

Human sperm motility, capacitation and acrosome reaction are impaired by 2-arachidonoylglycerol endocannabinoid

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Summary. 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: Arosome 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 arachidonylethanolamide 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. (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 μ M. 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°C for 1, 2 and 4 hours and the motility, viability and acrosome reaction was evaluated as described below.

Assays during the swim-up technique.

The swim-up technique was performed according to 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 pyruvate, 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 μ M. 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% CO₂ 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.

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 μ L of sample with 10 μ L 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 μ L 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°C 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 (3x5 min) and mounted with 5 μ L of Vectashield mounting medium

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(Vector Labs, Burlingame, CA) with 4',6-diamidino-2-phenylindole (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, -20°C 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-phenylindole (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 488 nm line of an Argon ion laser. For DAPI detection the 405 nm 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.

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).

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 CatSper1 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;

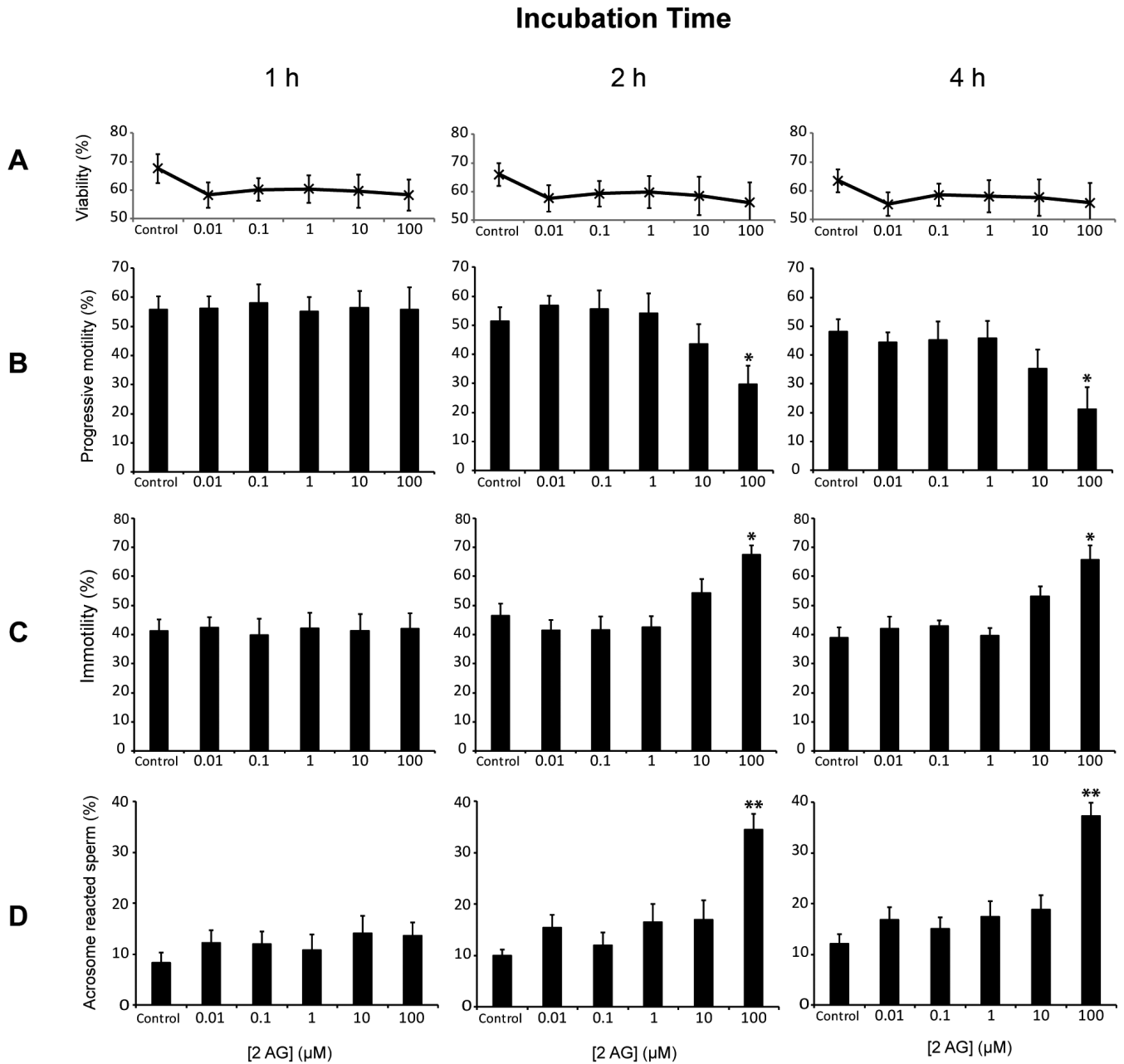


Fig. 1. 2AG effect on fresh sperm. The graphs show that 2AG did not cause a significant reduction in sperm viability compared to control after 1, 2 or 4 incubation hours (A). 2AG-100 μM only had an effect on sperm functions after 2 and 4 incubation hours, producing a significant decrease in progressive 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.

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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

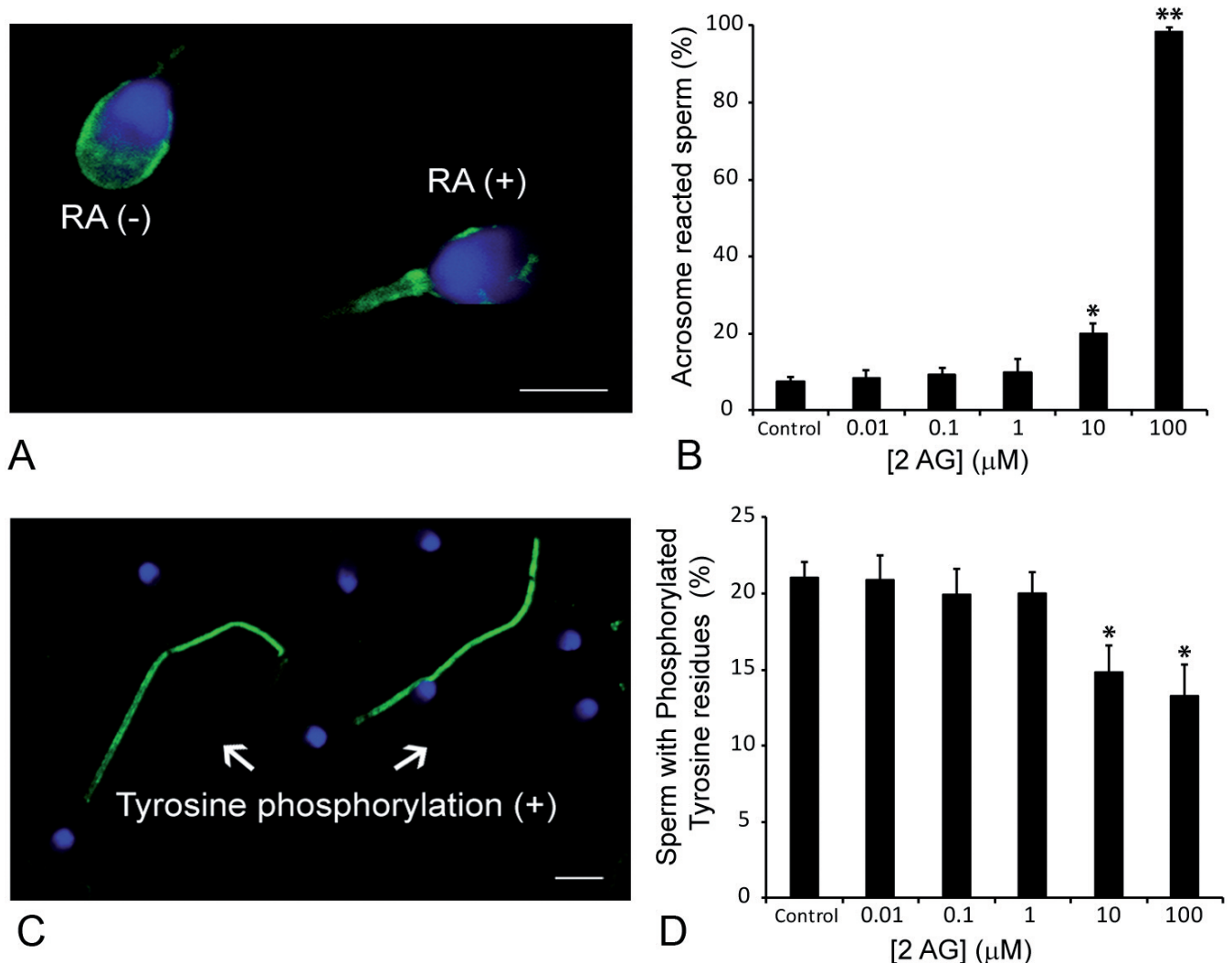


Fig. 2. 2AG effect during swim-up technique. The confocal micrograph shows an acrosome-reacted sperm (AR +, green-unlabeled) and an acrosome intact sperm (AR -, green-labeled) (**A**); and the percentage of acrosome-reacted sperm was only significantly higher with 2AG-10 μM and 100 μM (**B**). The spermatozoa 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 \pm SEM. Significant difference versus control, (*) $p < 0.05$. Scale bars: 5 μm .

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*, 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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