

## IMPACT OF AMPK ACTIVATOR METFORMIN ON SPERM QUALITY

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### SUMMARY

Semen cryopreservation allows crucial management of animal genetic diversity. However, the freeze-thaw process causes biochemical and physical alterations, impairing sperm energy-dependent functions. Currently, many chemicals are added to the media to enhance frozen-thawed sperm quality during artificial insemination. The aims of this study was to determine the effects of Metformin (Metf) on fresh chicken sperm motility and ability to perform acrosome reaction, and to evaluate Metf's effects on the functions of cryopreserved sperm. Chicken semen was diluted and incubated at 35°C in media supplemented with or without different doses of 5'-AMP-Activated Protein Kinase (AMPK) activator, Metf (0,5 to 5 mM). We then looked for the concentration improving the most sperm quality to use it in the cryopreservation media used for chicken sperm. Our results show that 1 mM Metf is the concentration giving the best results regarding sperm quality. AMPK $\alpha$  phosphorylation, viability, acrosome reaction ability (AR), and various motility parameters, were negatively affected by the freeze-thaw process, and that Metf partially restored them. Sperm quality improved (mean increased by 23% for motility, by 10% for viability) as well as AMPK $\alpha$  phosphorylation (mean increased by 30%). Moreover, fluorescent intensity levels of phospho-AMPK were also stronger with Metf than in the control. These results show that the presence of Metf in fresh semen has a positive impact on the quality of sperm and helps reducing the gradual decline in sperm motility caused by cryopreservation by partially restoring several essential sperm functions, and thus leads to a better overall quality of cryopreserved sperm.

**Keywords:** Acrosome reaction, chicken sperm, sperm motility, AMPK, Metformin

### INTRODUCTION

Metformin (Metf) is a major actor in the treatment of type II diabetes, and 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 Metf can be used as an antineoplastic agent. Indeed, Metf restricts the growth and proliferation of various neoplastic cells both *in vitro* and *in vivo*. These results were described in different tumors, such as pulmonary

adenocarcinoma (Wu *et al.*, 2010), gastric (Kato *et al.*, 2012), ovarian (Rattan *et al.*, 2011; Yasmeen *et al.*, 2011; Shank *et al.*, 2012), prostate and colon cancer (Zakikhani *et al.*, 2008), bladder neoplastic cells (Zhang *et al.*, 2013) and different types of breast cancer (Zakikhani *et al.*, 2006, 2010; Hirsch *et al.*, 2009). Metf can also be used in the treatment of an ovulatory infertility in women (Palomba *et al.*, 2006), inducing ovulation and increasing pregnancy rates (Lord *et al.*, 2003; Creanga *et al.*, 2008). However, if the effects of Metf 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 sperm. It is a valuable technique used to conserve precious genetic material 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 sperm (Curry, 2000; Watson, 2000; Neild *et al.*, 2005; John Morris *et al.*, 2012). Cryopreservation causes permanent damage to sperm such as loss of motility, reduction of DNA integrity, damage to the acrosome and plasma membrane, and apoptosis (Curry, 2000; Watson, 2000; Neild *et al.*, 2005; John 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).

AMP-activated protein kinase (AMPK) 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 (Hardi, Hawley, 2001; Hardi *et al.*, 2003, 2006). AMPK is a heterotrimeric protein consisting of a catalytic  $\alpha$ -subunit and two regulatory subunits,  $\beta$  and  $\gamma$ , with different species and tissue-specific isoforms. AMPK is phosphorylated by upstream kinases, including Liver kinase B1 (LKB1 also known as serine/threonine kinase 11 (STK11)), Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase (CaMKK) and transforming growth factor beta-activated kinase 1 (TAK1) (Woods *et al.*, 2003; 2005; Momcilovic *et al.*, 2006). AMPK is also activated by Metf (Zhou *et al.*, 2001). Recently, it has been established that AMPK is present in sperm and that its activation affects the sperm quality in species such as boar, mouse, stallion, or chicken. Metf improves the quality of boar (Hurtado de Llera *et al.*, 2012) and mouse frozen-thawed sperm (Bertoldo *et al.*, 2014) through AMPK activation. However, the role of Metf in chicken sperm has not been thoroughly studied. Based upon these interesting characteristics of Metf, this study evaluates its influence on sperm quality before and after cryopreservation by adding it directly into semen.

## MATERIALS AND METHODS

### Animals

The animals used were 28-55 week-old adult *Gallus domesticus* at the Institut National de la Recherche Agronomique (INRA) in 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, Quinn, 1937). Sperm samples were prepared as described by Blesbois *et al.* (2008). Fresh sperm samples were incubated in the presence or absence of different doses of Metf (Calbiochem) (0.5, 1, 2 and 5 mM). Stock solutions of Metf were prepared in deionized water. The most efficient concentrations affecting sperm parameters (viability, motility, and acrosome reaction) were chosen to perform the experiments on frozen sperm.

### Sperm cryopreservation and thawing procedures

The semen was diluted 1:1 with Lake PC (Sigma-Aldrich) in the presence or absence of 1 mM Metf and 11% glycerol based cryoprotectant in Lake PC (Lake, 1978). The semen was cryopreserved and thawed according to the method of Seigneurin and Blesbois (1995).

### Sperm viability

SYBR-14/PI (LIVE/DEAD sperm viability kit; Molecular Probes) was used to assess sperm membrane integrity before freezing and after thawing. 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) (Blesbois *et al.*, 2008). In this experiment, the parameters measured were percentage of motile sperm (%), and rapid cells (percentage of motile sperm with VAP > 50  $\mu\text{m/s}$ , in %).

### Acrosome reaction (AR) assessment with FITC-PNA

The completion of the acrosome reaction was detected by FITC-conjugated peanut agglutinin (FITC-PNA) (Sigma-Aldrich) binding (Horrocks *et al.*, 2000). The sperm having completed their acrosome reaction were identified and counted under fluorescence microscopy. A minimum of 100 sperm 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. 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-Thr172-AMPK $\alpha$  (63 kDa) (Millipore, Billerica, MA) or in anti-total AMPK $\alpha$  (63 kDa) (Millipore, Billerica, MA) (final dilution 1:1000) overnight at 4°C. Finally, the membranes were further incubated for 1 h in (HRP)-conjugated secondary goat anti-rabbit antibody (Cell Signaling, Danvers, MA) (final dilution 1:2000). The intensity of bands in the signal was analyzed using Odyssey Software, version 1.2 (LICOR Biosciences, Lincoln, Nebraska, USA).

### Immunocytochemistry

Total AMPK $\alpha$  and phospho-Thr172-AMPK $\alpha$  were localized in the chicken sperm by immunocytochemistry. Sperm cells were fixed in paraformaldehyde (Sigma-Aldrich) (4%) for 4 min, spread onto a Poly-L-lysine slide and then air-dried at room temperature. Sperm cells were washed in PBS (Sigma-Aldrich) and blocked with PBS supplemented with 10% goat serum (Sigma-Aldrich) for 30 min at room temperature. The samples were then incubated overnight at 4°C with an anti-AMPK $\alpha$  or an anti-phospho-Thr172-AMPK $\alpha$ , diluted 1:50 in PBS-1% goat serum, and then incubated with biotinylated goat anti-rabbit IgG (H+L) (Southern biotech, USA) (1:100 in PBS-1% goat serum) for 1 h at 4°C. Then sperm cells were washed with PBS and incubated with Cy2-Streptavidin (Southern biotech) (1:200 in PBS) for

45 min at room temperature in the dark and incubated with 4',6'-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) (0.05  $\mu$ g/mL) for 10 min. The presence of total AMPK $\alpha$  and phospho-Thr172-AMPK $\alpha$  in sperm was examined by fluorescence microscopy. Negative control immunostaining was also performed at the same time by omitting the primary antibody.

### Statistical analyses

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

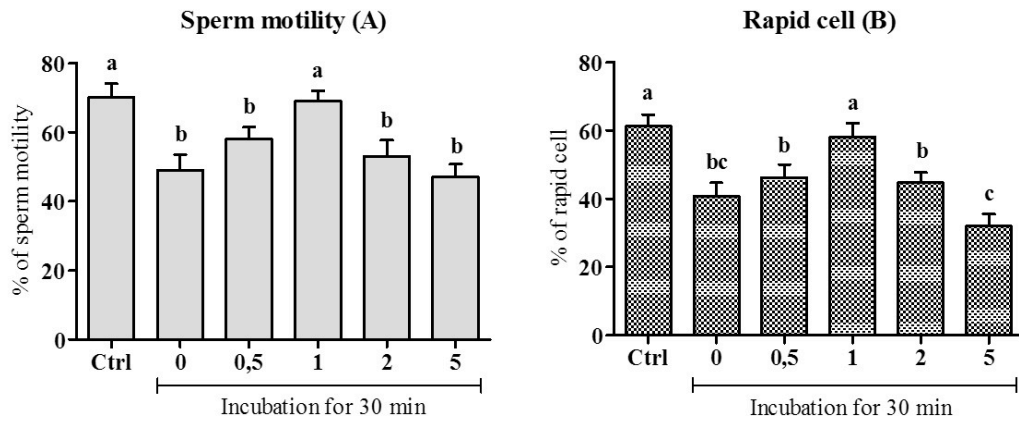
## RESULTS

### Metformin significantly increases the percentage of motile sperm

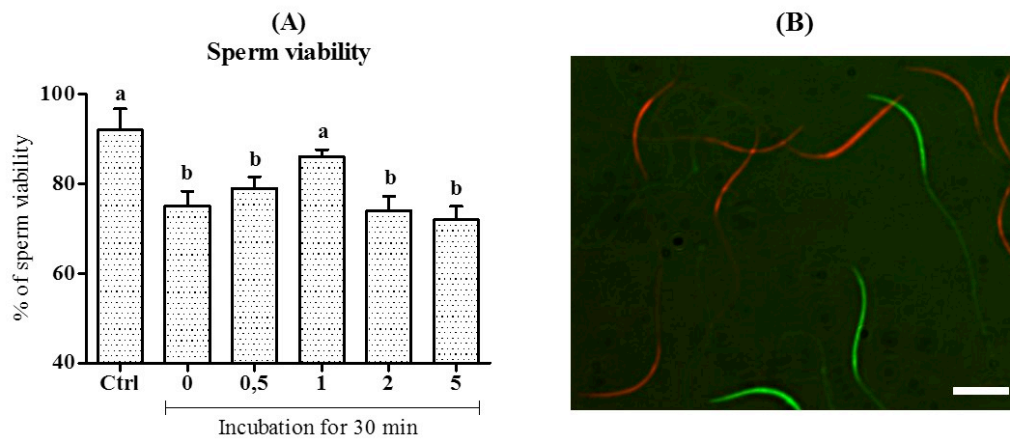
To evaluate the effect of Metf on fresh sperm motility, sperm were incubated in BPSE in the presence or absence of Metf with different concentrations for 30 min at 35°C. As observed in Figure 1, the percentage of sperm motility (Figure 1A) and rapid sperm (Figure 1B) is significantly reduced after 30 min of incubation compared with the negative control (Ctrl) which has not undergone incubation. However, after having treated the sperm with Metf, the sperm motility and rapid sperm parameters significantly increased (by about 41%) with 1 mM Metf, but did not change with other concentrations. Moreover, the percentage of rapid sperm tended to significantly decrease with increasing concentration of Metf (5 mM Metf,  $P = 0.04$ ) compared with other concentrations of Metf, but there was no difference compared to positive control (0 mM) (Figure 1B).

### Metformin significantly increases the percentage of sperm viability

The effect of Metf in sperm viability was studied in order to correlate it with motility studies and to know whether Metf treatment might cause side effects that lead to germ cells death. According to the results shown in figure 2A, sperm viability was sensitive to time of incubation: after 30 min, it significantly decreased compared with control which has not been incubated. However, in the presence of 1 mM Metf, the decrease of sperm viability after 30 min of incubation was greatly limited; although other Metf doses did not produce a similar effect.



**Figure 1.** Effect of Metformin on sperm motility (A) and rapid sperm (B). Sperm were incubated in the presence of Metf at 0.5, 1, 2, and 5 mM for 30 min. The positive control (0 mM) comprised sperm incubated for 30 min in the absence of Metf. The negative control (Ctrl) comprised sperm that did not incubate for 30 min. Values are means  $\pm$  SEM (n = 10). Different letters above bars indicate values that were statistically significantly different at P < 0.05.

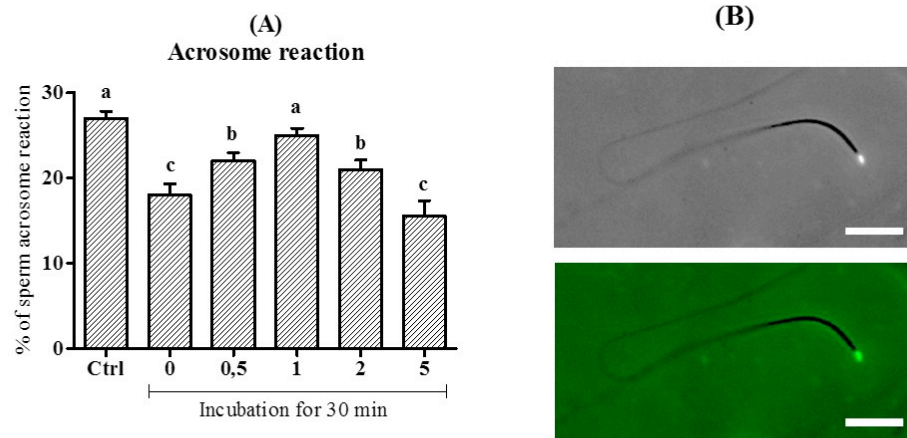


**Figure 2. Effect of Metformin on sperm viability.** (A) Sperm were incubated in the presence of Metf at 0.5, 1, 2, and 5 mM for 30 min. The positive control (0 mM) comprised sperm incubated for 30 min in the absence of Metf. The negative control (Ctrl) comprised sperm that did not incubate for 30 min. Values are means  $\pm$  SEM (n = 10). Different letters above bars indicate values that were statistically significantly different at P < 0.05. (B) Picture of sperm viability coloration with SYBR-14/PI: the red fluorescence shows dead sperm and the green fluorescence shows live ones. Bars indicate 20  $\mu$ m.

### Metformin significantly increases the percentage of sperm acrosome reaction

The ability of spermatozoa to undergo the acrosome reaction was also negatively affected after 30 min of incubation compared to the negative control

which has not been incubated (P < 0.01). However, the acrosome reaction rate was significantly increased by Metf at 0.5 mM (mean increase  $\sim$  22%), at 1 mM (mean increase  $\sim$  39%) and at 2 mM (mean increase  $\sim$  17%) compared with the positive control (0 mM), but not at 5 mM Metf (Figure 3A).



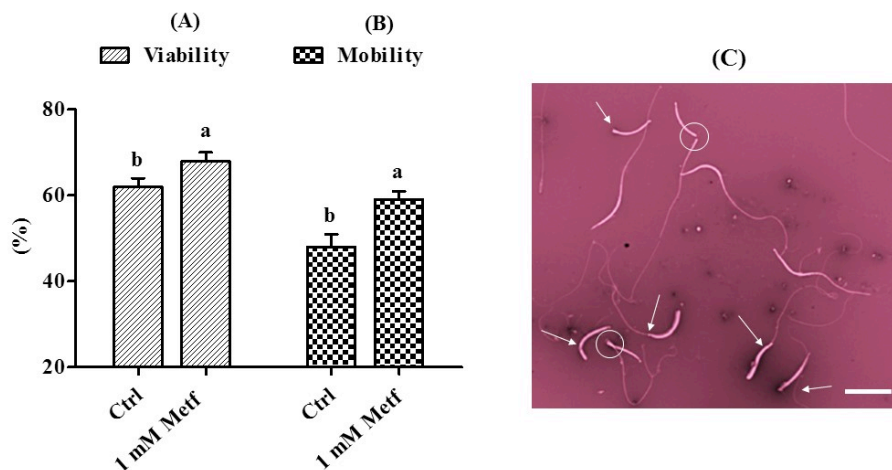
**Figure 3. Effect of Metformin on sperm acrosome reaction.** (A) Sperm were incubated in the presence of Metf at 0.5, 1, 2, and 5 mM for 30 min. The positive control (0 mM) comprised sperm incubated for 30 min in the absence of Metf. The negative control (Ctrl) comprised sperm that did not incubate for 30 min. Values are means  $\pm$  SEM (n = 10). Different letters above bars indicate values that were statistically significantly different at  $P < 0.05$ . (B) Picture of sperm acrosome reaction coloration with FICT-PNA: acrosome reaction is characterized by the green fluorescence of the acrosomal region. Bars indicate 20  $\mu$ m.

#### Effect of Metformin supplementation in cryopreservation media on spermatozoa

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

Sperm parameters were assessed 15 min after

thawing. The results show that the sperm viability of the samples treated with Metf slightly increased (by 10%) compared with control without Metf (Figure 4A). In addition, results obtained for motility were higher than those of the control without Metf. The percentage of motile sperm treated with Metf increased by 23% compared with control without Metf (Figure 4B). The decrease of sperm quality is also examined below by coloring cells with eosin-nigrosin in figure 4C.

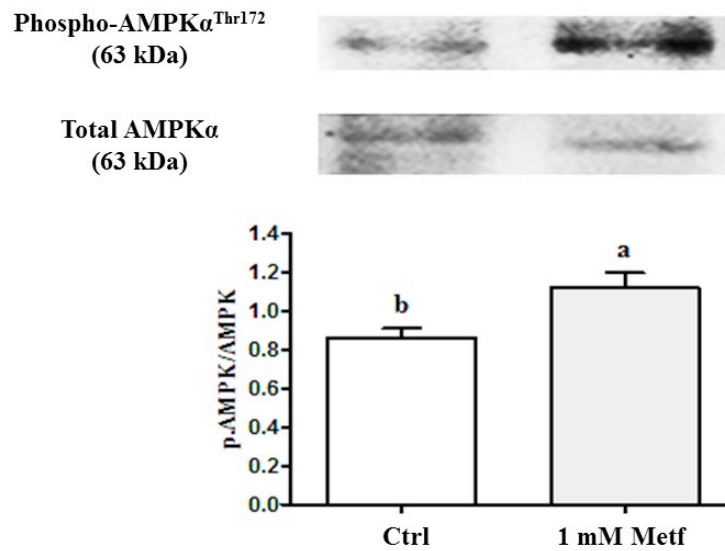


**Figure 4. Effect of Metformin on frozen-thawed sperm viability (A) and motility (B).** Sperm were incubated in the presence of 1 mM Metf for 30 min before cryopreservation. The control (Ctrl) comprised sperm cryopreserved in the absence of Metf. Values are means  $\pm$  SEM (n = 6). Different letters above bars indicate values that were statistically significantly different at  $P < 0.05$ . (C) Coloration with eosin-nigrosin shows that sperm morphology can be altered by deformations of the head or the flagella (highlighted by white arrows and circles), which can be either missing or misshapen. Bars indicate 10  $\mu$ m.

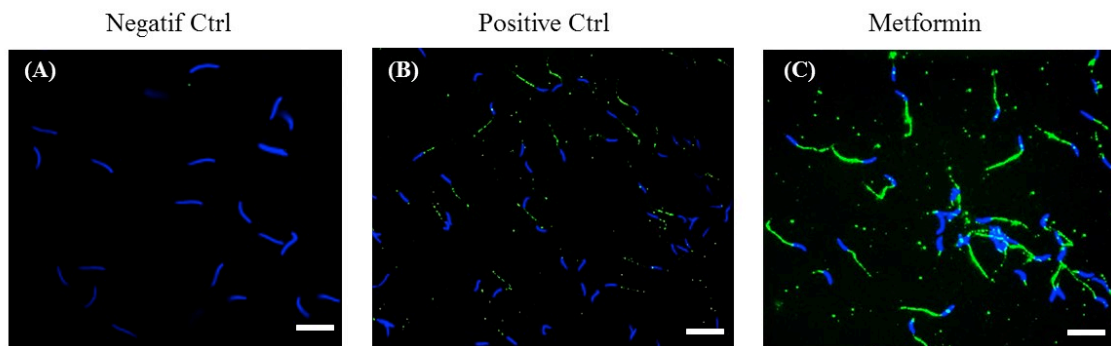
**Phosphorylation of AMPK in frozen/thawed spermatozoa after Metformin treatment**

Western blot analyses using antibodies against phospho-Thr172-AMPK $\alpha$  and total AMPK $\alpha$  (as Ctrl)

were performed on chicken sperm incubated in the absence or presence of 1 mM Metf during freezing and thawing (Figure 5). The AMPK phosphorylation was increased by 30% with Metf after the freeze-thaw process compared with control without Metf.



**Figure 5.** Metformin effects 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-AMPK $\alpha$  and anti-AMPK $\alpha$  antibody. Bands for phospho-Thr172-AMPK $\alpha$  were detected at 63 kDa (top lanes). Total AMPK $\alpha$  was used as loading control (63 kDa) (bottom lanes) and the phosphorylated protein AMPK $\alpha$  (Thr172)/total AMPK $\alpha$  ratio is shown at the bottom. Cryopreserved sperm were either treated in the presence of 1 mM Metf (in dark gray) or without anything for the Ctrl (in white). Values represent means  $\pm$  SEM from 6 different experiments. Different superscripts indicate significant differences between Ctrl and Metf in frozen-thawed semen ( $P < 0.05$ ).



**Figure 6.** Metformin effects on the staining of phosphor-AMPK in chicken spermatozoa. Sperm cells were incubated with 1 mM Metf at 35°C for 30 min and then treated with antibodies to phospho-Thr172-AMPK $\alpha$  and with secondary antibody conjugated to biotin (see Materials and Methods). Immunofluorescence staining of phospho-Thr172-AMPK (green) was conducted in chicken spermatozoa. Nuclei were stained with DAPI (blue). Negative control (Negative Ctrl): primary antibody was not added (**6A**), positive control (Positive Ctrl): sperm incubated without Metf (**6B**), sperm incubated with Metf in figure **6C**. Bars indicate 120  $\mu$ m.

### Metformin effects on the staining of phospho-AMPK in chicken spermatozoa

We focused on the effects of Metf treatment on AMPK localization in spermatozoa at 30 min. The staining of phospho-Thr172-AMPK protein was more visible in cells treated with Metf (Figure 6C) than in cells treated with vehicle alone as in control (Figure 6B). Specifically, in the Metf-treated cells, the staining of phospho-Thr172-AMPK in the flagellum was stronger than the one in the control.

### DISCUSSION

There have been few reports assessing the effect of Metf on the viability of fresh or cryopreserved spermatozoa *in vitro*. This study figured that treatment of fresh or cryopreserved chicken spermatozoa with Metf presented 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 5 mM treatment with Metf in fresh spermatozoa for two h. Furthermore, they reported a complete inhibition with very high concentrations (between 10 and 20 mM) (Hurtado de Llera *et al.*, 2012). Another study of Bertoldo *et al.* (2014) also showed that treatment of fresh mouse spermatozoa with 5 mM Metf decreased sperm motility, but not sperm viability.

In this study, 1 mM Metf treatment led to a significant increase in the percentage of viable, motile, and rapid spermatozoa (VAP > 50  $\mu\text{m/s}$ ). However, Metf with a high concentration of 5 mM did not affect sperm motility and viability but caused a significant reduction of the number of rapid spermatozoa. Moreover, the spermatozoa acrosome reaction was affected by Metf. The acrosome reaction occurred by fusion of the sperm head cytoplasmic membrane and the underlying outer acrosomal membrane, so that the acrosome content was released (Okamura, 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 Metf; this

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

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

In order to explain the positive action of Metf on fresh and frozen sperm functions, this study investigated the effects of Metf on AMPK phosphorylation. Recently, I showed the presence of the AMPK $\alpha$  protein in chicken sperm. AMPK presence in the acrosome, midpiece and flagellum of chicken spermatozoa is in relation to its possible function in spermatozoa motility and acrosome reaction process (Nguyen *et al.*, 2014). In this study, an increased AMPK phosphorylation in frozen/thawed spermatozoa with 1 mM Metf was measured. This indicated that the positive action of Metf on chicken sperm functions is done through AMPK activation. Metf was known as an indirect activator 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. In this study, Metf was shown to allow phospho-Thr172-AMPK $\alpha$  in sperm to be clearly seen through a strong stain. Therefore, the data strongly suggest that AMPK phosphorylation has a central role in regulating the improvement of metabolic



functions and that ATP production is needed to ensure high energy consuming process such as spermatozoa motility and acrosome reaction.

However it is possible that Metfas 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, Driscoll, 2010). Sperm membranes are enriched in polyunsaturated fatty acids (PUFAs) in mammals (Dandekar *et al.*, 2002) and bird species (Blesbois, Hermier, 2003), and 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 (GR) and catalase (CAT) are the major antioxidant enzymes naturally present 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 LPO have been shown after chicken sperm cryopreservation (Partyka *et al.*, 2012). Previous studies have provided evidence that Metf exerts an anti-inflammatory effect on non-alcoholic steato-hepatosis mice by preventing depletion in GPx, SOD, and catalase, and by decreasing ROS and MDA (Buldak *et al.*, 2014). Metf could also directly reduce ROS production via inhibition of complex I. Indeed, the inhibition of complex I by Metf 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 Metf on sperm quality is made through both AMPK-dependent and AMPK-independent pathways (Kita *et al.*, 2012).

## CONCLUSIONS

The results in this study demonstrated that Metf increased the quality of fresh chicken spermatozoa. Furthermore, chicken sperm cryopreserved in the presence of Metf have improved post-thaw motility and viability. This is the first assessment of the effect of Metf 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.

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## ẢNH HƯỞNG CỦA CHẤT KÍCH HOẠT AMPK METFORMIN ĐẾN CHẤT LƯỢNG TINH TRÙNG

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### TÓM TẮT

Bảo quản lạnh tinh dịch cho phép quản lý sự đa dạng di truyền động vật. Tuy nhiên, quá trình đông lạnh - giải đông đã làm thay đổi hình dạng, đặc tính sinh hóa, nguồn năng lượng... dẫn đến suy giảm chất lượng tinh trùng. Hiện nay, nhiều thành phần được thêm vào môi trường đông lạnh để nâng cao chất lượng tinh trùng đông lạnh - giải đông trong quá trình thụ tinh nhân tạo. Mục tiêu của nghiên cứu này là xác định tác động của Metformin (Metf) đến khả năng di chuyển của tinh trùng gà và khả năng thực hiện phản ứng acrosome, đồng thời đánh giá tác động của Metf đến các chức năng của tinh trùng bảo quản lạnh. Tinh dịch gà đã được pha loãng và ủ ở 35°C trong môi trường có hoặc không bổ sung chất kích hoạt AMPK, Metf ở các nồng độ khác nhau (từ 0,5 đến 5 mM) và nồng độ cải thiện chất lượng tinh trùng tốt nhất sẽ được bổ sung vào môi trường bảo quản lạnh tinh trùng gà. Kết quả cho thấy 1 mM Metf là nồng độ cho kết quả tốt nhất về chất lượng tinh trùng. Phosphoryl hóa AMPK $\alpha$ , khả năng sống, khả năng phản ứng acrosome (AR) các thông số di động đều bị ảnh hưởng tiêu cực bởi quá trình đông lạnh - giải đông và Metf đã phục hồi một phần tiêu cực đó. Chất lượng tinh trùng được cải thiện (trung bình tăng 23% đối với di động, 10% đối với khả năng sống) cũng như phosphoryl hóa AMPK $\alpha$  (trung bình tăng 30%). Hơn nữa, cường độ huỳnh quang của phospho-AMPK cũng mạnh hơn với Metf so với trong đối chứng. Các kết quả này cho thấy sự có mặt của Metf trong tinh dịch tươi có tác động tích cực đến chất lượng tinh trùng và giúp hạn chế sự giảm dần khả năng di chuyển tinh trùng do việc bảo quản lạnh và bằng cách phục hồi các chức năng của tinh trùng đã dẫn đến chất lượng tinh trùng đông lạnh được bảo quản tốt hơn.

**Từ khóa:** *Phản ứng Acrosome, tinh trùng gà, chuyển động của tinh trùng, AMPK, Metformin*