Physiol. Res. 48: 119-128, 1999

Inhibitory Effect of Gossypol on Basal and Luteinization Factor-Stimulated Progesterone Synthesis in Porcine Granulosa Cells

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Received September 21, 1998
Accepted November 9, 1998

Summary

Gossypol, a polyphenolic aldehyde, inhibits steroidogenesis and the reproductive system in both sexes. The present study was undertaken to investigate whether gossypol may affect progesterone biosynthesis in cultured porcine granulosa cells isolated from small (1-2 mm) follicles (SGC). SGC were cultured with gossypol, NO donor S-nitroso-N-acetylpenicillamine (S-NAP) or the specific NO-synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME), in the presence or absence of follicular fluid isolated from large (5-8 mm) follicles (LFF) or conditioned media (CM) of granulosa cells isolated from large follicles (LGC). Gossypol enhanced the nitrite content in culture media of SGC and inhibited basal progesterone secretion by SGC. S-NAP (10^{-3}M) inhibited progesterone secretion and enhanced the formation of cGMP by SGC. L-NAME had no effect on progesterone accumulation by SGC. The stimulatory effect of LFF or CM media on progesterone production by SGC in culture was also inhibited by S-NAP (10^{-3}) and gossypol (10^{-4}M). Moreover, gossypol inhibited forskolin-stimulated progesterone secretion, as well as substrate-enhanced conversion of 22-OH-cholesterol and pregnenolone to progesterone. These results suggest that the inhibitory effect of gossypol on progesterone secretion in culture of SGC may be mediated *via* NO generation.

Key words

Gossypol • Luteinization stimulator • Nitric oxide • Follicular fluid • Granulosa cell culture

Introduction

Gossypol, a polyphenolic aldehyde extracted from cotton plants, which inhibits spermatogenesis and interferes with testicular steroidogenesis (Hadley *et al.* 1981), has been used in fertile men in China as an effective male contraceptive agent for many years (Segal 1985). Gossypol has also exhibited anticancer and antiproliferative effects on a variety of human cancer epithelial cell lines, including breast, prostate, uterus, pancreas and colorectal cell lines (Wang and Rao 1984, Rao *et al.* 1985, 1987, Benz *et al.* 1988, Band *et al.* 1989, Wu *et al.* 1989). In addition, the antiproliferative effects of gossypol on human breast cancer cells were reported to be mediated through mechanisms independent of estrogenic responses (Hu *et al.* 1993). Gossypol is also known to affect reproductive function in females (Yang and Wu 1987), but in contrast to males, experience with its action on females is rather limited. It has been reported that gossypol inhibited implantation and decreased the concentration of progesterone in circulating blood of female rats (Lin *et al.* 1987).

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Gossypol is also capable of altering basal and substrate-stimulated progesterone (P_4) secretion by bovine luteal cells (Gu *et al.* 1990). However, no studies on the effect of gossypol on granulosa cell steroidogenesis has come to our attention.

Steroidogenesis of porcine granulosa cells is controlled by many intrafollicular factors. One of them, i.e. the luteinization stimulator (LS), was partially purified from the follicular fluid of large porcine follicles (Channing *et al.* 1982, Kolena and Channing 1985, Khan *et al.* 1988). A stimulatory effect on progesterone secretion, comparable with the activity of follicular fluid (LFF), was observed in conditioned media (CM) of porcine granulosa cells isolated from large follicles (LGC) (Danišová and Kolena 1992).

Data are presented here to demonstrate that gossypol inhibits steroidogenesis of porcine granulosa cells and that this effect may be mediated by nitric oxide.

Methods

Materials

Porcine FSH (FSH-F-1) was generously supplied by NIAMDD, NIH (Bethesda, MD, USA). Insulin, thyroxine, gossypol, 8-Br-cAMP, 22-hydroxycholesterol, pregnenolone and Medium 199 with Earle's salts were purchased from Sigma Chemical Co (St. Louis, MO, USA). S-nitroso-N-acetylpenicillamine (S-NAP) and forskolin were purchased from RBI (Natick, MA, USA). N-nitro-L-arginine methyl ester (L-NAME) was from Fluka (Switzerland), fungizone (amphotericin B) from Serva (Heidelberg, Germany), penicillin and streptomycin from Spofa (Prague, CR). [^{125}I]-Progesterone was obtained from the Institute of Radioecology and Exploitation of Nuclear Technique (Košice, SR). Tissue culture multiwell plates (Cat No 76-063-05) were purchased from Flow Labs (Rockwille, MD, USA).

Cell culture

Porcine ovaries from approximately 6-month-old animals were obtained at a local slaughterhouse and transported to the laboratory in physiological saline plus antibiotics on ice. Granulosa cells were aspirated from large ovarian follicles (5-8 mm in diameter) with a needle and syringe (Kolena and Channing 1972). LGC were washed twice in serum-free media and incubated at a density of 2.0-3.0 x 10^6 viable cells per ml. Cell viability was estimated by counting with 0.06 % trypan blue in a hemocytometer. LGC were cultured in a volume of 0.5 ml at 37 ^{0}C for 3 days in an atmosphere of 5 % CO_2, 95 % air, using tissue culture multiwell plates containing Medium 199 with Earle's salts and HEPES buffer (250 mmol . l^{-1}). The culture medium was supplemented with L-glutamine (1 mmol . l^{-1}), porcine FSH (1 mU . ml^{-1}), thyroxine (0.1 mmol . l^{-1}), insulin (0.04 $\mu g.ml^{-1}$) and the antibiotics fungizone (0.25 $\mu g.ml^{-1}$), penicillin (100 $U.ml^{-1}$) and streptomycin (100 $\mu g.ml^{-1}$). The culture medium was supplemented with 10 % fetal calf serum. The cells were cultured for 3 days in the presence of specific agents. At the end of the incubation period, the conditioned media (CM) were collected and assayed for progesterone. The follicular fluid was collected from 5-8 mm large follicles (LFF) by aspiration and centrifugation at 1000 x *g* for 15 min. The activity of LS in CMs or LFF was determined by adding aliquots to the cultures of granulosa cells isolated from small (1-2 mm) follicles (SGC). SGC were cultured under the same experimental conditions as described above and the concentration of progesterone was determined in the culture medium..

Progesterone and cGMP assay

Progesterone in culture media was quantified by [^{125}I]-progesterone radioimmunoassay using a specific antiserum against 11-OH-progesterone succinyl-BSA (kindly donated by Dr. Tománek, Research Institute of Animal Production, Prague) (Kolena and Channing 1985). cGMP content in culture media was analyzed by direct RIA (commercial kit).

Nitrite assay

The accumulation of nitrite in the medium was measured by mixing 100 µl of conditioned medium with 200 µl of water and 300 µl of Griess reagent (1 % sulfanilamide and 0.1 % naphthylenediamine in 5 % phosphoric acid). After incubation for 10 min at room temperature, the absorbance was measured at 540 nm (A_{540}) (Green *et al.* 1982). Nitrite concentrations were calculated by comparing the A_{540} of standard solutions of sodium nitrite prepared in the same culture medium.

Statistics

The results from the experiments (run in quadruplicate) were expressed as means ± S.E.M. Each experiment was replicated three times with different pools of granulosa cells. Statistical significance was analyzed by ANOVA followed by Bonferroni's multiple range test.

Results

Addition of 8-Br-cAMP, insulin or forskolin to the culture of porcine granulosa cells isolated from large follicles (5-8 mm) markedly enhanced LS formation as

well as progesterone secretion by porcine granulosa cells isolated from small follicles (SGC) (Danišová and Kolena 1992). Figure 1 presents the effect of preovulatory follicular fluid (LFF) and of the conditioned media (CM) obtained from cultivation of LGC with 8-Br-cAMP (0.1 mM), insulin (5 $\mu g.ml^{-1}$) or forskolin (10 μM) on basal progesterone secretion in cultures of porcine granulosa cells obtained from small follicles (SGC). LFF significantly enhanced progesterone production by SGC ($p<0.001$). The same effect was observed by addition of CM with enhanced LS production (CM-cAMP, CM-I, CM-F) ($p<0.05$).

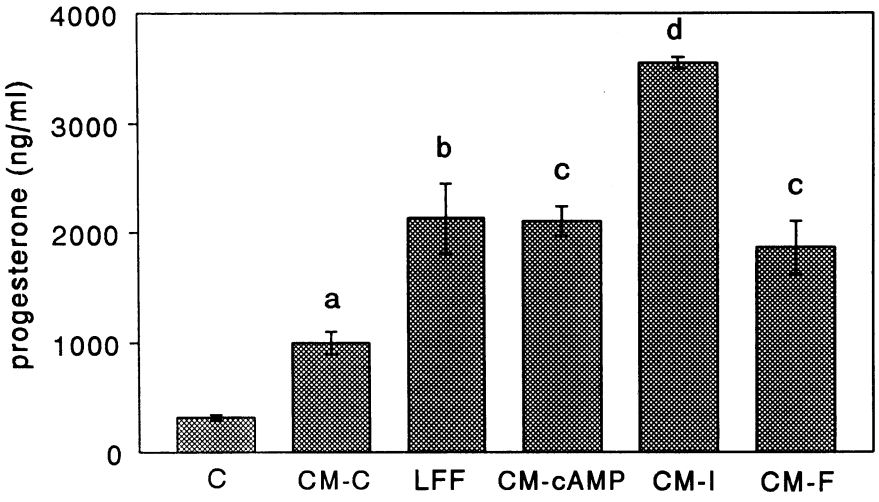

Fig. 1. *Effect of follicular fluid isolated from large follicles (LFF) or conditioned media (CM) of granulosa cells isolated from large follicles (LGC) on P_4 production by SGC. CM were collected after 3-day incubation of LGC with or without 8-Br-cAMP (0.1 mM, CM-cAMP), insulin (5 $mg.ml^{-1}$, CM-I) or forskolin (10 μM, CM-F). SGC were cultured for 3 days in a medium supplemented with L-glutamine (1 mmol . l^{-1}), FSH (1 mU . ml^{-1}), thyroxine (0.1 mmol . l^{-1}), insulin (0.04 $\mu g.ml^{-1}$), fungizone (0.25 $\mu g.ml^{-1}$), penicillin (100 $U.ml^{-1}$), streptomycin (100 $\mu g.ml^{-1}$) and 10 % fetal calf serum. The values are the means ± S.E.M. of 4 estimations. $^{a}p<0.05$ vs control (C), $^{b}p<0.001$ vs control (C), $^{c}p<0.05$ vs control conditioned media (CM-C), $^{d}p<0.001$ vs CM-C*

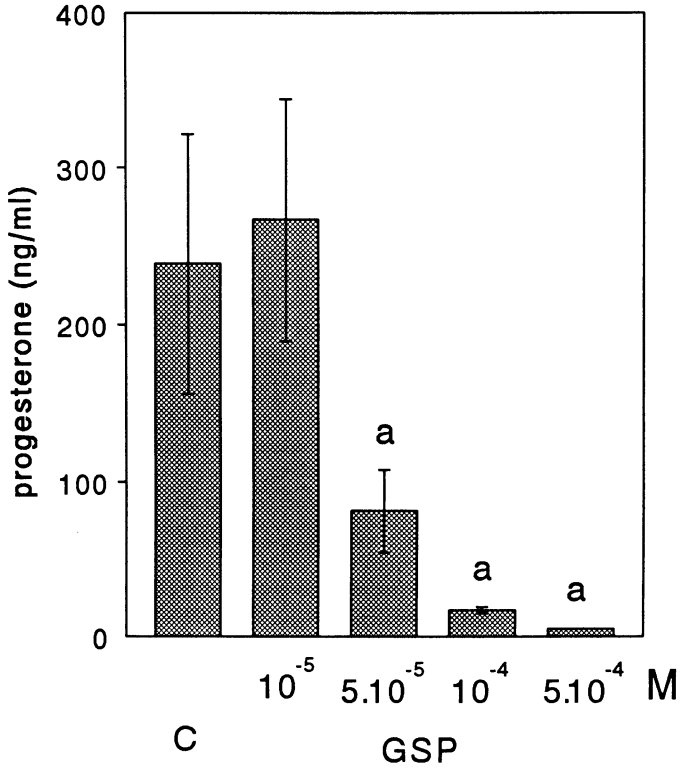

Fig. 2. *Effect of increasing doses of gossypol (GSP) on P_4 production by cultures of SGC. For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.*

Gossypol (10^{-5}, 5.10^{-5}, 10^{-4} and 5.10^{-4} M) caused a dose-dependent decrease in progesterone secretion by SGC after 3-day incubation (Fig. 2). The highest dose of gossypol (5.10^{-4} M) induced an approximately 50-fold inhibition of progesterone secretion. Gu *et al.* (1990) indicated that the viability of cells treated with gossypol even at 170 μM did not show a difference from that of the controls. We assume that antisteroidogenic effect of gossypol in cultured porcine granulosa cells may not be caused by the cytotoxic effect of gossypol. Endogenously produced NO has been reported to inhibit steroidogenesis in granulosa and luteal cells (Olson *et al.* 1996, Jablonka-Sharif and Olson 1997). To evaluate whether the inhibitory influence of gossypol on P_4 production by SGC resulted from gossypol activation of NO synthase, the concentration of nitrite, the main degradation product of nitric oxide, was measured. Figure 3 shows that gossypol significantly ($p<0.05$) increased the content of nitrite. The majority of the effects of NO were found to be mediated by NO activation of guanylate cyclase, resulting in the formation of cGMP (Koesling *et al.* 1991). In our study, however, gossypol in a 5.10^{-4} M concentration failed to increase the cGMP content in culture media of SGC significantly (Fig. 4). The same result was observed in the presence of LFF or LFF and gossypol.

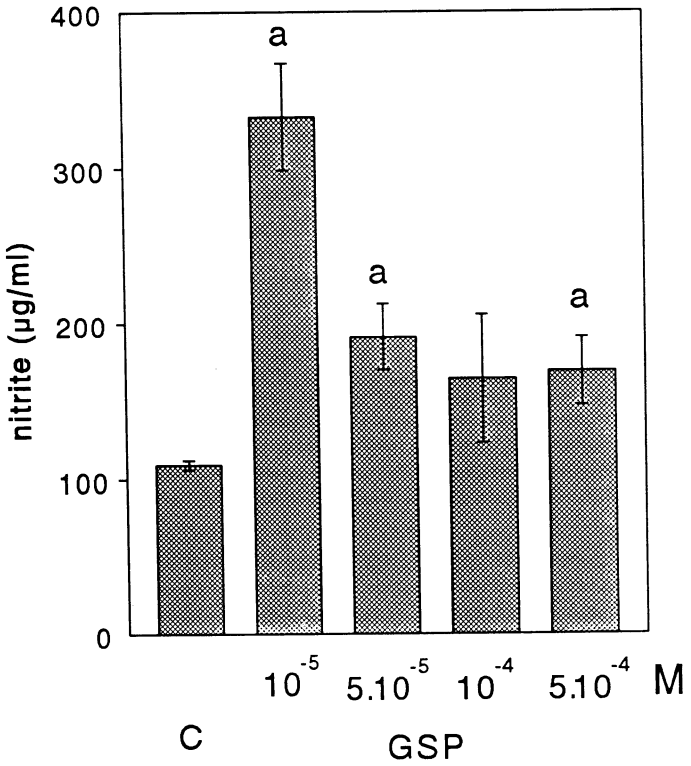

Fig. 3. *Stimulatory effect of gossypol on accumulation of nitrite in culture media of immature granulosa cells. For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.*

In further experiments, the effects of exogenously added NO donor (S-NAP) or NO synthase inhibitor (L-NAME) were determined. S-NAP in the 10^{-3} M concentration significantly ($p<0.05$) inhibited progesterone accumulation by SGC. The specific NO synthase inhibitor L-NAME, at both 10^{-4} and 10^{-3} M concentrations, caused no change in progesterone secretion (Fig. 5). S-NAP, in the 10^{-3} M concentration at which it lowered the secretion of progesterone, also enhanced cGMP content ($p<0.05$) in cultured media of SGC (Fig. 6). This stimulatory effect of S-NAP was not changed in the presence of LFF in cultures of granulosa cells.

Figure 7 presents the effect of L-NAME, S-NAP and gossypol on LFF-stimulated the P_4 production by SGC. The luteinization stimulator present in the LFF caused a 4-fold increase of progesterone secretion by SGC. The specific NO synthase inhibitor, L-NAME (10^{-4} and 10^{-3} M), exerted no effect on this stimulation. On the other hand, at the concentration of 10^{-3} M, the NO donor S-NAP decreased the stimulatory effect of LFF on progesterone production by granulosa cells to the level of control values ($p<0.05$). Gossypol at the concentration of 10^{-4} and 5.10^{-4} M inhibited the LFF-stimulated progesterone production by SGC ($p<0.05$). These findings suggest that gossypol inhibited the LFF stimulatory activity on SGC steroidogenesis.

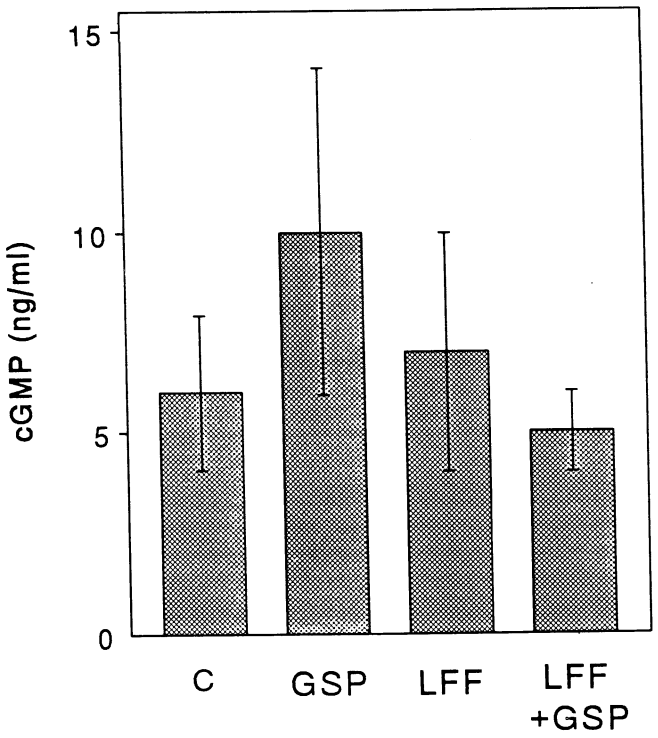

Fig. 4. *Effect of gossypol (GSP) and LFF (25 %) on accumulation of cGMP in SGC culture media. For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.*

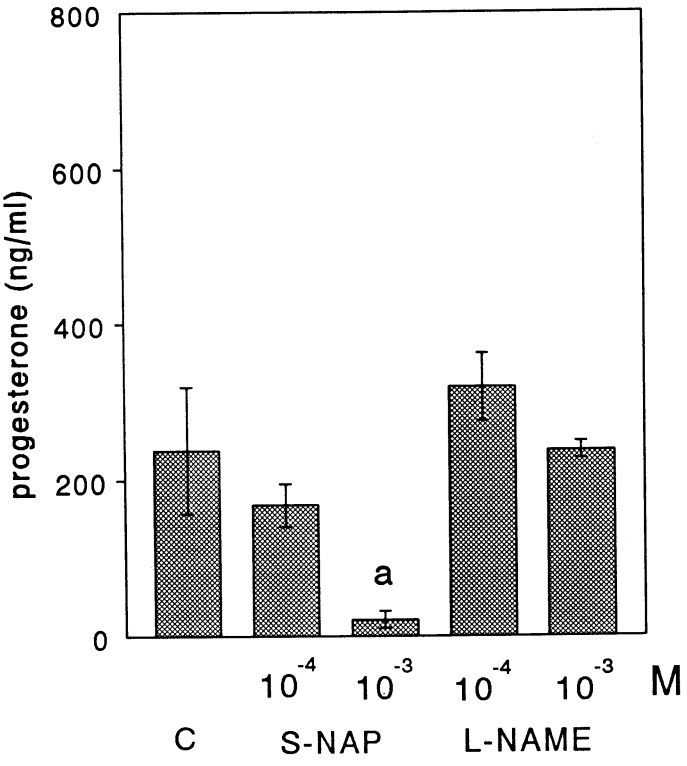

Fig. 5. *Progesterone production by immature porcine granulosa cells in response to S-NAP and L-NAME. For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.*

A further series of experiments was performed to determine the involvement of NO in the generation of the luteinization stimulator in granulosa cells (Fig. 8). Granulosa cells isolated from large follicles (LGC) were incubated with L-NAME (10^{-4} and 10^{-3} M), S-NAP (10^{-5}, 10^{-4} and 10^{-3} M) and gossypol (10^{-5}, 5.10^{-5}, 10^{-4} and 5.10^{-4}M). Conditioned media of LGC (125 µl) were added to the culture of granulosa cells isolated from small follicles (SGC). The respective parts of progesterone added in 125 µl of conditioned media to cultures of SGC were CM-C 102 ng, CM-L-NAME 108 and 103 ng, CM-S-NAP 115 ng, CM-GOSS 117 ng CM obtained by addition of S-NAP (10^{-3} M) or gossypol (5.10^{-4} M) to the LGC culture significantly ($p<0.05$) inhibited the secretion of P_4 in cultures with SGC. These findings suggest that LS formation in LGC may be affected in the presence of NO or gossypol.

Figure 9 presents some possible sites in steroidogenesis at which gossypol may inhibit progesterone secretion by SGC. Progesterone production was enhanced by forskolin (10 μM), 22-OH-cholesterol (2 $\mu g.ml^{-1}$) and pregnenolone (10^{-6} M). Gossypol at both concentrations, 25 and 100 μM, significantly ($p<0.05$) inhibited small granulosa cell progesterone production stimulated by forskolin as well as the conversion of 22-OH-cholesterol and of pregnenolone to progesterone.

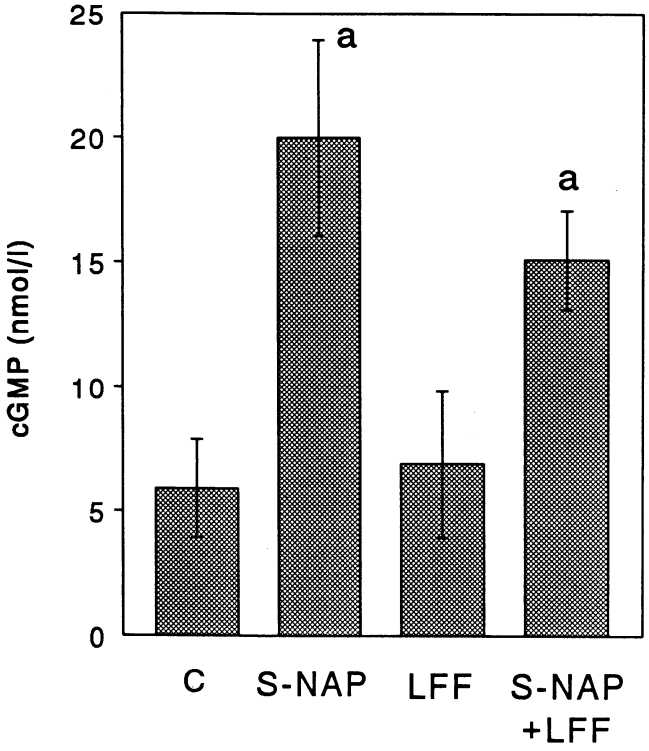

Fig. 6. *Effect of S-NAP and LFF on production of cGMP by immature granulosa cells. For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.*

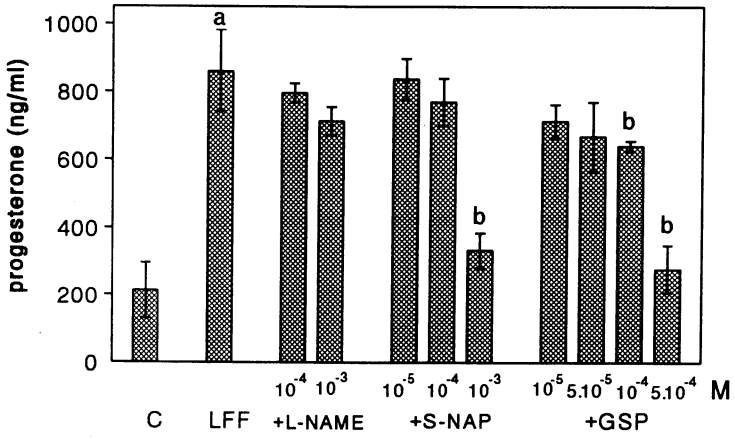

Fig. 7. *Effect of L-NAME , S-NAP and gossypol (GSP) on LFF stimulated progesterone production by SGC. LFF was used in 25 % concentration. For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.* $^{a}p<0.05$ *vs control (C),* $^{b}p<0.05$ *vs LFF*

Discussion

Steroidogenesis of porcine granulosa cells is modulated by steroid hormones, neurotransmitters, growth factors, components of the extracellular matrix and by some nonsteroidal factors produced locally by the ovary (Channing *et al.* 1982, Schomberg *et al.* 1983, Vranová *et al.* 1997). Follicular fluid isolated from large preovulatory follicles (5-8 mm) was found to stimulate steroidogenesis and maturation of granulosa cells from small porcine follicles (Ledwitz-Rigby and Rigby 1979, Kolena and Channing 1985). Our results indicated a comparable stimulatory activity of the conditioned media from large granulosa cells incubated with cAMP, forskolin or insulin. This finding is in agreement with our previous study (Danišová and Kolena 1992).

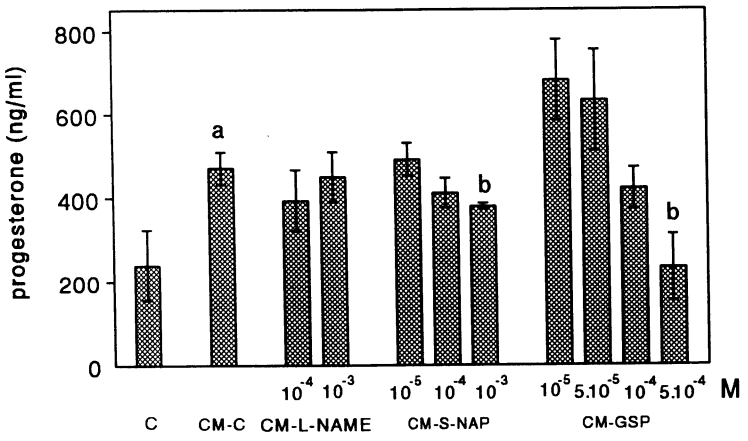

Fig. 8. *The changes in SGC progesterone secretion stimulated by granulosa cell conditioned media (CM) in the presence of various concentrations of L-NAME, S-NAP or gossypol. CM media were used in 25 % concentrations. For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.* $^{a}p<0.05$ *vs control (C),* $^{b}p<0.05$ *vs CM-C.*

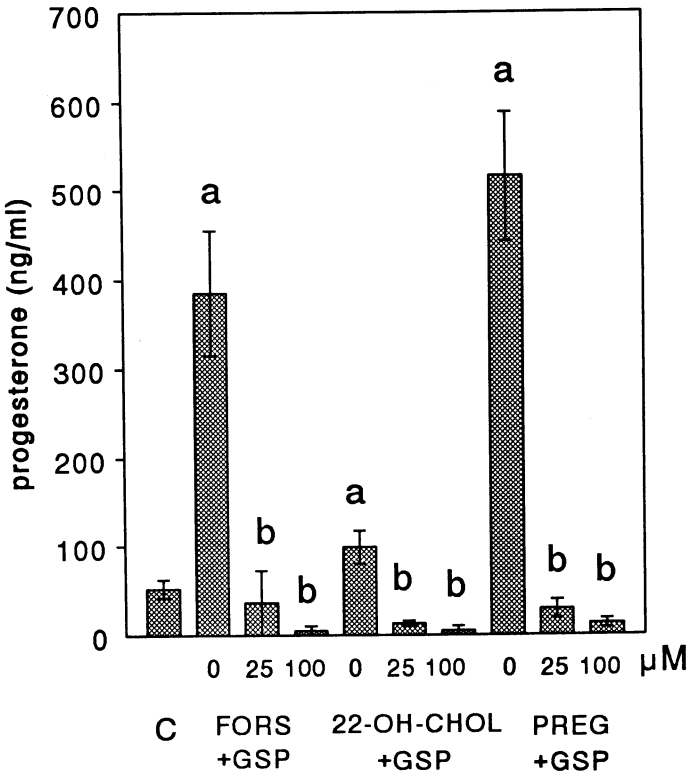

Fig. 9. *Inhibitory effect of gossypol (0, 25 and 100 μM) on the substrate-stimulated progesterone production by SGC. SGC were incubated with forskolin (10 μM), 22-OH-cholesterol (2 μg.ml*$^{-1}$*) or pregnenolone (10*$^{-6}$ *M). For details see legend to Figure 1. Values are means ± S.E.M. of 3 estimations.* $^{a}p<0.05$ *vs control (C),* $^{b}p<0.05$ *vs forskolin, 22-OH-cholesterol or pregnenolone.*

Gossypol, an antifertility agent in both sexes (Poso *et al.* 1980), was shown to inhibit progesterone secretion in cultured bovine luteal cells (Lin *et al.* 1985, Lei *et al.* 1985, Gu *et al.* 1990). We studied the effect of gossypol on porcine granulosa cell steroidogenesis and its mechanism of action. Our results indicated that gossypol markedly inhibited progesterone synthesis by granulosa cells in culture. The mechanism of the gossypol action is not clear. Gossypol was found to disrupt spermatogenesis by inhibiting lactate dehydrogenase-X in bovine and rat testes (Olgiati and Toscano 1983, Lin *et al.* 1987). Gu *et al.* (1990) reported that inhibition of bovine luteal cell steroidogenesis by gossypol may be due to its effect on adenylate cyclase and 3ß-hydroxysteroid dehydrogenase. On the other hand, gossypol was shown to interfere with key steroidogenic enzymes such as 5α-reductase and 3α-hydroxysteroid dehydrogenase in the rat testes (Moh *et al.* 1993), and to inhibit 5α-reductase in the canine prostate gland (Moh *et al.* 1992). Gossypol was found to inhibit specifically basal and estrogen-stimulated DNA

synthesis in human breast carcinoma cells (Hu *et al.* 1993).

Our studies provide evidence that nitric oxide may be involved in the regulation of granulosa cell progesterone production by gossypol. NO is both an intracellular and intercellular mediator and it exists in several cell types either in constitutive or inducible forms, which can be activated by a number of agents (Nathan 1992). NO inhibited steroidogenesis in cultured rat granulosa cells (Dave *et al.* 1997) as well as in cultured human granulosa-luteal cells (Van Voorhis *et al.* 1994). NO appears to have a number of cellular targets and effects, one of which is the activation of soluble guanylate cyclase. Soluble guanylate cyclase constitutes one of two major synthetic pathways for cGMP and failed to be hormonally stimulated (Schmidt *et al.* 1993). There are indeed Ca^{2+}-dependent mechanisms of activation of certain forms of nitric oxide synthase producing NO, which activates soluble guanylate cyclase (Murad *et al.* 1978, Böhme *et al.* 1978).

In agreement with these data, our experiments indicate that the NO donor S-NAP significantly inhibited basal and LS-stimulated progesterone production by SGC and increased cGMP content in SGC culture. Similar results were obtained with gossypol. It decreased basal and LS-stimulated granulosa cell progesterone production and enhanced nitrite concentration in culture media. In our experiments, cGMP accumulation in culture media of granulosa cells isolated from small follicles was not changed by the influence of gossypol. However, it cannot be ruled out that nitric oxide in granulosa cells is acting by a pathway which is independent of the guanylate cyclase signal system.

Cholesterol is a major substrate for progesterone synthesis. It is first converted to pregnenolone by the side-chain cleavage enzyme complex. Pregnenolone is then converted to progesterone by the 3ß-hydroxysteroid dehydrogenase-isomerase complex (3ß-HSD) (Azhar and Menon 1981). The present study showed that gossypol decreased small granulosa cell progesterone production as well as conversion of 22-OH-cholesterol and pregnenolone to progesterone. Although 3ß-HSD is not considered a rate-limiting step for steroidogenesis, it plays a key role in the synthesis of progesterone in luteal cells. Gu *et al.* (1990) reported that substrate-stimulated progesterone production was decreased by gossypol inhibition of 3ß-HSD in bovine luteal cells. Gossypol inhibited hCG-stimulated cAMP formation and hCG- and cAMP-stimulated progesterone production in cultured rat luteal cells (Wang *et al.* 1987). Moreover, gossypol inhibited hCG- and forskolin-induced intracellular cAMP formation and progesterone secretion in bovine luteal cells *in vitro* (Gu *et al.* 1990). Gossypol inhibited the activity of cytochrome $P450_{SCC}$ which is considered a rate-limiting enzyme in cleavage of the side-chain of cholesterol. Since NO is known to inhibit cytochrome $P450_{SCC}$ in granulosa luteal cells directly, the observed gossypol action may be due to modulation of this hem-containing enzyme by NO (Van Voorhis *et al.* 1994).

Our results clearly illustrate that gossypol inhibited the basal and LS-stimulated progesterone synthesis in porcine small granulosa cells. A relatively similar action of gossypol with the NO donor S-NAP suggests that gossypol may act *via* NO generation. More experiments are needed to evaluate the possibility that NO may be a second messenger in gossypol action in porcine granulosa cell steroidogenesis. In the light of these results, it is reasonable to conclude that gossypol may probably have multiple inhibitory effects on progesterone biosynthesis in porcine granulosa cells.

Acknowledgments

We are grateful to Dr. A. Sirotkin, from the Research Institute of Animal Production, for his expert assistance with the cGMP radioimmunoassay. This work was partly supported, by the Slovak Grant Agency for Science, Grant VEGA 2/4134/97.

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