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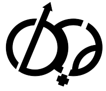

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Effects of chlorogenic acid on the maturation and fertilisation of bovine oocytes and their embryonic development with a comparative bovine granulosa cell co-culture

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Abstract

Chlorogenic acid (CGA) plays several biological roles, but lacks studies that demonstrate how this phenolic compound affects animal reproduction. The aim of the present study was to evaluate the effects of different CGA concentrations on bovine oocyte maturation and embryo development *in vitro*. This study also evaluates co-culture systems involving bovine granulosa cells (BGC) from fed with CGA containing plant, *Pittosporum Undulatum*. The ovaries were recovered after slaughter and the oocytes were removed, maturated, *in vitro* fertilized and cultured in medium containing CGA in 5 different concentrations 1.25; 2.5; 5; 10; 20 µm and a control group (0 µm) for seven days. Selected oocytes (n = 1040) were matured in any of the 5 treatment or control groups. Significantly lower (P < 0.05) maturation rates were observed for the highest CGA concentrations 10 µm, and 20 µm, compared to the control group (Control = 93.4 ± 2.1% *vs*. 10 µm = 80.9 ± 2.2%; 20 µm 77.9 ± 3.3%). We observed that the higher the concentration of CGA present, the lower the rate of cleavage and development after 3 and 7 days, respectively. It was observed that the significant difference recorded in regards to embryonic development were evident between control and group (20; 51.1 ±5.6 *vs*. 19.4 ± 2.2%). In respects to the study involving co-culture of embryos with BGC the only difference recorded involved the block rate. No differences (P > 0.05) were identified between control and experimental groups in relation to the progesterone production by BGC. These results suggest that CGA may affect oocyte maturation and inhibit the progression of meiosis and consequently the entire embryo development *in vitro*.

Keywords: blastocysts, bovine, chlorogenic acid, IVF, IVM, P. undulatum; progesterone.

Introduction

Chlorogenic acid (CGA) is a bioactive phenolic compound formed by the esterification of trans-cinnamic acids (caffeic, coumaric, and ferulic acid) with quinic acid, comprising a group of isomers mainly 3-O-caffeoylquinic acid (3-CQA), 4-O-caffeoylquinic acid (4-CQA), and 5-O-caffeoylquinic acid (5-CQA; Hao *et al*., 2016). It is widely distributed in plants such as coffee, tea, miscellaneous vegetables (Gordon and Wishart, 2010) and also *Pittosporum undulatum*, one of the most successful invader plants of the Azores archipelago used mainly as alternative feedstuff for cattle (Lourenço *et al*., 2011; Nunes *et al*., 2014). Presently the only CGA commercially available is the 5-CQA which has been extensively studied due to its wide range of pharmacological properties including anti-rust, anti-oxidant, anti-bacterial, and anti-viral. It has also been associated with glucose absorption inference and modulating gene expression of antioxidant enzymes, among other biological activities (Gugliucci *et al*., 2009; Liu *et al*., 2016). Besides its described pharmacological properties, CGA also acts on the metabolism of arachidonic acid cascade inhibiting cyclooxygenase 2 (COX-2; Shan *et al*., 2009), which can interfere with the reproductive physiology namely in the maturation of oocytes, as well as, cleavage (Calder *et al*., 2001) and embryo development after fertilisation (Thatcher *et al*., 1984). This may be due to a direct effect of CGA on an oocyte or an indirect effect on the metabolism of the granulosa cells surrounding the oocytes. As known, the maturation process is one of the most important stages of *in vitro* embryo production since it is at this time that the oocyte obtains the capacity to be fertilised. Several nuclear changes take place, including a cytoplasmic series of biochemical activities established by a complex cascade of phosphorylation and dephosphorisation of proteins involved in the regulation of meiosis (Meinecke *et al*., 2001). Among which stand out the mitogen activated protein kinase (MAPK) family (Tian *et al*., 2002), triggering signal transduction pathways. During maturation, proteins maturation-promoting factor (MPF) complex and MAPK or/and extracellular signal-regulated kinase (ERK) pathway are involved in the regulation of a variety of growth and differentiation pathways through several phosphorylation cascades (Katz *et al*., 2007).

In the present study, oocyte maturation and IVF were performed in conjunction with a granulosa cell co-culture obtained from bovine fed with and without *P. undulatum*. Furthermore, oocyte maturation and *in vitro* embryo culture were performed using different concentrations of CGA without co-culture. Levels of progesterone produced by granulosa cells from both animal groups (with and without *P. undulatum*) were also evaluated.

Material and Methods

Experimental design

Experiment 1 was designed to test the effect of CGA on oocyte maturation and embryo development produced *in vitro* where the maturation and culture

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media were supplemented with different concentrations of CGA. For such purpose, after collection, oocytes (n = 1040) were divided into six groups: 0 (control, without CGA; (n = 221); 1.25 µm (n = 170); 2.5 µm (n = 130); 5 µm (n = 164); 10 µm (n = 163) and 20 µm (n = 192). The groups were maturated without co-culture for 24 h as afore described followed by IVF. Presumptive embryos were checked on day 3 for cleavage and on day 7 for morula/blastocyst development.

In experiment 2, the media was not supplemented with CGA, but embryos were produced in co-culture using bovine granulosa cells recovered from animal fed for six weeks with *P. undulatum* (experimental group) and without (control group). For the control and experimental group, 150 and 218 oocytes were respectively processed.

Chemicals

All the chemicals and reagents used in this study were obtained from Sigma-Aldrich (St. Louis, Mo, USA) unless stated otherwise.

Ovaries

Ovaries were obtained at a local abattoir from slaughtered cows, trimmed of adhering tissue and transported to the laboratory in Dulbecco's phosphate buffered saline (DPBS) at 37°C within 2 h post-slaughtering. Upon arrival at laboratory, all ovaries were rinsed once with 70% alcohol, and followed by a wash with fresh warm DPBS.

Immature oocytes collection

Cumulus oocytes complexes (COCs) were collected by aspiration from antral follicles (2 - 8 mm diameter) with an 18-gauge needle. The aspiration method was applied to avoid disruption of the COCs; the needle and syringe were primed with approximately 0.25 - 0.5 ml of washing medium consisting of TCM 199 buffered with Hepes, supplemented with 2% Fetal Bovine Serum (FBS), 0.3 mg/ml glutamine and 50 µg/ml gentamycin. COCs and follicular fluid were slowly expelled into a 10 ml tube and maintained there for at least 5 min to allow for sedimentation of the COCs. The precipitate was taken into a sterile petri dish for COC morphological evaluation. Good quality COCs covered by at least four layers of compacted cumulus cells and evenly granulated ooplasm were selected for maturation (Santos *et al*., 2008).

In vitro *embryo production*

Fresh COCs were divided randomly in groups and washed twice in TCM-199 Hepes medium supplemented with 2% FBS, 0.3 mg/ml glutamine, 50 µg/ml gentamycin and matured in TCM-199 supplemented with 10% FBS, 5 µg/ml or 0.02 IU/ml of FSH-LH (Stimufol, Belgium), 1 µg/ml estradiol-17β, 0.15 mg/ml glutamine, 22 µg/ml sodium pyruvate, 50 µg/ml gentamycin. The stock solution of CGA was prepared in 0.2% dimethyl sulfoxide (DMSO) and diluted in maturation medium ranging from 7 µg/ml to 1.4 µg/ml CGA. A final concentration per group consisted of 1.25µm, 2.5 µm, 5 µm, 10 µm, 20 µm. After 24 h under 5% CO2 in a humidified atmosphere at 38.5ºC, oocytes considered to be maturated by means of cumulus expansion were placed for insemination in fertilisation TALP medium (Faheem *et al*., 2011). Briefly, thawed semen was washed three times by centrifugation, twice in sperm-TALP medium (4 ml each time) and once in IVF-TALP medium supplemented with 10 µg/ml heparin, 6 mg/ml bovine serum albumin (BSA, essentially fatty acid free), 22 µg/ml Na-pyruvate, 50 µg/ml gentamycin and 20 µg/ml of nystatin. After removing the supernatant the sperm pellet was homogenized with 0.25-0.5 ml of remaining IVF-TALP medium for adjusting the sperm concentration to 1×10^6 sperm/ml. Oocytes and sperm were co-cultured in 50 µl of fertilisation medium (10-15 oocytes/ droplet) for 22-24 h at 38.5ºC in 5% CO2 in air. Presumptive zygotes were denuded separately by vortex, washed and cultured in TCM-199 with Hepes supplemented with 3 mg/ml BSA (Fr. V), 22 µg/ml Na-pyruvate, 10 µl/ml NEAA (MEM, non-essential amino acids), 20 µl/ml EAA (BME, essential amino acids), 50 µg/ml gentamycin, 20 µg/ml of nystatin and CGA in different concentrations: 0 (control group/without CGA), 1.25µm, 2.5 µm, 5 µm, 10 µm, 20 µm. All Presumptive zygotes were incubated at 38.5ºC in 5% CO2 in air. Cleavage rate was determined after 3 days of fertilisation (day 0) and embryonic development was evaluated on day 7 of culture.

Bovine granulosa cells culture

Monolayer granulosa cell culture for co-culture were prepared on the day of oocyte maturation, in which granulosa cells were aspirated from follicular fluid of follicles 2-8 mm diameter from bovines fed with *P. undulatum* (experimental group) or animals not fed with this plant (control group). Follicular fluid was then centrifuged at 200×g for 10 min, and re-suspended the pellet in granulosa culture medium, which consisted of TCM-199 without Hepes containing 10% FBS, 50 µg/ml gentamycin and 20 µg/ml of nystatin. The clumps of cells were mechanically broken down by repeated aspiration through an 18 gauge needle attached to a 5 ml syringe. Cell concentration and viability was determined and measured using a haemocytometer after cell staining with Trypan Blue method (0.4%, w/v) and adjusted to 1×10^6cells/ml by adding granulosa culture medium, TCM-199 without Hepes (M5017) and gentamicin (5 µg/L gentamycin). Drops of 100 µl of this dilute suspension were placed in a 60 mm Petri dish (Nunc®, Denmark) covered with mineral oil, permitting a confluent monolayer of cells to be formed at the base of the droplets during the next 2 days before zygotes were transferred. Half of the medium was replaced before placing the embryos in co-culture and every 48 h.

Cells from these two groups were used separately to produce embryos, as well as, to evaluate their ability to produce progesterone. For such purpose the cells were divided into three culture flasks in a

concentration of 1 × 10^6 cells/ml, in 5 ml and cultivated for 24, 48 and 72 h. After which, supernatant aliquots of 1 ml were collected separately from each flask and stored at -20ºC for further determination of P4 using an Enzyme-Linked Immunosorbent Assay (ELISA) method (Progesterone ELISA kit, K0299, Abnova®).

Statistical analysis

In experiment 1 data were analysed by one-way analysis of variance (ANOVA) and expressed as mean ± standard error of the mean (SEM) calculated from the collected oocytes for each group. Percentile data were normalized through arc sine transformation and then submitted to homogeneity test, followed by variance analysis (ANOVA one-way) with post-hoc Fisher's least significant difference (LSD) test. Data from the experiment 2 were analysed by Student's t-test and expressed as mean ± standard error of the mean (SEM) calculated from collected oocytes for each group. Differences in progesterone concentration in the granulosa culture medium were determined by repeated measures analysis of variance (ANOVA). All analyses were performed using the IBM SPSS v.20 Statistics Program (SPSS Inc. Chicago, IL). For all analyses comparisons were considered significantly different when P value of ≤0.05.

Results

In vitro *maturation and embryo development without co-culture cells*

In experiment 1, significant differences (P < 0.05) in the maturation rates were observed between the groups with high concentration of CGA (20 µm) and the other groups (0, 1.25, 2.5, 5 µm). In regards to the lover CGA concentrations groups (0, 1.25, 2.5, 5 µm), no statistical differences were observed (Table 1) in any of the studied parameters. Statistical differences (P < 0.05) were observed only for the highest CGA concentration. The blocking ratio of the embryos was not affected by CGA, and no statistical difference was observed among the groups.

Culture medium CGA supplementation caused a negative impact on embryonic development. No statistical differences were observed between CGA = 20 µm (19.4 ± 2.2%) and CGA = 10 µm (21.2 ± 1.2%), these two concentrations resulted in statistically lower values when compared to the lowest CGA concentrations employed. The highest embryonic production was observed during CGA = 0 and 1.25 µm, resulting in 51.1 ± 5.6 and 50.5 ± 3.3 percentage of produced embryos.

Table 1. Rates of development embryonic *in vitro* of different concentrations of CGA.

Concentration CGA (µm)	No. of oocytes	Maturation (%)	Cleavage (%)	Blocked (%)	Developed embryo (%)
0	221	93.4 ± 2.1[a,b]	78.3 ± 4.8[a]	27.1 ± 4.4[a]	51.1 ± 5.6[a]
1.25	170	94.1 ± 2.0[a,b]	73.8 ± 3.4[a]	22.0 ± 3.8[a]	50.5 ± 3.3[a]
2.5	130	91.0 ± 0.7[a,b]	67.5 ± 2.4[a]	26.6 ± 0.8[a]	40,5 ± 1.4[a,b]
5	164	86.6 ± 0.7[b,c]	67.4 ± 3.7[a]	33.2 ± 3.6[a]	34.2 ± 3.7[a,b,c]
10	163	80.9 ± 2.2[c,d]	54.6 ± 1.9[a,b]	33.1 ± 0.7[a]	21.2 ± 1.2[b,c]
20	192	77.9 ± 3.3[d]	48.75 ± 1.8[b]	29.4 ± 1.9[a]	19.4 ± 2.2[c]

Numbers in the same column with different letters ([a,b,c,d]) differ significantly at P < 0.05; Data are expressed in percentage (%) as mean ± SEM, standard error of means; developed embryos classified as morula, early blastocyst and blastocyst stages.

Embryo production in co-culture

When co-culture of granulosa cells and oocytes were observed for maturation and continued development after IVF it was evident that maturation, cleavage and embryo development rates were statistically similar when cultivated in granulosa cells obtained from animals fed with and those fed without *P. undulatum* (Table 2), being 52.5 ± 1.7% for the control *vs*. 43.1 ± 1.4% for the experimental group. Yet, it was observed in Table 2 that a number of embryos blocked in the experimental group (33.1 ± 3.4%) was higher when compared to the control group (10.7 ± 1.6%), being that difference statistically significant (P < 0.05).

Progesterone production by medium cultured bovine granulosa cells (BGC)

Progesterone produced by the granulosa cells after 72 h of culture resulted in similar concentrations among both groups, being 7.6 ± 0.2 ng/ml 8.6 ± 0.8 ng/ml, respectively for the control and experimental group. In each group 1 x 10^6 cells were placed in a petri dish drop (Table 3).

Table 2. Rates of development embryonic *in vitro* of experimental and control groups with bovine granulosa cells.

Group	No. of oocytes	Maturation (%)	Cleavage (%)	Blocked (%)	Developed embryo (%)
Control	150	90.4 ± 0.5[a]	94.5 ± 2.8[a]	10.7 ± 1.6[a]	52.5 ± 1.7[a]
Experimental	218	93.7 ± 1.8[a]	91.4 ± 3.8[a]	33.1 ± 3.4[b]	43.1 ± 4.4[a]

Numbers in the same column with different letters ([a,b]) differ significantly at P < 0.05; Data are expressed in percentage (%) as mean ± SEM, standard error of means; developed embryos classified as morula, early blastocyst and blastocyst stages.

Table 3. Progesterone levels in granulosa cells culture media during 72 h.

Culture hours	Progesterone concetration (ng/ml)	
	Control group	Experimental group
24	6.5 ± 1.3[a]	7.8 ± 1.0[a]
48	8.1 ± 1.0[a]	6.9 ± 1.6[a]
72	7.6 ± 0.2[a]	8.6 ± 0.8[a]

Results represent as mean ± SEM. Different letters in the same columns are statistically different (P < 0.05).

Discussion

CGA properties are not yet fully studied, to our knowledge this is the first study that reports the effect of different concentrations on oocyte maturation and embryo development in cattle. In the present study, experiment 1, when 5μm CGA concentrations or higher were added to the maturation medium a decrease in the oocyte maturation rate was observed. Although there is no scientific evidence in this work to suggest that this reduction is due to the CGA suppression of ERK´s/MAPK protein phosphorylation, second Kang *et al*. (2011) one can speculate that this is the reason that leads to inhibiting the progression of meiosis leading to the deficiency in oocyte maturation.

For groups associated with lower concentrations (2.5 μm and 1.25 μm and 0 μm) no differences were observed. This leads us to believe that these concentrations of CGA are not sufficient enough to produce interference in the ERK / MAPK pathways.

Several studies have shown a higher cell death for in vitro produced embryos up to the blastocyst stage due to the lack of several paracrine factors in the *in vitro* conditions (Pakrasi and Jain, 2008). With this study we can verify that high concentrations of CGA (10 and 20 μm) in the culture medium cause the rate of cleavage and embryonic development to decrease when compared with the control group (0 μm, without addition of CGA) or with the results of the experiment 2. This information leads us to believe that the rates of cell death in these two periods are greater when compared to non-experimental *in vitro* embryos primarily because of the CGA manipulation. Only embryos that were divided beyond 16 cells were considered as not blocked, which were found inversely proportional to the amount of CGA added to the maturation/culture medium. Although the mechanisms by which CGA reduces embryonic development is not fully understood, a hypothesis moves toward the effect of cyclooxygenases (COXs) and particularly the effect of COX-2 expression, which is highly induced by a variety of stimuli and observed during certain periods of embryonic development. This may occur through interdiction signalling of ERK and p38 MAPK that are required for bovine embryonic development (Madan *et al*., 2005), namely for the production of prostaglandins that play an important role in embryonic development by increasing the number of cells (Pakrasi and Jain, 2008).

In the production of embryos co-cultured with BGC (experiment 2), contrary to expectations, no differences were observed between the control (using granulosa cells recovered from cows without *P. undulatum* in the fed) and experimental groups (using granulosa cells recovered from cows feed with *P. undulatum*) in relation to cleavage and embryo development. Our results comparing the two groups were similar and this can be due to the fact that CGA present in the *P. undulatum* does not accumulate in BCG. The CGA metabolism, as well as, bioavailability in ruminants is still unknown; however, it is known that in monogastrics only a small proportion of CGA are absorbed without hydrolysis and approximately 1% of the ingested CGAs are found intact (Oliveira and Bastos, 2011). Given the particularities of bovine digestive physiology, we believe that the bioavailability of CGA is still lower and not enough to accumulate in the granulosa cells. In summary, the addition of CGA at high concentrations, i.e., more than 20 μm to the maturation and culture medium, may affect oocyte maturation, inhibit meiosis progression and consequently all *in vitro* embryonic development.

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