Can Tho University Journal of Science
website: sj.ctu.edu.vn

DOI: 10.22144/ctu.jen.2017.026

Metformin - An agent stimulating motility and acrosome reaction in chicken sperm

Nguyen Thi Mong Diep
Faculty of Biology-Agricultural Engineering, Quy Nhon University, Vietnam

Article info.

Received 27 Aug 2016
Revised 10 Dec 2016
Accepted 29 Jul 2017

Keywords

Acrosome reaction, AMPK, chicken sperm, Metformin, sperm motility

ABSTRACT

Sperms have main functions of motility and acrosome reaction, they promote their essential role of the oocyte fertilization. Currently, many chemicals are added to the media to enhance sperm quality during artificial insemination. Metformin, commonly used for the treatment of type II diabetes, possesses properties impacting cell metabolism control that has not been assessed yet in sperm. The aims of this experiment were to determine the effects of Metformin on fresh chicken sperm motility and ability to perform acrosome reaction, and evaluate Metformin's effects on the functions of cryopreserved sperms. Theresults showed that the presence of Metformin in fresh semen has a positive impact on the quality of sperms and helps reducing the gradual decline in sperm motility caused by cryopreservation.

Cited as: Diep, N.T.M., 2017. Metformin - An agent stimulating motility and acrosome reaction in chicken sperm. Can Tho University Journal of Science. Vol 6: 47-55.

1 INTRODUCTION

Metformin (*1,1-diMetforminhylbiguanide hydrochloride*) is a major actor in the treatment of type II diabetes, and it is the only drug of the biguanide class currently used. It promotes insulin-stimulated glucose uptake in muscle (Bailey, 1993) and lowers hepatic glucose output (Hundal*et al.*, 1992). It also affects lipid metabolism, lowering plasma triglycerides (Cusi*et al.*, 1996), and free fatty acids (Abbasi *et al.*, 1997), the latter possibly due to inhibition of catecholamine-stimulated lipolysis (Flechtner-Mors *et al.*, 1999). In addition, it has also been shown that Metformin can be used as an antineoplastic agent. Indeed, Metformin restricts the growth and proliferation of various neoplastic cells both in vitro and in vivo. These results were described in different tumors, such as bladder neoplastic cells (Zhang *et al.*, 2013), gastric (Kato *et al.*, 2012), ovarian (Shank *et al.*, 2012; Rattan *et al.*, 2011; Yasmeen *et al.*, 2011), pulmonary adenocarcinoma (Wu *et al.*, 2010), endometrial (Cantrall*et al.*, 2010), prostate and colon cancer (Zakikhani*et al*, 2008), and different types of breast cancer (Zakikhani*et al.*, 2010; 2006; Hirsch *et al.*, 2009). Metformin can also be used in the treatment of an ovulatory infertility in women (Palomba*et al.*, 2006), inducing ovulation and increasing pregnancy rates (Creanga*et al.,* 2008; Lord *et al.*, 2003). However, if the effects of Metformin on several organs have been broadly studied, little is known about its effects on the male germinal cells.

Cryopreservation is the most convenient technique for the long-term storage of sperms. It is a valuable technique used to conserve precious genetic materials for domestic and endangered species and manage infertility in humans. However, despite the advancements made over the years, in most species, the post-thaw quality and function of sperm are impaired when compared with fresh sperms (Curry, 2000; Watson, 2000; Neild *et al.*, 2005; Morris *et al.*, 2012). Cryopreservation causes permanent damage to sperms such as loss of motility, reduced DNA integrity, damage to the acrosome and plasma membrane, and apoptosis (Curry, 2000; Watson, 2000; Neild *et al.*, 2005; Morris *et al.*,

2012). This is why the extender composition and the nature of the external cryoprotectant compounds are of critical importance for sperm survival following cryopreservation (Bucak*et al.*, 2013; Cordova *et al.*, 2014). Various antioxidants have been added into the cryopreservation media and have improved sperm function such as motility and membrane integrity in numerous species (Branco *et al.*, 2010; Garcez*et al.*, 2010; Chhillar*et al.*, 2012).

AMPK (5' adenosine monophosphate-activated protein kinase) is the downstream component of a protein kinase cascade that acts as a sensor of cellular energy charge. AMPK activation stimulates catabolic pathways that produce adenosine triphosphate (ATP) and simultaneously inhibits ATP-consuming anabolic pathways, thus adjusting the cellular energy balance (Hardie and Hawley, 2001; Hardie*et al.*, 2003, 2006). AMPK is a heterotrimeric protein consisting of a catalytic α-subunit and two regulatory subunits, β and γ, with different species and tissue-specific isoforms. AMPK is phosphorylated by upstream kinases, including STK11 (LKB1), Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) and TAK1 (Woods *et al.*, 2003; 2005; Momcilovic*et al.*, 2006). AMPK is also activated by Metformin (Zhou *et al.*, 2001). Recently, AMPK has been shown in sperms and its activation affects the sperm quality in some species such as boar, mouse, stallion, or chicken. Metformin improves the quality of boar (Hurtado de Llera*et al.*, 2012) and mice frozen-thawed sperm (Bertoldo*et al.*, 2014) through AMPK activation. However, the role of Metformin in chicken sperm has not been thoroughly studied. Based upon these interesting characteristics of Metformin, the study evaluated its influence on sperm quality before and after cryopreservation by adding it directly into semen.

2 MATERIALS AND METHODS

Chemicals and reagents

All chemicals were from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise noted. Metformin (1,1-dimethylbiguanide hydrochloride) was from Calbiochem (Billerica, Massachusetts). Stock solutions of Metformin were prepared in deionized water. Complete mini EDTA-free, protease inhibitor cocktail tablets were from Roche diagnostics (Mannheim, Germany). Tris/glycine buffer (10X), Tris/glycine/SDS buffer (10X), and Precision Plus Protein All Blue Standards (Catalog #161-0373) were from Bio-Rad (Hercules, California) and anti-AMPKα from Millipore (Billerica, MA), anti-phospho-Thr172-AMPKα and anti-rabbit IgG (H+L) (DyLight 680 Conjugate) antibodies from Cell Signaling technology, Inc (Danvers, MA). SYBR-14/PI (LIVE/DEAD sperm viability kit) was from Molecular Probes (Saint Aubin, France). The LPO-586 kit was from Oxis Research (Burlingame, CA, US).

Animals

The animals used were 28-55-week-old adult *Gallus domesticus* at the Unit Poultry Experimentation of National Institute of Agricultural Research (INRA) in Tours, France. All the animals were housed in individual battery cages under a 14L/10D photoperiod and fed a standard diet of 12.5 MJ/day.

Semen collection

Semen was routinely collected twice a week by the abdominal massage method (Burrows and Quinn, 1937). Sperm concentration was determined by light absorption of semen with a photometer (IMV, L'Aigle, France) at a wavelength of 530 nm. The semen was gently mixed after collection from each male and split into two groups for the fresh and frozen treatments. Fresh sperms were diluted in Beltsville Poultry Semen Extender (BPSE) (Sexton, 1977) to get a final sperm concentration of 1 x 10^9 cells/ml. For all experiences with fresh sperms, sperm samples were incubated in the presence or absence of different doses of Metformin (0.5, 1, 2 and 5mM). Then, the concentrations affecting sperm parameters (viability, motility, and acrosome reaction) were chosen in the most positive way to perform the experiments on frozen sperms.

Sperm cryopreservation

The semen was diluted 1:1 with Lake PC in the presence or absence of 1mM Metformin and 11% glycerol based cryoprotectant in Lake PC (Lake, 1978). The diluted semen and cryoprotectant were then equilibrated for 10min at 4°C. After equilibration, the semen was transferred to 0.5 ml plastic freezing straws (IMV, L'Aigle, France) which were sealed and finally frozen from +4 to -35°C at -7°C/min and from -35 to -140°C at -20°C/min using a programmable Minidigitcool 1400 freezer (IMV, L'Aigle, France). The straws were then plunged into liquid nitrogen (-196°C).

Thawing procedures

Sperms were thawed for 4 minutes in a water bath adjusted to 4°C. After thawing, the straws were quickly opened and semen transferred to a glass beaker. Semen was progressively diluted (6 times 2 minutes) with Lake PC (Lake, 1978) at 4°C to final dilution of 1:19. Glycerol was removed by centrifugation (15 minutes at 700 G, 4°C). After removal

of the supernatants, the resulting pellets were re-suspended in 100 ml of Lake PC (Lake, 1978). Concentration of sperm was estimated at a wavelength of 530 nm. Concentrations were close to 1 x 10^9 cells/ml.

Sperm viability

SYBR-14/PI was used to assess sperm membrane integrity before freezing and after thawing. The red fluorescence from PI shows dead sperms while the green fluorescence from SYBR-14 shows those whose plasma membrane is intact (PMI), which are therefore alive. A total of 300 sperms per slide were counted (two slides/sample = 1 replicate) under fluorescence microscopy (Zeiss Axioplan 2; Zeiss Gruppe, Jena, Germany) and a total of six replicates/treatment examined. All preparations were analyzed by the same observer.

Analysis of sperm motility by computer-assisted sperm analysis (CASA) system

The sperm motility parameters were evaluated by the computer-assisted sperm analysis (CASA) system with an HTM-IVOS (Hamilton-Thorn Motility Analyzer, IVOS) (Blesboiset *al*, 2008). In this experiment, the parameters measured were percentage of motile sperm (%), and rapid cells (percentage of motile sperm with VAP > 50µm/s, in %).

Acrosome reaction (AR) assessment with FITC-PNA

The completion of the acrosome reaction was detected by FITC-conjugated peanut agglutinin (FITC-PNA) binding (Horrocks*et al.*, 2000). The sperms having completed their acrosome reaction were identified and counted under fluorescence microscopy (Zeiss Axioplan 2; Zeiss Gruppe, Jena, Germany). A minimum of 100 sperms was counted for each sample (two slides/sample = 1 replicate) and a total of six replicates/treatment examined. Acrosome reaction was characterized by the green fluorescence of the acrosomal region. All preparations were analyzed by the same observer.

Western–Blotting

For western-blotting experiments, total proteins from chicken sperm were extracted in lysis buffer (10mM Tris, 150mM NaCl, 1mM EGTA, 1mM EDTA, 100mM sodium fluoride, 4mM sodium pyrophosphate, 2mM sodium orthovanadate, 1% Triton X-100, 0.5% NP40 containing a protease inhibitor cocktail with EDTA). Cell lysates were centrifuged at 12000g for 30minutes at 4°C and the protein concentration in each supernatant was determined by a colorimetric assay (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA). The proteins were then separated by 10% SDS-PAGE (SDS Polyacrylamide Gel Electrophoresis) and transferred onto nitrocellulose membrane (Whatman Protran,Dassel, Germany). Afterwards, the membranes were incubated in anti-phospho-Thr172AMPKα (62kDa) or in anti-total AMPKα (62kDa) diluted in 5% BSA in TBS-Tween 0.1% as primary antibodies (final dilution 1:2000) overnight at 4°C. Finally, the membranes were further incubated for one hour in (HRP)-conjugated secondary goat anti-rabbit antibody (final dilution 1:2000). The intensity of bands in the signal was analyzed using Odyssey Software, version 1.2 (LICOR Biosciences, Lincoln, Nebraska, USA).

Statistical analyses

Differences between treatments were analyzed by 1-way ANOVA and Bonferroni's multiple comparisons using GraphPad Prism version 5.0d for Mac (GraphPad Software, San Diego, CA). The minimum level of significance retained was $P < 0.05$.

3 RESULTS

3.1 Metformin significantly increasing motile sperm percentage

To evaluate the effect of Metformin on fresh sperm motility, sperms were incubated in BPSE for 30 minutes without or with Metformin at different concentrations (0, 0.5, 1.0, 2.0, and 5.0mM). As observed in Figure 1, the percentage of sperm motility (Figure 1A) and rapid sperm (Figure 1B) is significantly reduced after 30minutes of incubation compared with the positive control (Ctrl) which has not undergone incubation However, after having treated the sperms with Metformin, the sperm motility and rapid sperm parameters significantly increased (by about 41%) with 1mM Metformin, but did not change with other concentrations. Moreover, the percentage of rapid sperm tended to significantly decrease with increasing concentration of Metformin (5mM Metformin, $P = 0.04$) compared with other concentrations of Metformin, but there was no difference compared to Ctrl (Figure 1B).

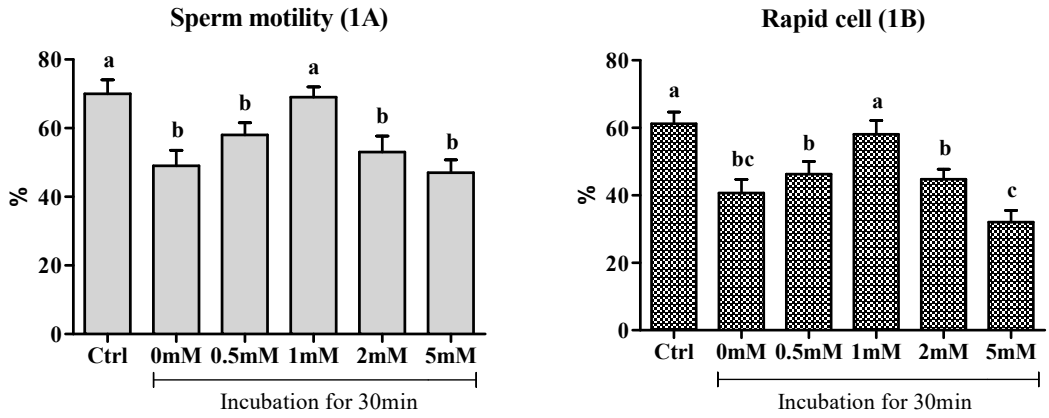

Fig. 1: Effect of Metformin on sperm motility (2A) and rapid sperm (2B)

Values are means ± SEM (n = 10). Different letters above bars indicate values that were statistically significantly different at P < 0.05

3.2 Metformin significantly increasing the percentage of sperm viability and acrosome reaction

The effect of Metformin in sperm viability was studied in order to correlate it with motility studies and to know whether Metformin treatment might cause side effects that lead to germ cells death. According to theresults in Figure 2A, sperm viability is sensitive to time of incubation: after 30minutes, the percentage of sperm viability significantly decreases compared with positive control. However, sperm viability of incubation after 30 minutes was greatly reduced in the presence of 1mM Metformin compared with control without Metformin; while the other Metformin doses did not affect sperm viability.

The ability of spermatozoa to undergo the acrosome reaction was also negatively affected after 30minutes of incubation for the positive control as well as for the treated samples ($P < 0.01$). However, the acrosome reaction rate was significantly increased by Metformin at 0.5mM (mean increase ~ 22%), at 1mM (mean increase ~ 39%) and at 2mM (mean increase ~ 17%) compared with the control (Figure 2B).

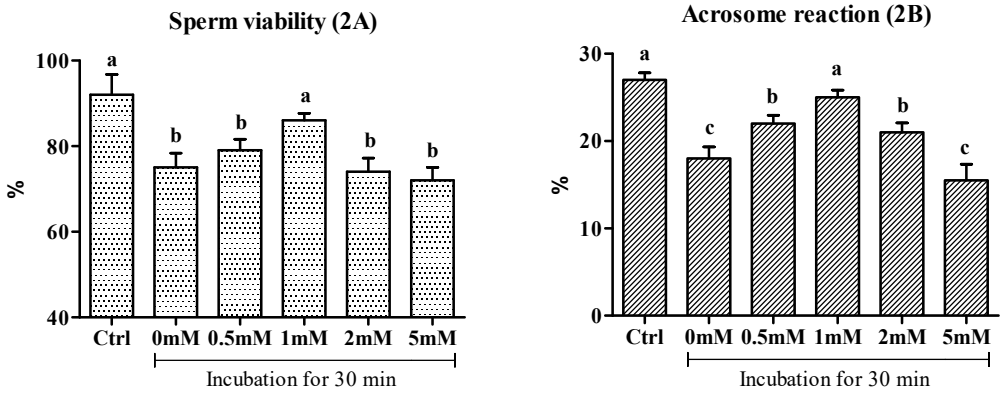

Fig. 2: Effect of Metformin on the sperm viability (2A) and acrosome reaction (2B)

Values are means ± SEM (n = 10). Different letters above bars indicate values that were statistically significantly different at P < 0.05

3.3 Effect of Metformin supplementation in cryopreservation media on spermatozoa

Based on the results obtained from experiments with fresh sperms and according to the observation, 1mM Metformin is the most effective dose to improve chicken sperm quality. Therefore, the potential effect of 1mM Metformin on cryopreserved sperm was tested.

Sperm parameters were assessed 15minutes after thawing. The results show that the sperm viability

of the samples treated with Metformin slightly increased (by 10%) compared with control without Metformin (Figure 3A). In addition, results obtained for motility were higher than those of the control without Metformin. The percentage of motile sperm treated with Metformin increased by 23% compared with control without Metformin (Figure 3B).

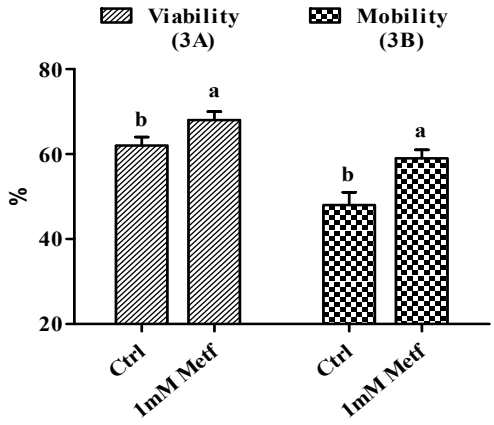

Fig. 3: Effect of Metformin on the frozen-thawed sperm viability (3A) and motility (3B)

Values are means ± SEM (n = 6). Different letters above bars indicate values that were statistically significantly different at $P < 0.05$.

3.4 Phosphorylation of AMPK in frozen/thawed spermatozoa after Metformin treatment

Western-blot analyses using antibodies against phospho-Thr172-AMPKα and total AMPKα (as control) were performed on chicken sperm incubated in the absence or presence of 1mM Metformin during freezing and thawing (Figure 4). The AMPK phosphorylation was increased by 30% with Metformin after the freeze-thaw process compared with control without Metformin.

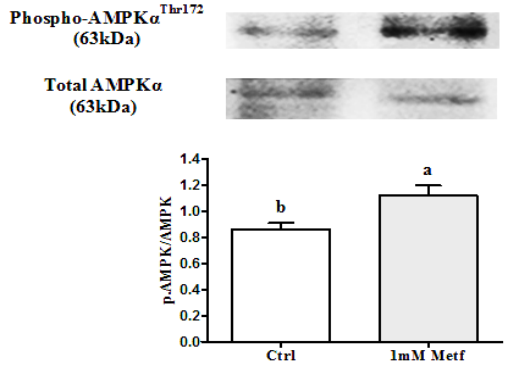

Fig. 4: Effects of Metformin on AMPK phosphorylation in frozen-thawed chicken sperm

Sperm lysates were prepared and resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-Thr172-AMPKα and anti-AMPKα antibody. Bands for phospho-Thr172-AMPKα were detected at 63kDa (top lanes). Total AMPKα was used as loading control (63kDa) (bottom lanes) and the phosphorylated protein AMPKα (Thr172)/total AMPKα ratio is shown at the bottom. Cryopreserved sperms were either treated in the presence of 1mM Metformin (in dark gray) or without anything for the Ctrl in white. Values represent means ± SEM from 6 different experiments. Different superscripts indicate significant differences between Ctrl and Metformin in frozen-thawed semen ($P<0.05$).

4 DISCUSSION

There are few reports assessing the effects of Metformin on the viability of fresh or cryopreserved spermatozoa in vitro. This study figured that treatment of fresh or cryopreserved chicken spermatozoa with Metformin presents a beneficial effect on motility, viability, and acrosome reaction. The results obtained with fresh spermatozoa in the present study differed from those of Hurtado de Llera *et al.* (2012) who observed a partial reduction in motility of boar spermatozoa following 5mM treatment with Metformin in fresh spermatozoa for two hours. Furthermore, they reported a complete inhibition with very high concentrations (between 10 and 20 mM). Another study of Bertoldo *et al.* (2014) also showed that treatment of fresh mouse spermatozoa with 5mM Metformin decreased sperm motility, but not sperm viability.

In this study, 1mM Metformin treatment leads to a significant increase inthe percentage of viable, motile, and rapid spermatozoa (VAP >50 µm/s). However, Metformin with a high concentration of 5mM does not affect sperm motility and viability but causes a significant reduction of the number of rapid spermatozoa. Moreover,the spermatozoa acrosome reaction is affected by Metformin. The acrosome reaction occurs by fusion of the sperm head cytoplasmic membrane and the underlying outer acrosomal membrane, so that the acrosome content is released (Okamura and Nishiyama, 1978). In most mammalian species, acrosome reaction occurs only in capacitated spermatozoa (Yanagimachi, 1994; Baldi *et al.*, 2000), and capacitation requires specific environments and different substrates. But in chicken spermatozoa, the acrosome reaction can be induced very rapidly in vitro (Horrocks *et al.*, 2000) without previous capacitation (Lemoine *et al.*, 2008). Unlike in mice sperm (Bertoldo *et al.*, 2014), there is no modification in acrosome reaction by Metformin;

the study indicates that Metformin significantly increases chicken sperm acrosome reaction at 0.5, 1 and 2mM but not 5mM, which means that the effect of Metformin is not the same in all animal species.

Thestudy is also the first showing a positive effect of Metformin on the capacity of mature sperm to restore their biological functions after cryopreservation. Metformin indeed improved sperm motility, acrosome reaction and viability in frozen-thawed chicken sperm. These results differ from those obtained with stallion sperm, where Metformin did not affect sperm viability and motility after cryopreservation (Cordova *et al.*, 2014). However, in addition to the use of highly different doses of Metformin in the two studies, the work on stallion sperm by Cordova *et al.* in 2014 used a very specific hypo-metabolic medium of sperm storage, with restricted access to energetic substrate that greatly limits the potential comparisons with thisstudy. In accordance with a previous study on epididymal mice sperm (Bertoldo *et al.*, 2014), Metformin showed a low but significant positive effect on sperm viability after cryopreservation.

In order to explain the positive action of Metformin on fresh and frozen sperm functions, this studyinvestigated the effects of Metformin on AMPK phosphorylation. Recently, it showed the presence of the AMPKα protein in chicken sperm. AMPK presence in the acrosome, midpiece and flagellum of chicken sperm is in relation toits possible function in sperm motility and acrosome reaction process (Nguyen *et al.*, 2014). In this study, an increased AMPK phosphorylation in frozen/thawed sperms with 1mM Metformin was measured. This indicates that the positive action of Metformin on chicken sperm functions is done through AMPK activation. Metformin was known as an indirect activation of AMPK which inhibits complex I of the mitochondrial respiratory chain, suggesting an AMPK activation through the increase of the AMP/ATP ratio (Owen *et al.*, 2000). As presented, AMPK protein acts as a sensor that detects the cell energy state and subsequently regulates metabolism; when AMPK becomes activated it stimulates catabolic pathways that produce ATP and simultaneously inhibits ATP-consuming anabolic pathways. Therefore, thedata strongly suggest that AMPK phosphorylation has a central role in regulating the improvement of metabolic functions and ATP production needed to ensure high energy consuming process such as sperm motility and acrosome reaction.

However it is possibile that Metformin is a molecule of the biguanide family, and has the ability to decrease reactive oxygen species (Ouslimani *et al.*, 2005; Piwkowska *et al.*, 2010; Esteghamati *et al.*, 2013) and to activate the transcription factor to increase expression of antioxidant genes (Onken and Driscoll, 2010). Sperm membranes are enriched in polyunsatured fatty acids in mammalian (Dandekar *et al.*, 2002) and bird species (Blesbois and Hermier, 2003), sperms are very susceptible to lipid peroxidation (LPO) with subsequent alterations of structure and functions (Griveau *et al.*, 1995). Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase and catalase are the major antioxidant enzymes naturally presenting in mammalian and bird semen to protect sperm from lipid peroxidation and to maintain its integrity against ROS (Surai *et al.*, 1998). Reductions in SOD, GPx, catalase activities, and increases in ROS and lipid peroxidation have been shown after chicken sperm cryopreservation (Partyka *et al.*, 2012). Previous studies have provided evidence that Metformin exerts an anti-inflammatory effect on non-alcoholic steato-hepatosis mice by impeding depletion in GPx, SOD, and catalase, and by decreasing ROS and MDA (Buldak*et al.*, 2014). Metformin could also directly reduce ROS production via inhibition of complex I. Indeed, the inhibition of complex I by Metformin is known to reduce the number of electrons entering the electron transport chain, thus blocking NADH oxidation by complex I (Piwkowska *et al.*, 2010), and therefore reducing ROS production by both complex I and III (Ouslimani*et al.*, 2005). It suggests that the impact of Metformin on sperm quality is made through both AMPK-dependent and AMPK-independent pathways (Kita *et al.*, 2012).

5 CONCLUSIONS

Theresults demonstrate that Metformin increases the quality of fresh chicken sperm. Furthermore, chicken sperm has improved post-thaw motility and viability in the presence of Metformin. This is the first assessment of the effect of Metformin on chicken sperm through their influence on AMPK activity to reduce cryopreservation damages in avian sperm. Such data will most certainly be helpful to develop and improve semen handling and storage techniques in the near future.

ACKNOWLEDGEMENTS

This study was performed with the financial support of the French Agence Nationale de la Recherche (http://www.agence-nationale-recherche

.fr/), INRA (http://www.inra.fr), and the French National Science Infrastructure CRB-Anim funded by “Investissements d’avenir”, ANR-11-INBS-0003 (http://www.crb-anim.fr). Special thanks would be sent to Elisabeth Blesbois, Isabelle Grasseau, Sabine Alves (INRA) forhelp regarding the methodology and discussions, and the staff of the INRA experimental unit PEAT for the animal breeding and participation in semen collections.

REFERENCES

Abbasi, F., Kamath, V., Rizvi, A.A., Carantoni, M., Chen, Y.D., Reaven, G.M., 1997. Results of a placebo-controlled study of the metabolic effects of the addition of metformin to sulfonylurea-treated patients. Evidence for a central role of adipose tissue. Diabetes care. 20: 1863-1869.

Bailey, C.J., 1993. Metformin--an update. General pharmacology. 24: 1299-1309.

Baldi, E., Luconi, M., Bonaccorsi, L., Muratori, M., Forti, G., 2000. Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. Front Biosci. 5: E110-123.

Bertoldo, M.J., Guibert, E., Tartarin, P., Guillory, V., Froment, P., 2014. Effect of metformin on the fertilizing ability of mouse spermatozoa. Cryobiology. 68: 262-268.

Blesbois, E., Grasseau, I., Seigneurin, F., Mignon-Grasteau, S., Saint Jalme, M., Mialon-Richard, M.M., 2008. Predictors of success of semen cryopreservation in chickens. Theriogenology. 69: 252-261.

Blesbois, E., Hermier, D., 2003. Specificity of fatty acids in domestic birds spermatozoa. In:Vriese,D. (Eds).Male Fertility and Lipid Metabolism.Champaign IL. American Oil Chemist’s Society Press,pp: 73-85.

Branco, C.S., Garcez, M.E., Pasqualotto, F.F., Erdtman, B., Salvador, M., 2010. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology. 60: 235-237.

Bucak, M.N., Keskin, N., Taspinar, M., Coyan, K., Baspinar, N., Cenariu, M.C., Bilgili, A., Ozturk, C., Kursunlu, A.N., 2013. Raffinose and hypotaurine improve the post-thawed Merino ram sperm parameters. Cryobiology. 67: 34-39.

Buldak, L., Labuzek, K., Buldak, R.J., Kozlowski, M., Machnik, G., Liber, S., Suchy, D., Dulawa-Buldak, A., Okopien, B., 2014. Metformin affects macrophages' phenotype and improves the activity of glutathione peroxidase, superoxide dismutase, catalase and decreases malondialdehyde concentration in a partially AMPK-independent manner in LPS-stimulated human monocytes/macrophages. Pharmacological reports. 66: 418-429.

Burrows, W.H., Quinn, J.P., 1937. The collection of spermatozoa from the domestic fowl and turkey. Poultry Science. 16: 19-24.

Creanga, A.A., Bradley, H.M., McCormick, C., Witkop, C.T., 2008. Use of metformin in polycystic ovary syndrome: a meta-analysis. Obstet. Gynecol. 111, 959-968.

Chhillar, S., Singh, V.K., Kumar, R., Atreja, S.K., 2012. Effects of Taurine or Trehalose supplementation on functional competence of cryopreserved Karan Fries semen. Animal reproduction science. 135: 1-7.

Cordova, A., Strobel, P., Vallejo, A., Valenzuela, P., Ulloa, O., Burgos, R.A., Menarim, B., Rodriguez-Gil, J.E., Ratto, M., Ramirez-Reveco, A., 2014. Use of hypometabolic TRIS extenders and high cooling rate refrigeration for cryopreservation of stallion sperm: presence and sensitivity of 5' AMP-activated protein kinase (AMPK). Cryobiology. 69: 473-481.

Curry, M.R., 2000. Cryopreservation of semen from domestic livestock. Reviews of reproduction. 5: 46-52.

Cusi, K., Consoli, A., DeFronzo, R.A., 1996. Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. The Journal of clinical endocrinology and metabolism. 81: 4059-4067.

Dandekar, S.P., Nadkarni, G.D., Kulkarni, V.S., Punekar, S., 2002. Lipid peroxidation and antioxidant enzymes in male infertility. Journal of postgraduate medicine. 48 : 186-189.

Esteghamati, A., Eskandari, D., Mirmiranpour, H., Noshad, S., Mousavizadeh, M., Hedayati, M., Nakhjavani, M., 2013. Effects of metformin on markers of oxidative stress and antioxidant reserve in patients with newly diagnosed type 2 diabetes: a randomized clinical trial. Clinical nutrition. 32: 179-185.

Flechtner-Mors, M., Ditschuneit, H.H., Jenkinson, C.P., Alt, A., Adler, G., 1999. Metformin inhibits catecholamine-stimulated lipolysis in obese, hyperinsulinemic, hypertensive subjects in subcutaneous adipose tissue: an in situ microdialysis study. Diabet Med. 16(12): 1000-1006.

Garcez, M.E., dos Santos Branco, C., Lara, L.V., Pasqualotto, F.F., Salvador, M., 2010. Effects of resveratrol supplementation on cryopreservation medium of human semen. Fertility and sterility. 94: 2118-2121.

Griveau, J.F., Dumont, E., Renard, P., Callegari, J.P., Le Lannou, D., 1995. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. Journal of reproduction and fertility. 103: 17-26.

Hardie, D.G., Hawley, S.A., 2001. AMP-activated protein kinase: the energy charge hypothesis revisited. BioEssays. 23(12): 1112-1119.

Hardie, D.G., Hawley, S.A., Scott, J.W., 2006. AMP-activated protein kinase--development of the energy sensor concept. The Journal of physiology. 574: 7-15.

Hardie, D.G., Scott, J.W., Pan, D.A., Hudson, E.R., 2003. Management of cellular energy by the AMP-

activated protein kinase system. FEBS letters. 546: 113-120.

Hirsch, H.A., Iliopoulos, D., Tsichlis, P.N., Struhl, K., 2009. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer research. 69: 7507-7511.

Horrocks, A.J., Stewart, S., Jackson, L., Wishart, G.J., 2000. Induction of acrosomal exocytosis in chicken spermatozoa by inner perivitelline-derived N-linked glycans. Biochemical and biophysical research communications. 278: 84-89.

Hundal, H.S., Ramlal, T., Reyes, R., Leiter, L.A., Klip, A., 1992. Cellular mechanism of metformin action involves glucose transporter translocation from an intracellular pool to the plasma membrane in L6 muscle cells. Endocrinology. 131: 1165-1173.

Hurtado de Llera, A., Martin-Hidalgo, D., Gil, M.C., Garcia-Marin, L.J., Bragado, M.J., 2012. AMP-activated kinase AMPK is expressed in boar spermatozoa and regulates motility. PloS one. 7: e38840.

Kato, K., Gong, J., Iwama, H., Kitanaka, A., Tani, J., Miyoshi, H., Nomura, K., Mimura, S., Kobayashi, M., Aritomo, Y., Kobara, H., Mori, H., Himoto, T., Okano, K., Suzuki, Y., Murao, K., Masaki, T., 2012. The antidiabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. Molecular cancer therapeutics. 11: 549-560.

Kita, Y., Takamura, T., Misu, H., Ota, T., Kurita, S., Takeshita, Y., Uno, M., Matsuzawa-Nagata, N., Kato, K., Ando, H., Fujimura, A., Hayashi, K., Kimura, T., Ni, Y., Otoda, T., Miyamoto, K., Zen, Y., Nakanuma, Y., Kaneko, S., 2012. Metformin prevents and reverses inflammation in a non-diabetic mouse model of nonalcoholic steatohepatitis. PloS one. 7: e43056.

Lake, P.E., Stewart, J.M., 1978. Preservation of fowl semen in liquid nitrogen-an improved method. British poultry science. 19: 187-194.

Lemoine, M., Grasseau, I., Brillard, J.P., Blesbois, E., 2008. A reappraisal of the factors involved in in vitro initiation of the acrosome reaction in chicken spermatozoa. Reproduction. 136: 391-399.

Lord, J.M., Flight, I.H., Norman, R.J., 2003. Metformin in polycystic ovary syndrome: systematic review and meta-analysis. Bmj. 327: 951-953.

Momcilovic, M., Hong, S.P., Carlson, M., 2006. Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. The Journal of biological chemistry. 281: 25336-25343.

Morris, G.J., Acton, E., Murray, B.J., Fonseca, F., 2012. Freezing injury: the special case of the sperm cell. Cryobiology. 64: 71-80.

Neild, D.M., Brouwers, J.F., Colenbrander, B., Aguero, A., Gadella, B.M., 2005. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. Molecular reproduction and development. 72: 230-238.

Nguyen, T.M., Alves, S., Grasseau, I., Metayer-Coustard, S., Praud, C., Froment, P., Blesbois, E., 2014. Central role of 5'-AMP-activated protein kinase in chicken sperm functions. Biology of reproduction. 91: 121.Okamura, F., Nishiyama, H., 1978. The passage of spermatozoa through the vitelline membrane in the domestic fowl, Gallus gallus. Cell Tissue Res. 188: 497-508.

Onken, B., Driscoll, M., 2010. Metformin induces a dietary restriction-like state and the oxidative stress response to extend C. elegans Healthspan via AMPK, LKB1, and SKN-1. PloS one. 5: e8758.

Ouslimani, N., Peynet, J., Bonnefont-Rousselot, D., Therond, P., Legrand, A., Beaudeux, J.L., 2005. Metformin decreases intracellular production of reactive oxygen species in aortic endothelial cells. Metabolism: clinical and experimental. 54: 829-834.

Owen, M.R., Doran, E., Halestrap, A.P., 2000. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J. 348: 607-614.

Palomba, S., Orio, F., Jr., Falbo, A., Russo, T., Tolino, A., Zullo, F., 2006. Effects of metformin and clomiphene citrate on ovarian vascularity in patients with polycystic ovary syndrome. Fertility and sterility. 86: 1694-1701.

Partyka, A., Lukaszewicz, E., Nizanski, W., 2012. Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. Theriogenology. 77: 1497-1504.

Piwkowska, A., Rogacka, D., Jankowski, M., Dominiczak, M.H., Stepinski, J.K., Angielski, S., 2010. Metformin induces suppression of NAD(P)H oxidase activity in podocytes. Biochem Biophys Res Commun. 393 : 268-273.

Rattan, R., Giri, S., Hartmann, L.C., Shridhar, V., 2011. Metformin attenuates ovarian cancer cell growth in an AMP-kinase dispensable manner. J Cell Mol Med. 15: 166-178.

Sexton, T.J., 1977. A new poultry semen extender. 1. Effects of extension on the fertility of chicken semen. Poultry science. 56: 1443-1446.

Shank, J.J., Yang, K., Ghannam, J., Cabrera, L., Johnston, C.J., Reynolds, R.K., Buckanovich, R.J., 2012. Metformin targets ovarian cancer stem cells in vitro and in vivo. Gynecologic oncology. 127: 390-397.

Surai, P.F., Blesbois, E., Grasseau, I., Chalah, T., Brillard, J.P., Wishart, G.J., Cerolini, S., Sparks, N.H., 1998. Fatty acid composition, glutathione peroxidase and superoxide dismutase activity and total antioxidant activity of avian semen. Comp Biochem Physiol B Biochem Mol Biol. 120: 527-533.

Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Animal reproduction science. 60-61: 481-492.

Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., Carling, D., 2003. LKB1 is the upstream kinase in the AMP-activated

protein kinase cascade. Current biology : CB. 13: 2004-2008.

Woods, A., Dickerson, K., Heath, R., Hong, S.P., Momcilovic, M., Johnstone, S.R., Carlson, M., Carling, D., 2005. Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell metabolism. 2: 21-33.

Wu, N., Gu, H.J., Li, Q., 2010. Effects of antidiabetic drug metformin on the migration and invasion abilities of human pulmonary adenocarcinoma A549 cell line in vitro. Journal of thoracic disease. 2: 76-80.

Yanagimachi, R., 1994. Fertility of mammalian spermatozoa: its development and relativity. Zygote. 2: 371-372.

Yasmeen, A., Beauchamp, M.C., Piura, E., Segal, E., Pollak, M., Gotlieb, W.H., 2011. Induction of apoptosis by metformin in epithelial ovarian cancer: involvement of the Bcl-2 family proteins. Gynecologic oncology. 121: 492-498.

Zakikhani, M., Blouin, M.J., Piura, E., Pollak, M.N., 2010. Metformin and rapamycin have distinct effects on the AKT pathway and proliferation in breast cancer cells. Breast cancer research and treatment. 123: 271-279.

Zakikhani, M., Dowling, R., Fantus, I.G., Sonenberg, N., Pollak, M., 2006. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer research. 66: 10269-10273.

Zakikhani, M., Dowling, R.J., Sonenberg, N., Pollak, M.N., 2008. The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. Cancer prevention research. 1: 369-375.

Zhang, T., Guo, P., Zhang, Y., Xiong, H., Yu, X., Xu, S., Wang, X., He, D., Jin, X., 2013. The antidiabetic drug metformin inhibits the proliferation of bladder cancer cells in vitro and in vivo. International journal of molecular sciences. 14: 24603-24618.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J., Moller, D.E., 2001. Role of AMP-activated protein kinase in mechanism of metformin action. The Journal of clinical investigation. 108: 1167-1174.