). In another study, 1 mM L-carnitine supplementation in rooster semen cryopreservation mixture improved post-thaw live sperm ratio without apoptosis and membrane reorganization, live sperm ratio without lipid peroxidation and mitochondrial membrane potential, and decreased sperm proportion with detectable DNA fragmentation (Partyka *et al*., 2017). The effect of L-carnitine supplementation in a freezing mixture on fertility in chicken is not known.

Free amino acids in seminal plasma were found to be reduced in infertile patients in comparison to fertile persons, and it was suggested that these amino acids play a protective role for sperm in the hostile vaginal environment (Silvestroni *et al*., 1979). Among the free amino acids, glycine occurs at the second highest concentration in bull seminal plasma (Assumpção *et al*., 2005). Glycine is the smallest and simplest amino acid with only single hydrogen forming its side chain. Amino acids such as glycine, glutamine, histidine and proline have been employed during sperm cryopreservation in different species, such as ram (Khalili *et al*., 2010), stallion (Trimeche *et al*., 1999), goat (Kundu *et al*., 2001) as well as human (Renard *et al*., 1996). Combining amino acids with glycerol or DMSO have shown to improve post-thaw sperm motility in goat (Kundu *et al*., 2001).

To the best of our knowledge, there is no report about the inclusion of L-glycine during chicken semen cryopreservation on post-thaw sperm parameters, fertility and hatchability. Furthermore, the effect of adding L-carnitine during chicken semen cryopreservation on fertility is not known. Against this background, the present study was carried out to reveal the effect of inclusion of L-glycine and L-carnitine in cryopreservation mixture on post-thaw semen parameters, fertility and hatchability in chicken. The information from this study will help in better understanding of the effects of these compounds and possible inclusion during chicken semen cryopreservation.

MATERIAL AND METHODS

Experimental birds and husbandry

The experiment was carried out at the experimental poultry farm of ICAR- Directorate of Poultry Research, Hyderabad, India. PD6 line males derived from multicolored broiler population, which has been selected for shank length for six generations, were used in the experiments. This line is used as male parent line for production of a countrywide popular rural poultry variety *Gramapriya*. The birds were housed in individual cages in an open-sided house. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC/DPR/17/2).

Semen collection and processing for cryopreservation and quality assays

Adult PD6 roosters were trained to respond to abdominal massage technique (Burrows and Quinn, 1937) for the collection of semen. Semen was collected, pooled and kept on ice throughout the experiment. Collected semen was immediately brought to the laboratory over ice in a covered thermocol box, evaluated and processed for cryopreservation. In the laboratory, a portion of the semen was diluted four times in a semen diluent (Table 1) and was used for evaluation of semen quality parameters.

Fifteen PD6 line male birds aged 38 weeks were used for collecting semen and cryopreservation.

Table 1. Composition of semen diluent (Sasaki *et al*., 2010)

Components	g/100 ml
D (+)-glucose	0.2
D (+)-trehalose dehydrate	3.8
L-glutamic acid, monosodium salt	1.2
Potassium acetate	0.3
Magnesium acetate tetrahydrate	0.08
Potassium citrate monohydrate	0.05
BES	0.4
Bis-Tris	0.4
Distilled water	Up to 100 ml
pH	6.8

The pooled semen samples were initially evaluated for sperm concentration. The samples were diluted with a cryoprotectant-free diluent so that the sperm concentration was 4000 x 10^6/ml. The samples were equilibrated at 5 °C for 30 min and were diluted in 1:1 proportion with the diluent containing 8 % dimethyl sulfoxide (DMSO) so that the final concentration of DMSO was 4 % and the final sperm concentration was 2000 x 10^6/ml in each treatment. The effect of supplementing L-glycine (Invitrogen, USA, Cat. No. 15527-013) at 5 and 15 mM concentrations and L-carnitine at 1 mM concentration (SRL Pvt. Ltd., India, Cat. No. 0348283) along with DMSO on cryopreserved semen were studied. During the preliminary trials in the laboratory it was observed that L-glycine concentrations above 15 mM adversely affected the *in vitro* sperm motility and viability. The L-carnitine concentration was selected based on information from previous reports. The semen mixed with test compounds of different treatments was immediately loaded into 0.5 ml French straws and sealed with polyvinyl chloride powder. The filled straws were placed 4.5 cm above the level of liquid nitrogen (LN_2) on a styrofoam raft floating on LN_2 in a thermocol box and exposed to nitrogen vapours for 30 minutes, then the straws were plunged into LN_2, transferred into canisters and stored at -196 °C. Semen straws were stored for a minimum of ten days before further evaluation. Cryopreserved semen after thawing at 5 °C for 100 sec in ice water (Sasaki *et al*., 2010) was evaluated on ten different occasions for progressive sperm motility, live and abnormal sperm, MTT dye reduction test and seminal plasma lipid peroxidation.

Sperm motility and concentration

Sperm motility was recorded as a percentage of progressively motile sperm by placing a drop of diluted semen on a Makler chamber and examining under 200 x magnification. The percentage of sperm with normal, vigorous and forward linear motion was subjectively assessed and scored. The sperm concentration was estimated by measuring optical density using colorimeter (Taneja and Gowe, 1961).

Live and abnormal sperm

Percentages of live and abnormal sperm were estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al*., 1953). Semen smear was prepared by mixing one drop of semen with two drops of Eosin-Nigrosin stain and then air dried. Slides were evaluated under high magnification (1000 x). All completely or partially pink-stained sperm were considered as dead and unstained sperm as live. The percentage of live sperm was determined by counting at least 200 sperm cells. The same slides were used for estimating the abnormal sperm percentage that was showing different morphological abnormalities evaluated at 1000 x magnification.

MTT dye reduction test

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction test was carried out in duplicate samples as described by Hazary *et al*. (2001). Briefly, in a tube, 900 µl Nacl–TES, 100 µl of 100 mM glucose, 30 µl semen sample (2 million sperm/µl) and 50 µl of 4 mM MTT dye were added, mixed and incubated for one hour in a shaking water bath at 37 °C. After incubation, 200 µl of 10 % sodium dodecyl sulfate (SDS) in 0.01 M hydrochloric acid (HCl) solution was added to each tube, mixed well and allowed to stand for one hour. The optical density of each sample was measured against blank at 570 nm in a colorimeter (CL 157, Elico Ltd, Hyderabad, India) and the dye reduction activity of sperm was calculated.

Seminal plasma lipid peroxidation

Lipid peroxidation in seminal plasma was measured by thiobarbituric acid method (Hsieh *et al*., 2006). Semen samples were centrifuged at 7000 rpm

for 5 min to separate seminal plasma. To each test tube, 0.9 ml of distilled water and 0.1 ml of seminal plasma were added followed by addition of 0.5 ml of thiobarbituric acid reagent. The tubes were incubated for one hour in a boiling water bath. After cooling the tube contents, absorbance was measured against blank at 534 nm using UV double beam spectrophotometer (Metstar MUV-61PCS, India).

Fertility trials

Fertility trial was conducted by inseminating PD-3 line hens four times at two days interval. Fifty-five PD-3 line females aged 44 weeks were divided equally into five groups with 11 hens per group. The semen straws were thawed at 5 °C for 100 sec in an ice water (Sasaki *et al*., 2010) and inseminated in hens per vagina with a sperm concentration of 200 million/0.1 ml. The control group was inseminated with freshly collected semen (200 million sperm/0.1 ml). Eggs were collected from the second day of the first insemination and stored at 15 °C until incubation. The eggs were incubated at standard conditions in automatic setter/hatcher incubator (VJ Equipments, Pune, India). The eggs were candled on the 18th day of incubation for embryonic development and fertile eggs were transferred into setter compartment. Infertile eggs were broken open to confirm the absence of embryonic development. The chicks hatched on the 21st day of incubation were counted for calculating hatchability.

Statistical analysis

Data were analyzed using SAS 9.2 software and $P < 0.05$ level was considered as significant. Statistical analyses of semen parameters, fertility and hatchability were performed by one-way ANOVA with Tukey's post hoc test. Data having percentage values were arcsine-transformed and analyzed.

RESULTS

The cryopreserved semen samples had significantly ($P < 0.05$) lower post-thaw sperm motility, live sperm and MTT dye reduction activity compared to control (Table 2). The post-thaw abnormal sperm percentage was significantly higher ($P < 0.05$) in cryopreserved semen. However, there was no difference in seminal plasma lipid peroxidation and fertility in comparison to control.

L-carnitine supplementation in the cryopreservation mixture significantly ($P < 0.05$) lowered post-thaw sperm motility, live sperm, MTT dye reduction activity and fertility.

Table 2. Effect of L-Glycine and L-Carnitine on post-thaw semen parameters, fertility and hatchability of cryopreserved chicken semen

Parameters	Control (fresh semen)	4 % DMSO	4 % DMSO + L-Carnitine 1mM	4 % DMSO + L-Glycine 5mM	4 % DMSO + L-Glycine 15mM
Progressive sperm motility (%)	78.0 ± 0.8^{a}	21.5 ± 0.8^{bc}	24.0 ± 1.3^{b}	21.5 ± 0.8^{bc}	19.0 ± 1.0^{c}
Live sperm (%)	89.75 ± 1.45^{a}	24.00 ± 0.54^{bc}	27.55 ± 1.07^{b}	24.90 ± 0.78^{bc}	22.15 ± 1.01^{c}
Abnormal sperm (%)	1.5 ± 0.18^{b}	2.15 ± 0.18^{a}	2.05 ± 0.11a^{b}	2.25 ± 0.11^{a}	2.35 ± 0.18^{a}
MTT dye reduction test (nM of MTT Formazan/min/million sperm)	95.14 ± 2.31^{a}	39.3 ± 1.55^{b}	40.7 ± 1.9^{b}	39.61 ± 1.46^{b}	40.26 ± 0.93^{b}
Seminal plasma lipid peroxidation (nM MDA/ml)	1.5 ± 0.06	1.37 ± 0.03	1.35 ± 0.04	1.39 ± 0.04	1.41 ± 0.03
Fertility (%)	96.05 ± 2.06^{a}	78.14 ± 5.46^{ab}	59.34 ± 7.68^{b}	54.73 ± 10.04^{b}	51.45 ± 7.43^{b}
Hatchability on FES (%)	77.03 ± 3.98	78.65 ± 3.98	84.32 ± 8.87	69.54 ± 8.77	76.10 ± 8.91
No. of eggs incubated	87	86	85	84	82

Values are mean ± SE.
^{a,b,c} Figures bearing different superscripts in a row differ significantly ($P < 0.05$).
DMSO - Dimethylsulfoxide
FES - Fertile Egg Set
MDA - Malondialdehyde

L-glycine supplementation significant ($P < 0.05$) reduced post-thaw sperm motility, live sperm and MTT dye reduction activity. The reduction in motility and live sperm were progressive with increasing L-glycine concentration. The abnormal sperm percentage was significantly higher ($P < 0.05$) in both the concentrations of L-glycine tested. There was no difference in post-thaw seminal plasma lipid peroxidation between L-glycine treatments and control. The fertility from L-glycine supplemented treatments was significantly ($P < 0.05$) lower compared to the control.

Post-thaw semen parameters and fertility of the L-gycine and L-carnitine treatments were not significantly ($P > 0.05$) different in comparison with the cryoprotectant only (4 % DMSO) treatment. No difference in hatchability on fertile egg set (FES) was observed between the treatments.

DISCUSSION

L-carnitine is concentrated at high levels in the seminal plasma and there exists a correlation between total seminal carnitine concentration and sperm motility (Agarwal and Said, 2004). Dietary supplementation of L-carnitine in roosters has been shown to improve sperm concentration and reduce lipid peroxidation (Neuman *et al*., 2002; Zhai *et al*., 2007) and this may be due to the antioxidant and anti-apoptotic action of L-carnitine (Qi *et al*., 2006). Based on the available information it was hypothesized that adding L-carnitine into the cryopreservation mixture may help in improving the post-thaw semen parameters. In the present study addition of L-carnitine into the cryodiluent mixture did not improve post-thaw semen parameters or fertility. This result is in contrast to improvement in post-thaw sperm motility, membrane functionality, sperm viability and decreased lipid peroxidation by the inclusion of L-carnitine at 1 and 2 mM concentrations (Fattah *et al*., 2017). However, at higher concentrations of 4 and 8 mM the total sperm motility was reduced and sperm lipid peroxidation was increased (Fattah *et al*., 2017). Another chicken semen cryopreservation study (Partyka *et al*., 2017) has indicated that L-carnitine at 1 or 5 mM improved post-thaw live sperm percentage without lipid peroxidation, high mitochondrial potential and reduced sperm ratio with DNA fragmentation. In both the earlier studies effect of L-carnitine on fertility from cryopreserved semen was not documented. In human semen cryopreservation, inclusion of L-carnitine improved post-thaw sperm motility and viability but had no effect on sperm DNA oxidation (Banihani *et al*., 2014). In contrast, Duru *et al*. (2000) had reported no effect of acetyl L-carnitine supplementation on post-thaw human sperm motility or membrane integrity. Semen extender supplemented with L-carnitine (2 mM) had no effect on post-thaw sperm motility and pregnancy rate in bovine (Sarıözkan *et al*., 2014). Thus, the beneficial effects of L-carnitine supplementation during semen cryopreservation were not observed across the species or vary with the concentration in chicken. L-carnitine plays important role in β-oxidation of long-chain free fatty acids in mitochondria, wherein L-carnitine transports free fatty acids and derivative of acyl-CoA into mitochondria. The production of ATP and supply of energy for movement of sperm is modulated by L-carnitine. In the present experiment MTT dye reduction test indicating mitochondrial activity was not altered by supplementation of L-carnitine. Furthermore, seminal plasma lipid peroxidation was also not reduced in the L-carnitine supplemented group. The differing results from other reports on L-carnitine in chicken may be due to a difference in breed, diluent or cryopreservation protocols employed (Abouelezz *et al*., 2015; Long, 2006).

In the present study L-glycine in the cryodiluent mixture did not provide any additional advantages in terms of post-thaw semen parameters or fertility. There is no published literature on the use of L-glycine in chicken semen cryopreservation process for comparison. In the striped bass fish sperm L-glycine at all concentrations tested (25 -100 mM) was found to increase the post-thaw sperm motility (He and Woods, 2003). Glycine has also been used as a component in the red jungle fowl semen extender (RFE) and a fertility of 57 % was reported (Rakha *et al*., 2016). The 5 mM concentration of L-glycine tested in the present study was similar to the concentration present in the RFE diluent, however, this as well as higher concentration in the present study resulted in a deleterious effect on post-thaw sperm motility and live sperm. Earlier reports have indicated that the cryoprotective action by amino acids was manifested at lower

concentrations (Kundu *et al*., 2001; Khalili *et al*., 2010). In the biosynthetic process of glutathione in the cell glycine as well as glutamate is involved (Wu *et al*., 2009) and Rakha *et al*. (2016) had suggested that the presence of higher level of glycine might have supported the Indian red jungle fowl sperm against lipid peroxidation of sperm plasma membrane during cryopreservation process. In the present study, there was no difference in seminal plasma lipid peroxidation in treatments having glycine, when compared with control or 4 % DMSO group. The exact mechanism of cell protective action of glycine is not known. It was suggested that small neutral amino acids, such as glycine, stabilize cell membrane protein tertiary structure through their physicochemical effects; furthermore, their metabolism is not required for producing beneficial effects (Baines *et al*., 1990). Similarly, another study suggested that by interaction with the phosphate groups in the sperm plasma membrane phospholipids amino acids could form a layer on the sperm surface (Anchordoguy *et al*., 1988). Glycine inclusion in striped bass semen cryopreservation had increased the sperm mitochondrial function and ATP content (He and Woods, 2003). The authors have quoted two hypothesis for higher mitochondrial function and ATP content; glycine after crossing sperm plasma membrane provides a positive effect on mitochondria (Flipse, 1956) or glycine binding to its receptors on plasma membrane triggers signal transduction that finally protects mitochondrial function and sperm ATP content.

In the present study, there was no improvement in the sperm mitochondrial activity in the glycine supplemented treatments in contrast to that reported on striped bass. Though glycine receptor on sperm membrane in other species has been reported with a role in acrosome reaction (Melendrez and Meizel, 1995), their presence in rooster sperm is yet to be documented.

CONCLUSION

In conclusion, results of the present study indicated that addition of L-carnitine and L-glycine to cryopreservation mixture did not improve post-thaw semen quality and fertility in chicken. Therefore inclusion of these compounds during chicken semen cryopreservation may not provide any additional advantage during the cryopreservation process.

Conflict of Interest

None of the authors have any conflict of interest to declare.

REFERENCES

Abouelezz, F. M. K., Castaño, C., Toledano-Díaz, A., Esteso, M. C., López-Sebastián, A., Campo, J. L. & Santiago-Moreno, J. 2015. Effect of the interaction between cryoprotectant concentration and cryopreservation method on frozen/thawed chicken sperm variables. *Reproduction in Domestic Animals*, 50(1), 135–141.

Agarwal, A. & Said, T. M. 2004. Carnitines and male infertility. *Reproductive Biomedicine Online*, 8(4), 376–384.

Aliabadi, E., Jahanshahi, S., Talaei-Khozani, T. & Banaei, M. 2018. Comparison and evaluation of capacitation and acrosomal reaction in freeze-thawed human ejaculated spermatozoa treated with L-carnitine and pentoxifylline. *Andrologia*, 50(2). https://doi: 10.1111/and.12845

Anchordoguy, T., Carpenter, J. F., Loomis, S. H. & Crowe, J. H. 1988.Mechanisms of interaction of amino acids with phospholipid bilayers during freezing. *Biochimica et Biophysica Acta*, 946(2), 299–306.

Assumpção, T. I., Torres Júnior, R. A. A., Sousa, M. V. & Ricart, C. A. O. 2005. Correlation between fertility and levels of protein, sugar and free amino acids in seminal plasma of Nelore bulls. *Arquivo Brasileiro de Medicina Veterináriae Zootecnia*, 57(1), 55–61.

Banihani, S., Agarwal, A., Sharma, R. & Bayachou, M. 2014. Cryoprotective effect of L-carnitine on motility, vitality and DNA oxidation of human spermatozoa. *Andrologia*, 46(6), 637–641.

Baines, A. D., Shaikh, N. & Ho, P. 1990. Mechanisms of perfused kidney cytoprotection by alanine and glycine. *American Journal of Physiology*, 259(1 Pt 2), F80–F87.

Bieber, L. L. 1988. Carnitine. *Annual Review of Biochemistry*, 57, 261–283.

Bremer, J. 1983. Carnitine--metabolism and functions. *Physiological Reviews*, 63(4), 1420–1480.

Bucak, M. N., Tuncer, P. B., Sarıözkan, S., Başpınar, N., Taşpınar, M., Çoyan, K., Bilgili, A., Akalın, P. P.,

Büyükleblebici, S., Aydos, S., Ilgaz, S., Sunguroğlu, A., & Oztuna, D. 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. *Cryobiology*, 61(3), 248–253.

Burrows, W. H. & Quinn, J. P. 1937. The collection of spermatozoa from the domestic fowl and turkey. *Poultry Science*, 16(1), 19–24.

Campbell, R. G., Hancock, J. L. & Rothschild, L. 1953. Counting live and dead bull spermatozoa. *Journal of Experimental Biology*, 30, 44–49.

Cerolini, S., Kelso, K. A., Noble, R. C., Speake, B. K., Pizzi, F. & Cavalchini, L. G. 1997. Relationship between spermatozoan lipid composition and fertility during aging of chickens. *Biology of Reproduction*, 57(5), 976–980.

Chatterjee, S. & Gagnon, C. 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Molecular Reproduction and Development*, 59(4), 451–458.

Duru, N. K., Morshedi, M., Schuffner, A. & Oehninger, S. 2000. Semen treatment with progesterone and/or acetyl-L-carnitine does not improve sperm motility or membrane damage after cryopreservation-thawing. *Fertility and Sterility*, 74(4), 715–720.

Fattah, A., Sharafi, M., Masoudi, R., Shahverdi, A., Esmaeili, V. & Najafi, A. 2017. L-Carnitine in rooster semen cryopreservation: Flow cytometric, biochemical and motion findings for frozen-thawed sperm. *Cryobiology*, 74, 48–153.

Flipse, R. J. 1956. Metabolism of glycine by bovine spermatozoa. *Science*, 124, 228.

Hazary, R. C., Chaudhuri, D. & Wishart, G. J. 2001. Application of an MTT reduction assay for assessing sperm quality and predicting fertilising ability of domestic fowl semen. *British Poultry Science*, 42(1), 115–117.

He, S. & Woods, L. C. 2003. Effect of glycine and alanine on short term storage and cryopreservation of striped bass (*Morone saxatilis*) spermatozoa. *Cryobiology*, 46(1), 17–25.

Hinton, B. T., Snoswell, A. M. & Setchell, B. P. 1979. The concentration of carnitine in the luminal fluid of the testis and epididymis of the rat and some other mammals. *Journal of Reproduction and Fertility*, 56(1), 105–111.

Hsieh, Y. Y., Chang, C. C. & Lin, C. S. 2006. Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. *International Journal of Biological Sciences*, 2(1), 23–29.

Holt, W. V. 2000. Basic aspects of frozen semen storage. *Animal Reproduction Science*, 62(1-3), 3–22.

Khalili, B., Jafaroghli, M., Farshad, A. & Paresh-Khiavi, M. 2010. The effects of different concentrations of glycine and cysteine on the freezability of Moghani ram spermatozoa. *Asian-Australasian Journal of Animal Sciences*, 23(3), 318–325.

Kundu, C. N., Das, K. & Majumder, G. C. 2001. Effect of amino acids on goat cauda epididymal sperm cryopreservation using a chemically defined model system. *Cryobiology*, 42(1), 21–27.

Kutluyer, F., Kayim, M., Öğretmen, F., Büyükleblebici, S. & Tuncer, P. B. 2014. Cryopreservation of rainbow trout *Oncorhynchus mykiss* spermatozoa: effects of extender supplemented with different antioxidants on sperm motility, velocity and fertility. *Cryobiology*, 69(3), 462–466.

Li, Z., Lin, Q., Liu, R., Xiao, W. & Liu, W. 2010. Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. *Journal of Andrology*, 31(5), 437–444.

Long, J. A. 2006. Avian Semen Cryopreservation: What Are the Biological Challenges? *Poultry Science*, 85(2), 232–236.

Melendrez, C. S. & Meizel, S. 1995. Studies of porcine and human sperm suggesting a role for a sperm glycine receptor/Cl- channel in the zona pellucida-initiated acrosome reaction. *Biology of Reproduction*, 53(3), 676–683.

Neuman, S. L., Lin, T. L. & Hester, P. Y. 2002. The effect of dietary carnitine on semen traits of white leghorn roosters. *Poultry Science*, 81(4), 495-503.

Partyka, A., Łukaszewicz, E. & Niżański, W. 2012. Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. *Theriogenology*, 77(8), 1497–1504.

Partyka, A., Rodak, O., Bajzert, J., Kochan, J. & Niżański, W. 2017. The effect of L-carnitine, hypotaurine, and taurine supplementation on the quality of cryopreserved chicken semen. *BioMedical Research International*, Article ID 7279341. https://doi.org/10.1155/2017/7279341

Qi, S. N., Zhang, Z. F., Wang, Z. Y., Yoshida, A. & Ueda, T. 2006. L-carnitine inhibits apoptotic DNA fragmentation induced by a new spin-labeled derivative of podophyllotoxin via caspase-3 in Raji cells. *Oncology Reports*, 15(1), 119–122.

Rakha, B. A., Ansari, M. S., Akhter, S., Hussain, I. & Blesbois, E. 2016. Cryopreservation of Indian red

jungle fowl (*Gallus gallus murghi*) semen. *Animal Reproduction Science*, 174, 45–55.

Renard, P., Grizard, G., Griveau, J. F., Sion, B., Boucher, D. & Le, L. D. 1996. Improvement of motility and fertilization potential of postthaw human sperm using glutamine. *Cryobiology*, 33(3), 311–319.

Sarıözkan, S., Bucak, M. N., Tuncer, P. B., Büyükleblebici, S. & Cantürk, F. 2014. Influence of various antioxidants added to TCM-199 on post-thaw bovine sperm parameters, DNA integrity and fertilizing ability. *Cryobiology*, 68(1), 129–133.

Sasaki, K., Tatsumi, T., Tsutsui, M., Niinomi, T., Imai, T., Naito, M., Tajima, A. & Nishi, Y. 2010. A method for cryopreserving semen from Yakido roosters using n-methylacetamide as a cryoprotective agent. *The Journal of Poultry Science*, 47(4), 297–301.

Silvestroni, L., Morisi, G., Malandrino, F. & Frajese, G. 1979. Free amino acids in semen: measurement and significance in normal and oligozoospermic men. *Archives of Andrology*, 2(3), 257–261.

Taneja, G. C. & Gowe, R. S. 1961. Spermatozoa concentration in the semen of two breeds of fowl estimated by three different methods. *Poultry Science*, 40(3), 608–615.

Trimeche, A., Yvon, J. M., Vidament, M., Palmer, E. & Magistrini, M. 1999. Effects of glutamine, proline, histidine and betaine on post-thaw motility of stallion spermatozoa. *Theriogenology*, 52(1), 181–191.

Wu, G., Bazer, F. W., Davis, T. A., Kim, S. W., Li, P., Marc Rhoads, J., Carey Satterfield, C., Smith, S. B., Spencer, T. E. & Yin, Y. 2009. Arginine metabolism and nutrition in growth, health and disease. *Amino Acids*, 37(1), 153–168.

Zhai, W., Neuman, S. L., Latour, M. A. & Hester, P. Y. 2007. The effect of L-Carnitine on semen traits of white leghorns. *Poultry Science*, 86(10), 2228–2235.