Physiol. Res. 50: 627-630, 2001

RAPID COMMUNICATION

Gossypol Inhibits Follicle-Stimulating Hormone- and Epidermal Growth Factor-Stimulated Expansion of Oocyte-Cumulus Complexes from Porcine Preovulatory Follicles

J. KOLENA, S. VRŠANSKÁ, E. NAGYOVÁ[1], M. JEŽOVÁ

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic and [1]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Liběchov, Czech Republic

Received May 7, 2001
Accepted June 20, 2001

Summary

The role of gossypol in the cumulus expansion of oocyte-cumulus complexes (OCC) isolated from large antral porcine follicles was investigated. Marked suppression of cumulus expansion stimulated with follicle-stimulating hormone (FSH) and epidermal growth factor (EGF) was observed in the presence of different concentrations of gossypol. Comparable inhibitory effects were obtained in the presence of NO donor, S-nitroso-N-acetylpenicillamine or sodium nitroprusside, suggesting that the inhibitory effect of gossypol may be mediated *via* NO generation. The inhibitory effect of gossypol on cumulus expansion of OCC was accompanied by inhibition of progesterone secretion of OCC and the decrease of [^{125}I]EGF binding to granulosa cells.

Key words

Gossypol – Cumulus expansion – Progesterone secretion– Epidermal growth factor receptors

Gossypol is a polyphenolic compound naturally occurring in the pigment of cotton plants. Gossypol first attracted attention as a potential male contraceptive agent as a result of many studies in China (Segal 1985). Although the mechanism of contraceptive action and the toxicology of gossypol are not well defined, a special feature of the action of gossypol is that this drug is selectively effective on testicular tissues (Sang 1983). Gossypol has been shown to disrupt spermatogenesis and to interfere with steroidogenesis in testicular cells (Hadley *et al.* 1981). Moreover, gossypol has exhibited antitumor effects against several tumor cell lines in culture (Wang and Rao 1984). Gossypol is also known to affect reproductive functions in females, but in contrast to males, investigation of its effects in female has been limited (Yang and Wu 1987). Gossypol inhibited implantation and decreased the concentration of blood progesterone in female rats (Lin *et al.* 1987) and inhibited progesterone secretion in cultured bovine luteal and porcine granulosa cells (Gu *et al.* 1990, Vranová *et al.* 1999).

© 2001 Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
Fax +420 2 24920590
E-mail physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

At the time of follicular antrum formation, the granulosa cell population becomes divided into mural layers, inner layers of granulosa cells and cumulus cells surrounding the oocyte. In response to the ovulatory surge of gonadotropins, the cumulus cell-oocyte complex (OCC) expands by depositing an extensive extracellular matrix between the cumulus cells enriched in hyaluronan (Salustri *et al.* 1989). Extracellular matrix components appear to be important for ovulation and sperm-egg interaction, thereby contributing to successful fertilization (Chen *et al.* 1993). *In vitro*, cumulus expansion can be induced by FSH, cAMP, adenylylcyclase activators and EGF (Eppig 1979). In pigs, EGF-induced expansion of porcine OCC originates only from large antral follicles (Procházka *et al.* 1997). Our previous findings that gossypol inhibits basal as well as stimulated progesterone synthesis in granulosa cells (Vranová *et al.* 1999) led us to examine the effects of gossypol on FSH and EGF-induced cumulus expansion of OCC isolated from large antral follicles.

The porcine ovaries used for isolation of oocyte-cumulus complexes (OCC) were transported to the laboratory in a thermos at 30 °C. The OCC were isolated from 5-8 mm follicles, washed three times in M199 with Earle's salts buffered with 20 mmol/l $NaHCO_3$ and 6.25 mmol/l HEPES and supplemented with 10 % fetal calf serum, 0.91 mmol/l sodium pyruvate, 1.62 mmol/l calcium lactate and antibiotics, and cultured for 24 h at 38 °C in an atmosphere of 5 % CO_2 and 95 % air in the above medium. Groups of 10 porcine OCC were cultured in 24-well dishes (with well diameter of 15 mm) using 0.5 ml of media per well (Ježová *et al.* 2001). The degree of expansion was assessed after 24 h incubation according to a subjective scoring system from 0 to +4 as follows: 0 – no expansion; +1 – separation of only the outermost layer of cumulus cells; +2 – further expansion involving the outer half of the cumulus oophorus; +3 – further expansion up to, but not including, the corona radiata; +4 – complete expansion including the corona radiata cells (Downs 1989).

Table 1. Effect of gossypol (GSP), S-nitroso-N-acetylpenicillamine (S-NAP) and sodium nitroprusside (SNP) on cumulus expansion of the oocyte-cumulus complexes isolated from large (5-8 mm) porcine follicles.

		Degree of cumulus expansion				
	n	**+4**	**+3**	**+2**	**+1**	**0**
Control	50					50
FSH (1 μg/ml)	73	43	16	1	1	12
FSH+GSP (10^{-6}M)	17		14			3
FSH+GSP (10^{-5}M)	19	2	13	2		2
FSH+GSP (10^{-4}M)				4		28
FSH+S-NAP (10^{-3}M)	23		10			13
FSH+SNP (10^{-3}M)	7		6			1
EGF (10 ng/ml)	62	37	8	5	1	11
EGF+GSP (10^{-6}M)	26	1	8			17
EGF+GSP (10^{-5}M)	57	8	15	5		29
EGF+GSP (10^{-4}M)	39		4	5		30
EGF+S-NAP (10^{-3}M)	16		6	4		6
EGF+SNP (10^{-3}M)	9		3			6

At the end of the incubation period, the OCC media were collected for progesterone determination. The level of progesterone in the medium was determined by the [^{125}I]- progesterone radioimmunoassay method without extraction (Vranová *et al.* 1999).

The EGF receptor content was estimated by determining the binding of [^{125}I]EGF to granulosa cells (Buck and Schomberg 1988). The cells in culture were washed with a fresh medium and then incubated with [^{125}I]EGF (about 90 000 cpm) in 10 mmol/l PBS and 0.1% BSA for 2 h at 24 °C. EGF was radiolabeled to a specific activity of approximately 134 μCi/μg. Non-specific binding was measured in the presence of excess unlabeled EGF (200 ng). The medium was aspirated off at the end of incubation, the cells were washed four times and solubilized in 0.1 N NaOH.

After 24 h incubation in the presence of FSH or EGF, more than 58 % OCC expanded to the +4 stage (Table 1). Suppression of cumulus expansion stimulated by FSH and EGF was obtained in the presence of different gossypol concentrations (10^{-6}, 10^{-5} and 10^{-4} M, $P<0.01$). The stimulatory effect of FSH and EGF on the cumulus expansion of OCC isolated from large porcine follicles was accompanied by increased cumulus cell progesterone production during 42 h incubation of OCC from 1.8±0.2 ng/1 OCC to 6.9±0.8 and 4.2±0.2 ng/1 OCC, respectively. Gossypol (10^{-5} and 10^{-4} M) caused a significant ($P<0.05$) decrease in progesterone secretion by OCC (3.5±0.3 and 1.1±0.06 ng/1 OCC in FSH or 3.1±0.6 and 1.6±0.3 ng/1 OCC in EGF experiments, n=4). We assume that the antisteroidogenic effect of gossypol in cultured cumulus cells may not be caused by the cytotoxic effect of gossypol. Gu *et al.* (1990) and our data (Ježová *et al.* 2001) indicated that the viability of granulosa cells treated with gossypol at the above concentration did not differ from that of the controls. The mechanism of this gossypol action is not clear. Endogenously produced NO has been reported to inhibit steroidogenesis in luteal and granulosa cells (Olson *et al.* 1996, Vranová *et al.* 1999). The effects of endogenously added NO donor were determined in further experiments. S-nitroso-N-acetylpenicillamine (S-NAP) and sodium nitroprusside (SNP) in the 10^{-3} M concentration inhibited cumulus expansion of OCC (Table 1). This concentration of the NO donor significantly decreased progesterone secretion by granulosa cells (Vranová *et al.* 1999). NO is both an intracellular and intercellular mediator and it is synthesized in several cell types by a constitutive or inducible enzyme, which can be activated by different agents (Nathan 1992). NO appears to have a number of cellular targets, one of them concerns the activation of soluble guanylylcyclase. Soluble guanylylcyclase constitutes one or two major synthetic pathways for cGMP (Schmidt *et al.* 1993). There are indeed Ca^{2+}-dependent mechanisms of activation of certain forms of nitric oxide synthase producing NO, which activate soluble guanylylcyclase (Murad *et al.* 1978). We have previously shown that EGF-induced cumulus expansion was inhibited by calcium channel blockers – gallopamil, verapamil and norverapamil – indicating the important role of calcium mobilization in the stimulatory effect of EGF on cumulus expansion of OCC (Ježová *et al.* 2001). EGF receptors were demonstrated in cumulus and granulosa cells of all stages of porcine follicles (Singh *et al.* 1995). According to our results, the inhibitory effect of gossypol on the cumulus expansion of OCC was accompanied by a decreased number of EGF receptors in granulosa cells isolated from large porcine follicles. The [^{125}I]EGF binding activity of about 1845±144 fg bound EGF/10^6 cells in controls decreased to 36±6 and 661±67 fg bound EGF/10^6 cells (n=4, $P<0.05$) after 72 h treatment of granulosa cells with 10^{-4} M of gossypol or 10^{-3} M of SNP, respectively. In the light of these and previous results, it is reasonable to conclude that gossypol may probably have a multiple inhibitory effect on cumulus expansion of OCC.

Acknowledgements

This work was supported, in part, by Grant 2/7179/20 from VEGA and Grant A 5045102/01 from GA AS CR.

References

BUCK AP, SCHOMBERG DW: [^{125}I]Iodo-epidermal growth factor binding and mitotic responsiveness of porcine granulosa cells are modulated by differentiation and follicle-stimulating hormone. *Endocrinology* **122:** 28-33, 1988.

CHEN L, RUSSELL PT, LERSEN WJ: Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol Reprod Dev* **34:** 87-93, 1993.

DOWNS SM: Specificity of epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus in vitro. *Biol Reprod* **41:** 371-379, 1989.

EPPIG JJ: FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* **281:** 483-484, 1979.

GU Y, LIN YC, RIKIHISA Y: Inhibitory effect of gossypol on steroidogenic pathways in cultured bovine luteal cells. *Biochem Biophys Res Commun* **169**: 455-461, 1990.

HADLEY MA, LIN YC, DYM M: Effects of gossypol on the reproductive system of male rats. *J Androl* **2**: 190-199, 1981.

JEŽOVÁ M, SCSUKOVÁ S, NAGYOVÁ E, VRANOVÁ J, PROCHÁZKA R, KOLENA J: Effect of intraovarian factors on porcine follicular cells: cumulus expansion, granulosa and cumulus cell progesterone production. *Animal Reprod Sci* **65**: 115-126, 2001.

LIN YC, CHITCHAROENTHUM M, RIKIHISA Y: Effect of gossypol on spermatozoal lactate dehydrogenase-X (LDH-X) in male rats. *Contraception* **36**: 581-592, 1987.

MURAD F, MITTAL CK, ARNOLD WP, KATSUKI S, KIMURA H: Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv Cyclic Nucleotide Res* **9**: 145-158, 1978.

NATHAN C: Nitric oxide as a secretory product of mammalian cells. *FASEB J* **6**: 3051-3064, 1992.

OLSON LM, JONES-BURTON CM, JABLONKA-SHARIFF A: Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression. *Endocrinology* **137**: 3531-3539, 1996.

PROCHÁZKA R, KALÁB P, MIYANO T: EGF stimulated expansion of porcine oocyte-cumulus complexes is affected by the size of the donor follicle. *Theriogenology* **47:** 199, 1997.

SALUSTRI A, YANAGISHITA M, HASCALL VC: Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. *J Biol Chem* **264:** 13840-13847, 1989.

SANG GW: Effect of gossypol on male reproduction. In: *Hormones in Normal and Abnormal Human Tissues.* Walter de Gruyter, Berlin, 1983, Vol. III, pp 215-249.

SCHMIDT HHHW, LOHMANN SM, WALTER U: The nitric oxide and cGMP transduction system: regulation and mechanism of action. *Biochim Biophys Acta* **1178**: 153-175, 1993.

SEGAL SJ: *Gossypol, a Potential Contraceptive for Men.* Plenum Press, New York, 1985.

SINGH B, RUTLEDGE JM, ARMSTRONG DT: Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles. *Mol Reprod Dev* **40:** 391-399, 1995.

VRANOVÁ J, JEŽOVÁ M, SCSUKOVÁ S, KOLENA J: Inhibitory effect of gossypol on basal and luteinization factor-stimulated progesterone synthesis in porcine granulosa cells. *Physiol Res* **48:** 119-128, 1999.

WANG Y, RAO PN: Effect of gossypol on DNA synthesis and cell cycle progression of mammalian cells in vitro. *Cancer Res.* **44**: 35-38, 1984.

YANG YQ, WU XY: Antifertility mechanisms of gossypol acetic acid in female rats. *J Reprod Fert* **80**: 425-429, 1987.

Reprint requests

J. Kolena, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovak Republic. Fax: +421 2 54774247. E-mail: ueenkole@savba.sk.