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# Human Sperm Motility, Capacitation and Acrosome Reaction are impaired by 2-arachidonoylglycerol Endocannabinoid.


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**ABSTRACT**

The endocannabinoids are cannabinoids synthesized by mammalian tissues. These compounds are closely related to the regulation of the male reproductive system. However, little is known about the effects produced by 2-arachidonoylglycerol (2AG) on *in vitro* human sperm functions. This study was undertaken to determine the effects produced by 2AG on fresh human sperm and in the capacitation technique. Semen samples from healthy young men were exposed to different concentrations of 2AG before and during capacitation technique. In this work, we have demonstrated that 2AG induces the spontaneous acrosome reaction and reduces progressive motility in fresh human sperm. During the capacitation technique, sperm becomes more sensitive to low concentrations of 2AG, triggering the acrosome reaction and inhibiting protein phosphorylation. In summary, 2AG affects the *in vitro* functionality of human sperm by reducing motility, inhibiting capacitation and triggering the acrosome reaction.

**KEY WORDS:** acrosome reaction, arachidonoylglycerol, endocannabinoid, noladin, protein phosphorylation

## INTRODUCTION

Marijuana is the most widely consumed illicit drug worldwide, used by about 180 million people (UNDOC, 2013). In addition, the active principle of marijuana is used as a therapeutic compound to treat diseases such as multiple sclerosis (Vaney *et al.*, 2004; Zajicek and Apostu, 2011) and Alzheimer's disease (Campbell and Gowran, 2007; Martin-Moreno *et al.*, 2011), and to mitigate the adverse effects of cancer chemotherapy (Cotter, 2009; Van Ryckeghem and Van Belle, 2010). Thus, many people are also exposed to the components of marijuana, without having consumed the drug for recreational purposes. The substances responsible for the pharmacological effects of the marijuana plant (*Cannabis sativa*) are jointly referred to as cannabinoids. However, mammalian tissues also produce cannabinoids, called endocannabinoids, including 2-arachidonoylglycerol (2AG) and arachidonoylethanolamide or anandamide (AEA) (Mechoulam *et al.*, 1995; Howlett *et al.*, 2002). Cannabinoids and endocannabinoids produce their effects by binding to two types of receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). CB1 and CB2 distribution is highly diverse, and includes mammals (Mouslech and Valla, 2009), fish (Yamaguchi *et al.*, 1996), invertebrates (Matias *et al.*, 2001) and higher plants (Faure *et al.*, 2009). In humans, CB1 is more abundant in the central nervous system (Matsuda *et al.*, 1990) and peripheral tissues such as heart (Thakur *et al.*, 2005), liver (Howlett *et al.*, 2010), uterus (Fride, 2004) and testis (Cacciola *et al.*, 2008), whereas CB2 is primarily found in the immune system (Howlett *et al.*, 2002). Initially it was thought that human sperm only expressed CB1 (Rossato *et al.*, 2005), but the presence of CB2 has also subsequently been demonstrated in the sperm plasma membrane (Agirregoitia *et al.*, 2010).

Endocannabinoids and their receptors form part of the endocannabinoid system, which is involved in many functions of several systems, including the reproductive system

(Schuel and Burkman, 2005; Wang *et al.*, 2006; Taylor *et al.*, 2007). For example, in the male reproductive system, cannabinoids affect spermatogenesis by lowering LH and testosterone levels (Kolodny *et al.*, 1974; Cone *et al.*, 1986). Furthermore, AEA has been shown to reduce sperm motility and affect the acquisition of motility in the epididymis (Rossato *et al.*, 2005; Ricci *et al.*, 2007). Endocannabinoids are also intimately related to capacitation and the acrosome reaction. For example, it has been demonstrated that AEA blocks the acrosome reaction in human (Rossato *et al.*, 2005), boar (Maccarrone *et al.*, 2005) and sea urchin spermatozoa (Chang *et al.*, 1993). Furthermore, capacitation produces biochemical and molecular changes that lead to hyperactivated sperm motility (Suarez and Ho, 2003), intracellular calcium accumulation (Baldi *et al.*, 1991), cytoskeleton reorganization (Brener *et al.*, 2003; Francou *et al.*, 2013) and increased protein phosphorylation (Visconti *et al.*, 2011). In this respect, Maccarrone et al. (Maccarrone *et al.*, 2005) have demonstrated that AEA inhibits calcium-related changes in boar spermatozoa capacitation. Furthermore, methanandamide (an AEA analog) produces a significant reduction in the percentage of human sperm with hyperactivated motility (Schuel *et al.*, 2002). However, little is known about the effects produced by 2AG on *in vitro* human sperm functions. Thus, the main objective of this study was to determine the effects produced by this potent endocannabinoid on fresh human sperm and in the capacitation process.

## MATERIALS AND METHODS

*Samples collection*

Semen samples were collected from healthy donors (aged 18-35 years old; n=15) after 3-4 days of sexual abstinence. Samples were allowed to liquefy at 37°C for 30 min before processing. Assessment of sperm concentration, semen volume, liquefaction, pH, motility, viability and morphology confirmed that samples were normozoospermic, in accordance with WHO guidelines (World Health Organization, 2010). Donors gave written consent to use their semen samples for scientific purposes. The study protocol and informed consent were reviewed and approved by the Institutional Review Board.

*Assays on fresh sperm***.**

Seminal plasma was removed from each fresh sample by washing twice with HEPES sodium salt (21 mmol/l, Sigma Aldrich). The sample was divided into aliquots and then incubated with different concentrations of 2AG (Cayman Chemical Company, MI, USA): 0.01, 0.1, 1, 10 and 100 µM. Control was performed by omitting the endocannabinoids. The concentration of organic vehicle was maintained at a constant level in all experiments. Samples were incubated at 37 ºC for 1, 2 and 4 hours and the motilily, viabiliy and acrosme reaction was evaluated as described below.

*Assays during the swim-up technique.*

The swim-up technique was performed according to Ricci *et. al*. (Ricci *et al.*, 2009). Thus, each sample was divided into six Falcon tubes and then washed in HAM F-10 (Gibco, Invitrogen) supplemented with HSA (1% Human Serum Albumin, 0.003% sodium piruvate, 0.36% sodium lactate, 0.2% sodium bicarbonate) at 37°C and

centrifuged at 300 *g* for 10 min. The supernatant was discarded and the pellet resuspended in 0.5 ml of fresh media adding 2AG to obtain concentrations of 0.01, 0.1, 1, 10 and 100 µM. The addition of 2AG was omitted for the control and the concentration of organic vehicle was maintained at a constant level in all experiments. These samples were incubated at an angle of 45 degrees and 37°C in a 5% $CO_2$ atmosphere for 2 hours. Sperm was selected by carefully collecting the top supernatant layer prior the assessment of tyrosine phosphorylation and evaluation of acrosome reaction.

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*Motility and Viability*.

Motility was graded as progressive motility, non-progressive motility, and immotility; using a phase contrast microscope in accordance with WHO guidelines (World Health Organization, 2010). Sperm viability was assessed using the eosin-nigrosin technique (Bjorndahl *et al.*, 2003; World Health Organization, 2010). Eosin-nigrosin staining was performed by mixing 10 µL of sample with 10 µL of eosin Y solution (0.5 % w/v diluted in 0.9 % w/v of sodium chloride; CI 45380, Panreac química SAU, Barcelona, ES). Samples were incubated for 1 min before adding 10 µL of nigrosin, which acted as a contrast. A slide extension was made and allowed to dry at room temperature. A count of live sperm (white, without eosin staining) and dead sperm (pink, with eosin staining) was performed by bright field microscopy (40x magnification) using 500 sperm cells for each sample.

*Evaluation of acrosome reaction*

Acrosomal status was evaluated in fresh and swim-up selected sperm. The technique used was fluorescein isothiocyanate-conjugated Pisum sativum agglutinin labeling

(FITC-PSA)(Cross and Meizel, 1989). For this, the fixed spermatozoa (methanol, -20 ºC for 15 min) were incubated with FITC-PSA (1:20 dilution; L0770, Sigma Aldrich) for 30 min at room temperature in the dark. Samples were washed with PBS (3 x 5 min) and mounted with 5 µL of Vectashield mounting medium (Vector Labs, Burlingame, CA) with 4',6-diamidino-2-phenyilindole (DAPI, 1% v/v; Sigma-Aldrich). FITC fluorescence was evaluated in 400 sperm cells from each sample using a confocal microscope LEICA SP2 (Leica Microsystems).

*Phosphorylation of tyrosine residues*

Phosphorylation of tyrosine residues was only evaluated in swim-up selected sperm. Indirect immunofluorescence was carried out on fixed sperm (methanol, -20ºC for 15 min). Samples were blocked with 1% BSA in PBS for 30 min at room temperature and subsequently incubated with the monoclonal anti-phosphotyrosine antibody (Sigma-Aldrich; dilution 1:500). The antiserum was diluted in 1% BSA in PBS and incubated overnight at 4°C. Samples were washed in PBS for 15 min (3x5 min) and afterwards incubated for 1 h at room temperature with DyL 488-conjugated donkey anti-mouse IgG (Jackson Immuno Research Europe Ltd, Suffolk, UK). Samples were then rinsed in PBS for 15 min and mounted with 5 µL of Vectashield mounting medium (Vector Labs) with 4',6-diamidino-2-phenyilindole (DAPI, 1% v/v; Sigma-Aldrich). Negative controls were performed by omitting either primary or secondary antibody. In addition, the specificity of anti-phosphotyrosine antibody was demonstrated using a specific phosphotyrosine antibody inhibitor (O-phospho-L-tyrosine conjugated to BSA, Sigma-Aldrich). The fluorescence signal was evaluated in 400 sperm cells from each sample using a confocal microscope LEICA SP2 (Leica Microsystems).

*Image processing and statistical analysis*

Immunofluorescence was analyzed using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems). DyLight 488 and FITC were excited using the 488nm line of an Argon ion laser. For DAPI detection the 405nm line of a diode-coupled laser was used. Immunofluorescence images were then processed with Leica TCS SP2-PC software.

To examine differences between the means, an independent ANOVA and Bonferroni post-hoc test was performed using SPSS software (V15.0, IBM). Data are expressed as the mean ± SEM and $p < 0.05$ was considered statistically significant.

## RESULTS

### Effects of 2AG on fresh sperm: viability, motility and acrosome reaction.

Statistical analysis showed that none of the 2AG tested concentrations caused a significant reduction in sperm viability compared to control (Fig. 1A). However, 2AG-100 μM had important effects on motility when the sample was incubated during 2 and 4 hours. Progressive motility was significantly lower in 2AG-100 μM than control (Fig.1B), and the immotile sperm was significantly higher in 2AG-100 μM than control (Fig. 1C). Non-progressive motility was not altered by 2AG concentrations analyzed (no significant differences with control).

A high percentage of spermatozoa with an intact acrosome are desirable in fresh samples. Treatment of the samples with progressive concentrations of 2AG caused an increase in the percentage of acrosome-reacted sperm, which was statistically significant for 2AG-100 μM (during 2 and 4 incubation hours). However, 2AG had no effect on acrosomal exocytosis at concentrations below 100 μM (no significant differences with control) (Fig. 1D).

Our data demonstrated that 1 hour incubation of 2AG produces no significant effects on *in vitro* sperm functions. However, no significant differences between 4 hours *versus* 2 hours incubation were found (Fig. 1). Thus, we selected 2 hours as optimal incubation time to observe the 2AG effects on sperm during swim-up experiments.

**Fig 1. near here**

**2AG effects on swim-up technique: tyrosine phosphorylation and acrosome reaction.**

The endocannabinoid effect on swim-up technique was evaluated by adding different concentrations of 2AG to capacitation culture media. The percentage of acrosome-reacted sperm was evaluated by FITC-PSA technique and confocal images were obtained (Fig. 2A). The percentage of acrosome reacted sperm was higher with 2AG-10 μM (20.0 ± 2.6 %) and 2AG-100 μM (99.3 ± 0.2 %) than control (7.51 ± 1.2 %) (Fig. 2B).

Confocal analysis shows phosphorylation of tyrosine residues is produced mainly in flagellar proteins of human spermatozoa (Fig 2C). No immunofluorescent signal was found in negative controls. The graph shows that the percentage of sperm with tyrosine phosphorylation decreased when 2AG was added during capacitation technique (2AG-10 μM, 14.8 ± 1.8 %; 2AG-100 μM, 13.3 ± 2.0 %; control; 21.0 ± 1.0 %) (Fig. 2D).

**Fig 2. near here**

## DISCUSSION

Our data show that 2AG (100 μM) decreased progressive motility without affecting the viability of fresh sperm. Other authors demonstrated that from a concentration of 0.1μM, anandamide decreases human sperm motility (Rossato *et al.*, 2005). Moreover, 2AG levels are high in mouse spermatozoa isolated from the caput epididymis, where these do not move, and decreases dramatically in spermatozoa isolated from the cauda epididymis (Cobellis *et al.*, 2010). These studies suggest that endocannabinoids exert a tight control on sperm motility. However, the mechanism by which these compounds affect motility is poorly understood. In this sense, a recent study has shown that 2AG inhibits the cationic channel of sperm (CatSper) (Miller *et al.*, 2016) and the CatSperI expression is related with human sperm motility (Tamburrino *et al.*, 2015). Thus, these authors demonstrated that CatSperI protein expression was reduced in asthenozoospermic samples and was significantly correlated with total and progressive motility.

Our results also show that the highest concentration of 2AG caused an increase in the percentage of acrosome-reacted sperm. In this sense, an early acrosome reaction, prior to contact with the oocyte, will abrogate sperm fertilizing ability (Yanagimachi, 1989). In contrast to the effect of 2AG, anandamide inhibits the acrosome reaction in human (Schuel *et al.*, 2002; Rossato *et al.*, 2005), boar (Maccarrone *et al.*, 2005) and sea urchin spermatozoa (Schuel *et al.*, 1994). However, the molecular mechanisms by which endocannabinoids affect the acrosome reaction are still poorly understood. In this respect, several studies have shown that anandamide is also able to bind transient receptor potential vanilloid 1 (TRPV1), a nonselective cation channel activated by capsaicin (De Petrocellis *et al.*, 2001; Van Der Stelt and Di Marzo, 2004; Francavilla *et*

*al.*, 2009). Additionally, activation of TRPV1 stabilizes the acrosomal membrane and inhibits the spontaneous acrosome reaction (Wang *et al.*, 2006). In contrast to anandamide, 2AG does not have the capacity to activate TRPV1 (Szallasi and Blumberg, 1999; Van Der Stelt and Di Marzo, 2004). Moreover, an increase in intracellular calcium is a molecular requirement to trigger the acrosome reaction (Baldi *et al.*, 1996; Breitbart, 2002), and this increase mainly comes through calcium channels in the plasma membrane of sperm cells . Interestingly, endocannabinoids have the capacity to regulate the opening of voltage-sensitive calcium channels (Chemin *et al.*, 2001). In fact, different CB1 or CB2 agonists may play a key role in the calcium influx by producing antagonistic effects with respect to the acrosome reaction.

Sperm protein phosphorylation was lower when 2AG (10 and 100 μM) was added during the swim-up technique than in control. The increase in phosphorylation of tyrosine residues is one of the main molecular events that occur during capacitation (Visconti *et al.*, 1995; Barbonetti *et al.*, 2010). Moreover, our results are consistent with previous reports which have shown that anandamide reduces the percentage of capacitated sperm in boar (Maccarrone and Wenger, 2005) and human (Rossato *et al.*, 2005). Regarding the cellular mechanism, several studies have demonstrated that activation of cannabinoid receptors inhibits adenylyl cyclase, the enzyme necessary to increase intracellular levels of cAMP (Vogel *et al.*, 1993; Visconti *et al.*, 1995; Howlett *et al.*, 2002). In turn, low levels of cAMP inhibit activation of protein kinase A (PKA) which, through other phosphorylases, triggers the protein phosphorylation cascade observed during capacitation (Visconti *et al.*, 2002; Harrison, 2004). Thus, 2AG could inhibit capacitation through the adenylyl cyclase/cAMP/PKA pathway.

In conclusion, our results suggest that *in vitro* studies 2AG decreases motility, triggers the acrosome reaction and inhibits protein phosphorylation. Thus, this endocannabinoid could impair fertility due to the important effects it has on sperm physiology.

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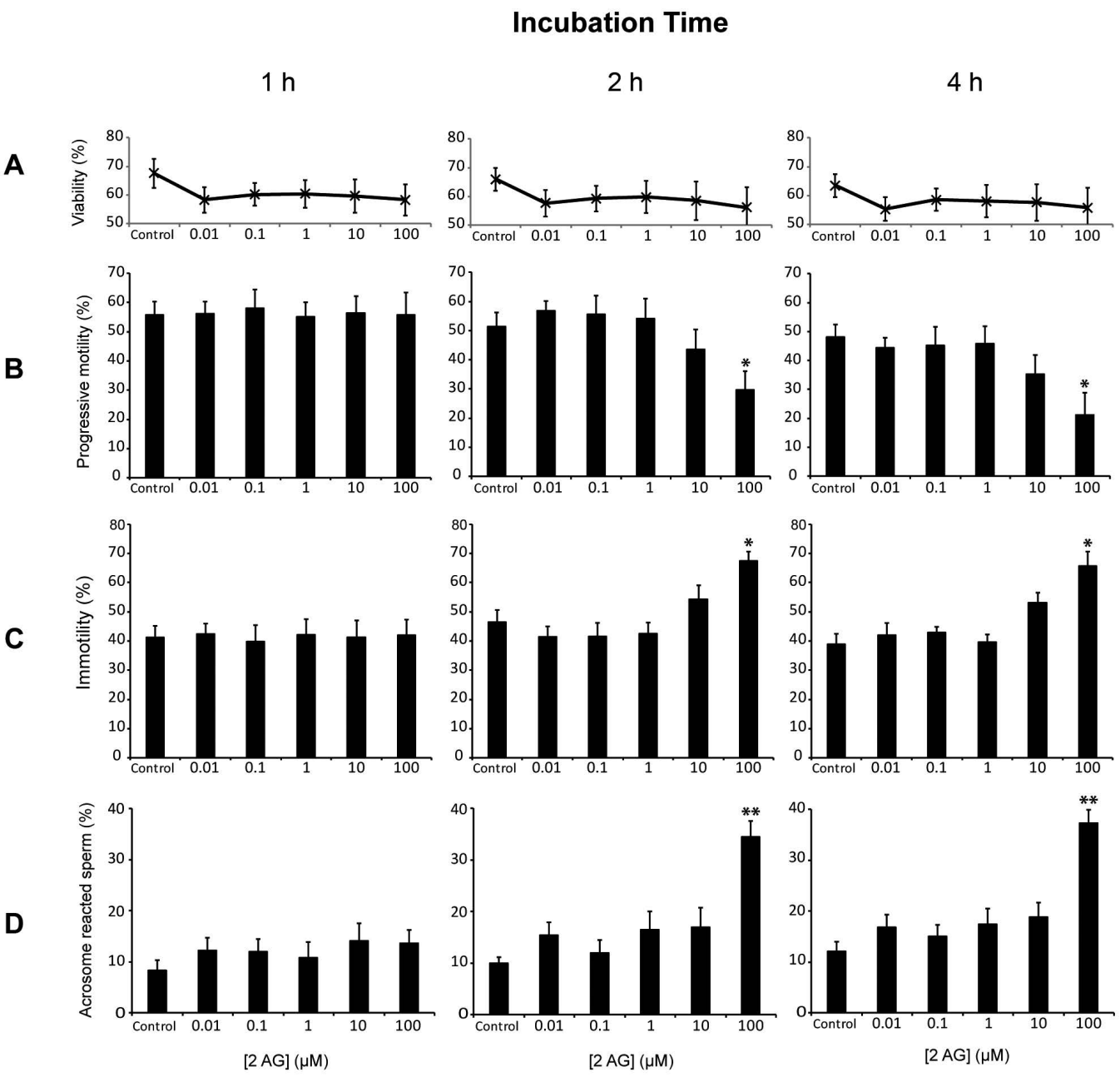
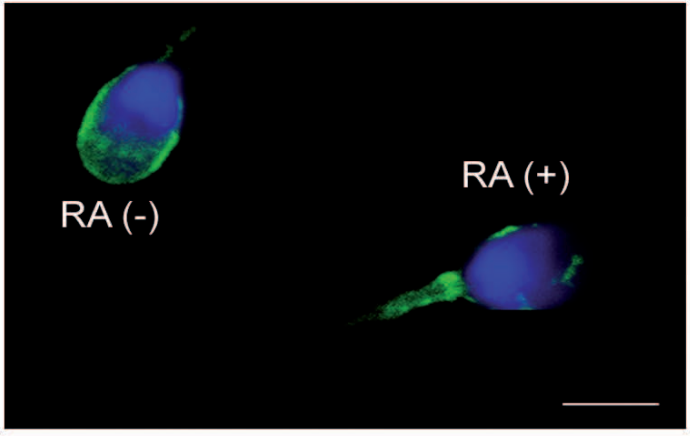
## FIGURE LEGENDS

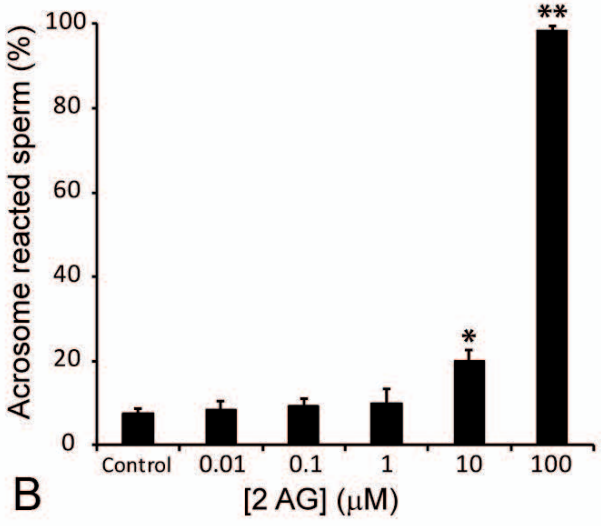
**Figure 1.** 2AG effect on fresh sperm**.** The graphs show that 2AG did not cause a significant reduction in sperm viability compared to control after 1,2 or 4 incubation hours **(A).** 2AG-100 µM only had an effect on sperm functions after 2 and 4 incubation hours, producing a significant decrease in progressive motility **(B)**, a significant increase in immotility **(C)** and acrosome reacted sperm **(D).** Values are mean ± SEM. Significant difference *versus* control, (*) $p < 0.05$; (**) $p < 0.01$.

**Figure 2.** 2AG effect during swim-up technique**.** The confocal micrograph shows an acrosome-reacted sperm (AR +, green-unlabeled) and an acrosome intact sperm (AR -, green-labeled) **(A)**; and the percentage of acrosome-reacted sperm was only significantly higher with 2AG-10 µM and 100 µM **(B)**. The spermatozoa with tyrosine phosphorylation (green-labeled flagellum) and without tyrosine phosphorylation (unlabeled) were observed by confocal analysis **(C).** The percentage of sperm with tyrosine phosphorylation was lower than control, when 2AG-10 µM and 100 µM were added **(D).** Values are mean ± SEM. Significant difference *versus* control, (*) $p < 0.05$. Scale bars 5µm.

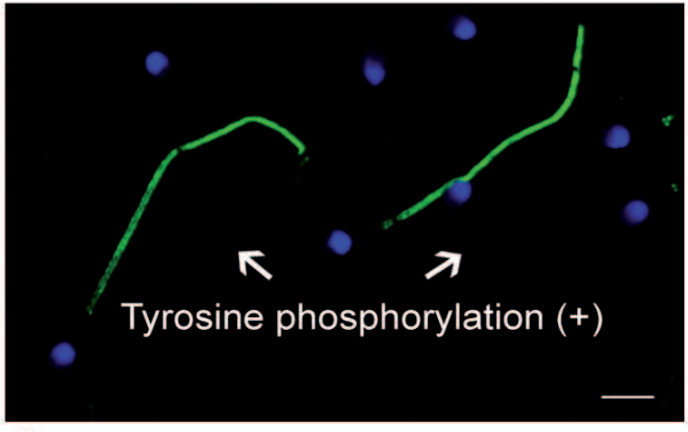

Incubation Time
1 h
2 h
4 h
A
B
C
D
Viability (%)
Progressive motility (%)
Immotility (%)
Acrosome reacted sperm (%)
Control
0.01
0.1
1
10
100
[2 AG] (μM)
*
**


RA (-)
RA (+)

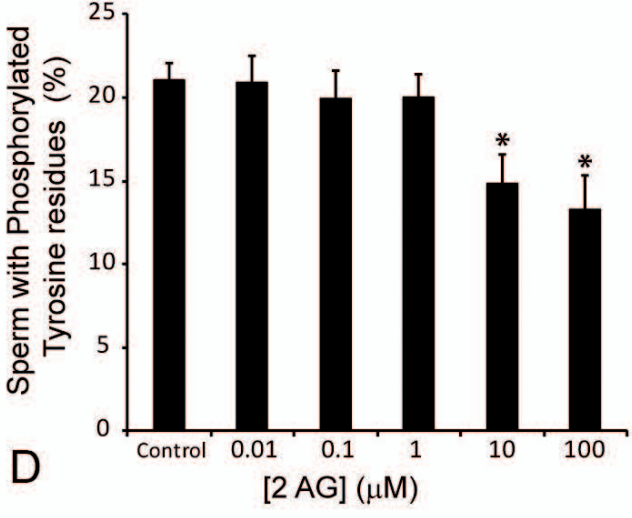
A


Acrosome reacted sperm (%)
100
80
60
40
20
0
**
*
Control
0.01
0.1
1
10
100
[2 AG] (μM)

B


Tyrosine phosphorylation (+)

C


Sperm with Phosphorylated Tyrosine residues (%)
25
20
15
10
5
0
*
*
Control
0.01
0.1
1
10
100
[2 AG] (μM)

D