

Effect of Met-Anandamide on Prevention of Hyperactivation, Cryo-Capacitation and Acrosome Reaction in Ram Semen Cryopreservation

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Abstract

Cryopreserved spermatozoon has some defects on membrane causing hyper activation, cryo-capacitation and timeless (early) acrosome reaction that lead to low fertility. While low fertility is generally accepted as a consequence of cryopreservation of sperm cells, a lot of studies are being carried out on cryopreservation techniques to improve spermatozoon membrane integrity and also viability. Some studies point out that using materials such as Cannabinoids can be effective in achieving this aim. The main effects of endocannabinoids on spermatozoa are the inhibition of motility, capacitation and acrosome reaction. The aim of this study was to determine the effect of soluted Met-anandamide in extender on post thawing sperm motility, capacitation and acrosome reaction in ram semen cryopreservation. For this aim, ten ejaculated collected from five mature Bakhtiari rams were diluted with extender containing Met-anandamide, equilibrated and then cryopreserved in liquid nitrogen vapour (approximately -125 to -130°C). Cryopreserved semen samples were preserved in liquid nitrogen until evaluation. Post thawing sperm analyses revealed that the Cannabinoids agonists affected the motility of ram sperm after thawing ($P<0.05$), resulting in an increase in the AEA (arachidonoylethanolamide) concentration (10, 100 and 1000 nM) which significantly reduced sperm motility. subsequent freezing and thawing. On the other hand, the degrading enzyme of endocannabinoid FAAH may have had a reduction effect on sperm motility and increased capacitation and early acrosome reaction ($P<0.05$). In conclusion, endocannabinoid system may be used to improve ram sperm freezing.

Keywords: Ram, Sperm, Cryopreservation, Met-anandamide, FAAH, Capacitation

Met-Anandamidin Koç Spermasının Dondurulmasında Hiperaktivasyon, Kryo-Kapasitasyon ve Akrozom Reaksiyonunun Önlenmesi Üzerine Etkisi

Özet

Dondurulmuş spermatozoon membranında oluşan bazı hasarlar hiperaktivasyona, kryo-kapasitasyona ve zamansız (erken) akrozom reaksiyonuna neden olarak düşük fertiliteye yol açabilmektedir. Genel olarak; düşük fertilitenin sperm hücrelerinin dondurulmasının bir sonucu olarak oluştuğu kabul edilirken, spermatozoon membran bütünlüğünü sağlama ve aynı zamanda canlılığını artırmaya yönelik dondurma teknikleri üzerinde pek çok yeni çalışma yapılmaktadır. Bu amaçla, bazı araştırmalar Cannabinoid gibi maddelerin kullanımının etkili olabileceğini göstermektedir. Endocannabinoidlerin spermatozoa üzerine asıl etkisi motiliteyi inhibe etmesi, kapasitasyon ve akrozom reaksiyonudur. Sunulan çalışmanın amacı, koç semen dondurulmasında Met-anandamid solüsyonu içeren sulandırıcıyla sulandırmanın çözünme sonrası sperm motilitesi, kapasitasyonu ve akrozom reaksiyonu üzerine etkisini araştırmaktır. Bu amaçla, 5 adet ergin Bakhtiari koçundan elde edilen 10 ejakulat Met-anandamid içeren sulandırıcı ile sulandırılarak, ekilibre edilmesinin ardından sıvı azot buharında (yaklaşık -125 ila -130°C) donduruldu. Dondurulmuş sperma örnekleri değerlendirilene kadar sıvı azot içerisinde muhafaza edildi. Çözünme sonrası sperm analizleri, Cannabinoid agonistlerinin çözünme sonrası koç sperm motilitesini etkilemesi ($P<0.05$) sonucu AEA (arakidonoletanamid) konsantrasyonundaki (10, 100 ve 1000 nM) artışın sperm motilitesini önemli derecede azalttığını gösterdi. Öte yandan, endocannabinoid FAAH enzim degradasyonu sperm motilitesini azaltıcı ve kapasitasyonu ve erken akrozom reaksiyonunu ise artırıcı etki göstermiş olabilir ($P<0.05$). Sonuç olarak, endocannabinoid sistem koç spermının dondurulmasında daha iyi sonuç almak amacıyla kullanılabilir.

Anahtar sözcükler: Koç, Sperm, Kryporeservasyon, Met-anandamid, FAAH, Kapasitasyon



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INTRODUCTION

In most species, such as sheep, a lower fertility is generally determined as a consequence of cryopreservation of sperm cells and in order to improve the sperm viability in cryopreservation. However, sperm cryopreservation induces some defects such as formation of intracellular and extracellular ice crystals [1], osmotic [2-5] and chilling injury [6], cryo-capacitation and early acrosome reaction that reduced the functional life span of sperm and lead to low fertility [6,7]. Therefore, many attempts are being developed in cryopreservation techniques to improve sperm viability.

Anandamide (arachidonoyl ethanolamide, AEA) is a member of a group of endogenous lipids, collectively termed 'endocannabinoids'. It binds to type-1 and type-2 receptors of cannabinoid (CB1R and CB2R), thus having many functions in the central and peripheral nervous systems [8,9]. Such functions inactivation by cellular uptake through an AEA membrane transporter (AMT), completed by degradation to arachidonic acid and ethanolamine by the fatty acid amide hydrolase (FAAH) [10,11].

Some researchers believe that Cannabinoid receptor Type 1 (CB1) is effective in sperm membrane stability. They argue that endocannabinoids have an effect on CB1 receptors and then inhibit the adenylate cyclase/cAMP/PKA pathway which was earlier activated during the acrosome reaction [12-15]. They argue that the role of CB1 receptors in rapid hyper polarization of the cell membrane is also essential. However, other researchers have revealed that stimulation of vanilloid type 1 receptors (a non-selective cationic channel that belongs to the vanilloid-type transient receptor potential family), in the sperm membrane has been effective in preventing the acrosome reaction [16,17].

However, the endocannabinoids, by effect on their sperm receptors, lead to sperm remains in non-capacitated situation until they enter the female reproductive tract; concurrent with the reduction in sperm concentration endocannabinoids, and when the sperm reaches to oocyte and binds to the zona pellucida, sperm capacitation occurred [18-20]. Exactly, it prevents inappropriate capacitation and acrosome reaction, and then increases the chance of the sperm reaching to the egg. Now, due to the early capacitation problem in the process of freezing and given the need to preserve the reproductive potential of sperm after freezing and thawing, it can be effective to employ a substance that delays capacitation and acrosome reaction of sperm.

Therefore, the aim of present study is to examine the impact of different doses of Met-anandamide as a non-metabolized analog anandamide and FAAH as hydrolyzing enzyme of anandamide, on the quality of frozen Lori-Bakhtiari sheep sperm. Findings of the present research can help us to understand the beneficial or detrimental

effects of the cannabinoid system on the quality of sperm after freezing and thawing. It is worth mentioning that this is the first time that Met-anandamide has been added to the freezing media for improving fertility potential of sperm.

MATERIAL and METHODS

Animals

In this study, the samples give from five 4 to 5 years rams (*Ovis aries*) that had bred for giving sperm for artificial insemination in the Lori-Bakhtiari research flock at Shooli station in Shahrekord.

Semen Extenders and Treatment Groups

The base extender used in this study (Tris base; TB) was composed of 200 mM Tris (Hydroxymethyl)-aminomethan (Merck, Darmstadt, Germany), 27.8 mM D-Glucose (Sigma; G6152), 94.7 mM Citric acid (Sigma; C1909), 100 IU/ml Penicillin (sigma) and 0.05 mg/ml streptomycin (Sigma). The osmolality and pH were adjusted to 320 mOsm and 7.2, respectively. Freezing medium was prepared by adding 25% egg yolk (egg yolk lipids were solubilized by adding 0.035% (w/v) sodium dodecyl sulfate (SDS) [21] and adding 7% Glycerol to Tris base medium. Five different extenders were designated by the addition of different concentrations of AEA (Met-anandamide) as following 10 nM; 100 nM AEA; 1000 nM AEA and FAAH (0.025 IU/ml). No addition group was considered as control .

Semen Collection and Processing

Ten ejaculates from each ram were collected by artificial vagina twice a week during the breeding season from five mature Bakhtiari rams known to have good fertility. Only normospermic ejaculates (volume >0.75 ml, sperm concentration >3×10⁹ sperm/mL, motility >70%, abnormal sperm <10%) were used during this study. To eliminate individual differences, semen samples from the five rams were pooled. Each pooled sample was divided into five equal aliquots and diluted with extenders containing different AEA concentrations or FAAH. The diluted semen was gradually cooled to 4°C for 2 to 3 h. The cooled semen was loaded into 0.5-mL French straws (Biovet, L'Agile France), semen samples loaded to straws were exposed to liquid nitrogen vapor for 10 min (3 cm above LN2 level, approximately -125 to -130°C), plunged into liquid nitrogen (LN2), and stored in LN2 until thawed and used for the evaluation of sperm parameters.

Evaluation of Sperm after Freezing-Thawing

- **Sperm motility:** Sperm motility parameters were measured by CASA Hamilton, with the following settings: Image collection speed, 20 frames per second; analysis time per frame, less than 15 seconds; sperm velocity that can be analyzed, 0-180 µm/s; Number of vision fields that were selected, 10/samples; magnifying power of micro-

scope (object lens), 40X measurements were done in sperm miter chambers 10 μ m depth. The sperm motility was measured as fast progressive motility (class A) in percentage. The studied motion parameters was as following: VSL, VCL, VAP, ALH, BCF, LIN, STR and WOB [22].

- **Sperm viability:** For viability, the hypo-osmotic swelling (HOS) test and eosin-nigrosin staining were used according to Revel and Mortimer methods, respectively [23,24] and then were performed in each aliquot. In HOS test, 25 mL semen was added to 200 ml hypo-osmotic solution (100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate). After 30 minutes of incubation at room temperature, the mixtures were homogenized and evaluated with an inverted light microscope. A total of 200 spermatozoa were assessed in four different microscopic fields. The sperm percentage with curved and swollen tails was recorded.

In eosin-nigrosin staining technique, 0.67% eosin Y and 10% nigrosin dissolved in 0.9% sodium chloride in distilled water. Approximately equal volumes of sperm sample and stain were blended. The suspension was incubated for 30 sec. at room temperature (20°C). Then, a 12 μ l droplet was transferred with the pipette to a labeled microscope slide where it was smeared by sliding a cover slip in front of it. Two smears were taken from each sample. The smears were air dried and evaluated directly. At least 200 sperms were assessed for each preparation at a magnification of 400x. Spermatozoa that were unstained or white were considered live, while those that indicated red or pink coloration in the head region were classified as dead.

- **Capacitation Status:** Acrosome reaction was assessed with chlortetracycline (CTC) staining as described by Perez et al.[25] with little modification. A CTC working solution (750 mM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM D, L-cysteine at a pH of 7.8. Five microliters of semen were mixed with 20 mL CTC working solution. After 20 seconds, the reaction was stopped by the adding 5 mL 1% (v/v) glutaraldehyde to 1 M Tris-HCl, pH 7.8. Smears were prepared on a clean microscope slide and examined under an epifluorescent microscope (Olympus). Stained spermatozoa shows three types of patterns: 1) uniform fluorescence head, or un-

capacitated sperm (Noncapacitated pattern); 2) post-acrosomal region without fluorescence, capacitated sperm (Capacitated pattern); and 3) Fluorescence-free head or a thin fluorescence band on the equatorial segment (sperm that underwent an acrosome reaction [acrosome reacted pattern]). In this study 200 sperm were assessed in each smear.

Statistical Analysis

Each treatment was replicated ten times. For each, three straws were thawed and pooled for sperm parameters evaluation. To evaluate differences between groups, we used analysis of variance (ANOVA) for comparisons of means after evaluate the normality of our data by Kolmogorov-Smirnov test. When statistical differences were revealed with the ANOVA test, the mean of the treatments was compared using Duncan's multiple range test (DMRT), and a confidence level of $P < 0.05$ was regarded to be significant. Statistical evaluations were conducted using the Statistical Package for Social Studies software (Version 20; SPSS, Chicago, IL). Information on CTC staining was analyzed using the GENMOD procedure of SAS (Version 9.0, SAS Institute Inc., USA) to fit a generalized linear model with a logit link function.

RESULTS

The findings are shown separately and interpreted comprehensively below.

Sperm Motility

CASA evaluation revealed that the presence of AEA in freezing medium affected motility of rams sperm after thawing. Increases in AEA concentration reduced sperm motility significantly by using high concentration of AEA compared to the low concentrations of AEA and control group. In addition, FAAH had a positive impact on increasing the sperm motility compared to the control group and all levels of AEA ($P < 0.05$) (Table 1). These effects take place through the change in the number of sperm with fast progressive motility.

Table 1. Effect of different concentration of Met-anandamide and FAAH in freezing medium on motility (mean \pm SE) of frozen-thawed ram sperm (evaluated by CASA)

Table 1. Dondurulmuş-çözdürülmüş koç sperminin motilitesi (ortalama \pm SE) üzerine farklı Met-anandamid ve FAAH konsantrasyonunun etkisi (CASA ile değerlendirme)

Groups	Concentration	Motile Sperm	Progression (%)			
			Fast Progressive	Slow Progressive	Non- Progressive	Non- Motile
Anandamide	10 nM	50.37 \pm 5.69 ^a	19.76 \pm 6.58 ^{ab}	23.35 \pm 1.34	6.65 \pm 0.69	50.23 \pm 2.94 ^{ab}
	100 nM	38.79 \pm 3.05 ^{ab}	12.5 \pm 2.51 ^a	18.46 \pm 1.16	7.22 \pm 0.71	61.81 \pm 3.24 ^a
	1000 nM	31.19 \pm 3.18 ^b	6.37 \pm 0.95 ^a	16.57 \pm 1.96	7.65 \pm 1.01	69.41 \pm 3.12 ^a
FAAH	0.025 IU/ml	65.67 \pm 2.37 ^c	26.26 \pm 1.33 ^b	30.67 \pm 3.69	9.15 \pm 0.58	33.93 \pm 2.37 ^b
Control		43.74 \pm 4.01 ^a	19.49 \pm 4.7 ^{ab}	17.15 \pm 0.76	6.5 \pm 0.83	56.86 \pm 4.31 ^{ab}

^{a,b,c} Numbers with different superscripts in the same column differ significantly ($P < 0.05$)

In some motility, parameters of sperm which are associated with velocity including VCL, VSL, VAP and BCF, significant differences were found between AEA groups and control group; and groups containing high concentrations of AEA (100nM and 1000nM) and FAAH group were significantly different as well ($P<0.05$); as in the presence of FAAH, sperm moves faster and beats cross frequency (BCF) and the mean angle degree (MAD) increased. These parameters decrease in the presence of high concentrations of AEA. High concentration of AEA significantly reduces the percentage of sperms with linearity (LIN) and straightness (STR) moving and also reduces sperm wobble ($P<0.05$) (Table 2).

Sperm Viability

Eosin-Nigrosin and HOS tests indicated that AEA did not affect viability of sperm ($P>0.05$) (Fig. 1); while FAAH had an impact on vitality of ram sperm significantly

($P<0.05$) compared to 1000nM AEA group.

Acrosomal Status

The findings are shown in Fig. 2. They revealed that Met-anandamide in 1.000 mg/ml concentration can protect ram sperm against induced cryo-capacitaction and acrosome reaction significantly ($P<0.05$) subsequent freezing and thawing and can maintain the sperm viability by delaying capacitation and acrosome reaction. Our findings suggest that FAAH stimulates acrosome reaction in spermatozoa significantly ($P<0.05$) (Fig. 2).

DISCUSSION

Poor sperm motility after cryopreservation is associated with a significant reduction in fertility, pointing to the importance of preserving motility in cryopreserved sperm. Despite the availability of intricate assisted reproductive

Table 2. Effect of different concentration of Met-anandamide and FAAH in freezing medium on motility pattern (mean \pm SE) of frozen-thawed ram sperm (evaluated by CASA)

Tablo 2. Dondurulmuş-çözdürülmüş koç sperminin motilite modelleri (ortalama \pm SE) üzerine farklı Met-anandamid ve FAAH konsantrasyonunun etkisi (CASA ile değerlendirme)

Groups	Concentration	Sperm Motility Pattern								
		VCL (μ m/s)	VSL (μ m/s)	VAP (μ m/s)	MAD ($^{\circ}$)	ALH (μ m)	BCF (Hz)	LIN (%)	WOB (%)	STR (%)
Anandamide	10 nM	30.28 \pm 2.62 ^{ab}	12.68 \pm 2.09 ^{ab}	19.33 \pm 2.05 ^{ab}	6.85 \pm 1.25 ^a	1.94 \pm 0.08 ^{ac}	0.16 \pm 0.05 ^{ab}	37.26 \pm 2.33 ^a	54.73 \pm 1.80 ^a	56.83 \pm 2.09 ^{ab}
	100 nM	25.94 \pm 2.21 ^a	9.52 \pm 1.45 ^a	15.43 \pm 1.52 ^a	6.40 \pm 1.18 ^a	1.70 \pm 0.08 ^{ab}	0.15 \pm 0.04 ^a	28.79 \pm 1.54 ^b	46.83 \pm 1.24 ^{bc}	46.69 \pm 1.86 ^{cd}
	1000 nM	21.57 \pm 0.89 ^a	7.47 \pm 0.56 ^a	12.79 \pm 0.74 ^a	3.49 \pm 0.29 ^a	1.45 \pm 0.07 ^b	0.07 \pm 0.01 ^a	27.16 \pm 1.16 ^b	44.89 \pm 0.86 ^c	45.25 \pm 1.16 ^c
FAAH	0.025 IU/ml	38.23 \pm 3.44 ^b	17.80 \pm 1.91 ^b	23.35 \pm 2.08 ^b	12.52 \pm 1.70 ^b	2.26 \pm 0.10 ^c	0.33 \pm 0.05 ^b	39.12 \pm 2.17 ^a	55.35 \pm 1.83 ^a	61.34 \pm 1.80 ^a
Control		30.28 \pm 2.99 ^{ab}	12.12 \pm 2.42 ^{ab}	19.73 \pm 2.52 ^{ab}	7.78 \pm 1.17 ^{ab}	1.92 \pm 0.11 ^{ab}	0.21 \pm 0.05 ^{ab}	33.66 \pm 1.98 ^{ab}	51.48 \pm 1.95 ^{ab}	53.79 \pm 2.01 ^{bd}

^{a,b,c,d} Numbers with different superscripts in the same column differ significantly ($P<0.05$)

Fig 1. Comparison of sperm vitality of frozen-thawed ram sperm between treatments and control groups using Eosin-Nigrosin test and hypoosmotic swelling test (HOS test)

^{a,b} Columns with the same pattern and different superscripts differ significantly ($P<0.05$)

Şekil 1. Dondurulmuş-çözdürülmüş koç sperminin sperm canlılığının Eosin-Nigrosin testi ve hypoosmotic swelling testi (HOS test) kullanılarak karşılaştırılması

^{a,b} Aynı model ve farklı harf taşıyan sütunlar arasındaki fark istatistiksel olarak önemlidir ($P<0.05$)

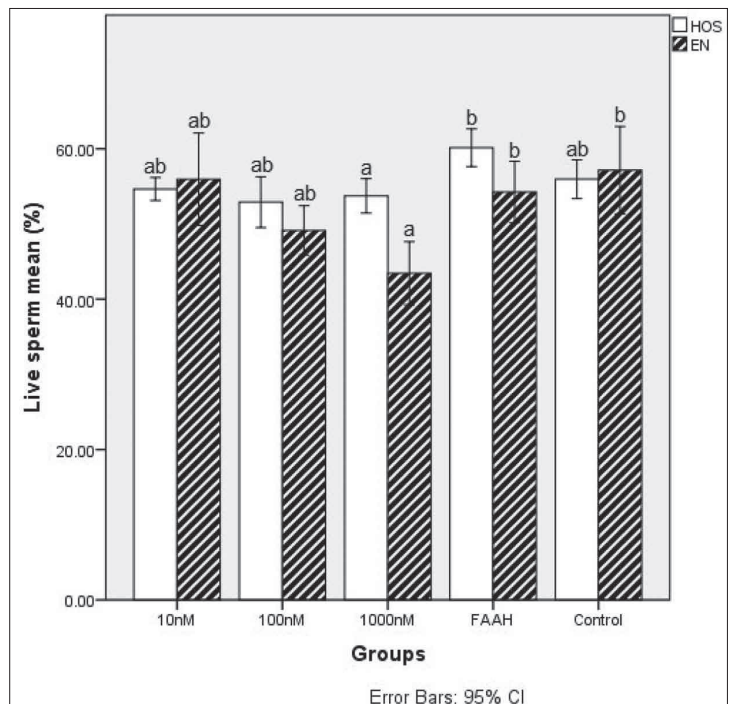
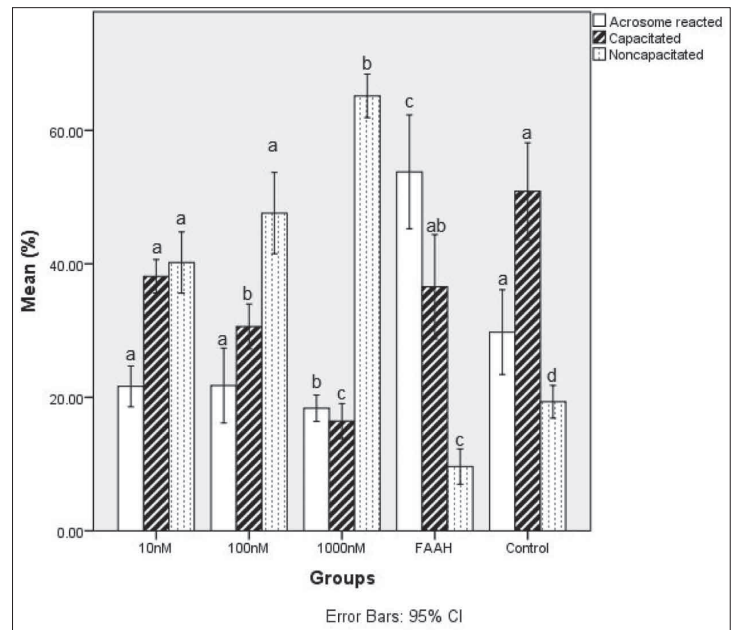


Fig 2. Effect of different concentration of Met-anandamide and FAAH in freezing medium on acrosome reaction and capacitation status of frozen-thawed ram sperm (evaluated by chlortetracycline test)

^{a,b,c,d} Columns with the same pattern and different superscripts differ significantly ($P < 0.05$)

Şekil 2. Dondurulmuş-çözdürülmüş koç sperminin akrozom reaksiyonu ve kapasitasyon durumu üzerine dondurma medyumundaki farklı Met-anandamid ve FAAH konsantrasyonunun etkisi (chlortetracycline testi ile değerlendirme)

^{a,b,c,d} Aynı model ve farklı harf taşıyan sütunlar arasındaki fark istatistiksel olarak önemlidir ($P < 0.05$)



techniques, the cryosurvival and pregnancy rates with frozen semen are lower than those with fresh semen [26-28]. In this study, we observed a detrimental impact of cryopreservation on overall sperm quality. Regardless of whether the spermatozoa was treated with endocannabinoids before freezing, significant decreases in the percentage of motile spermatozoa, motion characteristics, and in the percentage of viable spermatozoa with intact acrosomes were observed.

It was also revealed through CASA evaluation that presence of AEA in freezing medium influenced motility of ram sperm after thawing. Here increases in AEA concentration reduced sperm motility significantly by using high concentration of AEA compared to the low concentrations of AEA and control group. These impacts take place through the change in the number of sperm with rapid progressive motility. AEA reduced some motility parameters of sperm that are associated with velocity like VCL, VSL, VAP and BCF and in the presence of high concentrations of AEA sperm moves slower and beats cross frequency (BCF) and the mean angle degree (MAD) were reduced. High concentration of AEA significantly reduces the percentage of sperms with linearity (LIN) and straightness (STR) moving and also reduces sperm wobble. In addition to increasing the number of motile spermatozoa, AEA can improve sperm motion characteristics in preselected subpopulations of spermatozoa, which is consistent with other studies which found that AEA reduces motility of human sperm by mitochondrial activity reduction. Athanasiou et al.^[29] have revealed that cannabinoids inhibit mitochondrial membrane potential, oxygen consumption and ATP production by preventing the mitochondrial respiratory chain. Sperm motility, along with capacitation and acrosome reaction are all energy consuming processes. The sperm kinetic activity is

dependent on many factors such as integrity of all flagellar structural proteins, oxidation of energetic substrates and ATP production, transformation of chemical energy into mechanical movement, the activity of all enzymes involved in the flagellar beating [19,30]. The decline in mitochondrial activity would be expected to reduce energy supply, thus affecting different sperm functions as well as motility but also capacitation and acrosome reaction. If endocannabinoids inhibit energy metabolism in sperm, then it could be possible that the inhibition of all sperm functions by endocannabinoids could origin from the same mechanism of action, i.e. reduction of the energetic reserves on which all these processes rely on [19].

To this respect, Rossato et al.^[19] have shown that AEA induced a dose-dependent decrease of the sperm mitochondrial activity that paralleled the reduction of sperm motility without affecting viability and Whan et al.^[20] have shown that Delta-9 tetrahydrocannabinol reduce human sperm motility. These studies are consistent with our results and our findings are consistent with these studies.

Considering our observations, such findings support the hypothesis that cryopreservation somehow precociously triggers the signal transduction pathway leading to capacitation, and that cryo-capacitation is partly responsible for the reduced fertility of thawed semen. Physiological capacitation is now accepted to be regulated by intracellular signaling pathways that results in the tyrosine phosphorylation of various sperm proteins [12-15]. The signaling pathways are not completely clear, although protein kinase A and protein tyrosine kinases appear to be involved. In the present study, the reduction of sperm capacitation of frozen-thawed ram sperm at the exposure of AEA in freezing medium confirms the above information. The results in Fig. 2 reveal that Met-anandamide in 1000

mg/ml concentration can protect ram sperm against induced cryo-capacitation and acrosome reaction significantly subsequent freezing and thawing and can maintain sperm viability by delaying the capacitation and acrosome reaction.

In this study AEA inhibited ram sperm acrosome reaction which is consistent with other studies which suggest AEA inhibits capacitation-induced acrosome reaction [19]. Based on the inhibitory effects of endocannabinoids on sperm capacitation, available substantially homogeneous data indicate that CB1 activation reduces sperm ability to undergo acrosome reaction both in vertebrate and invertebrates [19,20,31-33]. More importantly, endocannabinoids do not affect fertility of eggs; this shows that these effects are directed just to sperm [31,32]. The mechanisms activated by endocannabinoids determining the prevention of the sperm acrosome reaction are still unclear. It is well-known that the regulation of ion fluxes through the ion channels of sperm plasma membrane are essential for the acrosome reaction, and the most essential regulator appears to be calcium (Ca^{2+}) signaling [34,35]. In this respect, Rossato et al. [19] have revealed that the endocannabinoid AEA do not change the intracellular Ca^{2+} concentrations in human sperm, thus possibly ruling out any interfering effect of endocannabinoids on Ca^{2+} signaling. It is also known that CB1 is a receptor of G-protein coupled that has been revealed to inhibit adenylate cyclase activity [36,37]. Adenylate cyclase/cAMP/protein kinase A pathway plays an important role in the sperm capacitation and acrosome reaction: intracellular cAMP levels rise during acrosome reaction, cell permanent cAMP analogues induce the acrosome reaction, pharmacological inhibitors of cAMP-dependent PKA reduce this exocytotic event supporting a role for the adenylate cyclase/cAMP/PKA system in the signaling pathway leading to acrosome reaction of sperm and egg fertilization [12-15]. The inhibitory effects of endocannabinoids on this fundamental signaling pathway as recently described in mammalian sperm [33] may explain the negative actions of these agents activating the CB1 on sperm capacitation and acrosome reaction.

Furthermore, in inhibition of capacitation and acrosome reaction by endocannabinoids, many hypotheses may explain the inhibitory effects of endocannabinoids on mitochondrial function: endocannabinoids may interfere with mitochondrial electron transport [38,39], mitochondrial activity via depletion of NADH as previously shown [40], mitochondrial permeability transition pore complex as recently suggested [40]. The possible interference of endocannabinoids with glucose uptake and utilization for ATP production as previously suggested by experimental studies utilizing testicular extracts [41], seems not plausible since in many different cell types endocannabinoids have been shown to increase glucose uptake instead of decreasing it as we have recently demonstrated in human adipocytes [42] confirming recent observations in rat 3T3-L1 adipocytes [43]. Thus, an increase of AEA could be

completing an important duty via improving mechanisms involved in the obtaining of the sperm fertilizing capacity.

Findings of this study indicated that FAAH as hydrolyzing enzyme of anandamide had a positive effect on increasing sperm motility and motility parameters associated with velocity such as VCL, VSL, VAP and BCF. Findings also suggested that in the presence of FAAH, sperm moves faster and beats cross frequency (BCF) and the mean angle degree (MAD) was increased. Sperm capacitation and acrosome reaction are also stimulated with FAAH. Endocannabinoids can be metabolized inside the cell by multiple pathways: AEA is a substrate mainly for fatty acid amide hydrolase (FAAH), that breaks the amide bond and releases arachidonic acid (AA) and ethanolamine [44]. FAAH is bound to intracellular membranes [45]. Obstruction AEA degradation by URB97 (an inhibitor of FAAH) caused sperm capacitation and the pre-incubation of sperm with CB1 or TRPV1 antagonists inverted this result [46]. It is proposed that cellular uptake of AEA can be controlled with FAAH by maintaining and/or creating an inward concentration gradient that drives the facilitated diffusion of AEA through anandamide membrane Transporter (AMT). Although FAAH is not the only factor controlling AEA transport, its pivotal function in degradation of AEA may explain why it is modulated in some pathophysiological conditions.

In conclusion, the importance of the endocannabinoid system makes it an attractive target for improvement of mammals' sperm freezing methods and subsequent *in vitro* embryo production, artificial insemination and pharmacological interventions to control male fertility.

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