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Original Paper

Tetrahydrocannabinol Modulates *in Vitro* Maturation of Oocytes and Improves the Blastocyst Rates after *in Vitro* Fertilization

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Key Words
Oocyte • Maturation • THC

Abstract
Background/Aims: Among the assisted reproductive techniques, the *in vitro* maturation of oocytes (IVM) is less developed than other techniques, but its implementation would entail a qualitative advance. This technique consists in the extraction of immature oocytes from antral ovarian follicles with the patient under low hormone stimulation or without hormone to mature exogenously in culture media supplemented with different molecules to promote maturation. In this sense, we are interested in the role that cannabinoids could have as IVM promoters because cannabinoid's molecular pathway is similar to the one by which oocyte's meiosis resumption is activated. With the intention of advancing in the possible use of cannabinoids as supplements for the media for *in vitro* maturation of oocytes, we intend to deepen the study of the function of the phytocannabinoid Δ-9-tetrahydrocannabinol (THC) in the IVM process. ***Methods:*** By immunocytochemistry, we detected the location pattern of cannabinoid receptor type 1 (CB1) and type 2 (CB2) during oocyte maturation in presence or absence of THC, as well as, the staining pattern of p-AKT and p-ERK. We used a genetic/pharmacological approach generating knockout oocytes for CB1 and/or CB2 and they were incubated with THC during the oocyte maturation to visualize the physiological effects of THC, observing the rate of blastocyst achieved by oocyte. ***Results:*** This study confirms that the incubation of oocytes with THC during IVM accelerated some events of that process like the phosphorylation pattern of ERK and AKT and was able to increase the blastocyst rate in response to IVF. Moreover, it seems that both CB1 and CB2 are necessary to maintain a healthy oocyte maturation. ***Conclusion:*** Our data suggest that THC may be useful IVM supplements in clinic as is more feasible and reliable than any synthetic cannabinoid.

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Introduction

For years now, growing fertility problems and current changes in family patterns have resulted in a wider use of assisted reproduction techniques (ART) among couples trying to get pregnant [1–3]. Even so, the success of ARTs is not yet complete and the research work continues to improve these techniques. Among the ARTs, the *in vitro* maturation of oocytes (IVM) is less developed than other techniques, but its implementation would entail a qualitative advance. This technique consists in the extraction of immature oocytes from antral ovarian follicles with the patient under low hormone stimulation or without hormone. The oocytes are then exogenously matured in culture media supplemented with different molecules to promote maturation [4, 5].

The most important goal of IVM method is that could be the only hope or alternative for a high number of patients unable to tolerate high doses of gonadotropins, such as patients with polycystic ovary syndrome (PCOS) or ovarian hyperstimulation syndrome (OHSS) [6, 7]. In general, clinical pregnancy and implantation rates per embryo transfer have reached 35%–40% and 15%–20%, respectively, in infertile women with PCOS after IVM of immature oocytes [8]. Further candidates for oocyte IVM are patients whose ovarian tissue was frozen due to a pre-existing disease such as cancer, in whom hormone stimulation was not recommended or there was insufficient time to undergo a normal *in vitro* fertilization (IVF) cycle [9]. In addition, by avoiding controlled ovarian stimulation, the IVM procedure eliminates the need for frequent ultrasound monitoring and is thus less costly than conventional IVF [10].

The complexity lies in that the oocyte meiotic maturation is a complex process whereby immature oocytes acquire the characteristics required for successful fertilization and embryogenesis. For that, immature oocytes arrested at the diplotene of prophase I (designed as germinal vesicles -GV-) must resume meiosis until the metaphase II of meiosis (MII) [11].

However, although some mechanisms that lead the reactivation of meiosis are still to be explained, it is known that the oocyte maturation involves the activation of various signal transduction pathways that converge to activate maturation-promoting factor. Among others, there are some evidence that heterotrimeric G-proteins, which inhibit adenylate cyclase, can interact with both PI3K and MAPK to promoting the oocyte maturation [12]. In this sense, we are interested in the role that cannabinoids could have as IVM promoters. On the one hand, the molecular cascade that cannabinoids exerts when they activate the G-protein coupled cannabinoid receptors (inhibition of adenylate cyclase, reducing cAMP, and activation of PI3K/Akt and MAPK patways) is very similar to the one that occurs during oocyte meiotic resumption [13, 14]. On the other hand, some studies described the presence of the cannabinoid system in human [15–19], bovine [20] and murine [21] oocytes, and, at least in the two last species, synthetic cannabinoids were able to modulate the oocyte maturation [20, 21].

Being this the general content, with the intention of advancing in the possible use of cannabinoids as supplements for the media for *in vitro* maturation of oocytes, we intend to deepen the study of the function of the phytocannabinoid Δ-9-tetrahydrocannabinol (THC). The THC is called phytocannabinoid because it is a compound of the plant *Cannabis sativa*, and it has attracted particular attention since its synthetic analogs dronabinol and nabilone are licensed for medicinal use [22]. Even so, the use of phytocannabinoids as THC in clinic is more feasible and reliable than any synthetic cannabinoid [23, 24].

The aim of this study is to characterize the role of the phytocannabinoid THC in the IVM process. To that end, we used a genetic/pharmacological approach generating knockout oocytes for CB1 and/or CB2 receptors. They were modulated pharmacologically during the oocyte maturation to visualize the physiological effects of THC. Our results support the notion that the incubation of oocytes with THC during IVM accelerated some events of that process like the phosphorylation pattern of ERK and AKT and was able to increase the blastocyst rate in response to IVF. Moreover, it seems that both CB1 and CB2 are necessary to maintain a healthy oocyte maturation.

Materials and Methods

Experimental animals

The adult wild-type, *Cnr1*$^{-/-}$ [25], *Cnr2*$^{-/-}$ [26]and *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$ mice used in this study were kept in an animal house under controlled conditions of temperature (22 ± 1°C) and photoperiod (light/dark cycle 14 h:10 h). Animals were given free access to water and food. All experimental procedures using mice were approved by the University of the Basque Country (UPV/EHU, CEEA reference number: M20-2015-016-027-028-173) and were performed according to the *Guide for Care and Use of Laboratory Animals*, endorsed by the Society for the Study of Reproduction and European legislation.

Isolation and in vitro maturation of cumulus–oocyte complexes

Female 8 to 10 week old WT (C57BL/6xCBA) or KO on a C57BL/6N background (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$, and *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$) mice were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (Folligon, Intervet, Castle Hill, NSW, Australia), and ovaries were collected 46 to 48 h later. The ovaries were cleaned of any connective tissue and placed in handling medium M2 supplemented with 4 mg/ml bovine serum albumin fraction V. Antral follicles were punctured with 30-gauge needles, and immature cumulus–oocyte complexes (COCs) were collected in handling medium. Only COCs with 3 compact cumulus cells were used. COCs were matured for 17 h in TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor at 37°C under an atmosphere of 5% CO_2 in air with maximum humidity.

IVM supplementation with cannabinoid agonist THC

The THC stock solutions were prepared in DMSO. During maturation (17 h), COCs were incubated with different doses of THC (10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) to evaluate the effects of activation of cannabinoid receptor by this agonist. Once obtained the most efficient concentration for oocyte maturation, to see the phosphorylation pattern of ERK and AKT proteins and the effect on embryo development, the COCs were incubated with 10^{-7} M (100nM) of THC. COCs containing the same amount of DMSO were used as an incubation control.

Meiotic progression of mice oocytes undergoing IVM with THC

To determinate the impact of THC on germinal vesicle breakdown (GVBD), COCs from experimental group and control with DMSO at 2, 4, 6, 8, 12, 17h of IVM were used as described previously [27]. In sum, COCs were partially denuded in 0, 1% of hyaluronidase (Sigma H3506) and fixed in 4% paraformaldehyde (Panreac, Barcelona, Spain) for 20 min. Then, were washed twice in PBS and incubated in PBS containing Hoescht 33342 (0.01 mg/ml) for 15 min. Oocytes were then placed in glass slides and squashed with coverslip in order to visualize the nuclear stage under microscopy (Zeiss Axioskop, NY, USA).

Immunofluorescence

For these experiments, 3 females per genotype were used in 3 replicate trials. Once meiotic stages were established, to immunocytochemically localize the CB1 and CB2, 20 WT oocytes per stage were treated as previously described [17]. Briefly, *in vitro* matured oocytes in presence or absence of THC were washed in PBS supplemented with 1% polyvinyl alcohol (PVA) and fixed in 4% paraformaldehyde (Panreac, Barcelona, Spain) for 10 min at room temperature. The oocytes were then permeabilized by incubation in PBS with 10% (v/v) FCS and 1% Triton X-100 for 45 min at room temperature. After permeabilization, oocytes were incubated overnight at 4°C in PBS containing 1% PVA, 5% normal FCS serum, and 1:100 rabbit polyclonal anti-cannabinoid CB1 (Cayman Chemicals, Ann Arbor, MI, USA) and 1:400 rabbit polyclonal anti-cannabinoid CB2 (Cayman Chemicals, Ann Arbor, MI, USA). After incubation, oocytes were washed twice in PBS containing 1% PVA and incubated in PBS supplemented with 1% PVA, 5% FCS serum, and 1:500 goat polyclonal secondary antibody Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Next, the oocytes were washed 3 times in PBS–1% PVA. In all cases, nuclei were stained with Hoechst 33342 (0.01 mg/ml) during the second wash to facilitate the determination of the maturation stage of each oocyte [28]. Finally, oocytes were mounted in microdrops with Fluoromount G (EMS, Hatfield, United Kingdom) and examined by confocal microscopy (Fluoview FV500; Olympus, Tokyo, Japan). Negative controls were prepared in the same way omitting the primary antibody before addition of the secondary antibody.

To monitor the activation of pERK1/2 and pAKT signaling during oocyte maturation in the presence of cannabinoids, an average number of 20 oocytes was collected at 0 h, 10 min, 30 min and 1 h to evaluate the initial response, and at 17 h as the optimal time for maturation. Rabbit polyclonal phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) primary antibody was used at a 1:400 dilution and Rabbit phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA) 1:200 primary antibody was used at a 1:400 dilution. Finally, oocytes were mounted in microdrops with Fluoromount G (EMS, Hatfield, United Kingdom) and examined by confocal microscopy (LSM 800, Zeiss, NY, USA). Negative controls were performed in the same way, except for omission of the primary antibody before secondary antibody addition.

Isolation and in vivo maturation of COCs

MII oocytes were collected from 8- to 10-week-old oviducts of female WT (C57BL/6xCBA) or KO (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$) superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (Folligon), followed 48 h later by 7.5 IU of human chorionic gonadotropin (Veterin Corion;Divasa-Farmavic S.A., Spain,). Briefly, at 14 h after human chorionic gonadotropin administration, oviducts were removed from superovulated female mice and placed in a Petri dish containing M2 at 37°C. After washing, collected oviducts were placed in fresh M2 medium, and COCs were released from the ampulla with the aid of Dumont #55 forceps and washed in new M2 medium until fertilization.

In vitro fertilization

Sperm from C57BL/6xCBA male mice aged 6 to 24 wk and of proven fertility were incubated for 1 h in human tubal fluid (HTF) medium under 5% CO_2 at 37°C for capacitation. After *in vitro* or *in vivo* maturation, COCs were transferred to a 500 µl equilibrated HTF drop and overlaid with mineral oil and a 1×10^6 concentration of spermatozoa. All *in vitro* fertilization experiments were repeated 9 times using 3 females per genotype.

Rates of oocyte nuclear maturation and fertilization

To assess whether adding THC affected rates of oocyte nucleus maturation and fertilization, the presence of the first polar body and pronuclear formation were identified respectively. At 24 h after fertilization, all presumptive zygotes that had not divided into 2 cells were fixed in 4% paraformaldehyde for 10 min and then stained with Hoechst 33342 (0.01 mg/ml) for observation with a immunofluorescence microscope (Zeiss Axioskop, NY, USA) under UV light.

In vitro culture of embryos

Five hours after *in vitro* fertilization, 10-25 presumptive zygotes from oocytes of female WT (C57BL/6xCBA) or KO (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$) were washed in HTF medium and cultured in 20-µl drops of equilibrated culture medium KSOMaa overlaid with mineral oil at 37°C under an atmosphere of 5% CO_2 in air with maximum humidity. Embryos were cultured for 5 d, and cleavage rates were assessed on d 1 (24 h after fertilization) and blastocysts on d 4 (96–100 h after fertilization).

TUNEL analysis

Apoptosis was determined by in situ DNA 3 end labelling of histological sections using a non-radioactive labelling method [Terminal deoxynucleotidyl transferasemediated dUTP nick-end labelling assay (TUNEL) from detection Kit, TUNEL POD (ROCHE). The control oocytes, the vehicle (DMSO) group and THC group were processed as per the manufacturer's instructions. The apoptotic cells appear in fluorescent green whereas the nucleus was marked in blue with Hoechst 33342.

Statistical analysis

All statistical tests were performed by using Graphpad software (GraphPad Software, Inc. La Jolla, CA 92037 USA). The mean and standard error of the mean of cleavage rates, blastocyst yields, the percentage change of blastocyst rate between the 4 genotypes were compared by 1-way ANOVA followed by multiple pairwise comparisons by using the Tukey's *post hoc* test in most data. Paired t-test was performed to compare the difference between treatments (without and with THC). Values of $P < 0.05$ were considered significant.

Statement of Ethics

All experimental procedures using mice were approved by the University of the Basque Country (UPV/EHU, CEEA reference number: M20-2015-016-027-028-173) and were performed according to the Guide for Care and Use of Laboratory Animals, endorsed by the Society for the Study of Reproduction and European legislation.

Results

CB1 receptor location-pattern during the resumption of meiosis and nuclear maturation of oocyte in presence or absence of THC

To analyse if the THC has any effect on the relocation of CB1 during the oocyte maturation, we incubated the immature oocytes with increasing concentrations of THC during 17h and we collected the oocytes at 10 min, 30 min, 1 h and 17 h to observe the CB1 location-pattern.

As it is known, in immature oocytes, CB1 is homogeneously localized over the GV and the receptor is relocated to the periphery of the oocyte after GVBD, when meiosis resume, which happens after more than one hour of maturation (Control of Fig. 1). However, the presence in the incubation media of any tested concentration of THC (from 10^{-9} M to 10^{-6} M) accelerated the relocation of CB1receptor from GV to the periphery of oocytes. Thus, the staining of CB1 in GV disappeared before 1 h of incubation (quicker than in control) and, specifically, the concentration of 10^{-7} M of THC led to accelerate that relocation before 30 min (Fig. 1).

CB2 receptor location-pattern during the resumption of meiosis and nuclear maturation in presence or absence of THC

To continue understanding the role of THC in oocyte maturation and after determining its effect on CB1 receptor relocation, we also examined the effect of THC on location of CB2 receptor. We performed the same experiment explained previously for CB1. CB2 showed a homogeneous distribution-pattern in the whole oocyte with more staining intensity in the periphery of the oocyte at all tested times both in absence or in presence of THC (Fig. 2).

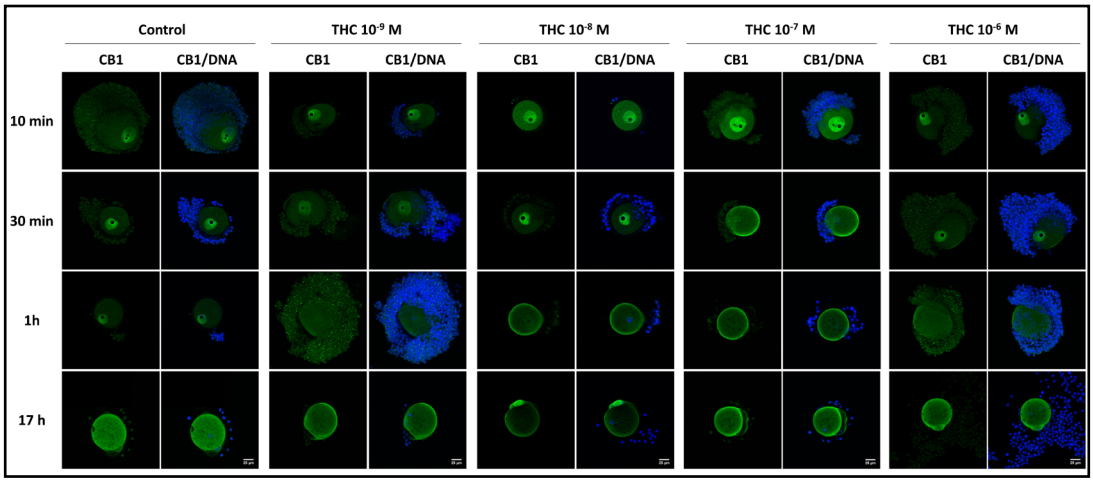


Fig. 1. Immunolocalization of CB1 during the maturation of mouse oocytes. Immature COCs were cultured in vitro in absence of THC and in presence of THC at 1 nM (10-9 M), 10 nM (10-8 M), 100 nM (10-7 M) and 1 μM (10-6 M) during 10 min, 30 min, 1h and 17 h. The distribution of CB1 is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had the same staining pattern; representative photomicrographs are shown. Scale bars, 25 μm.

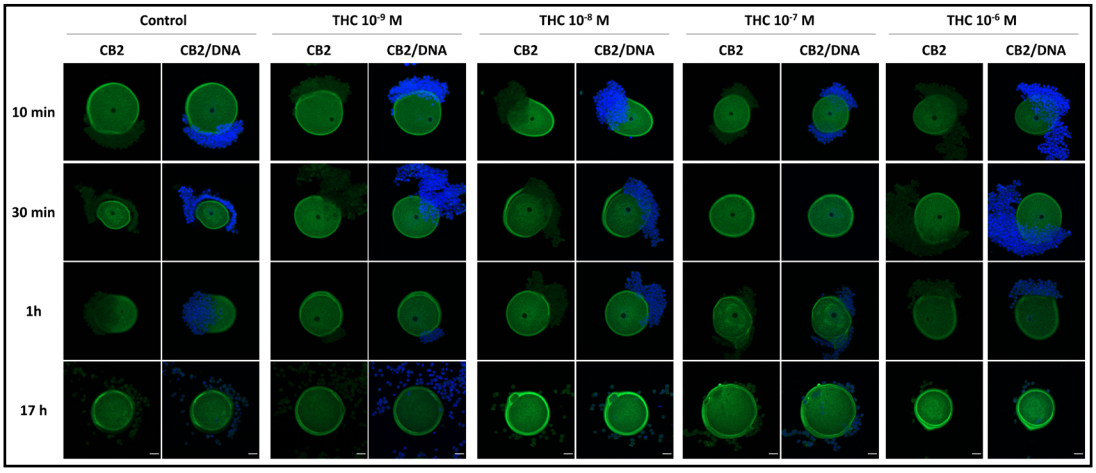


Fig. 2. Immunolocalization of CB2 during the maturation of mouse oocytes. Immature COCs were cultured in vitro in absence of THC and in presence of THC at 1 nM (10-9 M), 10 nM (10-8 M), 100 nM (10-7 M) and 1 µM (10-6 M) during 10 min, 30 min, 1h and 17 h. The distribution of CB1 is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 µm.

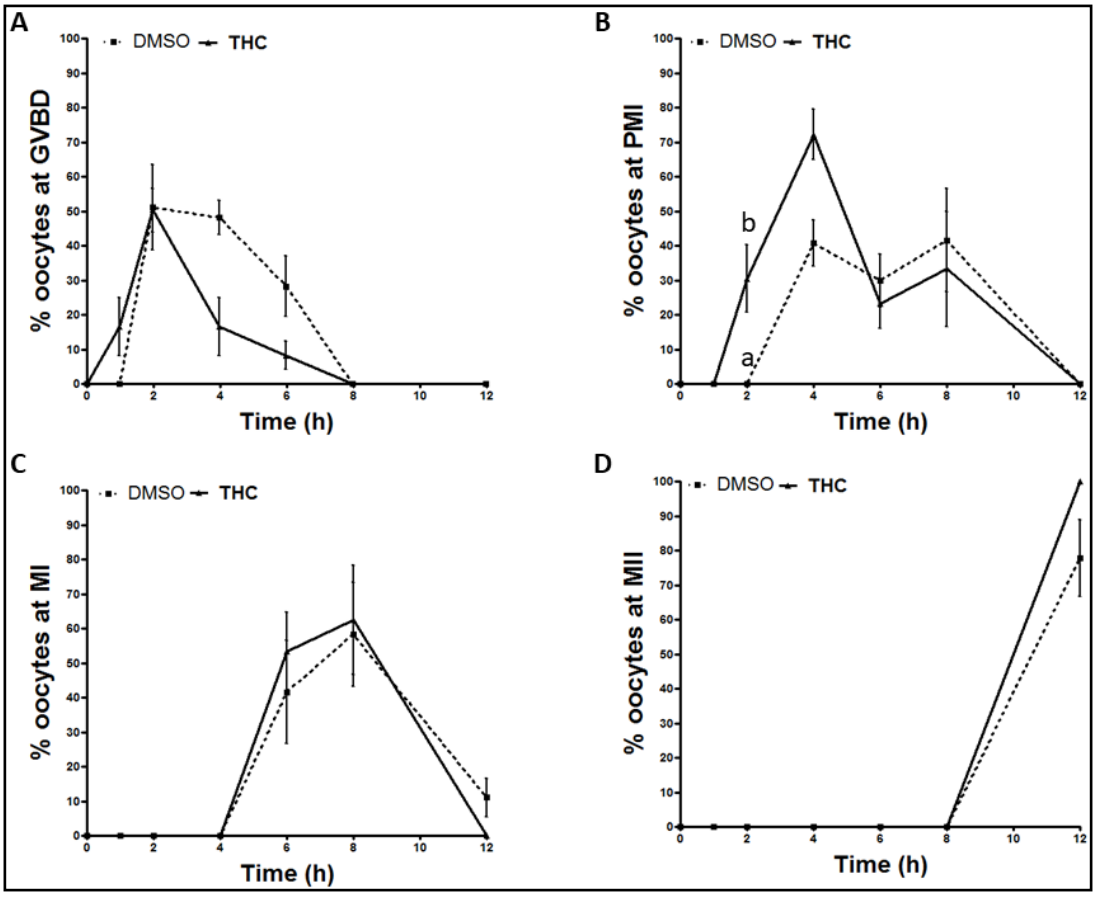


Fig. 3. Changes in nuclear maturation of oocytes. Results are expressed as percentage of oocytes at each stage of maturation at each point: (A) germinal vesicle break down, GVBD; (B) pro-metaphase I stage, PMI; (C) metaphase I stage, MI; (D) metaphase II stage, MII. n = 5 independent experiments of 15 oocytes per treatment. Significant differences between treatments are indicated with different letters; $p<0.05$ in all cases.

Meiotic progression of mice oocyte's exposed to THC

To determinate if THC has also some effect on oocyte nuclear maturation we fixed oocytes after 0, 2, 4, 6, 8 and 12h of IVM where media was supplemented with 10^{-7} M of THC or vehicle (DMSO). Oocytes nuclear stage was classified in germinal vesicle (GV), germinal vesicle break down (GVBD), pro-metaphase I (PMI), metaphase I (MI) and metaphase II (MII). At 0h all the oocytes were in GV stage (Fig. 3A) and after 2h of IVM a significant higher percentage of PMI was detected when we used THC compared with the oocytes maturated in presence of the vehicle (Fig. 3B). After 4h of IVM the majority of the oocytes were at PMI, but no differences were observed among treatments. Also after 8 h of IVM, all groups reached MI (Fig. 3C) at the same time. After 12 h of maturation a higher percentage of MII stage was found in THC experimental groups compared with control (Fig. 3D) but that difference was not significant. Finally, after 17 hours, all the oocytes of both treatments arrived at MII (data not shown).

Phosphorylation pattern of ERK1/2 during the resumption of meiosis and nuclear maturation in presence or absence of THC

As signaling via MAPKs plays a role in the oocyte maturation and that pathway is commonly modulated by cannabinoids, we used the cannabinoid agonist THC to determine whether the activation of the CB1 and CB2 receptors during oocyte maturation could affect the phosphorylation pattern of ERK1/2 compared with oocytes maturated in presence of the vehicle (DMSO).

According to the results obtained in the relocation of cannabinoid receptors during the maturation of oocytes, for these experiments we used the concentration of THC that generated the most changes regarding the relocation of the receptors in comparison to the control (10^{-7} M).

In immature oocytes, ERK1/2 was dephosphorylated but, after 17 h of incubation (the time required for mouse oocyte maturation), ERK1/2 was phosphorylated in oocytes. At 10 min, ERK1/2 appeared phosphorylated only in the granulose cells of oocytes incubated with THC. We did not observe the phosphorylation of granulose cells of control oocytes until 30 min. However, at that time, the COCs incubated with THC had already dephosphorylated its granulose cells but, instead, we could observe the presence of phosphorylated ERK1/2 inside the oocyte. At 1 h of incubation, the oocytes incubated with THC showed more intense phosphorylated ERK1/2 compared with the control and, at that time, the granulose cells of control COCs had already been dephosphorylated (Fig. 4A).

When we used oocytes from knock out mice where the cannabinoids receptors were absent (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$), we did not observe difference in the phosphorylation pattern of ERK1/2 between the oocytes matured in the presence or absence of THC (Fig. 4B, 4C and 4D).

Phosphorylation pattern of AKT during the resumption of meiosis and nuclear maturation in presence or absence of THC

The pathways mediated by AKT are also key in the oocyte maturation and that kinase is also modulated by cannabinoids, so, we used THC to determine whether the activation of the CB1 and CB2 receptors during oocyte maturation could affect the phosphorylation pattern of AKT compared with oocytes maturated in presence of the vehicle (DMSO).

We performed the same experiments as previously explained for pERK1/2. In this case, we observed few differences in the phosphorylation pattern of AKT in response to 10 and 30 min of treatment with THC, where the AKT was phosphorylated in granulose cells while it was undetectable in granulose cells exposed to vehicle. From that moment, the pattern of phosphorylation observed in the COCs treated with THC or vehicle was similar (Fig. 5A).

When we used oocytes from knock out mice where the cannabinoids receptors were absent (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$), we did not observe difference in the phosphorylation pattern of AKT between the oocytes matured in the presence or absence of THC (Fig. 5B, 5C and 5D).

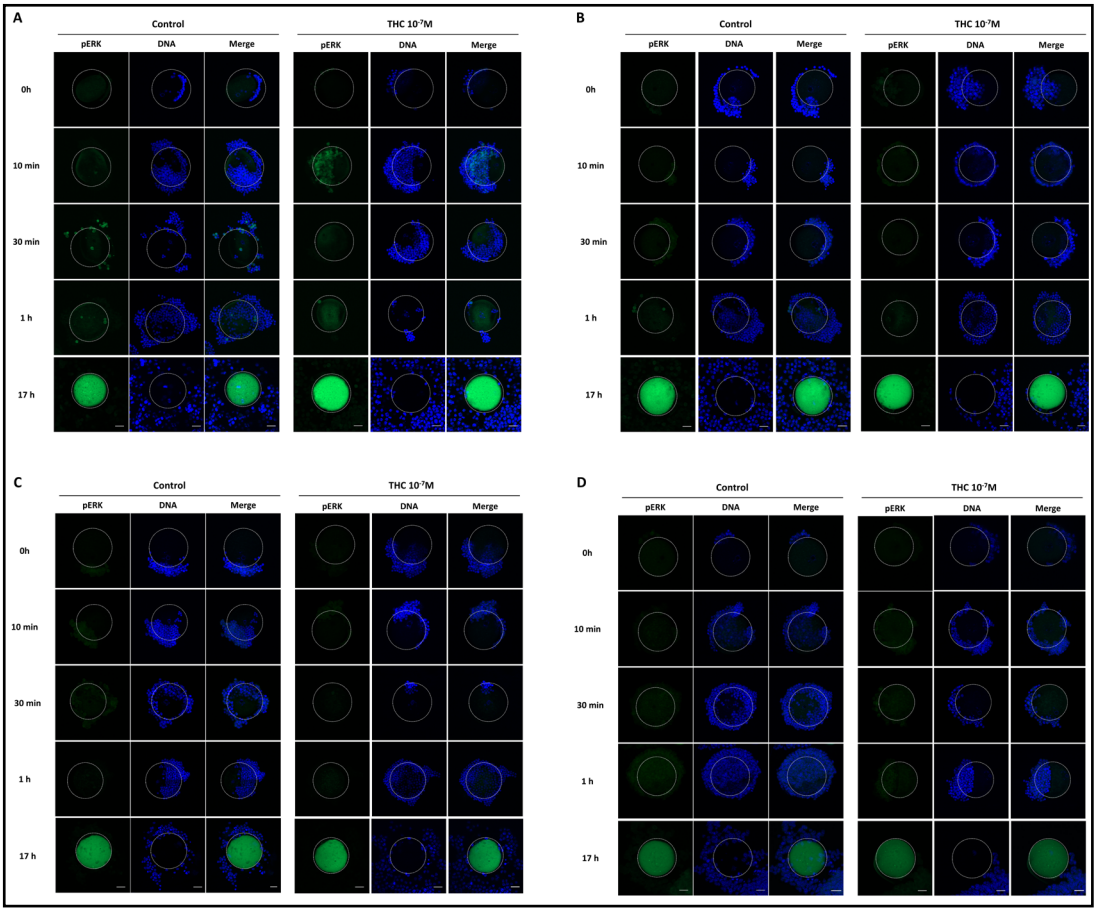


Fig. 4. Phosphorylation of ERK by THC during oocyte maturation. Immature GV oocytes from (A) wild type mice, (B) Cnr1$^{-/-}$ mice, (C) Cnr2$^{-/-}$ mice and (D) Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice were cultured in vitro in presence of 10-7 M of THC or in absence of it (control) for 17 h and the phosphorylation status of ERK (pERK) was observed at 0, 10 min, 30 min, 1 h and 17 h. pERK is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 µm.

Apoptosis analysis in oocytes exposed to THC during maturation

To check if the presence of THC, during the 17 hours it was in contact with the COCs, was harmful, the detection of apoptotic oocytes or granulosa cells was performed using the in situ TUNEL analysis. We did not observe any apoptotic cell in the COCs treated with THC during maturation or in those treated only with the vehicle (DMSO) (Fig. 6).

Blastocyst rate produced from oocytes matured in presence or absence of THC

Our next objective was to test whether exposure of COCs to THC during oocyte maturation would affect the maturation observing the fertilization and/or subsequent embryo development rate. For that purpose, we cultured, *in vitro*, immature COCs during 17 h (the average time to mature mice oocytes) in absence or presence of 10-7 M of THC and, then, we performed the *in vitro* fertilization (IVF).

The incubation with THC had no significant effect on rates of mature oocytes (MII), fertilized zygotes and 2-cell embryos compared to vehicle-treated oocytes. However, THC led to a significant improvement in blastocysts rate since twice as many embryos were produced when we used a 10^{-7} M concentration of THC, compared to vehicle (Fig. 7A).

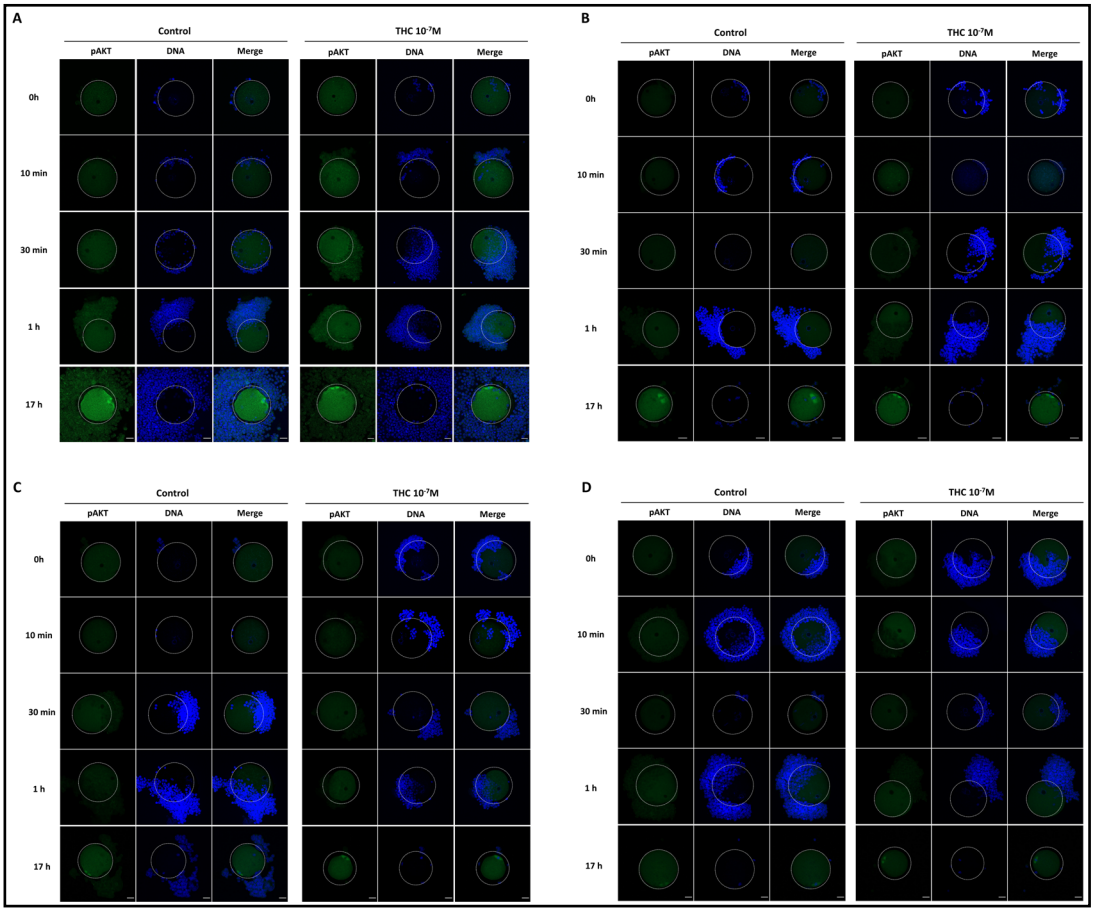
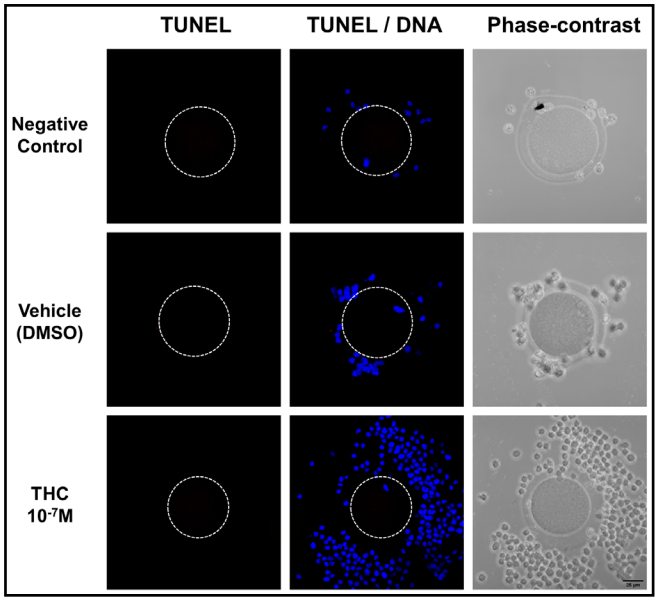


Fig. 5. Phosphorylation of AKT by THC during oocyte maturation. Immature GV oocytes from from (A) wild type mice, (B) Cnr1$^{-/-}$ mice, (C) Cnr2$^{-/-}$ mice and (D) Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice were cultured in vitro in presence of 10-7 M of THC or in absence of it (control) for 17 h and the phosphorylation status of AKT (pAKT) was observed at 0, 10 min, 30 min, 1 h and 17 h. pAKT is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 µm.

Fig. 6. Analysis of apoptotic cells, by TUNEL, in COCs treated with vehicle (DMSO) or THC. Apoptotic cells showed in green and DNA, stained by Hoechst 3342, in blue. Representative photomicrographs are shown.



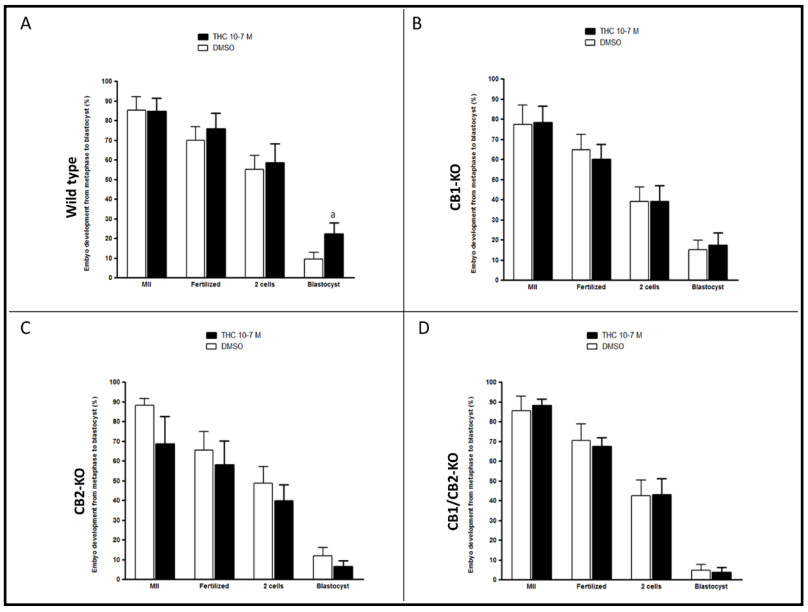


Fig. 7. Response to THC 10^{-7} M during oocyte maturation (17 h) measured as rates of metaphase II oocytes (MII), fertilized oocytes, 2 cell-stage embryos and blastocysts after IVF. The Fig. shown the embryo development observed for oocytes incubated with vehicle (DMSO) (white) and incubated with THC 10^{-7} M (black). The sperm always came from WT mice and the oocytes came from A) wild type (WT) mice, B) Cnr1$^{-/-}$ mice, C) Cnr2$^{-/-}$ mice and D) Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice. Results are the mean of % ± SEM of 10 independent experiments. Significant differences between treatments are indicated with different letters; $p<0.005$ in all cases.

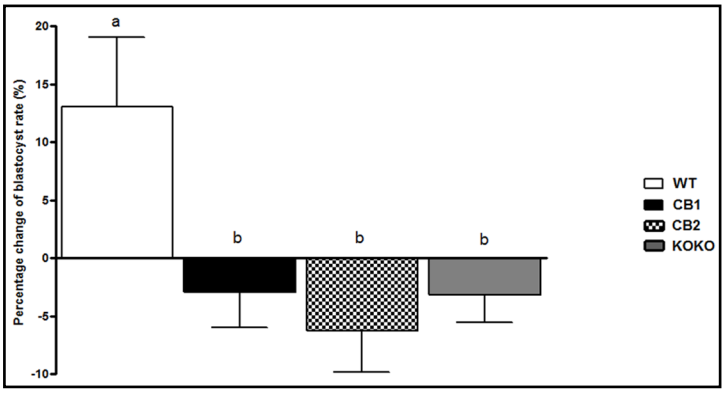


Fig. 8. Percentage change of blastocysts rate between control and treatment. Blastocysts rate changes using oocytes from wild type (WT) mice (white), Cnr1$^{-/-}$ mice (black), Cnr2$^{-/-}$ mice (dotted) and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice (grey). Results are the mean ± SEM of 10 independent experiments. Significant differences between treatments are indicated with different letters; $p<0.05$ in all cases.

To be able to know if the observed action of THC was carried out by CB1 and/or CB2 receptor, we also performed the IVM with COCs from knock out mice *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1–*$^{-/-}$*/Cnr2*$^{-/-}$, followed by the subsequent IVF done using sperm from wild type male mice. That way, we were able to verify that the effect observed on *in vitro* embryo development were effectively attributable to the absence of the cannabinoid receptor during maturation and not to the *in vitro* fertilization (IVF) or embryo culture processes.

We did not observe any improvement on blastocyst rate between the oocytes treated with THC or with vehicle for any genotype (Fig. 7B, C and D). In that sense, taking into account the percentage change of blastocyst rate between control and treatment for each genotype, we observed that the only genotype where incubation of oocytes with THC is beneficial for an increase in the number of blastocysts is the wild type (Fig. 8).

Discussion

Through systematic series of genetic and pharmacologic experiments, we examined whether the use of THC acting via CB1 and/or CB2 modulated oocyte maturation in a mouse

model, and we tried to elucidate the mechanisms of such actions. The choice of using the phytocannabinoid THC has to do with the controversy generated by the use of synthetic cannabinoids [23, 24], which is why it seems that the use of THC in the clinic could be more feasible and reliable. Our observations in wild type, *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$, and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice indicated that, when present during IVM of oocytes, the THC accelerated the relocation of CB1 receptor in oocytes. Moreover, the incubation of oocytes with THC during the IVM produced modulations in ERK1/2 and AKT phosphorylation and increased blastocyst rates achieved. Those effects seem to be achieved through both cannabinoid receptors studied (CB1 and CB2).

The first evidence that linked the endocannabinoid system and the oocyte maturation was that the endocannabinoid AEA (*N*-arachidonoylethanolamide) was present in human follicular fluid [29] and its concentration rose during oocyte maturation [30, 31]. In previous works, it was detected the Cnr1 mRNA transcript and protein of CB1 receptor in oocytes during *in vitro* and *in vivo* maturation [17, 20, 21]. Furthermore, CB1 receptor could be an indicator of oocyte nuclear maturation, because it was clearly localized over GV oocyte and it moves towards the periphery as it changes to the stage MII [17, 20, 21]. In the present work we saw how the presence of THC in the maturation media accelerated that relocation of CB1 receptor from GV to the periphery. That difference in relocation velocity is interesting because, in oocytes matured *in vivo*, CB1 was localized peripherally sooner than in oocytes matured *in vitro* [21] and, as is well established, the *in vitro* maturation process is delayed in comparison with *in vivo* maturation [32]. The presence of THC in the maturation media also accelerated the oocyte nuclear maturation of immature oocytes between 1 and 2 hours of incubation, similar times observed in the aforementioned acceleration of the relocation of CB1 receptor. The meiosis resumption also began earlier (around 2 h) when THC was present in the IVM media of bovine oocytes [20].

Although CB2 did not show any relocation difference during the IVM, we detect a peripheral location of this receptor did not previously found. So, as according to the classic theory of GPCR functionality, to exert its actions, a receptor needs to reach the cell surface [33], both CB1 and CB2 could have any involvement in oocyte maturation. Actually, there are data supporting the notion that GPCR-Gαi (such as CB1 and CB2) is a meiotic maturation inducer [34].

To know if the changes observed during the maturation of oocytes incubated with THC were observed in other processes of IVM, we analysed the phosphorylation-pattern of ERK1/2 and AKT, since those essential kinases regulate the oocyte meiosis progression [35]. Interestingly, the presence of THC during the oocyte maturation accelerated the phosphorylation-pattern of ERK1/2 and, to a lesser extent, the phosphorylation-pattern of AKT. Previously, it was described that the selective CB2 receptor agonist, JWH133, induced the ERK1/2 and AKT phosphorylation cascade in spermatogonia and their progression toward meiosis [36, 37]. In addition, in the previous functional experiments done with cows showed how the presence of THC during the IVM led to accelerate the phosphorylation-pattern of ERK1/2 and AKT kinases [20]. These results are in accordance with the fact that the activation (phosphorylation) of AKT stimulates the meiosis resumption, it is involved in the MI/MII transition and it regulates polar body emission and spindle organization [38–41]. In the same way, the prompt activation of ERK1/2 induces premature chromosome condensation and meiosis resumption as well as pronucleus breakdown [42]. It is interesting to highlight that the activation of CB2 by a treatment with JWH133 induced and accelerated the meiosis progression in fetal oocytes of mice, although they observed how also decreased the pool of primordial and primary follicles [43]. Even so our treatment with THC was *in vitro* and it only lasted 17 hours, and, that could be why we do not observe apoptosis in oocytes or granulosa cells as observed by De Domenico and co-workers [43] after systemic treatment with JWH133.

Finally, our last objective was to test whether all that changes described regarding the exposure of COCs to THC during oocyte maturation would affect the fertilization and/or subsequent embryo development rate in positive or negative manner. Although the incubation

with THC had no significant effect on rates of mature oocytes (MII), fertilized zygotes and 2-cell embryos compared to vehicle-treated oocytes, the THC led to an improvement in blastocysts rate (2 times more number of embryos). That improvement in the amount of blastocysts achieved had not been observed in previous experiments performed with THC in bovine oocytes [20], although it was observed using a synthetic agonist for CB1 in mice oocytes [21]. In addition, the experiments carried out by knockout mice for CB1 and/or CB2 receptors, confirmed that both receptors are involved in the modulation of oocyte maturation by THC since, when one of the two receptors is absent, the THC is not able to generate improvements in the ratio of blastocysts compared to the control.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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