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EFFECT OF NATURAL PLANT EXTRACTS ON PORCINE OVARIAN FUNCTIONS

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ABSTRACT

This report provides information about the impact of chosen natural plant extracts on basic ovarian functions. This article summarizes our results concerning the effect of selected plant extracts on proliferation, apoptosis and hormone secretion – release of progesterone (P4), testosterone (T) and leptin (L) on porcine granulosa cells (GC), We analyzed effects of ginkgo (GB), rooibos (RB), flaxseed (FL), green tea polyphenols (GTPP), green tea - epigallocatechin-3-gallate (EGCG), resveratrol (RSV) and curcumin (CURC) (0; 1; 10 and 100 μg.ml^{-1}) on markers of proliferation, apoptosis and secretory activity of porcine ovarian granulosa cells by using immunocytochemistry and EIA. It was demonstrated, that all these natural plants and plant molecules inhibited the accumulation of proliferation-related peptide (PCNA) and apoptosis-associated peptide (Bax) in cultured. Furthermore, it was observed that natural plant extracts altered progesterone, testosterone and leptin release in porcine ovarian cells. It is concluded, that GB, RB, FL, RSV, CURC, GTPP and EGCG can directly affect ovarian cells and therefore they could potentially influence ovarian functions.

Keywords: Natural plant, proliferation, apoptosis, hormone secretion, ovarian cells

INTRODUCTION

Nowadays, the study of natural plant and their substances with pharmacological activity has become an emerging trend in nutritional and pharmacologic research. Natural plant extracts represent a rich group used as remedies for human and animal diseases and for regulation of particular physiological processes **(Hammer *et al*., 1999; Nostro *et al*., 2000)**. These plant extracts are used due to their preventive, antibacterial and therapeutic effects, anticancer and apoptosis inducing-properties **(Blanko *et al*., 2003)**. *Ginkgo biloba* extract displays free radical scavenging and antioxidant actions **(Marcocci *et al*., 1994)**. In vitro experiments showed that Ginkgo extract and its components have significant anti-proliferative effects in ovarian cancer cells **(Ye *et al*., 2007)**. It is known that Rooibos tea contains abundant flavonoids **(Shimoi *et al*., 1996)**, aspalathin, chrysoeriol, orientin, isoorientin, vitexin, isovitexin, quercetin, isoquercitrin and rutin **(Duke *et al*., 2002)**. Rooibos tea is commonly used for treating cardiac arrhythmias, colic, diarrhea **(Duke *et al*., 2002)**, asthma **(Brown, 1995)** and hypertension **(Nakano, 1997)**. Flaxseed is a rich source of 3 components with demonstrated cardioprotective effects **(Chantal *et al*., 2009)**, can inhibit arrhythmogenesis during ischemia-reperfusion **(Ander *et al*., 2004)**, inhibit atherogenesis **(Prasad, 2005)**, and protect against vascular dysfunction during hypercholesterolemic conditions **(Dupasquier *et al*., 2006)**. RSV was chemically 3, 4', 5 – trihydroxystilbene, is one of the natural phytoalexins **(Hain *et al*., 1990)** and is found in grapevines, in soft fruits and hazelnut (Frémont, 2000). CURC is Curcuma longa L. extract and chemicaly is 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-eptadiene-3,5-dione **(Nadkarmi, 1976)**. Among effects of CURC extract is reduction of P secretion in mature follicle of swine GCs **(Nurcahyo and Kadarsih, 2003)**. Green tea is obtained from the leaves and the leaf buds of the plant *Camellia sinensis*. GTPP may induced reductions in the levels of sex steroids hormones as testosterone and estradiol and possible negative effects on reproductive performance **(Kao *et al*., 2000)** on granulosa cell functions **(Basini *et al*., 2005)** and *in vitro* fertilization in swine **(Spinaci *et al*., 2006)**. The main components of green tea are polyphenols. 50-80 % of polyphenols are represented by special flavonoids - catechins, especially epigallocatechin-3-gallate (EGCG) **(Fukai *et.al*., 1991; Khan *et al*., 2006)**. Long term consumption of green tea may influence the incidence of obesity, diabetes, and cardiovascular disease **(Kao *et al*., 2000)**. Direct effect of these natural plant and plant molecules on healthy ovarian cells functions remaines unknow. The aim of our studies was to analyze the effects of selected plants, GB, RB, FL, and plant substances GTPP, RSV and CURC on markers of proliferation, apoptosis and secretory activity of porcine ovarian granulosa cells.

MATERIAL AND METHODS

Granulosa cells were aspirated from the ovaries of Slovakian white gilts after slaughter at a local abattoir. The subsequet procedures followed standard protocols according to **Sirotkin, *et al*.(2008)**. After formed confluent monolayer and medium replacement experimental cells were cultured in the presence of GB (Shangai TECH Chemical Indutry Testing Co., Ltd), RB (Clanwilliam, South Africa), FL (MEDU s.r.o., Čenkovce, Slovakia), RSV, CURC, GTPP and EGCG (all from Changsha Sunfull Bio-tech. Co, Hunan China) alone at concentrations of 0; 1; 10 and 100 μg.ml^{-1}.
After removing the medium from chamber slides, cell were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2-7.4; 60 min) and held at 4°C to await immunocytochemistry. The medium from the plate wells was gently aspirated and frozen at -24 °C to await EIA. Concentrations of P4, T and L were determined in 25 μl aliquots of incubation medium by EIA, previously validated for use in culture medium, by using antisera against steroids (produced in the Institute of Animal Science, Neustadt, Germany) as previously described and characterised **(Sirotkin et al., 2008)**. Significant differences among the experiments were evaluated using Student´s T-test and one/two-way ANOVA folowed by paired Wilcoxon-Mann Whitney test, by using Sigma Plot 11.0 software (Systat Software, GmbH, Erkhart, Germany). Differences against control at $P<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Immunocytochemistry

In our study of plants extracts (GB, RB, FL, RSV and EGCG) significantly inhibited the percentage of cells containing PCNA at all doses added. Proliferation of GCs was diminished also after addition of CURC (at dose 10 μg.ml^{-1}) and GTPP (at doses 10 and 100 μg.ml^{-1}). These datas comfirmed antiproliferative activity on cancer cells **(Salganik, 2001; Kim *et al*., 2005; Chen *et al*., 2002; Xu *et al*., 2003; Wei *et al*., 2007; Zhang *et al*., 2008; Demark-Wahnefried *et al*., 2001; Thompson *et al*., 2005; Zhou *et al*., 2009),** on interstitial theca cells **(Wong *et al*., 2010),** in rat ovarian GCs **(Ortega *et al*., 2012),** on non-ovarian and swine ovarian cells **(Nurcahyo and Kadarsih, 2003; Huh *et al*., 2004; Spinella *et al*., 2006; Basini *et al*., 2005a,b)**. Our results are

not inline with **Pantsi *et al*. (2001)** reports of the cardio-protective properties of aqueous rooibos extracts via the inhibition of apoptosis. These observation is the first observation on healthy ovarian cells , that these plants and their molecules can directly suppress ovarian cell proliferation and therefore potentially inhibit ovarian follicle growth and development.

Table 1. The percentage of cells containing marker of proliferation PCNA in cultured ovarian granulosa cells cultured with and without (control) GB, RB, FL, RSV, CURC, GTPP and EGCG

Supplement	Doses of supplement added (μg.ml^{-1})			
	0 (control)	1	10	100
GB	45.21±1.07 (1900)	24.00±0.99* (500)	20.50±1.12* (400)	22.55±1.35* (550)
RB	45.21±1.07 (1900)	28.75±2.03* (400)	26.20±1.44* (500)	22.20±0.76* (500)
FL	45.21±1.07 (1900)	23.40±0.67* (500)	21.75±1.10* (400)	17.40±0.85* (500)
RSV	51.00±1.43 (1404)	36.30±1.20* (356)	39.50±1.50* (273)	34.00±10.00* (320)
CURC	51.00±1.43 (1404)	50.5±3.43 (867)	42.83±1.49* (743)	44.71±2.56 (823)
GTPP	49.86±1.3 (3311)	45.13±2.17 (903)	34.6±3.98* (597)	41.0±3.53* (658)
EGCG	49.86±1.3 (3311)	40.42±2.99* (844)	38.88±3.46* (907)	36.88±2.53* (909)

All the values represent % of cells containing particular antigen, means ± SEM, *- significant (P<0.05) differences with control (cells not treated with plant molecules). In the brackets is a number of counted cells.

In our study plant extracts (GB, RB, FL, RSV, CURC and GTPP) significantly stimulated the number of cells containing Bax at all used doses (except doses 1 and 10 μg.ml^{-1}of EGCG). Pro-apoptotic effect is confirmed on cancer lines **(Salganik, 2001; Chen *et al*., 2002; Xu *et al*., 2003; Wei *et al*., 2007; Zhang *et al*., 2008; Demark-Wahnefried *et al*., 2001; Thompson *et al*., 2005; Zhou *et al*., 2009; Chen and Huang, 1998; Zheng *et al*. 2004)**, in theca cells **(Wong *et al*., 2010)**, on rat ovarian GCs **(Ortega *et al*., 2012)** via activation of apoptotic peptide caspase 3/7. Our results not correspondent with **Wei *et al*. (2000)**; **Ni *et al*. (1996)**; **Fan *et al*. (2006)** who demonstated antiapoptotic effect of GB. These observation is the first demonstration, that these plants and their molecules can directly promote ovarian cell apoptosis and therefore potentially stimulated ovarian cell death and ovarian follicular atresia.

Table 2 The percentage of cells containing Bax The percentage of cells containing marker of apoptosis Bax in cultured ovarian granulosa cells cultured with and without (control) GB, RB, FL, RSV, CURC, GTPP and EGCG

Supplement	Doses of supplement added (μg.ml^{-1})			
	0 (control)	1	10	100
GB	31.49±0.95 (1950)	48.20±1.09* (500)	49.00±0.38* (400)	54.00±0.82* (450)
RB	31.49±0.95 (1950)	46.75±1.46* (400)	40.80±0.85* (500)	50.44±1.59* (450)
FL	31.49±0.95 (1950)	40.50±0.91* (400)	37.50±0.73* (400)	45.25±2.10* (400)
RSV	49.88±1.72 (1980)	67.50±1.04* (453)	67.00±3.34* (472)	66.50±2.02* (441)
CURC	49.88±1.72 (1980)	58.25±1.31* (949)	66.13±2.37* (886)	71.0±3.07* (921)
GTPP	49.8±1.24 (3939)	61.75±1.71* (886)	64.88±3.0* (890)	68.71±1.77* (809)
EGCG	49.8±1.24 (3939)	55.83±2.02 (728)	59.88±3.49 (874)	67.25±2.85* (908)

Legends as in Table 1.

Release of hormones

In our experiment, GB and RSV addition significantly decreased respectively CURC, GTPP and EGCG increased P4 release. This secretion was not affect after RB treatment. This is the first evidence for an involvement of GB in the control of ovarian hormone secretion. Stimulated P4 release found **Kolesarova *et al*. (2012)** after treatment of RSV alone and RSV in combination with mycotoxin – deoxynivalenol (DON). Inhibited P4 secretion was detected by **Ortega *et al*. (2012)** after RSV output on rat GCs, by **Basini *et al*. (2010)** in porcine GCs treatment of polymethoxystilben 2 – analogue of RSV, by **Nurcahyo and Kadarsih (2003)** after CURC treatment on porcine GC from large mature follicles. Therefore, our observations confirm some previous observations on the ability of some plant extracts to affect P4 release. Since P4 represents dominant steroid hormone of the *corpus luteum*, and suppressor of fecundity **(Sirotkin, 2014)** it can be proposed that GB, RB, RSV are a repressor of ovarian cell luteinisation.

Table 3 The secretion of P4 (ng.10^{-6} $cells^{-1}$.day^{-1}) in cultured ovarian granulosa cells cultured with and without (control) GB, RB, FL, RSV, CURC, GTPP and EGCG (EIA).

Supplement	Doses of supplement added (µg.ml^{-1})			
	0 (control)	1	10	100
GB	2.32 ± 0.35	0.18 ± 0.05*	2.77 ± 0.83	-
RB	2.00 ± 0.22	2.34 ± 0.10	1.29 ± 0.43	-
RSV	78.10±5.75	52.00±2.26*	50.80±3.03*	41.40±5.90*
CURC	145.00±6.88	250.00±5.00*	250.00±5.00*	62.10±6.90
GTPP	81.20±6.28	172.00±28.40	250.00±5.00*	146.70±47.30
EGCG	81.20±6.28	63.70±6.47	230.00±5.00*	101.00±17.80

All the values represent P4 release, means ± SEM, *- significant (P<0.05) differences with control (cells not treated with plant molecules).

In our study, T release was stimulated after RSV, CURC and GTPP respectively inhibited after GTPP and EGCG administration on porcine GCs. This is the first evidence about impact of these plant molecules on T release by swine ovaries. It might be hypothesised, that reduction in P4 outpout might indicate, that plant extracts can reduce ovarian cell luteinisation, which is characterised by promotion of P4 production and reduction in P4 derivates – androgens and estrogens. Both P4 and T have antiproliferative and proapoptotic properties, therefore they can suppress growth of ovarian follicles **(Sirotkin, 2014)**, whilst plant extracts can affect these processes.

Table 4 The secretion of T in pg.10^{-6} $cells^{-1}$.day^{-1}in cultured ovarian granulosa cells cultured with and without (control) GB, RB, FL, RSV, CURC, GTPP and EGCG (EIA).

Supplement	Doses of supplement added (µg.ml^{-1})			
	0 (control)	1	10	100
RSV	420.70±54.90	496.70±27.50	777.00±15.0*	1932.00±41.9*
CURC	787.00±82,90	596.00±91,80	549.00±88.40	1203.28±47.70*
GTPP	344.46±79.20	965.00±29.90*	154.00±20.50*	156.00±15.20*
EGCG	344.46±79.20	274.40±12.10*	270.60±45.90	297.00±50.80

Legends as in Table 3

In our study, all used plant decreased L release. Since leptin is considered as a hormonal stimulator of ovarian functions and fecundity **(Spicer, 2001; Ogunwobi and Beales, 2007; Sirotkin, 2014)**, it may be proposed, that the analysed plants can suppress ovarian functions via inhibition of leptin output.

Table 5. The secretion of L in ng.10^{-6} $cells^{-1}$.day^{-1} in cultured ovarian granulosa cells cultured with and without (control) GB, RB, FL, RSV, CURC, GTPP and EGCG (EIA).

Supplement	Doses of supplement added (µg.ml^{-1})			
	0 (control)	1	10	100
GB	1.61 ± 0.83	0.76 ± 0.54	0.18 ± 0.03*	1,67 ± 0.04
RB	1.87 ± 0.00	1.06 ± 0.27*	1.14 ± 0.33*	1.62 ± 0.11*
FL	4.17 ± 0.38	4.68 ± 0.52	2.82 ± 0.33*	4.3 ± 0.52

Legends as in Table 3

CONCLUSION

The present review suggest a possible inhibitory impact of GB, RB, FL, RSV, CURC, GTPP and EGCG on proliferation (accumulation of PCNA) and stimulatory influence on apoptosis (accumulation of Bax) in porcine granulosa cells. Also addition of these natural plant and natural molecules affect the release of P4,T and L. Our results suggest a direct effect of these plant extracts on proliferation, apoptosis and hormone release in porcine ovaries. Taken together, these data suggest that GB, RB, FL, RSV, CURC, GTPP and EGCG can suppress porcine reproductive (ovarian) function –inhibit ovarian cell proliferation, promote their apoptosis and alter release of hormones. These direct inhibitory action of medical and food plants on ovarian cells functions observed in our experiments should be validated by further *in vivo* experiments. If this action will be confirmed, the potential anti-reproductive action of these plant extracts should be taken into account by their consumption by humans and farm animals.

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