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# Exocannabinoids effect on *in vitro* bovine oocyte maturation via activation of AKT and ERK1/2

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## Abstract

**Endocannabinoids are known to mediate practically all reproductive events in mammals; however, little is known about their role in oocyte maturation. Through RT-PCR and immunocytochemistry, this study confirms the presence of CB1 and CB2 cannabinoid receptors in bovine oocytes and shows how exposure to the exogenous cannabinoids HU-210 and THC during their *in vitro* maturation (IVM) activates the phosphorylation of AKT and ERK1/2 proteins associated with the resumption of meiosis. Although supplementation with HU-210 or THC during IVM did not increase blastocyst yields, the expression of interferon tau (*IFNτ*) and gap junction alpha-1 protein (*GJA1*) was enhanced at the blastocyst stage. Our data suggest that cannabinoid agonists may be useful IVM supplements as their presence during oocyte maturation upregulates the expression in blastocysts of key genes for embryo quality.**
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## Introduction

The molecular mechanisms responsible for oocyte maturation are not fully understood and various molecules seem capable of modulating this process. Among these molecules, an important role of the cannabinoid compounds, fatty acid derivatives that exert their effects by binding to membrane cannabinoid receptor 1 (CNR1 or CB1) and cannabinoid receptor 2 (CNR2 or CB2), both Gi/o protein-coupled receptors, has been proposed (Devane *et al*. 1988, Matsuda *et al*. 1990). Many of the components of the endocannabinoid system have been identified in the ovary, oocyte, granulosa cells and embryos of mice, rats and humans (Wang *et al*. 2004, El-Talatini *et al*. 2009*a*, Peralta *et al*. 2011, López-Cardona *et al*. 2014, Agirregoitia *et al*. 2015, Miller *et al*. 2016). In effect, the best characterized endocannabinoid, anandamide (AEA), is present in human follicular fluid and its concentrations rise during oocyte maturation (Schuel *et al*. 2002, Wang *et al*. 2003, El-Talatini *et al*. 2009*b*). A recent study in cattle has identified AEA in oviductal fluid with highest concentrations found during the periovulatory period (Gervasi *et al*. 2013). However, the presence of AEA receptors on the oocyte has not yet been reported. In addition, cell signalling exerted by cannabinoids and the signalling that occurs when oocyte meiosis resumes both in humans (Peralta *et al*. 2011) and mice (Paria *et al*. 1995, Schmitt & Nebreda 2002) share several similar features.

In human clinical practice, immature oocytes harvested from antral ovarian follicles can be *in vitro* matured (IVM) in culture media supplemented with different molecules to promote maturation. Despite disappointingly low embryo production and pregnancy rates offered by this technique (Barnes *et al*. 1995, Barnes *et al*. 1996, Cha *et al*. 2000), IVM could be the only option for women intolerant to gonadotropins (Cha *et al*. 2000) wishing to undergo *in vitro* fertilization (IVF). Before any clinical trial is designed to improve IVM, research is needed in an animal model similar to the human model such as cow (Menezo & Herubel 2002). Humans and cows are single ovulators and show close similarity in ovarian function and oocyte characteristics (Van Hoeck *et al*. 2011). Indeed, bovine oocyte maturation shares features with the human model in terms of ovarian follicular dynamics and endocrine control (Menezo & Herubel 2002, Campbell *et al*. 2003). Some authors even proposed the bovine maturation model as a reliable surrogate method to screen for toxic agents or metabolic studies in human oocytes (Van Hoeck *et al*. 2011, Beker van Woudenberg *et al*. 2012, Santos *et al*. 2014).


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Despite this useful model, investigations to date examining the role of endocannabinoids in bovine reproduction events have mainly focused on the sperm physiology and sperm–oviduct interaction. These efforts have revealed that AEA induces sperm capacitation through CB1 and the transient receptor potential vanilloid 1 (TRPV1) activation, and modulates sperm release from the oviductal reservoir (Gervasi *et al*. 2011, 2016). Moreover, recently it has been demonstrated that endocannabinoid signalling governs sperm activation via sex hormone progesterone (Miller *et al*. 2016), a non-genomic signal that is also required for oocyte maturation (Revelli *et al*. 1998). Based on the above discussion, the aim of this study is to confirm the presence of cannabinoid receptors in bovine oocytes and whether the presence of exogenous cannabinoids, such as HU-210 ((6aR,10aR)-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol) or THC (Δ9-tetrahydrocannabinol), in the maturation medium activates pathways involved in oocyte maturation like protein kinase B (also known as AKT) and extracellular signal-regulated kinase (ERK1/2) phosphorylation and thus improves *in vitro* blastocyst quality without compromising embryo development.

## Materials and methods

Unless otherwise stated, all chemicals and media were purchased from Sigma Chemical Co.

### *Isolation and* in vitro *maturation of cumulus–oocyte complexes*

Immature cumulus–oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm) from the ovaries of matured heifers and cows collected at slaughter from a local abattoir. COCs (homogenous cytoplasm and intact cumulus cells) were matured in four-well dishes (Nunc, Roskilde, Denmark) in 500 µL TCM-199 maturation medium, supplemented with 10% (v/v) foetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF) in groups of 50 COCs per well for 24 h at 38.5°C under an atmosphere of 5% $CO_2$ in air with maximum humidity (Rizos *et al*. 2002).

### *IVM supplementation with cannabinoid agonists*

COCs were co-incubated with the cannabinoid agonists HU-210 and THC (concentration of 100 nM in 0.1% DMSO) in maturation medium, according to prior optimization experiments (López-Cardona *et al*. 2014). COCs incubated with and without DMSO served as controls. A total of five replicates were carried out.

### In vitro *fertilization*

After 24 h of maturation, the *in vitro* matured COCs were washed twice in fertilization medium before being transferred in groups of 50 to the wells of four-well plates each containing 250 µL fertilization medium (FERT-TALP medium (Merck) supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid–free bovine serum albumin (BSA) and 10 mg/mL heparin). Frozen bull semen was thawed in a water bath at 37°C for 50 s and motile spermatozoa separated on a density gradient (BoviPure, Nidacon International, Mölndal, Sweden), centrifuged and re-suspended in fertilization medium. Then, 250 µL of this suspension were added to each fertilization well to obtain a final concentration of $1 \times 10^6$ spermatozoa/mL. Gametes were co-incubated under 5% $CO_2$ in air at maximum humidity for 20 h at 38.5°C.

### In vitro *culture*

Presumptive zygotes were denuded by gentle vortexing for 3 min, washed twice in phosphate buffer saline (PBS) supplemented with 3% BSA and cultured in groups of 25 in 25 µL droplets of synthetic oviduct fluid (SOF) (Holm *et al*. 1999) supplemented with 5% FCS under mineral oil at 38.5°C in an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$, with maximum humidity. Cleavage rate was recorded on day 2 (48 h post insemination) and cumulative blastocyst yield was recorded on days 7, 8 and 9 post insemination.

### *Meiotic progression of bovine oocytes undergoing IVM with exogenous cannabinoids*

In order to determine the impact of HU-210 and THC on germinal vesicle (GV) breakdown, COCs from both experimental groups and control with DMSO at 2, 4, 6, 8 and 12 h of IVM ($n = 30$ per time point and group in three independent replicates) were used as described previously (Khatir *et al*. 1998). Briefly, COCs were partially denuded by vortexing during 3 min 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 10 µg/mL Hoechst 33342 for 15 min. Oocytes were then placed in glass slides and squashed with coverslip in order to visualize the nuclear stage under microscopy (Nikon Optiphot-2) (Coy *et al*. 2008).

### *Immunofluorescence*

Pools of 20 COCs were obtained from DMSO group for the different maturation stages: The first group was immediately fixed after removal from the follicle representing the GV stage in 100% of oocytes. The second and third groups were removed from maturation medium at 12 h for metaphase I (MI) and 24 h for metaphase II (MII) stages, representing 94 and 90% respectively of total oocytes evaluated. CB1 and CB2 receptors were immunocytochemically localized as described previously (Peralta *et al*. 2011). In brief, COCs were partially denuded and washed in PBS supplemented with 1% polyvinyl alcohol (PVA) and fixed in 4% paraformaldehyde for 10 min at room temperature. Next, they were 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% FCS and 1:100 rabbit polyclonal anti-cannabinoid CB1 receptor (Cayman Chemical) and anti-cannabinoid CB2 receptor (Cayman Chemical) antibodies. Following incubation, oocytes were washed twice in PBS with 1% PVA and incubated in PBS supplemented with 5% FCS and 1:250 goat polyclonal secondary antibody Alexa Fluor 488 (Molecular Probes) for 2 h at room temperature followed by washing again three times in PBS – 1% PVA. In all cases, nuclei were stained with Hoechst 33342 at a concentration of 0.01 mg/mL during the second wash to determine the maturation stage of each oocyte (Gutierrez-Adan & Pintado 2000). Finally, oocytes were mounted in microdrops with Fluoromount G (EMS, Hatfield, UK) and examined by confocal microscopy (Leyca TCS-SPE). Negative control were prepared using blocking peptide and omitting the primary antibody before adding the secondary antibody. To monitor AKT and ERK1/2 activation during oocyte maturation due to the presence of the cannabinoids, 20 oocytes were removed from the maturation medium at 0, 5 and 30 min, to evaluate the initial response, and 24 h, to evaluate if the initial response was remaining in order to produce an effect in the mature oocyte, following the same protocol described previously. The primary rabbit polyclonal antibodies against phosphorylated AKT and phosphorylated ERK1/2 (Cell Signaling Technology) were used at 1:100 dilution, and the secondary antibody goat polyclonal Alexa Fluor 488 (Molecular Probes) was used at 1:250. Fluorescence of phosphorylated AKT and phosphorylated ERK1/2 were analysed by confocal microscopy (Leyca TCS-SPE) and quantified using the ImageJ programme v. 1.45 (http://rsb.info.nih.gov/ij) (Huang *et al*. 2012). Fluorescence was evaluated separately in granulosa cells and oocytes. All experiments were performed in the same day, with the same method and using the same settings in the microscope to be able to compare the fluorescence among treatments. Data are expressed as a fold over vehicle phosphorylation.

### *RNA extraction, reverse transcription PCR and real-time PCR (qPCR)*

Gene expression was examined in oocytes and blastocysts as described previously (Bermejo-Alvarez *et al*. 2011). Pools of 20 oocytes were obtained for the maturation stages as performed for immunofluorescence: GV, MI and MII. For blastocysts, we used pools of 10 obtained on day 7 post-fertilization; three replicates of four samples per experimental group were carried out in both cases. Poly(A) RNA was extracted using the Dynabeads mRNA DIRECT Micro Kit (Ambion, Thermo Fisher Scientific Inc) following the manufacturer's instructions with minor modifications (Bermejo-Alvarez *et al*. 2010). After 10 min of incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and washed twice in washing buffer A and washing buffer B. RNA was eluted with Tris-HCl. After extraction, RT reaction was carried out following the manufacturer's instructions (Epicentre Technologies Corp, Madison, WI, USA) using poly(T) primers, random primers and MMLV High Performance Reverse Transcriptase enzyme in a total volume of 40 µL, to prime the RT reaction and produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. Next, the tubes were incubated at 25°C for 10 min to promote the annealing of random primers, at 37°C for 60 min to allow the RT of RNA and, finally, at 85°C for 5 min to denature the enzyme. Four cDNA samples were performed per experimental group. All qPCR analyses were run in the following conditions: 95°C for 3 min, 35 cycles at 94°C for 15 s, 54°C for 25 s and 72°C for 20 min, followed by a final extension step at 72°C for 5 min. qPCR analyses were carried out in duplicate in the Rotor Gene 6000 real-time cycler (Corbett Research, Sydney, Australia) by adding 5 ng of each sample to the PCR mix (GoTaq qPCR Master Mix, Promega) containing the specific primers selected to amplify the selected genes. The comparative cycle threshold (CT) method was used to quantify expression levels. Values were normalized to the endogenous control (housekeeping gene: histone *H2afz*). According to the comparative CT method, the ΔCT value was determined by subtracting the HK mean CT value for each sample from each gene CT value of the sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value, that is, the treatment with the lowest target expression, as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2-ΔΔCT (Murray *et al*. 2007). The primers used for qPCR are as follows: H2A histone family, member Z (*H2AFZ*), cannabinoid receptor type 1 (*CNR1*), cannabinoid receptor type 2 (*CNR2*), BCL2-associated X protein (*BAX*), BCL2-like 1 (*BCL2*), tumour protein p53 (*TP53*), glutathione peroxidase 1 (*GPX1*), superoxide dismutase 2, mitochondrial (*SOD2*), DNA cytosine-5-methyltransferase 3 alpha (*DNMT3A*), insulin-like growth factor 2 receptor (*IGF2R*), solute carrier family 2 (facilitated glucose 1 transporter), member (*SLC2A1*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), POU class 5 homeobox 1 (*Oct3/4*) (*POU5F1*), interferon tau (*IFNτ*) and gap junction protein, alpha 1 (former CX43) (*GJA1*). Primer sequences and approximate sizes of the amplified fragments of all transcripts are provided in Table 1. The presence or absence of mRNA for *CB1* and *CB2* was established in maturation stages; *H2AFZ* was used as the housekeeping gene. Foetal bovine organs like brain and spleen, obtained from the slaughterhouse were used as positive control for expression of *CNR1* and *CNR2* respectively. RT-PCR with total RNA without reverse transcriptase was used as negative control.

### *Data analysis*

All statistical tests were performed using the software package SigmaStat 3.5 (Jandel Scientific, San Rafael, CA, USA). Fluorescence intensity of phosphorylated AKT and phosphorylated ERK1/2, cleavage rates, blastocyst yields and relative mRNA abundances for candidate genes were compared by one-way ANOVA ($P < 0.05$) followed by multiple pair-wise comparisons using the Tukey method.

**Table 1** Details of the primers used for PCR and qPCR.

| Entrez gene symbol | Gene name | Accession no. | Forward primer (5′–3′) | Reverse primer (5′–3′) | Product length (bp) |
|---|---|---|---|---|---|
| *H2AFZ* | H2A histone family, member Z | NM_174809 | ACGGACGACTAGCCATGGACGTGTG | CCACCACCAGCAATTGTAGCCTTG | 209 |
| *CNR1* | Cannabinoid receptor 1 | NM_001242341.1 | GGTGAGGTTCACCTGGTCT | GAAGGGGTCCCCCGCTAA | 305 |
| *CNR2* | Cannabinoid receptor 2 | XM_010802588.1 | GAGGCTGGGATCACTTGGCATG | AGGGATGCGCTGGGAACAATTCG | 172 |
| *BAX* | BCL2-associated X protein | NM_173894.1 | CTACTTTGCCCAGCAAACTGG | TCCCAAAGTAGGAGAGGA | 158 |
| *BCL2* | BCL2-like 1 | BC147863.1 | GGAGCTGGTGGTTGACTTTC | CTAGGTGGTCATTCAGGTAAG | 517 |
| *TP53* | Tumour protein p53 | NM_174201.2 | CTCAGTCCTCTGCCATACTA | GGATCCAGGATAAGGTGAGC | 364 |
| *GPX1* | Glutathione peroxidase 1 | NM_174076.3 | GCAACCAGTTTGGGCATCA | CTCGCACTTTTCGAAGAGCATA | 116 |
| *SOD2* | Superoxide dismutase 2, mitochondrial | S67818.1 | GCTTACAGATTGCTGCTTGT | AAGGTAATAAGCATGCTCCC | 101 |
| *DNMT3A* | DNA cytosine-5-methyltransferase 3 alpha | AY271299 | CTGGTGCTGAAGGACTTGGGC | CAGAAGAAGGGGCGGTCATC | 318 |
| *IGF2R* | Insulin-like growth factor 2 receptor | NM_174352.2 | GCTGCGGTGTGCCAAGTGAAAAAG | AGCCCCTCTGCCGTTGTTACCT | 201 |
| *SLC2A1* | Solute carrier family 2 (facilitated glucose 1 transporter), member | NM_174602.2 | CTGATCCTGGGTCGCTTCAT | ACGTACATGGGCACAAAACCA | 68 |
| *GAPDH* | Glyceraldehyde 3-phosphate dehydrogenase | BC102589 | ACCCAGAAGACTGTGGATGG | AYGCCTGCTTCACCACCTTC | 247 |
| *POU5F1* | POU class 5 homeobox 1 (Oct3/4) | NM_174580.1 | CGAGTATCGAGAACCGAGTG | CAGGGTTCTCTCCCTAGCTC | 440 |
| *IFNτ* | Interferon tau | NM_001015511.3 | GCTATCTCTGTGCTCCATGAGATG | AGTGAGTTCAGATCTCCACCCATC | 359 |
| *GJA1* | Gap junction protein, alpha 1 (former *CX43*) | NM_174602.2 | TGCCTTTCGTTGTAACACTCA | AGAACACATGAGCCAGGTACA | 142 |

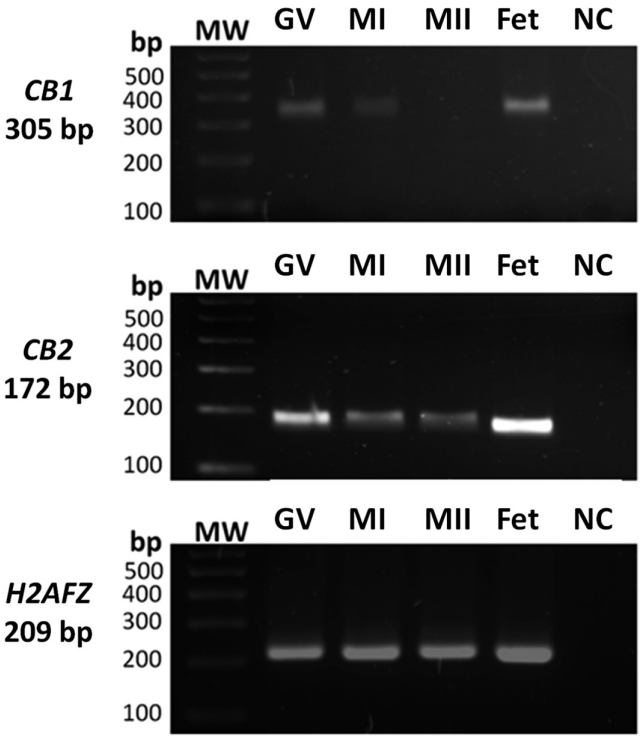


**Figure 1** *CB1* and *CB2* receptor expression during the nuclear maturation of bovine oocytes. *CB1* (305 bp), *CB2* (172 bp) and *H2AZF* (209 bp) mRNAs in stage GV, MII and MII oocytes matured *in vitro*. Foetus of bovine species (Fet) served as positive control for *CB1* and *CB2,* brain and spleen respectively. To validate the RT-PCR procedure, reactions with total RNA and without reverse transcriptase were used as negative control (NC). Molecular weights (MW; bp) are indicated on the left. $n=3$; a representative RT-PCR experiment is shown.

## Results

### *Expression dynamics and immunolocalization of the cannabinoid receptors CB1 and CB2 during bovine oocyte maturation*

During the *in vitro* maturation of bovine oocytes, *CB1* mRNA was detected in GV and MI oocytes but not MII stage oocytes. In contrast, the *CB2* transcripts appeared in oocytes in all stages following the resumption of meiosis (GV, MI and MII) (Fig. 1).

Immunofluorescence analysis revealed the presence of both CB1 and CB2 receptor proteins during IVM (Fig. 2). CB1 was evenly distributed in GV stage oocytes yet adopted a peripheral location after the resumption of meiosis in the MI stage and a more evident peripheral location in MII. In contrast, staining for the CB2 receptor was homogeneous across the cell in all the stages examined.

### *Exposure to HU-210 or THC during bovine oocyte maturation modifies subsequent AKT and ERK1/2 phosphorylation patterns*

No differences were found among control groups, with or without 0.1% DMSO, in phosphorylation pattern of

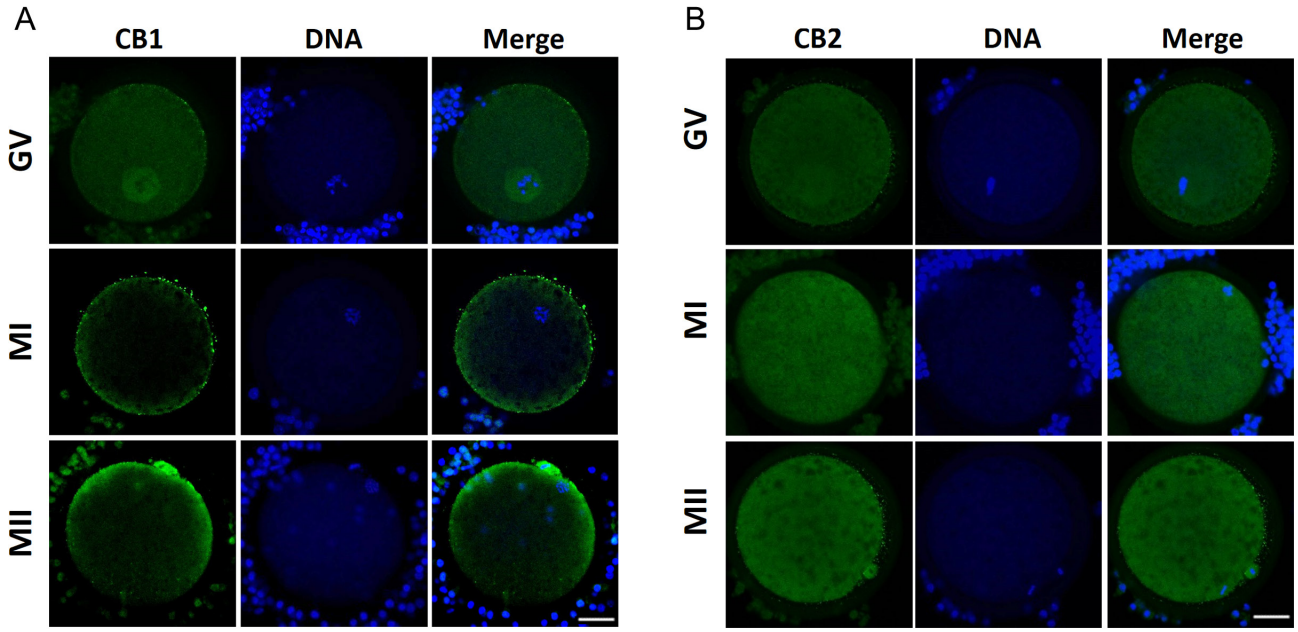


**Figure 2** CB1 and CB2 immunolocalization during the nuclear maturation of bovine oocytes. (A) Distribution of CB1 (shown in green) during the *in vitro* maturation of oocytes at the stages germinal vesicle (GV), metaphase I (MI) and metaphase II (MII). (B) Distribution of CB2 (green) during *in vitro* maturation of oocytes at the stages GV, MI and MII. Hoechst-labelled DNA (shown in blue). Scale bar represents 20 µm.

*AKT* and *ERK1/2* proteins, embryo development (day 9: 24.6 ± 2.6% vs 27.3 ± 4.7% respectively) and blastocysts gene expression. Based on this and on the fact that DMSO was the vehicle for both drugs, it was considered the most appropriate group to be used as a control.

In Fig. 3 it may be observed that no phosphorylation signal was produced for any of the proteins examined before the addition of the drugs (0′). In contrast, while staining for phosphorylated AKT was negligible in oocytes exposed to vehicle (DMSO), after 5 min of treatment with either HU-210 or THC, a clear AKT phosphorylation signal was detected in granulosa cells along with weaker staining in the oocyte. After 30 min, this staining pattern remained fairly stable. However, after 24 h of incubation (the time required for bovine oocyte maturation), pAKT was homogenously distributed across the oocyte and its signal in the granulosa cells diminished in both the HU-210 and THC groups while the absence of pAKT staining persisted in the vehicle group. The pAKT phosphorylation pattern at 24 h was, nevertheless, weaker in the HU-210 than THC group (Fig. 3A).

ERK1/2 phosphorylation patterns (Fig. 3B) in response to 5 min of treatment with the cannabinoid agonists included a pERK1/2 signal in granulosa cells, which was weaker in the vehicle group, and some signal across the oocyte in both the HU-210 and THC groups. However, after 30 min of incubation, pERK1/2 staining in the oocyte significantly differed between the vehicle and treatment groups whereas after 24 h, ERK1/2 phosphorylation similarly diminished in granulosa cells and increased over the oocyte in all the study groups (Fig. 3B).

### *Changes in velocity of meiotic progression of bovine oocyte's exposure to HU-210 or THC*

To analyse if the exocannabinoids have some effect on oocyte nuclear maturation we fixed oocyte after 0, 2, 4, 6, 8 and 12 h of IVM with media supplemented with HU-210 or THC. Oocytes nuclear stage was classified in GV, GVBD, PMI, MI and MII. As we can see in Fig. 4A, at 0 h all the oocytes were in GV stage, and after 2 h of IVM a higher percentage of GVDB was found when we used exogenous cannabinoids compared with the control group. After 6 h 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. 4C) at the same time, however, after 12 h of maturation a higher percentage of MII stage was found in both experimental groups compared with control (Fig. 4D).

### *Blastocyst rate and gene expression of embryo produced from oocytes matured in the presence of HU-210 or THC*

We next examined whether exposure to the cannabinoids during oocyte maturation would have impacts on fertilization and/or subsequent embryo development and quality. To first establish the effects of the cannabinoids during oocyte maturation, immature COCs were cultured *in vitro* for 24 h in the presence or absence of HU-210 or THC. The cannabinoids were then removed from the culture medium and IVF experiments performed on these matured COCs. Incubation with 100 nM of HU-210 or THC had no significant effects on the rates

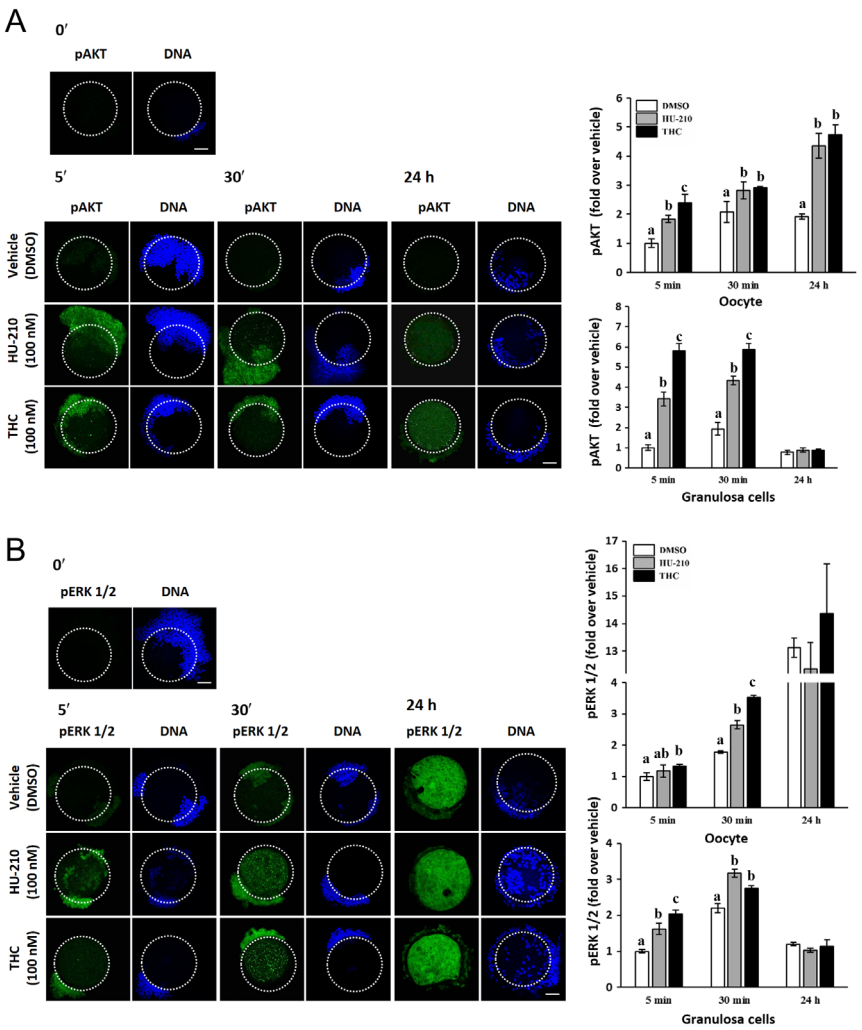


**Figure 3** AKT and ERK1/2 activation in response to HU-210 or THC supplementation during *in vitro* bovine oocyte maturation. Immature GV oocytes were cultured in the presence of 100 nM of each cannabinoid. The activation state of AKT or ERK1/2 was evaluated at 0, 5, 30 min and 24 h (the time needed for bovine oocyte maturation). (A) Phosphorylation state of AKT in response to the presence of HU-210 or THC during oocyte maturation. (B) Phosphorylation state of ERK1/2 (pERK1/2) in response to the presence of HU-210 or THC during oocyte maturation. Images depict Alexa488-labelled antigens in green. Hoechst-labelled DNA appears in blue, scale bar represents 20 µm. Normalized data was expressed as a fold over vehicle phosphorylation for both proteins and was analysed separately in oocyte and granulosa cells. Significant differences between treatments are indicated with different letters[a,b,c]; ($P<0.05$).

recorded of cleaved embryos or blastocysts on days 7, 8 and 9 post-IVF ($P>0.05$, Table 2).To determine effects on embryo quality, we examined the expression of mRNAs for key genes related to apoptosis, oxidative stress, methylation, metabolism, cell junctions, pluripotency and implantation. Expression patterns of the majority of these genes did not differ in embryos arising from oocytes maturated in the presence of THC or HU-210 compared with control (Fig. 5). However, we observed the upregulation in the treatment vs control groups ($P<0.05$) of the genes *GJA1*, also known as connexin 43, involved in cell signalling related to the formation of cell junctions, and *IFNτ* involved in cell signalling related to recognition for implantation. The expression of *IFNτ* was similarly upregulated in the HU-210 and THC groups while, although *GJA1* expression was also upregulated in both treatment groups ($P<0.01$, Fig. 5), this upregulation was significantly more pronounced in the THC group compared with the HU-210 group ($P<0.01$, Fig. 5).

## Discussion

The findings of our study indicate that, when present during the *in vitro* maturation of bovine oocytes, the exocannabinoids HU-210 and THC are able to modulate AKT and ERK1/2 phosphorylation. In addition to this effect we observed that the expression of two genes related to embryo quality (*IFNτ* and *GJA1)* was upregulated.

Exocannabinoids such as HU-210 and THC are pharmacologically more stable than endocannabinoids (such as AEA) and are thus the preferred choice for pharmacology assays (Pertwee 2006). Our selection of the bovine oocyte model was based on the fact that a high percentage of oocytes mature *in vitro* compared with other model species (Sirard *et al*. 2006, Moussa *et al*. 2015), and their similarities with human oocytes in terms of metabolic requirements, genome activation, interactions with the culture medium and early embryo development (Menezo *et al*. 2000, Van Hoeck *et al*. 2011).

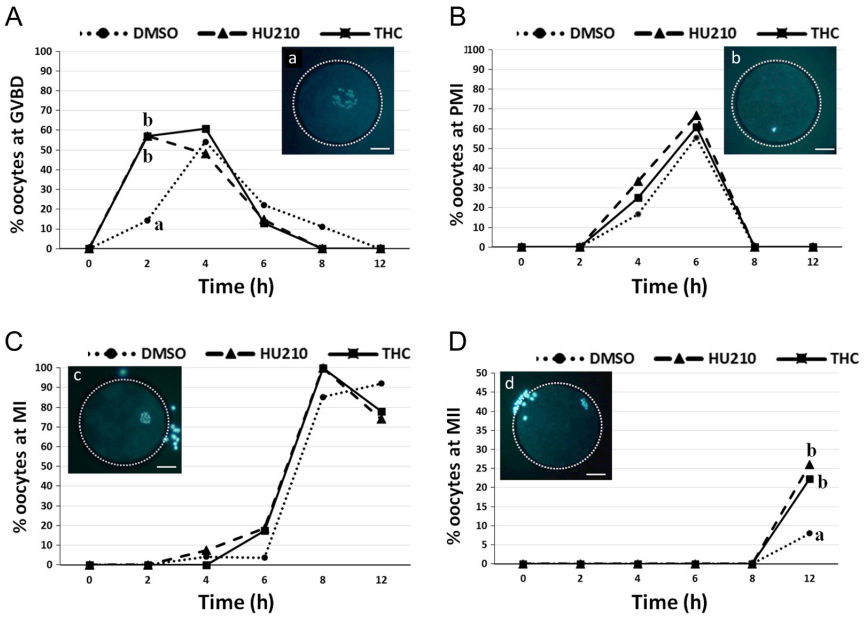


**Figure 4** Changes in nuclear status of oocytes throughout maturation. Results are expressed as percentage of oocytes at each stage of maturation at each time point. To simplify the analysis, four stages were chosen: germinal vesicle breakdown (A) (GVBD), defined by the absence of visible nuclear membrane; (B) pro-metaphase I stage (PMI), (C) defined by chromatin condensation; metaphase I stage (MI), (D) material with individual chromosomes and metaphase II stage (MII). Significant differences among treatments at the same time are indicated with different letters[a,b]; ($P<0.05$). Representative photomicrographs are shown. Scale bar represents 20 µm. (a) Representative image of GVBD, (b) representative image of oocyte with PMI, (c) representative image of MI, (d) representative image of oocyte with MII.

Maternal RNA is critical for an efficient meiotic cell cycle and ensuing fertilization and embryo development. Despite this, oocytes suffer a maternal RNA loss of around 20% during their maturation (Chen *et al*. 2013, Mehlmann 2013). This study detected the dramatic degradation of *CB1* mRNA in MII oocytes, as observed in the mouse model (López-Cardona *et al*. 2014). This event could play a critical role in acquiring oocyte competence for fertilization and subsequent embryo development. At the protein level, we noted changes only in the localization of the CB1 receptor, which moved towards the periphery of the oocyte as meiosis progressed, whereas the localization of the CB2 receptor remains unchanged. Given that receptors need to reach the cell surface in order to act (Cahill *et al*. 2007) (according to G-protein-coupled receptor theory), CB1 could be the active receptor during oocyte maturation, as reported for the mouse and human models (Peralta *et al*. 2011, López-Cardona *et al*. 2014), suggesting that the endocannabinoid system, acting via CB1, is a conserved mechanism in mammalian oocyte maturation.

Oocyte maturation involves the activation of various signal transduction pathways, including PI3K/AKT or MAPK pathways (Schmitt & Nebreda 2002, Cecconi *et al*. 2012, Conti *et al*. 2012), which culminate in the activation of maturation-promoting factor (MPF), a complex consisting of cdc2 kinase and cyclin B (Schmitt & Nebreda 2002). Myelin transcription factor 1 (MYT1), the major inhibitory kinase of the MPF complex, is phosphorylated and inhibited by AKT activity, thus promoting the resumption of meiosis (Okumura *et al*. 2002). In effect, studies in mouse and rat oocytes have shown that AKT activity blockage leads to a significant reduction in cdc2 activity and delayed meiosis resumption (Kalous *et al*. 2009) and that LH treatment induces higher phosphorylation levels of AKT (Carvalho *et al*. 2003). Cow oocytes are dependent on active AKT for successful MI/MII transition (Tomek & Smiljakovic 2005). Our results indicate that exposure of these oocytes to HU-210 or THC during their *in vitro* maturation enhances AKT phosphorylation both in granulosa cells and within the oocyte.

The capacity of FSH or EGF to activate ERK1/2 is well documented (Gonzalez-Robayna *et al*. 2000, Wayne *et al*. 2007). In cow, such phosphorylated ERK1/2 is necessary for meiosis resumption (Levesque & Sirard 1995, Su *et al*. 2003). Here, we show that cannabinoids

**Table 2** Cleavage rates (D2) and blastocyst yields at day 7 (D9), 8 (D8) and 9 (D9) with and without cannabinoids supplementation in IVM.

| | | Embryo cleavage | | Blastocyst yield | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | D2 | | D7 | | D8 | | D9 | |
| Group | *n* | *n* | (%±S.E.M.) | *n* | (%±S.E.M.) | *n* | (%±S.E.M.) | *n* | (%±S.E.M.) |
| DMSO | 190 | 146 | 77.3±4.2 | 28 | 14.9±1.4 | 42 | 23.0±2.7 | 45 | 24.6±2.6 |
| HU-210 | 227 | 180 | 77.9±3.3 | 32 | 15.6±3.5 | 50 | 23.1±2.0 | 56 | 25.0±1.3 |
| THC | 240 | 190 | 78.8±2.7 | 31 | 13.2±4.9 | 51 | 20.8±5.7 | 53 | 21.8±5.8 |

Values are the mean±S.E.M., 100 mM of HU210 or THC were used, $P>0.05$.

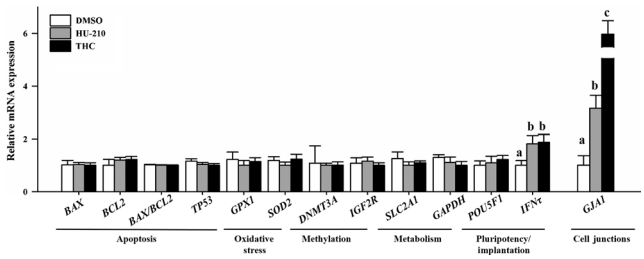


**Figure 5** Quantitative real-time PCR (qPCR). Expression patterns of key genes in bovine blastocysts arising from oocytes matured in the presence of the vehicle DMSO (control), HU-210 or THC. Bars with different superscripts indicate a significant difference among treatments ($P < 0.05$).

have a similar effect to EGF or other promotors of maturation (Conti *et al*. 2012) on the activation of ERK1/2 both in granulosa cells and in the oocyte. In those oocytes exposed to cannabinoids, meiosis resumption began earlier than in oocytes from the control group. This suggests a potential role for MAPK in the early stages of meiosis resumption in the bovine oocyte model through considerable acceleration of GVBD (Fissore *et al*. 1996, Kalous *et al*. 1993). On the contrary, in the mouse oocyte model, cannabinoids do not seem to affect the ERK1/2 phosphorylation status in early maturation stages in which MAPK is not involved (Verlhac *et al*. 1994, López-Cardona *et al*. 2014). Effectively, we observed here that after 24 h maturation, ERK1/2 reached its maximum activity in MII-arrested bovine oocytes, whereas in the mouse model, cannabinoids were found to reduce the phosphorylation of ERK1/2 (López-Cardona *et al*. 2014). Taken together, these results indicate differences among mammalian species in the dynamics of the MAPK pathway, as reported by others (Fissore *et al*. 1996).

In this study, the embryo development rate to the blastocyst stage was not affected by exposure to HU-210 or THC during IVM, although this treatment did have a beneficial effect on the expression of two genes related to embryo quality, *IFNτ* and *GJA1*. *IFNτ* is responsible for the establishment and maintenance of early pregnancy in cattle. Accordingly, bovine blastocysts produced *in vivo, in vitro* or through ICSI show the differential expression of *IFNτ* (Gutierrez-Adan *et al*. 2004, Arias *et al*. 2015) with *in vitro*-produced blastocysts featuring lower relative *IFNτ* transcription compared with their *in vivo* counterparts (Lonergan *et al*. 2003). Connexin43 (*GJA1*) is associated with the formation of cell junctions and several reports suggest that its expression is lower in blastocysts produced *in vitro* vs *in vivo* (Rizos *et al*. 2002, Gutierrez-Adan *et al*. 2004, Tesfaye *et al*. 2007). Hence, the significantly upregulated expression of both *IFNτ* and *GJA1* observed here for embryos supplemented with cannabinoids during oocyte maturation suggests that cannabinoids may have improved the quality of those embryos.

In conclusion, our findings indicate that exposure to cannabinoids during the *in vitro* maturation of oocytes activated AKT and ERK1/2 faster and it is possible that activation could serve to modulate PI3K/AKT and MAPK molecular pathways, probably via *CB1* activation, with positive effects on the expression of genes related to embryo quality and no repercussions on the embryo development. These findings have implications for improving IVM in cattle. In addition, the bovine oocyte model emerged as a useful tool for preclinical screening of exogenous cannabinoids targeted at improving the quality of human embryos produced through assisted reproduction techniques.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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