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Effect of quercetin on ovarian cells of pigs and cattle

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ABSTRACT

The bioflavonoid quercetin is a component of food with numerous biological effects, but its function in reproductive processes and mechanisms in various species remain unclear. The aim of this study was to examine the effect of quercetin on ovarian cells isolated from ovaries of two phytophagous mammalian species (*i.e.* pigs and cattle). There was analysis of the effect of quercetin (0, 1, 10, and 100 ng/mL) on cultured granulosa cells of pigs and cattle. Proliferation (PCNA) and apoptosis (bax) markers and release of progesterone (P4), testosterone (T), estradiol (E2), and IGF-I were quantified using quantitative immunocytochemistry, enzyme immunoassay, or radioimmunoassay. Treatments with quercetin reduced PCNA and bax accumulation and decreased P4 release from both granulosa cells of pigs and cattle. In cells of pigs, treatment with quercetin reduced T output, however, in cells of cattle quercetin increased T release. In cells of pigs, quercetin reduced IGF-I release. In cells of cattle, quercetin at smaller doses (1 or 10 ng/mL), promoted and at a large dose (100 ng/mL) reduced IGF-I secretions. There was no substantial E2 release from granulosa cells of pigs or cattle. These observations are the first to indicate there is a direct action of quercetin on basic ovarian cell functions (proliferation, apoptosis, and hormones release) which can be species-specific.

1. Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the most well-known flavonoids present in plants with phytoestrogen and antioxidant properties (Moutsatsou, 2007). It is present in large amounts in fruits and vegetables. There is the greatest quercetin concentration in onions, buckwheat, apples, blueberries, cherries, broccoli, grapes, leeks, lettuce, tomatoes, citrus fruits and wild herbs (Moutsatsou, 2007; Chen et al., 2010a; Anand et al., 2016; Kawai, 2018). Quercetin consumption by humans varies from 11 and 21 mg per day, while sometimes this consumption is less than the recommended amount (Nishimuro et al., 2015; Kawai, 2018; Pan et al., 2018; Yao et al., 2018). Although there has not been precise quantification of quercetin consumption by farm animals, it is an important flavonoid from a nutritional perspective (Santini et al., 2009). Quercetin has numerous effects on physiological state and health because of its antioxidant/anti-aging, anti-inflammatory, anti-proliferative, anti-carcinogenic, and cardio-protective properties (Boots et al., 2008; Chen et al., 2010a; Anand et al., 2016; Sharma et al., 2018). Through its pro-apoptotic and anti-proliferative

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properties, quercetin suppresses the functions of human non-ovarian (Hashemzaei et al., 2017; Sharma et al., 2018) and ovarian (Shen et al., 1999; Parvaresh et al., 2016; Zhang et al., 2018) cancer cells. The characteristics and mechanisms of quercetin action in reproduction have not been well defined and results from studies that have been conducted are inconsistent. Results of several studies indicate there are stimulatory actions of quercetin on ovarian functions: feeding quercetin reduced ovarian cell apoptosis, promoted proliferation, and increased ovarian weight, oocyte quality, and litter size in some animals (mice: Shu et al., 2011; Beazley and Nurminskaya, 2016 rabbits: Naseer et al., 2017). Results of other studies indicate there were different effects on ovarian folliculogenesis following quercetin feeding (rat: Chen et al., 2010b). Nevertheless, there appears to be a suppressive action of quercetin on female reproduction: feeding with quercetin disrupted estrous cycles, suppressed ovarian folliculogenesis and ovulation, increased ovarian follicular atresia, altered gonadotropin release, and decreased litter size in older mice (Shu et al., 2011). Results of previous studies, therefore, have been inconsistent regarding the effects of quercetin on female reproductive functions, which may be because of the different states of the reproductive system or animal species in the studies. Furthermore, the conduction of *in vivo* studies failed to lead to identification of the site and mechanisms of action of quercetin. The effects of quercetin on mice gonadotropin release (Shu et al., 2011) suggest that it regulates ovarian functions *via* hormonal regulators. In contrast, results of *in vitro* studies indicate there is quercetin uptake by Chinese hamster ovarian cells (Walgren et al., 2000) and direct quercetin actions on ovarian cell steroidogenesis, in which aromatase (human: Whitehead and Lacey, 2003; Rice et al., 2006) and progesterone (P4) release (pig: Santini et al., 2009) by cultured granulosa cells was inhibited. The action of quercetin on other ovarian cell functions (proliferation, apoptosis, release of peptide hormones) has not been reported.

The results from previous *in-vivo* experiments indicate quercetin is an important biologically active component of food of various phytophagous animals. More specifically, results indicate there are different actions on the reproductive processes due to different mechanisms of quercetin action on the gonads, but there have not been assessments of the mechanisms in different species.

The aim of the present study was to examine the direct effect of quercetin on basic ovarian cell functions in two phytophagous species with different fecundity (cow and pig) in which quercetin could be an important component of the diet affecting reproduction. There was analysis of markers of proliferation (proliferating cell nuclear antigen PCNA), apoptosis (bax), and release of progesterone (P4), testosterone (T), estradiol (E2) and IGF-I in cultured ovarian granulosa cells of pigs and cattle.

2. Materials and methods

2.1. Isolation and culture of granulosa cells

Granulosa cells were isolated from the ovaries of non-estrous cycling pubertal gilts at approximately 180 days of age and from the ovaries of estrous cyclic Czech Fleckvieh dairy cows that were 4–6 years of age that were slaughtered at a local abattoir. Cells were isolated and cultured as described previously (Sirotkin et al., 2017a, 2018). Briefly, isolated granulosa cells were cultured in DMEM/F12 1:1 (BioWhittaker™, Verviers, Belgium) with 10% fetal calf serum (BioWhittaker™) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration of 10^6 cells/mL medium in 16-well chamber slides (Nunc, Inc., International, Roskilde, Denmark, 200 μL/well) or in 24-well culture plates (Nunc; 1 mL suspension/well) until a 60% to 75% confluent monolayer was formed (3–5 days), at which point the medium was exchanged. The new medium had the same composition, but the cells were cultured in the presence of quercetin (AppliChem GmbH, Darmstadt, Germany) at concentrations of 0, 1, 10, and 100 ng/mL. These doses of quercetin correspond to those in blood concentrations of humans and what would be expected for concentrations in human (Nishimuro et al., 2015; Kawai, 2018; Pan et al., 2018; Yao et al., 2018) and animal (Walgren et al., 2000; Rodriguez Lanzi et al., 2018) tissues. Furthermore, these doses are comparable to the quercetin doses used in previous similar *in-vitro* experiments with ovarian cells (Rice et al., 2006; Jia et al., 2011; Nna et al., 2017). Quercetin was dissolved in culture medium immediately before addition to the cells. After 2 days culture with- and without quercetin, the cells and culture medium were processed as described previously (Sirotkin et al., 2017a; Sirotkin et al., 2017b) prior to conducting the enzyme immunoassay (EIA), radioimmunoassay (RIA), and immunocytochemistry procedures that have also been described previously (Sirotkin et al., 2017a). Cell viability (determined by Trypan blue staining) after culture ranged from 70% to 80%, and there were no significant differences in these indices between the control and experimental groups.

2.2. Immunocytochemical analysis

Following washing and fixation, the cells were incubated in blocking solution (1% goat serum in phosphate-buffered saline – PBS) at +20 °C for 1 h to block nonspecific binding of antiserum. The cells were subsequently incubated in the presence of mouse monoclonal antibodies against either PCNA (marker of proliferation; Naryzhny and Lee, 2001; Liebermann and Hoffman, 2007) or bax (marker of apoptosis; Harada and Grant, 2003; Liebermann and Hoffman, 2007 Ugarte-Uribe and García-Sáez, 2017) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. PC-10 and B-9, respectively, dilution 1:500 in PBS) for 2 h at +20 °C. To detect the binding sites of the primary antibody, the cells were incubated in secondary pig or goat antibody against mouse IgG labeled with fluorescein isothiocyanate (FITC) or horseradish peroxidase (both from Sevac, Prague, Czech Republic, dilution 1:1000) for 1 h. The FITC was detected using fluorescent microscopy. The DAB was visualized using staining with DAB-substrate (Roche Diagnostics GmbH, Manheim, Germany). Following DAB-staining, the cells on chamber-slides were washed in PBS, covered with a drop of glycergel mounting medium (DAKO, Glostrup, Denmark), and the coverslip was attached to a microslide. There was determination of the presence and localization of PCNA and bax in cells by assessing FITC green fluorescence or DAB-peroxidase brown staining. Cells processed without the primary antibody were used as a negative control. The ratio of DAB-HRP-stained cells to the total cell number

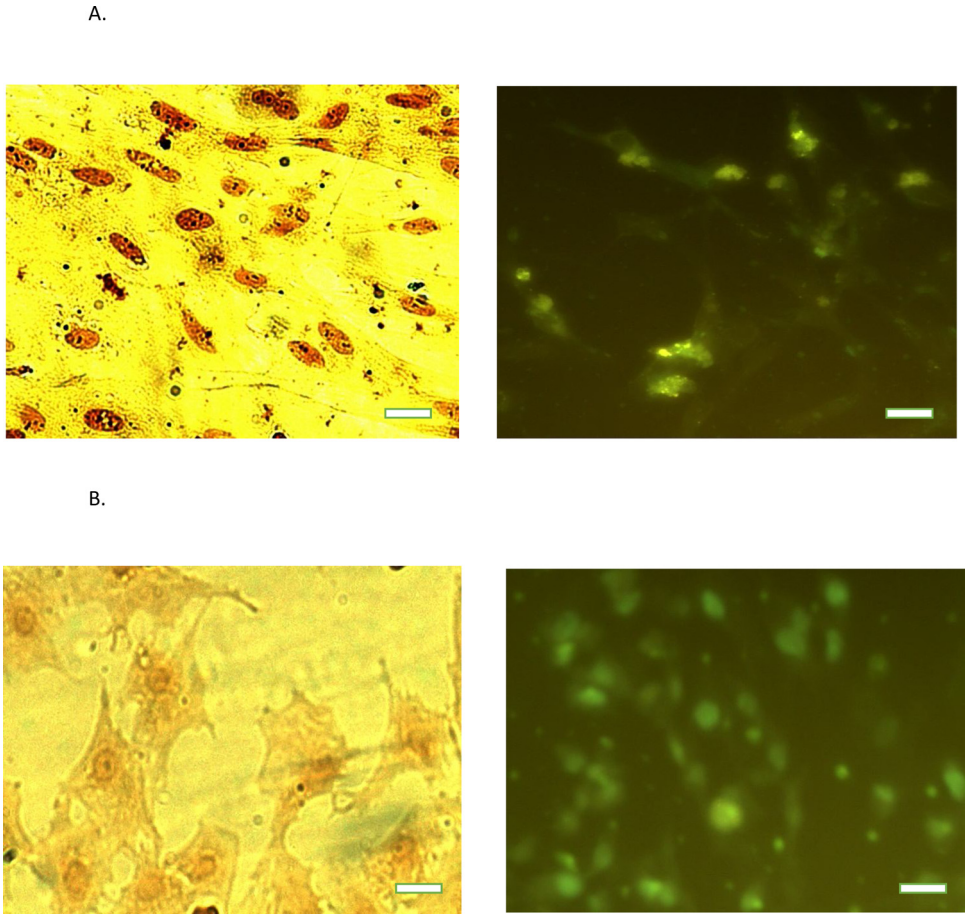

Fig. 1. Immunocytochemistry images of cultured granulosa cells of pigs containing PCNA (A) and bax (B) detected using either horseradish peroxidase and DAB substrate (left, brown staining) or FITC (rights, green fluorescence); Scale bars: 1 cm = 20 μm.

was calculated. The presence of both PCNA and bax in the cells after culture is documented by the inclusions in Fig. 1.

2.3. Immunoassay

Accumulation of the hormones P4, T, and E2 was determined in 25–100 μL aliquots of the incubation medium by EIA as described previously (Münster, 1989; Prakash et al., 1987). All EIAs were validated for use in aliquots of the culture medium. The assay sensitivity of P4 was 0.12 ng/mL and cross-reactivity of the antiserum to pregnenolone, androstenediol, testosterone, estradiol, and cortisol was less than 0.001%. Intra- and inter-assay coefficients of variation did not exceed 8% and 13%, respectively. The sensitivity of T was 10 pg/mL. The antiserum cross-reacted < 96% with dihydrotestosterone, < 3% with androstenedione, < 0.01% with progesterone and estradiol, < 0.02% with cortisol, and < 0.001% with corticosterone. Inter- and intra-assay coefficients of variation were 12.3% and 6.8%, respectively. Estradiol concentrations were evaluated using the procedures described by Münster (1989) with an assay sensitivity of 5 pg/mL. The cross-reactivity of the E_2 antiserum was < 2% to estrone, ≤ 0.3% to estriol, ≤ 0.004% to T, and ≤ 0.0001% to P_4 and cortisol. The inter- and intra-assay coefficients of variation did not exceed 16.6% and 11.7%, respectively. Concentrations of IGF-I were determined in incubation medium by using RIA kits from DRG Instruments GmbH (Marburg, Germany) according to the manufacturer's instructions with the IGF-I extraction procedure. No cross-reactivity of antiserum to IGF-I was detected (< 0.0001%) for IGF-II, insulin, proinsulin, and growth hormone. The maximum intra- and inter-assay coefficients of variation were 3.4% and 8.2%, respectively. The sensitivity of the assay was 0.8 ng/mL. All RIAs were validated for use with diluted aliquots of culture medium.

2.4. Statistical analysis

The data from this study are reported as the means of values obtained in at least three separate experiments performed on separate days with different groups of granulosa cells, each obtained from at least ten animals. Each experimental group of granulosa cells was processed using three chamber-slide wells and four culture plate wells. The proportions of granulosa cells containing

specific immunoactivity were calculated from at least 1000 cells per chamber (=at least 3000 cells per sample). Assays of hormones in the incubation medium were performed in duplicate. The values of blank control were subtracted from the value determined by EIA/RIA in cell-conditioned medium to exclude any non-specific background (less than 17% of total values). Rates of hormone secretion were calculated using 10^6 viable cells/day or mg follicular tissue/day. Significant differences between experiments and groups were evaluated using a one-way analysis of variance followed by use of the Duncan's or chi-square tests utilizing Sigma Plot 11.0 software (Systat Software, GmbH, Erkrath, Germany). The $P < 0.05$ value was considered to be statistically significant.

3. Results

The cells after culture contained immunoreactive PCNA and bax. The PCNA was localized predominantly in the cell nuclei, while bax was observed in both the cytoplasm and nuclei (Fig. 1). Furthermore, cultured cells released substantial amounts of P4, T and IGF-I. Quantification of E2 in the culture medium of granulosa cells from pigs indicated the values were less than the assay detection limit.

Addition of quercetin to the culture medium of granulosa cells from pigs resulted in a decrease in the percentage of cells containing PCNA (at a dose 10 or 100 ng/mL, Fig. 2A) and bax (at doses 1 and 10 ng/mL, Fig. 2B). Furthermore, quercetin addition to the medium resulted in an inhibition of the release of P4 (at all doses added, Fig. 2C), T (at doses 1 and 10 ng/ml, Fig. 2D), and IGF-I (at 10 or 100 ng/mL, Fig. 2E).

Quercetin addition to the incubation medium for granulosa cells of cattle resulted in a lesser proportion of both PCNA (at dose 100 ng/mL, Fig. 3A)- and bax (at 1 and 100 ng/mL)-positive cells (Fig. 3B). The P4 release was inhibited by addition of quercetin to the medium at all doses (Fig. 3C), while T secretion was stimulated by addition of quercetin (added at 1 or 10 ng/mL, Fig. 3D). Quercetin, when added at doses of 1 or 10 ng/mL, had a stimulatory effect on IGF-I release, while a dose of 100 ng/mL inhibited IGF-I release (Fig. 3E) by cultured granulosa cells of cattle. Similar to what occurred with granulosa cells of pigs, granulosa cells of cattle released an amount of E2 that was less than the assay detection limit.

4. Discussion

The small amount of E2 produced by cultured granulosa cells indicated luteinization of these cells was occurring in culture, which is associated with increased P4 and decreased E2 production (Sirotkin, 2014). The functions of quercetin in altering PCNA, bax, P4, T, and IGF-I release by cultured ovarian cells indicate that this flavonoid can modulate ovarian cell proliferation, apoptosis, and both steroid and peptide hormone release. Previously, only the direct effect of quercetin on ovarian steroidogenesis has been reported (Whitehead and Lacey, 2003; Rice et al., 2006; Santini et al., 2009). The results from the present study confirm the results from previous studies. Furthermore, the present study is the first where results indicate quercetin directly affects ovarian cell proliferation and apoptosis and IGF-I release. The PCNA protein is considered a marker and regulator of the S-phase of the cell cycle (Naryzhny and Lee, 2001; Liebermann and Hoffman, 2007). Quercetin may suppress cell proliferation by inhibitory actions during the S-phase of mitosis. These observations are not consistent with the observations of Shu et al. (2011) where it was observed that there was a quercetin-induced increase in mice ovarian cell proliferation *in vivo*, while the present and previous results indicate quercetin suppresses proliferation not only of cancer cells (Shen et al., 1999; Zhang et al., 2018), but also of healthy ovarian cells. In contrast to actions on cancer cells (Shen et al., 1999; Zhang et al., 2018), in the present study where there was examination of healthy pig and cattle granulosa cells, quercetin did not promote, but rather suppressed apoptosis. The quercetin-induced decrease in both proliferation and apoptosis markers might indicate that quercetin inhibits ovarian cell turnover in both species where there were assessments in the present study. This effect may provide an explanation of the capacity of quercetin to suppress mice ovarian follicullogenesis and ovulation *in vivo* (Shu et al., 2011). The potential anti-reproductive action of dietary quercetin in some phytophagous mammalian species might explain some effects of nutrition on the fecundity in these species (described in a subsequent section of this manuscript). Furthermore, this anti-reproductive quercetin effect should be considered when there is use of this compound in research and in its application for clinical purposes.

The inhibitory influence of quercetin on aromatase in human granulosa cells led to luteinization of these cells in culture (Whitehead and Lacey, 2003; Rice et al., 2006) and a resulting increase in P4 release by cultured granulosa cells of pigs as has been previously reported (Santini et al., 2009). The results of the present study confirm that quercetin functions to inhibit ovarian P4 production from granulosa cells of pigs and cattle. Furthermore, the present study is the first where quercetin affected ovarian T and IGF-I release. In contrast to the effects of proliferation and apoptosis, the action of quercetin on hormones was species-dependent: in pigs, quercetin had an inhibitory effect on T secretion, while in cattle quercetin promoted T secretion. There were substantial species-dependent differences in quercetin effects on IGF-I release: in pigs, quercetin inhibited IGF-I release, while in cows, there was a biphasic action of quercetin (quercetin at the smaller doses promoted and in larger doses suppressed IGF-I secretion. The previous observations suggest that quercetin mainly suppress ovarian steroidogenesis in pigs, cows, and humans, but promotes this process in rabbits. In addition, the effective doses of quercetin were different in different species indicating that cells isolated from ovaries of various species had varying responses to quercetin. Understanding the causes and biological significance of such species-specific differences in quercetin action on ovarian hormones requires additional studies. It is possible, that ovarian cells of different species have different numbers of estrogen receptors which are involved in mediation of phytoestrogen quercetin effects (Sirotkin and Harrath, 2014). Furthermore, in some species there may exist negative feedback mechanisms that inhibit the cell response to the regulator, if this regulator is present in amounts greater than optimal for cellular responses (Sirotkin, 2014). This mechanism might explain the biphasic action of quercetin (stimulatory effect smaller doses and inhibitory actions of larger doses) on IGF-I release by

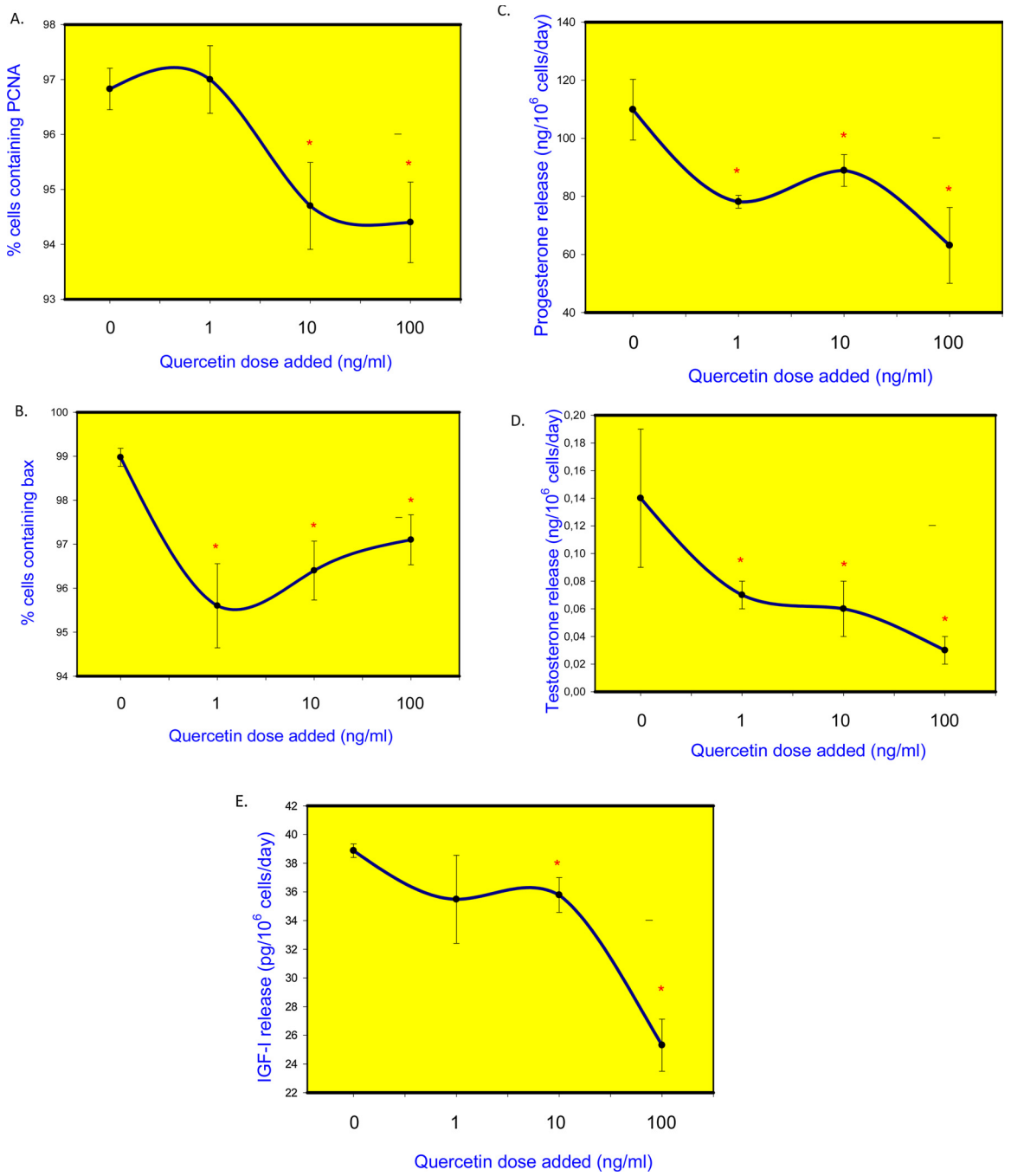

Fig. 2. Effect of quercetin (0, 1, 10, and 100 ng/mL medium) on proliferation (accumulation of PCNA, 1A), apoptosis (accumulation of bax, 1B), and release of progesterone (P4) (1C), testosterone (T) (1D), and IGF-I (1E) from cultured granulosa cells of pigs; Data from quantitative immunocytochemistry (1A, 1B), EIA (1C, 1D), and RIA (1D); Values represent means ± SEM; *Indicates difference ($P < 0.05$) for effect of quercetin: differences between cells cultured with quercetin (1, 10, and 100 ng/mL medium) and control (quercetin at 0 ng/mL medium).

granulosa cells of cattle. It should also not be ignored that consumption of a large amount of fresh food containing quercetin in the spring of the year by prolific phytophagic rabbits may affect reproduction. Rabbits can consume large amounts of this phytoestrogen, resulting in a positive effect on the reproductive system in this species. The stimulatory action of dietary quercetin on rabbit ovarian functions and fecundity in the summer season of the year has been documented (Naseer et al., 2017). In contrast, in less prolific animals in which there are not seasonal reproduction patterns, changes in quercetin consumption (pigs and cattle) can suppress steroidogenesis. The mechanisms of such suppression remain unknown but it should not be discounted that the hyper-activation of ovarian steroid hormone receptors by the phytoestrogen, quercetin, can induce negative feedback down-regulation of endogenous

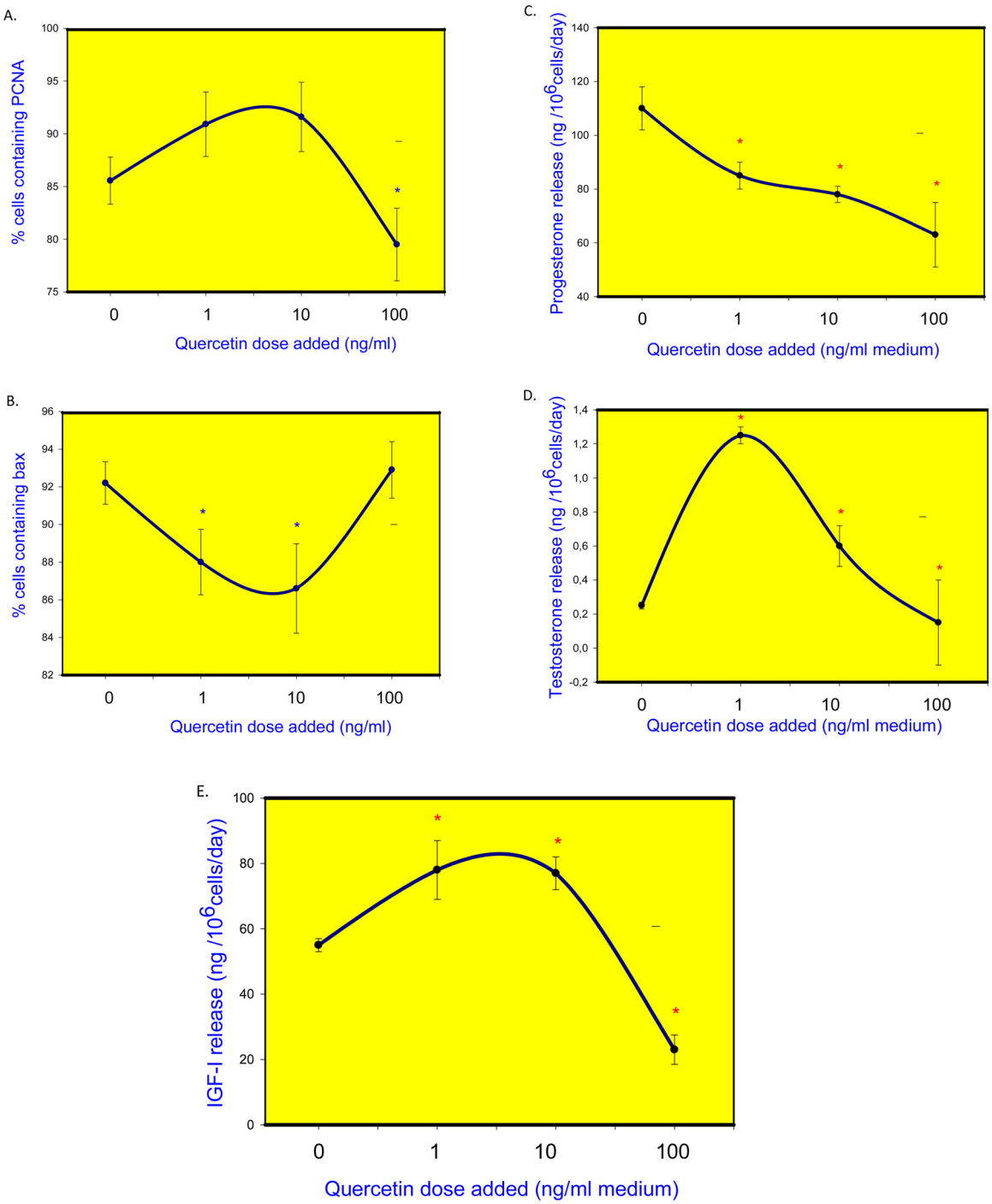

Fig. 3. Effect of quercetin (0, 1, 10, and 100 ng/mL medium) on proliferation (accumulation of PCNA, 2A), apoptosis (accumulation of bax, 2B), and release of progesterone (P4) (2C), testosterone (T) (2D), and IGF-I (2E) from cultured granulosa cells of cattle; Data using quantitative immunocytochemistry (1A, 1B), EIA (1C, 1D), and RIA (1D); Legends same as for Fig. 1.

steroid hormone secretions. In cattle this feedback mechanism only relates to P4, but in pigs both P4 and T secretions are affected.

The functional interrelationships and biological significance of hormonal events affected by quercetin remain unclear. Nevertheless, because P4 is a precursor of T (Sirotkin, 2014), changes in T release may occur because of changes in P4 synthesis or a reduction in P4 release may occur because of conversion to T. Furthermore, IGF-I and steroid hormones are stimulators of ovarian functions including ovarian cell proliferation, steroidogenesis, follicullogenesis, and fecundity (Scaramuzzi et al., 2011; Sirotkin, 2014). Quercetin, therefore, may reduce steroid hormone release, ovarian cell proliferation, and fecundity by reducing the release of

IGF-I, thus, there is less of a stimulatory action of this hormone. In contrast, the suppression of apoptosis by quercetin in the present experiment cannot be explained by the suppression of IGF-I release, which does not promote, but rather inhibits ovarian cell apoptosis (Sirotkin, 2014).

The effects of quercetin in the present study represent the first time that the effect of the flavonoid, quercetin, on basic ovarian cell functions has been reported, including proliferation, apoptosis, and release of ovarian T and IGF-I. Furthermore, this is the first comparative study of quercetin action on ovarian cells in different mammalian phytophagous species. The direct suppressive action of quercetin on ovarian cell functions in some species (pigs, cattle, humans) indicates its potential negative effect on female reproduction, which has been confirmed with results from some *in vivo* experiments in mice (Shu et al., 2011). If *in vivo* experiments with humans and farm animals were conducted, there would likely be similar conclusions regarding the anti-reproductive action of quercetin. There should be, therefore, consideration of consumption of quercetin-containing foods and therapeutic application of quercetin in human and large farm animal nutrition and medicine. There has been observation of the anti-apoptotic quercetin action on healthy ovarian cells and its pro-apoptotic action on ovarian cancer cells previously (Shen et al., 1999; Parvaresh et al., 2016; Zhang et al., 2018), thus, providing further evidence for its potential applicability in the selected suppression of cancer cells by ovarian cancer prevention and treatment. The stimulatory action of quercetin in mice and rabbit reproduction observed previously (Shu et al., 2011; Beazley and Nurminskaya, 2016; Naseer et al., 2017), however, indicates the potential applicability of this compound as a biostimulator of reproduction in other species.

Taken together, the results from the present represent the first for a direct action of quercetin on basic ovarian cell functions (proliferation, apoptosis, and hormones release), which appear to be species-specific.

Conflict of interest

None.

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