Effects of the AMP-Kinase modulators AICAR, Metformin and Compound C on chicken spermatozoa viability, motility and acrosome reaction

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Abstract

Spermatozoa are highly specialized cells whose main properties include motility and acrosome reaction. These processes require a high level of energy related to the intracellular ATP / 5'AMP ratio. AMPK (5'-AMP activated protein kinase) is a sensor of this ratio and its numerous roles include regulation of glucose, lipid, and protein metabolisms. When AMPK is activated, it stimulates catabolic pathways that produce ATP and simultaneously inhibits ATP-consuming anabolic pathways that adjust the cellular energy balance. In the study, we investigated the intracellular localization of AMPK in chicken spermatozoa using immunofluorescence and examined the effects of AMPK activators AICAR and Metformin (Met) or inhibitor Compound C (CC) on spermatozoa motility and ability to achieve the acrosome reaction. Our results show that AMPK protein is expressed in the acrosome, midpiece and flagellum of spermatozoa. Spermatozoa motility (CASA) and ability to accomplish acrosome reaction were inhibited by CC without effect on viability (Sybr14/PI). At the opposite, Met or AICAR stimulated the motility and the ability to achieve the acrosome reaction. These results show that AMPK stimulation may improve some essential functions of chicken spermatozoa.

Introduction

Sperm functions are critically controlled through the phosphorylation state of specific proteins. The 5'-AMP activated protein kinase (AMPK) is a serine/threonine protein kinase that is a sensor of energy metabolism in the cells. AMPK activation stimulates catabolic pathways that produce ATP and simultaneously inhibits ATP-consuming anabolic pathways, thus adjusting the cellular energy balance [1, 2].
Viability, motility and acrosome reaction are important factors influencing fertilization quality. Better knowledge of signal transduction pathways regulating these indicators is important to improve in vitro fertilization. In mammals, very few studies show signal transduction pathways regulating sperm motility and acrosome reaction such as PKA, PKC, MAPK3/1 or PIK3 and MAPK14 [3, 4]. Moreover, a recent study has shown that an AMPK protein in boar spermatozoa was regulating sperm mobility [5]. In birds, Ashizawa et al. showed the presence of PKC in spermatozoa and its possible inhibitory effect on acrosome reaction [6]. In 2009, Lemoine et al. showed that PKA and MAPK1 are positively involved in acrosome reaction and in the motility of chicken [7]. In our study, we demonstrate that AMPK protein is present in chicken sperm and is involved in the regulation of chicken spermatozoa viability, mobility and acrosome reaction by using pharmacological activators (AICAR and Met) and inhibitor of AMPK (CC).

Materials and methods

1. **Chemicals and reagents**

6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo [1,5-a] pyrimidine (CC), AICA riboside (AICAR) and 1,1-dimethylbiguanide hydrochloride (Met) were obtained from

Calbiochem. Stock solution of Met and AICAR were prepared in deionized water and stock solution of CC was prepared in dimethylsulfoxide (DMSO). PI/Sybr 14 (LIVE/DEAD sperm viability kit) was purchased from Molecular Probes.

1. Animals and semen collection

The animals used were 22-55 weeks-old adult Gallus domesticus. The semen was collected by the dorso-abdominal massage method [8]. After removing seminal plasma, sperm pellets were diluted in Beltsville Poultry semen extender (BPSE) [9] to a final sperm concentration of 500.10^6cells/ml. Sperm samples were then incubated at 35°C for 25 min in presence or absence of CC or Met or AICAR. The corresponding controls with the vehicles alone were measured in parallel.

2. Spermatozoa quality assessment

The spermatozoa viability was measured by Sybr 14 and PI (propidium iodide). The spermatozoa mobility parameters were evaluated by the computer-assisted sperm analysis (CASA) system with an HTM-IVOS (Hamilton-Thorn Motility Analyzer, IVOS) [10] and the accomplishment of acrosome reaction was detected by FITC-conjugated peanut agglutinin (FITC-PNA) in presence of Ca^{2+} and inner perivitelline layer (IPVL) [7].

3. Immunocytochemistry

Sperm cells were fixed in formaldehyde, and blocking with goat serum in PBS (Sigma). The sperm cells were then incubated overnight at 4°C with anti-phospho AMPKα (in PBS-goat serum) and then incubated with anti-rabbit IgG H+L (Southern biotech) (in PBS-goat serum). Sperm cells were incubated with Cy2-Streptavidin (Southern biotech) (in PBS) and incubated with DAPI (Sigma). Another experiment was carried out at same time in which sperm cells were incubated without anti-rabbit IgG and used as negative controls.

4. Statistical analysis

The data are expressed as mean ± S.E.M. Statistical analysis used SPSS v16.0. The significance of the difference between the treatments was calculated by Student t-test or analysis of variance (ANOVA). The level of significance was set at $p<0.05$.

Results

Localization of AMPK in chicken spermatozoa

The expression of AMPK protein in chicken spermatozoa was assessed with indirect immunofluorescence using anti-AMPKα as primary antibodies. Figure 1A, shows that AMPK protein is observed in the acrosome region, in the midpiece and in the entire flagellum. Rabbit IgG was used as a negative control and showed no positive signal in Figure 1C.

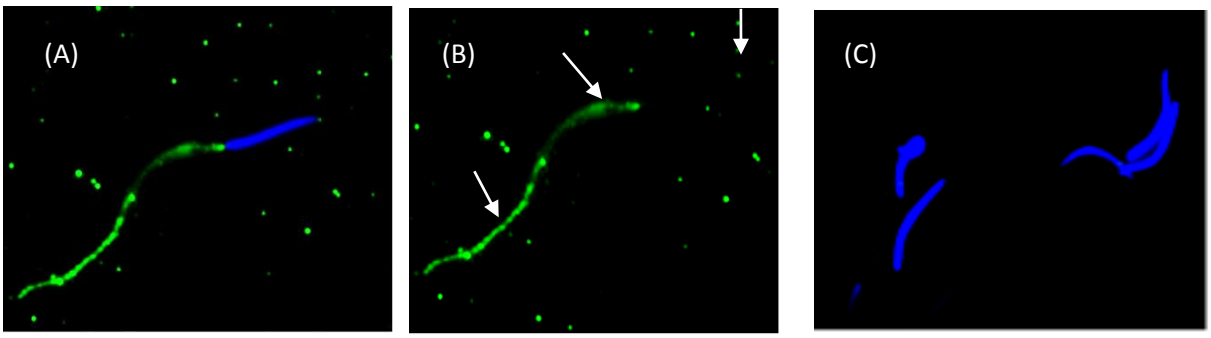

Figure 1 **: *Immunolocalization of AMPK in chicken sperm.*** *Images show the AMPK localization (green) and DAPI labelled nuclei (blue) (Fig.2A). AMPK localization (green) (Fig.2B). Negative control with anti-rabbit IgG (Fig.2C).*

Modulators effects on sperm viability

Before working with these concentrations, we assayed four different concentrations for each modulator and decided to keep the one giving the best results. Regarding the time factor, we chose to make our measurements after 25 min of incubation because AMPK expression on sperm quality is very clear at this time.

The percentage of spermatozoa viability assessed using PI/SYBR14 was significantly increased under AICAR (by 80%, n=10) and under Met (by 79%, n=10) compared to control samples (Fig. 2A). Treatment with AMPK inhibitor CC did not affect spermatozoa viability.

Modulators effects on sperm mobility

Our results indicate that sperm motility was significantly increased in the presence of Met (~ 65%, n=10) and ~ 58% (n=10) under AICAR compared to the control sample. In contrast, incubation with CC significantly decreased the percentage of motile spermatozoa to ~ 33% (n=10) compared to the control (DMSO). Data are shown in Fig. 2B.

Modulators effects on sperm acrosome reaction

Fig. 2C shows that the percentage of successful acrosome reaction was significantly increased by Met and AICAR after 25 min of incubation. Treatment of spermatozoa with CC significantly decreased the acrosome reaction rate: it is at ~ 15 % whereas it is at ~ 20 % in control (DMSO) sample.

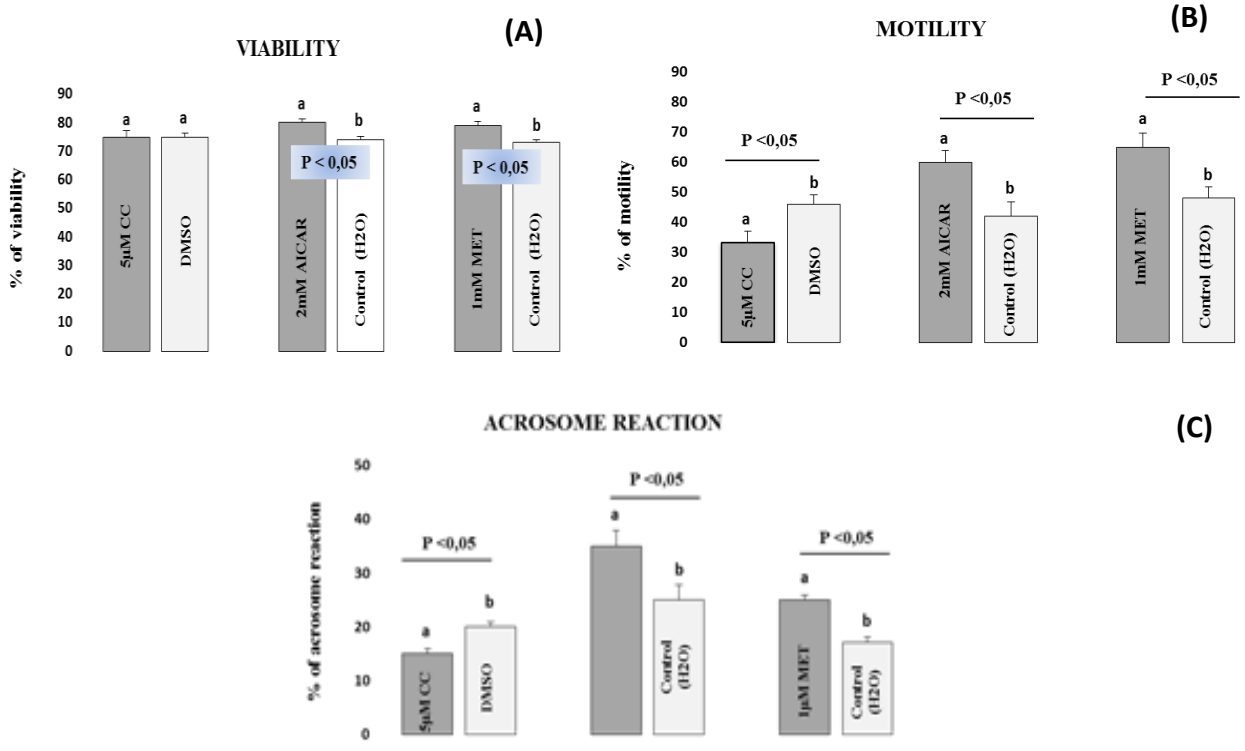

***Figure 2* : Effect of AMPK modulators in the percentage of viable, motile spermatozoa and in the percentage of spermatozoa acrosome reaction**. *Spermatozoa were incubated with an AMPK modulators (in black) and with SHAM (in white).*
Experiment was performed 10 times; values (%) are mean ± SEM.
a,b: significantly different (P< 0.05)

Discussion

AMPK was shown for the first time in mammalian tissues by Cheung et al. in 2000 [2]. It was also shown that it could be stimulated by uptake of glucose and oxidation of fatty acids [2].
The present study demonstrated that AMPK protein is expressed in chicken spermatozoa lysates. The presence of AMPK proteins in the spermatozoa was confirmed by immunocytochemistry. AMPKα is present at high concentration in the flagellum and it also found in the acrosome and in the midpiece. In mammalian, AMPK proteins were shown in spermatozoa by Bragado et al. in 2012 [5], but none were found in the flagellum.
We also showed that AMPK inhibitor (CC) negatively affects sperm motility and rate of acrosome reaction but not sperm viability. These results are in agreement with earlier studies by Bragado et al. [5], which showed that CC in chicken spermatozoa may reduce sperm motility but does not affect viability. Our results also suggest that AMPK activators (Met and AICAR) stimulate sperm viability, motility and rate of acrosome reaction.
In summary, we have shown that the AMPK pathway could be involved in the regulation of spermatozoa functions.

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