

# The Action of Benzene, Resveratrol and Their Combination on Ovarian Cell Hormone Release

(resveratrol / benzene / ovary / progesterone / oxytocin / prostaglandin F.)

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**Abstract.** The aim of our study was to examine the direct influence of plant polyphenol resveratrol and oil-related environmental contaminant benzene on ovarian hormone release, as well as the ability of resveratrol to prevent the effect of benzene. Porcine ovarian granulosa cells were cultured with and without resveratrol (0, 1, 10 or 100 µg/ml) alone or in combination with 0.1% benzene. The release of progesterone, oxytocin and prostaglandin F was measured by enzyme immunoassay (EIA). Benzene promoted the release of progesterone, oxytocin and prostaglandin F. Resveratrol, when given alone, stimulated both progesterone and prostaglandin F, but not the oxytocin output. Moreover, resveratrol prevented and even inverted the stimulatory action of benzene on all analysed hormones. These observations demonstrate the direct influence of both benzene and resveratrol on porcine ovarian hormone release, as well as the ability of resveratrol to prevent the benzene action on the ovary.

## Introduction

Resveratrol (3,5,49-trihydroxystilbene, R), a polyphenolic compound of plants and plant products including red wines, possesses phytoestrogenic, antioxidant, anti-proliferation and pro-apoptotic properties. Due to these activities, its therapeutic action against various kinds of cancer, cardiovascular, metabolic, mental and reproductive disorders has been expressed (Ortega and Duleba, 2015; Rauf et al., 2016; Varoni et al., 2016; Nguen et al., 2017). The direct action of R on ovarian cell proliferation (Ortega and Duleba, 2015), viability (Morita et al., 2012) and apoptosis (Ortega and Duleba, 2015; Macedo et al., 2017) has been demonstrated. Furthermore, R can affect ovarian secretory activity – reduce the plasma anti-Mullerian hormone, insulin-like growth factor 1 (Ergenoglu et al., 2015) and insulin (Cabello et al., 2015) levels, promote steroidogenic enzyme expression and progesterone release by cultured ovarian cells (Kolesarova et al., 2012; Morita et al., 2012). Other studies, however, demonstrated the anti-steroidogenic action of R (Cabello et al., 2015; Ortega and Duleba, 2015). The action of R on non-steroid ovarian hormones requires further examination.

Resveratrol was able to prevent the toxic influence of ionizing radiation (Said et al., 2016), chemotherapeutic agents (Rauf et al., 2016; Atli et al., 2017), chromium (Banu et al., 2016) and mycotoxin deoxynivalenol (Kolesarova et al., 2012) on ovarian cells. On the other hand, the ability of R to protect against more ubiquitous environmental contaminants such as benzene (B) has not been studied yet.

The adverse effect of B on animal and human reproduction, fertility and cancerogenesis has been well documented (Protano et al., 2012; Sirotkin and Harrath, 2017). This effect on women can be due to the inhibitory influence of B on the plasma gonadotropin and oestradiol levels (Chen et al., 2001; Reutman et al., 2002;

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Abbreviations: B – benzene, EIA – enzyme immunoassay, O – oxytocin, P – progesterone, PGF – prostaglandin F, R – resveratrol.

Alvaggi et al., 2014). In *in vitro* conditions, B was able to reduce progesterone and IGF-I (but not testosterone) release by isolated mouse ovaries (Sirotkin et al., 2017), but other studies failed to demonstrate a direct B effect on cultured rabbit ovarian cells (Földešiová et al., 2017). Therefore, it remains unclear whether B affects reproduction via central gonadotropin release or by targeting ovarian cells or ovarian hormones directly.

The aim of the present *in vitro* experiments was to examine (1) whether B can directly affect ovarian cell functions, in particular ovarian hormone release, (2) whether R possesses this capacity, and (3) whether R can prevent the B effect on the ovary. For these purposes, we analysed the effect of the addition of B, R and their combination on the release of steroid and non-steroid hormones (progesterone (P), oxytocin (OT) and prostaglandin F (PGF)) by cultured porcine ovarian granulosa cells.

## Material and Methods

### Isolation and culture of granulosa cells

Granulosa cells were isolated from ovaries of non-cycling pubertal gilts, about 180 days of age, and cultured as it was described previously (Pavlová et al., 2013; Sirotkin et al., 2014, 2015). After three days of pre-culture and formation of 60–75% confluent monolayer, cells were cultured with R (Changsha Sunfull Bio-tech. Co, Hunan, China) at a concentration of 0, 1, 10 or 100 µg/ml medium with and without 0.1% benzene. After 2-day culture, the culture medium was aspirated and stored at –14°C to await enzyme immunoassay (EIA).

### Immunoassays

The EIA for P, OT and PGF was based on the method described by Prakash et al. (1987) with our modifications (Kotwica et al. 1993, 1994; Skarzynski et al., 1999). The cross-reactivity of the antisera used against OT and P4 have been previously reported by Kotwica et al. (1993, 1994) and those against PGFM by Homanics and Silvia (1988). The range of the standard curve, the intra- and inter-assay coefficients of variation, and the relationship between the added and measured hormone concentration ( $N = 4$ ), expressed as the coefficient of regression, were as follows: for P4: 0.37–25 ng/ml, 8.7 %, 10.2 % and  $R = 0.96$ ; for OT: 3.9–1000 pg/ml, 9.8 %, 10.8 % and  $R = 0.94$ ; for PGFM: 62.5–2000 pg/ml, 8.2 %, 11.9 %, and  $R = 0.98$ , respectively. The PGFM concentration reliably reflected the PGF2a level ( $R = 0.92$ ;  $P < 0.001$ ) (Skarzynski et al., 1999). All the assays were previously validated for use by a serial dilution test.

### Statistics

The present results summarize the data of three experiments performed on separate days with separate groups of granulosa cells, each obtained from 8–15 ani-

mals. Each experimental group was represented by four culture wells. Hormones in the samples of incubation medium were analysed in duplicates. The values of blank controls (serum-supplemented medium incubated without cells) were subtracted from the specific values determined by EIA in cell-conditioned medium to exclude any non-specific background (less than 10 % of total values). Rates of secretion were calculated using  $10^6$  viable cells/day. Significant differences between the experiments and groups were evaluated using two-way analysis of variance (ANOVA), followed by Duncan's test using Sigma Plot 11.0 software (Systat Software, GmbH, Erkrath, Germany). A value of  $P < 0.05$  was considered as statistically significant.

## Results

All the cell cultures in our experiments produced substantial amounts of P, OT and PGF, whilst this production was affected by the presence of both R and B in the culture medium.

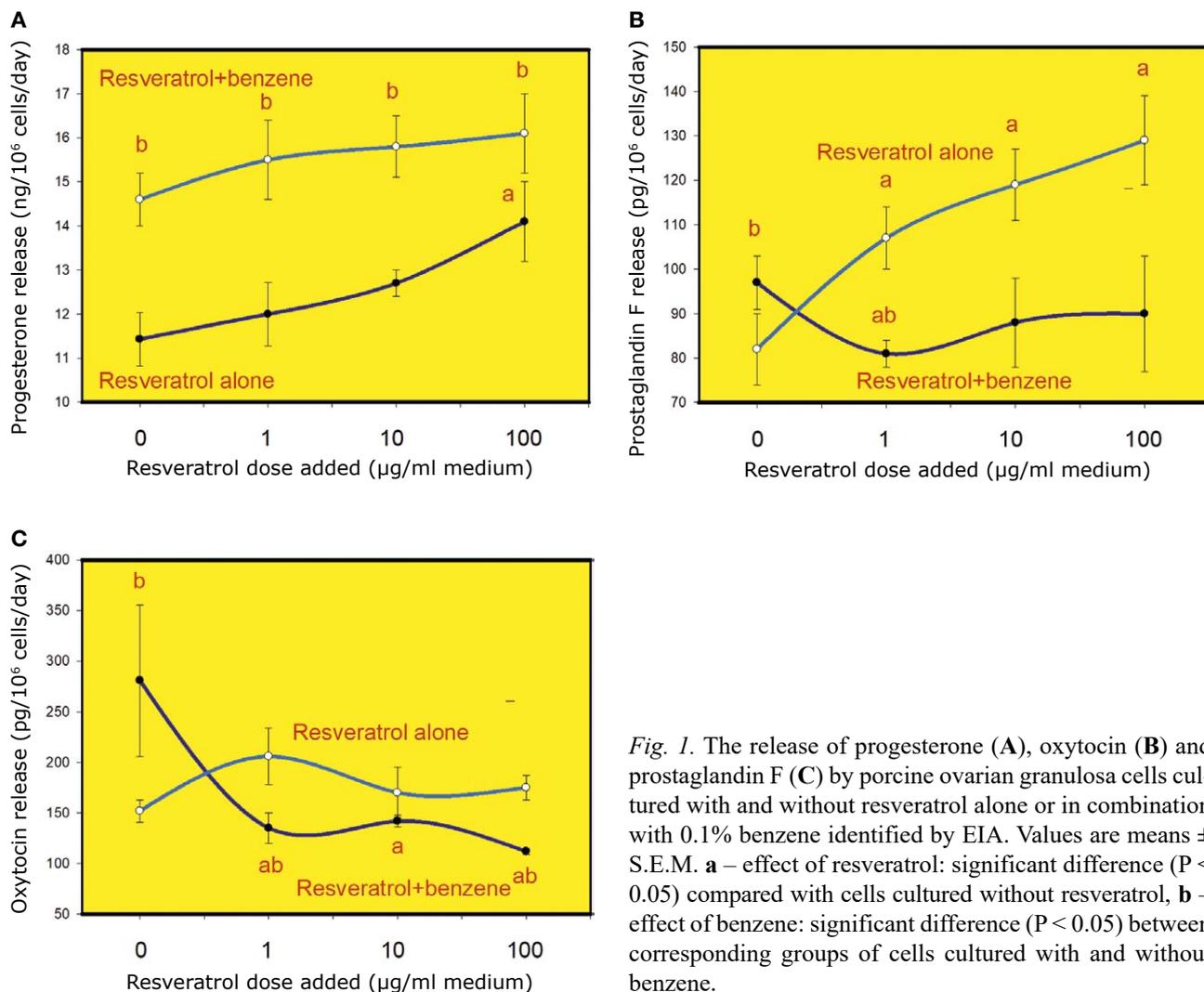
Resveratrol, when given alone, promoted release of both P (at a dose of 100 µg/ml, from  $11.4 \pm 0.6$  up to  $14.1 \pm 0.9$  ng/ $10^6$  cells/day,  $P < 0.05$ , Fig. 1A) and PGF (at all doses added, from  $82 \pm 8$  up to  $129 \pm 10$  pg/ $10^6$  cells/day,  $P < 0.01$ , Fig. 1C). No R influence on the OT output was found.

Benzene addition to cells cultured without R (R at a dose of 0 ng/ml) resulted in a significant increase in the P (from  $11.4 \pm 0.6$  up to  $14.6 \pm 0.6$  ng/ $10^6$  cells/day,  $P < 0.05$ , Fig. 1A), OT (from  $152 \pm 11$  up to  $281 \pm 75$  pg/ $10^6$  cells/day,  $P < 0.01$ , Fig. 1B) and PGF (from  $82 \pm 8$  up to  $97 \pm 6$  pg/ $10^6$  cells/day,  $P < 0.01$ , Fig. 1C) release. Moreover, in the presence of R, B did not affect the P level (Fig. 1A). The presence of R also returned the OT (Fig. 1B) and PGF (Fig. 1C) release to and even below the control level.

## Discussion

Our results demonstrate the ability of B to promote the release of P, OT and PGF by cultured porcine ovarian cells. These observations confirm the previous report (Sirotkin et al., 2017) on the direct action of B on mouse ovarian cell steroidogenesis, although B exerted an opposite effect on the P release by mouse and porcine ovaries. In some species, B can display an anti-androgenic effect (men: Rosati et al., 2017), although in other species (mice: Sirotkin et al., 2017), no B action on gonadal androgen has been observed. It is not to be excluded that in the pig, B can also affect reproduction due to its anti-androgenic effect, but this hypothesis requires further experimental validation.

Furthermore, our observations are the first demonstration that B can affect the non-steroid ovarian hormones. Since there is mutual support of P, OT and PGF release by ovarian cells (Sirotkin, 2014), it is not to be excluded that the B effects on some hormones are primary and the others are secondary. All these hormones



*Fig. 1.* The release of progesterone (A), oxytocin (B) and prostaglandin F (C) by porcine ovarian granulosa cells cultured with and without resveratrol alone or in combination with 0.1% benzene identified by EIA. Values are means  $\pm$  S.E.M. **a** – effect of resveratrol: significant difference ( $P < 0.05$ ) compared with cells cultured without resveratrol, **b** – effect of benzene: significant difference ( $P < 0.05$ ) between corresponding groups of cells cultured with and without benzene.

play an important role in the control of ovarian cell proliferation, apoptosis, folliculogenesis, fertility and malignant transformation (Sirotkin, 2014). Therefore, it is highly probable that the known B action on human reproduction, fertility and cancerogenesis (Protano et al., 2012; Sirotkin and Harrath, 2017) is due to the B influence on hormonal regulators of ovarian functions and malfunctions. Therefore, the correction of hormonal alterations induced by B could potentially prevent the negative effects of B on reproductive functions and health.

In our experiments, R when given alone stimulated both P and PGF, but not OT release by granulosa cells. These P-stimulating effects of R confirm the previous reports concerning the ability of R to promote ovarian steroidogenesis and P release in other species (Kolesarova et al., 2012; Morita et al., 2012), but not the indications of anti-steroidogenic action of R (Cabello et al., 2015; Ortega and Duleba, 2015). The R action on ovarian steroidogenesis could be explained by its phytoestrogenic activity and resulted ability to bind and up- or down-regulate ovarian steroid hormone synthesis and reception. Therefore, the nature of the R effect on steroidogenesis probably depends on the species, as well as

on the stage of ovarian cycle or ovarian cell source (van Duursen, 2017).

Our observations of the R stimulatory action on PGF (but not OT) output represent the first demonstration of the R influence on non-steroid hormones. The mechanisms of R action require further studies. It is possible that the R action on P can be due to the ability of phytoestrogen R to bind to ovarian steroid hormone receptors, which in turn influence the P output via positive or negative feedback mechanisms. Ovarian PGF is under the stimulatory control of steroid hormones (Sirotkin, 2014). It is therefore not to be excluded that the up-regulation of PGF output by R observed in our experiments might be the consequence of an increased P output. On the other hand, R added at doses of 1 or 10 μg/ml promoted PGF but not P release, indicating independent regulation of P and PGF by R. It is not to be excluded that R affects ovarian P and PGF via the mammalian target of rapamycin (mTOR) intracellular signalling system. At least the ability of R to affect the mTOR system, and the influence of mTOR regulators on ovarian hormone release, have been well documented (Pavlová et al., 2013; Sirotkin et al., 2014, 2015; Sirotkin, 2016).

The ability of R to prevent the toxic influence of ionizing radiation (Said et al., 2016), chemotherapeutic agents (Rauf et al., 2016; Atli et al., 2017), chromium (Banu et al., 2016) and deoxynivalenol (Kolesarova et al., 2012) on ovarian cells has been reported previously. In our experiments, R prevented and even inverted the action of B on P, OT and PGF. Our observations are the first demonstration that R can neutralise the B action on ovarian functions. The fine mechanisms of interrelationships between R and B require further studies, although the involvement of ovarian hormones, steroid receptors and mTOR system in mediating such interrelationships may not be excluded (see above). From the practical viewpoint, our observations suggest a protective action of R, R-containing plants and R-containing functional food against environmental contaminants. Such R action and application is worth of further studies. Nevertheless, the results of our experiments represent the first demonstration of the direct influence of both B and R on the release of various ovarian hormones, as well as the ability of R to prevent the B action on the ovary.

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