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Effects of steroids on mouse oocyte maturation *in vitro*

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Summary. Oestrone and dihydrotestosterone had no significant action. Other steroids inhibited maturation. The stage of maturation most affected and the median effective concentration (MEC) at this stage varied with different steroids. The predominant effect of pregnenolone (MEC = 6·4 μM), progesterone (MEC = 5·3 μM), androstenedione (MEC = 28·0 μM) and oestradiol-17β (MEC = 23·0 μM) was to block maturation after the resumption of meiosis but before completion of the first meiotic division. Testosterone was also effective at this stage (MEC = 23·5 μM) but at higher concentrations it prevented germinal vesicle breakdown (MEC = 40 μM) without causing oocyte degeneration. The inhibitory actions of all steroids were reversible in oocytes exposed for 4 or 18 h.

Introduction

Several authors have investigated the effects of oestrogen and progesterone on mammalian oocyte maturation *in vitro*. Oestradiol-17β (176·2 μM) reduced significantly the incidence of maturation of cumulus-enclosed rabbit and bovine oocytes (Robertson & Baker, 1969), denuded porcine oocytes at concentrations of 3·67 and 36·7 μM (McGaughey, 1977) and denuded mouse oocytes at a concentration of 100 μM (Nekola & Smith, 1974) and 36·7 μM (Eppig & Koide, 1978). Progesterone (102 μM) has been reported to enhance the maturation of denuded bovine and rabbit (Robertson & Baker, 1969), cumulus-enclosed rabbit (Bae & Foote, 1975) and denuded oocytes from gonadotrophin-treated prepubertal rhesus monkeys (Gould & Graham, 1976). Progesterone (31·8 μM) has also been found not to affect maturation of corona-enclosed human (Shea, Baker & Latour, 1975) or denuded porcine (McGaughey, 1977) oocytes in culture. It may, in addition, reverse the inhibition of porcine oocyte maturation caused by oestradiol-17β (McGaughey, 1977). On the other hand, progesterone (10 and 31·8 μM) has been shown to decrease maturation frequency of denuded mouse (Nekola & Smith, 1974; Eppig & Koide, 1978) and corona-enclosed rabbit oocytes (Smith, Tyler & Erickson, 1978).

In the above studies, the results for progesterone are conflicting and concentration–response relationships were not determined for either steroid. Considering, also, the changing concentrations of steroids within maturing follicles, especially during the preovulatory period, and the possibility that these fluctuations might play a role in regulating the resumption of meiosis, we have examined the effects of various concentrations of several steroids on mouse oocyte maturation *in vitro*.

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Materials and Methods

Swiss albino (CD-1) mice, were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. At 5–10 weeks of age the mice were killed by cervical dislocation and the ovaries were removed.

Culture procedures

At each time 2–4 mice were killed depending on the number of treatment groups per experiment. For each treatment, 4 ovaries (from 2 or 4 different mice) or 6 ovaries from 3 different mice were pooled in an embryological watch glass containing 2·5–3·0 ml control (steroid-free) medium or medium containing one concentration of the steroid being tested. A pair of watch glasses containing the medium for a particular treatment was equilibrated for 2 h at 37°C in an atmosphere of 100% humidity and 5% CO_2 in air before killing the mice. Oocytes were obtained by puncturing, with a 30-gauge needle, the largest visible (antral or early antral) follicles in the isolated ovaries. Healthy-looking oocytes were collected in a finely-drawn Pasteur pipette and washed in the second watch glass containing equilibrated medium. Oocytes with obvious signs of degeneration, presumably released from atretic follicles, were not cultured. Washed oocytes (10–20/chamber, and from 20–70/replicate depending on the number of follicles/ovary) were then cultured for 10–20 h in a 4-chamber slide (Lab Tec) containing 0·8 ml medium/chamber or in 4·0 ml medium in a 35 × 10 mm tissue culture dish (Falcon Plastics). There were 3–6 replicate experiments for each treatment group.

At the end of each culture procedure, the stage of oocyte maturation was determined with the aid of a dissecting microscope. Oocytes were classified as mature, i.e. polar body (pb) extruded; meiotically inactive, i.e. germinal vesicle (gv) retained; or as having resumed but not completed the first meiotic division, i.e. having undergone germinal vesicle breakdown (gvbd). Cultured oocytes were scored as degenerate if they showed cytolysis, fragmentation, necrosis, vacuolization or loss of spherical shape. Some of the oocytes in each category were fixed, stained with Giemsa (Rafferty, 1970) and examined by conventional light microscopy to confirm the dissecting microscope diagnosis of stage of maturation.

Culture media

The control medium was a modification of the medium for mouse ova culture described by Biggers, Whitten & Whittingham (1971) in which the sodium pyruvate content was increased to 0·055 mg/ml. Polyvinylpyrrolidone (Sigma Chemical Co.) at a concentration of 4 mg/ml was substituted for bovine serum albumin (BSA) because preliminary experiments showed that this reduced the concentration of progesterone which blocked mouse oocyte maturation *in vitro* compared to that reported previously (Nekola & Smith, 1974).

Stock solutions of steroids (10 mM) in absolute ethanol were prepared and maintained at 4°C in sealed vials. Steroids were added to the culture medium so that the concentration of ethanol in a particular treatment was never greater than 1%. Media with lower concentrations of steroid were prepared from the stock solutions by serial dilution with the PVP medium described above. Initially, oocytes were cultured in a wide range of concentrations (100, 10, 1·0 and 0·1 μM) of each steroid and the results indicated a narrower range of concentrations for testing. The effects of pregnenolone, progesterone, androstenedione, testosterone, dihydrotestosterone, oestradiol-17β and oestrone were examined. Even using 10% dimethylsulphoxide, it was impossible to prepare solutions of cholesterol in absolute ethanol so that it could be mixed with culture medium in concentrations comparable to those tested for the above steroids.

Reversibility of steroid effects

The reversibility of steroid inhibition of maturation was tested in a series of experiments in which 3 groups, each containing approximately 50 oocytes, were treated in the following manner. In Group A oocytes were cultured for 4 h with steroid at one of the concentrations shown to be effective in the narrow range, then washed and cultured for a further 20 h in steroid-free control medium. In Group B oocytes were cultured for 24 h in the same concentration of steroid, and in Group C the oocytes were cultured for 24 h in control medium. The results of the 3 treatments were compared statistically for each steroid. Three replicate experiments were done for each steroid. Replicate experiments for progesterone, testosterone and oestradiol-17β were done at the same time. Consequently, the pooled control data are the same for each of these hormones (Table 2). In additional experiments oocytes were exposed to one of the 5 steroids for 18 h. Preliminary data indicated that oocytes cultured longer than 18 h in control medium containing PVP instead of BSA degenerated. Consequently, the maturation of oocytes cultured for 18 h in steroid-free, PVP-containing medium and then transferred to control medium containing fetal bovine serum (heat-inactivated, Microbiological Associates; 20% v/v) was compared to that of oocytes cultured for 18 h in steroid-containing, PVP medium and then transferred to steroid-free, serum-containing medium and cultured further for 24 h (Table 3). All treatments were tested in two replicate experiments.

Statistical analysis

The results of replicate experiments were pooled as inspection indicated that they were similar. For the different categories of oocyte maturation in each set of pooled data, overall χ^2 values were calculated for $2 \times r$ contingency tables and the degrees of freedom partitioned as described by Maxwell (1961).

To plot a concentration–response curve for inhibition of polar body formation, the data for each steroid (except pregnenolone) were normalized with respect to control values which were assigned a value of 100% maturation. The formula for normalization was $P = P^*/C$, where P^* = observed response, P = normalized response, and C = control response. The normalized responses were plotted against concentration for androstenedione, testosterone and oestradiol-17β and against log concentration for progesterone and testosterone according to which type of plot permitted best fitting of a straight line or smooth sigmoid curve. Attempts to linearize the concentration–response curves with transformations such as probit were unsuccessful. In the case of pregnenolone, polar body formation in the lowest concentration was significantly greater than in control medium. The data were therefore not normalized and were plotted against log concentration. The median effective concentration or the concentration of each steroid which blocked polar body formation in 50% of the oocytes was read from these curves for all steroids.

Results

The pooled raw data for the effect of various concentrations of single steroids (narrow ranges) on oocyte maturation are shown in Table 1. These data normalized to control values were plotted. The median effective concentration (MEC) of each steroid to inhibit maturation between gvbd and pb formation was: pregnenolone, 6·4 μM; progesterone, 5·3 μM; androstenedione, 28·0 μM; testosterone, 23·5 μM; oestradiol-17β, 23·0 μM. The MEC for the inhibition of gvbd by testosterone was similarly determined to be 40·0 μM. Neither oestrone nor dihydrotestosterone, at any of the concentrations tested, had any significant effect on mouse oocyte maturation *in vitro*. Increasing concentrations of pregnenolone reduced pb formation, blocking maturation after gvbd. The effect was statistically significant, however, only at 10 μM which also caused a

Table 1. Effects of steroids on mouse oocyte maturation *in vitro*

Steroid conc. (μM)	No. of exps	Oocytes: No. cultured	Oocytes: No. not degenerating after 16–20 h (%)*	Oocytes: No. with pb (%)†	Oocytes: No. with gvbd (%)†	Oocytes: No. with gv (%)†
Pregnenolone						
0·0	3	152	135 (89)	107 (79)	28 (21)	0 (0)
0·316	3	143	128 (90)	120 (94)‡	8 (6)‡	0 (0)
1·0	3	151	134 (89)	111 (83)	22 (16)	1 (1)
3·16	3	127	107 (84)	76 (71)	28 (26)	3 (3)
10·0	3	130	112 (86)	49 (44)‡	51 (45)‡	12 (11)‡
31·6	3	153	0 (0)	0 (0)	0 (0)	0 (0)
Progesterone						
0·0	3	119	102 (86)	84 (82)	14 (14)	4 (4)
0·8	3	142	118 (83)	94 (80)	17 (14)	7 (6)
1·6	3	135	92 (68)	58 (63)‡	33 (36)‡	1 (1)
3·2	3	125	100 (80)	58 (58)‡	37 (37)‡	5 (5)
6·4	3	129	106 (82)	41 (39)‡	48 (45)‡	17 (16)‡
12·8	3	111	42 (38)	2 (5)‡	26 (62)‡	14 (33)‡
Androstenedione						
0·0	3	182	146 (80)	122 (83)	23 (16)	1 (1)
10·0	3	157	120 (76)	83 (69)‡	31 (25)	7 (6)
20·0	3	170	140 (82)	87 (62)‡	37 (27)	16 (11)‡
40·0	3	186	107 (58)	27 (25)‡	59 (55)‡	21 (20)‡
60·0	2	107	1 (1)	0 (0)	0 (0)	0 (0)
Testosterone						
0·0	4	176	112 (64)	94 (84)	18 (16)	0 (0)
10·0	4	194	138 (71)	104 (76)	32 (23)	2 (1)
20·0	4	153	90 (59)	45 (50)‡	41 (46)‡	4 (4)
40·0	4	164	97 (59)	18 (19)‡	34 (35)‡	45 (46)‡
60·0	4	137	73 (53)	0 (0)‡	8 (11)	65 (89)‡
80·0	4	145	48 (33)	0 (0)‡	0 (0)‡	48 (100)‡
Dihydrotestosterone						
0·0	4	280	224 (80)	197 (88)	25 (11)	2 (1)
1·0	4	228	199 (87)	171 (86)	26 (13)	2 (1)
2·0	4	204	153 (75)	126 (83)	25 (16)	2 (1)
4·0	4	200	146 (73)	109 (15)	37 (25)	0 (0)
6·0	4	161	106 (66)	71 (67)	35 (33)	0 (0)
8·0	4	264	196 (74)	156 (79)	39 (20)	1 (1)
Oestradiol-17β						
0·0	6	289	217 (75)	167 (77)	43 (20)	7 (3)
10·0	6	219	129 (59)	81 (63)‡	39 (31)	8 (6)
15·0	6	237	151 (64)	102 (67)	39 (26)	10 (7)
20·0	6	241	108 (45)	62 (57)‡	39 (36)‡	7 (7)
25·0	6	283	86 (30)	25 (29)‡	54 (63)‡	7 (8)
30·0	6	255	106 (42)	20 (19)‡	82 (77)‡	4 (4)
Oestrone						
0·0	3	111	81 (73)	74 (92)	6 (7)	1 (1)
1·0	3	93	59 (63)	47 (80)	12 (20)	0 (0)
10·0	3	106	50 (47)	37 (74)	13 (26)	0 (0)
31·6	3	88	46 (52)	34 (74)	9 (20)	3 (6)

pb, Polar body; gv, germinal vesicle; gvbd, germinal vesicle breakdown.

* Of oocytes cultured.

† Of oocytes which did not degenerate.

‡ Significantly different from control value for a particular stage of maturation, $P = 0{\cdot}01–<0{\cdot}001$.

significant increase in the number of oocytes remaining at the gv stage. Oestradiol-17β reduced the incidence of pb formation, causing maturation arrest after gvbd (MEC = 23 μM) although oocytes were more sensitive to pregnenolone (MEC = 6·4 μM). In increasing concentrations of progesterone, pb formation decreased and the number of oocytes that were arrested after gvbd increased significantly, as did the number which remained meiotically inactive. The predominant effect was one of blocking maturation after gvbd (MEC = 5·3 μM). Similar results were obtained with androstenedione except that higher concentrations were required to reduce the incidence of pb formation (MEC = 28 μM). Increasing concentrations of testosterone also blocked pb formation (MEC = 23·5 μM) and increased the numbers of oocytes maturing only to the gvbd stage or remaining meiotically inactive. The predominant effect of higher concentrations of testosterone, however, was to prevent oocytes from resuming meiotic activity in culture (MEC = 40 μM) without increasing the incidence of oocyte degeneration.

Table 2 shows that the inhibition of maturation after 4 h exposure to pregnenolone, progesterone, androstenedione, testosterone or oestradiol-17β was reversed by culturing treated oocytes in steroid-free medium. The effects of culturing oocytes in these steroids for 18 h were also reversible (Table 3).

Table 2. Reversibility of inhibition of maturation in mouse oocytes exposed to steroids for 4 h

		Oocytes				
Treatment (conc.)	Exposure to steroid (h)	No. cultured	No. not degenerating after 16–20 h (%)*	No. with pb (%)†	No. with gvbd (%)†	No. with gv (%)†
Pregnenolone (10 μM)	24–	156	123 (79)	98 (80)	22 (18)	3 (2)
	24+	83	61 (73)	35 (57)‡	22 (36)‡	4 (7)
	4+, 20–	73	58 (79)	48 (83)	8 (14)	2 (3)
Progesterone (6·4 μM)	24–	284	143 (50)	109 (76)	32 (22)	2 (1)
	24+	108	50 (46)	21 (42)‡	19 (38)	10 (20)‡
	4+, 20–	90	56 (62)	42 (75)	14 (25)	0 (0)
Androstenedione (40 μM)	24–	156	123 (79)	98 (80)	22 (18)	3 (2)
	24+	82	49 (60)	27 (55)‡	14 (29)	8 (16)‡
	4+, 20–	57	45 (79)	38 (84)	6 (13)	1 (2)
Testosterone (60 μM)	24–	284	143 (50)	109 (76)	32 (22)	2 (1)
	24+	113	79 (70)	0 (0)‡	0 (0)‡	79 (100)‡
	4+, 20–	87	48 (55)	22 (46)	20 (42)‡	6 (12)‡
Oestradiol-17β (30 μM)	24–	284	143 (50)	109 (76)	32 (22)	2 (1)
	24+	93	45 (48)	3 (7)‡	21 (46)‡	21 (46)‡
	4+, 20–	87	44 (51)	28 (64)	16 (36)	0 (0)

pb, Polar body; gv, germinal vesicle; gvbd, germinal vesicle breakdown.
* Of oocytes cultured.
† Of oocytes which did not degenerate.
‡ Significantly different from 24 h– (control), $P = 0{\cdot}01–<0{\cdot}001$.

Discussion

The present results show that pregnenolone, progesterone, androstenedione, testosterone and oestradiol-17β reduce the incidence of spontaneous mouse oocyte maturation *in vitro* while oestrone and dihydrotestosterone are without significant effect. The effects of the inhibitory steroids on polar body formation are concentration dependent. The stage at which maturation is most inhibited varies with the steroid and its concentration. In contrast to the results of

Robertson & Baker (1969), Bae & Foote (1975) and Gould & Graham (1976), progesterone did not enhance maturation at comparable concentrations or at any concentration tested. Only in one instance (pregnenolone, 0·316 μM) was maturation higher than control values. The other steroids tested (all concentrations) either did not affect maturation or had a significant inhibitory action.

Table 3. Reversibility of inhibition of maturation in mouse oocytes to steroids for 18 h and then to steroid-free medium with PVP ± serum for 24 h

Treatment			Oocytes				
Steroid (conc.)	High mol. wt material	Exposure to steroid (h)	No. cultured	No. without signs of degeneration at time of scoring (%)*	No. with pb (%)†	No. with gvbd (%)†	No. with gv (%)†
None (control)	PVP 18 h	None	128	115 (90)	98 (85)	9 (8)	8 (7)
	PVP 42 h	None	71	14 (20)‡	13 (93)	1 (7)	0 (0)
	PVP 18 h and serum 24 h	None	57	55 (97)	52 (95)	2 (4)	1 (1)
Pregnenolone (10 μM)	PVP	18+	121	100 (83)	68 (68)‡	13 (13)	19 (19)
	PVP+ serum	18+, 24–	32	31 (97)	17 (55)	13 (42)	1 (3)
Progesterone (6·4 μM)	PVP	18+	110	103 (94)	58 (56)‡	28 (27)	17 (17)
	PVP+ serum	18+, 24–	45	45 (100)	18 (40)	23 (51)	4 (9)
Androstenedione (2 μM)	PVP	18+	103	86 (84)	45 (52)‡	36 (42)	5 (6)
	PVP+ serum	18+, 24–	41	38 (93)	9 (24)	29 (76)	0 (0)
Testosterone (30 μM)	PVP	18+	108	85 (79)	0 (0)‡	10 (12)	75 (88)
	PVP+ serum	18+, 24–	84	68 (81)	49 (72)	15 (22)	4 (6)
Oestradiol-17β (15 μM)	PVP	18+	103	86 (84)	22 (26)‡	55 (64)	9 (10)
	PVP+ serum	18+, 24–	64	59 (92)	24 (41)	33 (56)	2 (3)

pb, Polar body; gv, germinal vesicle; gvbd, germinal vesicle breakdown. Healthy-looking oocytes which did not exhibit pbs after 18 h in steroid were transferred to steroid-free, serum-containing medium and cultured 24 h longer.

* Of oocytes cultured.

† Of oocytes which did not degenerate.

‡ Significantly different from 18-h control value, $P = 0{\cdot}01 - < 0{\cdot}001$.

In the present experiments, a simple, chemically defined medium was utilized. It contained no serum or albumin (see 'Materials and Methods') and would not maintain the healthy appearance of mouse oocytes much beyond 24 h regardless of the stage of maturation reached during the culture period (see Table 3). The culture methods described were chosen to examine efficiently the effect of a single variable (one concentration of a specific steroid) on the process of nuclear maturation. No attempt was made to assess the normality of maturation of the cytoplasm or zona pellucida by testing the fertilizability, *in vitro* or *in vivo*, of oocytes cultured in the absence of steroids or maturing after removal from a steroid-containing environment.

Since values for the concentrations of steroids in mouse follicular fluid have not been published, it is difficult to know whether or not the present results have physiological significance for the regulation of meiotic maturation in mice. Antral fluid steroid concentrations have, however, been measured in human follicles of various sizes at different stages of the menstrual cycle (see McNatty, 1978, for a review). The concentrations of steroids which, in the present

experiments, interfere with mouse oocyte maturation *in vitro* are within the limits which can be considered physiological, at least for human follicles.

As follicular fluid steroid levels or steroid production by isolated follicles have been measured in greater numbers of mammalian species, it has become clear that the concentrations of several steroids fall precipitously in the immediate preovulatory period or following LH stimulation (Hay & Moor, 1973; Moor, Hay & Seamark, 1975; Hillensjö, Bauminger & Ahren, 1976; Eiler & Nalbandov, 1977) or fall to low or immeasurable levels after an initial increase (YoungLai, 1972; Mills & Savard, 1973; Patwardhan & Lanthier, 1976; Szoltys, 1976; Bahr, 1978; Lau, Saksena & Chang, 1978). It is possible that in animals which show decreasing concentrations of follicular fluid steroids after mating or LH stimulation, oocytes might be stimulated to resume meiosis by exposure to follicular fluid depleted of these hormones. Whether or not such a mechanism would be active physiologically would depend on whether the predominant effect of one or several steroids which disappeared from the follicular fluid was to block oocyte maturation at the germinal vesicle stage, i.e. testosterone in the present experiments. In follicles in which steroid concentrations first increase and then fall during the preovulatory period, the resumption of meiosis might be stimulated by other changes in follicular physiology, the subsequent fall in steroid levels being permissive for maturation. For example, the decline in steroid concentrations later in the preovulatory period might be necessary before maturation could proceed from gvbd to metaphase II. This would be consistent with our finding that several steroids blocked mouse oocyte maturation after gvbd had occurred.

In some species the secretion of progestagens increases late in the preovulatory period after the concentrations of other steroids have declined in follicles exposed to LH *in vitro* or *in vivo* (Hay & Moor, 1973; Moor *et al.*, 1975; Hillensjö *et al.*, 1976; McNatty, 1978). In contrast, progestagens in follicular fluid decrease as the concentrations of androgens and oestrogens decline in the pig (Eiler & Nalbandov, 1977) and rabbit (YoungLai, 1972; Patwardhan & Lanthier, 1976; Bahr, 1978), decrease during the preovulatory period in dissected intact rat (Szoltys, 1976) and rabbit (Mills & Savard, 1973) follicles, and decrease as androgens and oestrogens decline in whole hamster ovaries (Saidapur & Greenwald, 1978) on the day of pro-oestrus. If the rise in progesterone production by the various ovarian tissues studied occurs close to the time of ovulation when oocyte maturation would be complete, or nearly so, it would not be expected to interfere with the resumption of meiosis or facilitation of maturation induced by the earlier fall in other steroid concentrations in the follicle. When there is simultaneous decline of all steroids, however, the resumption of meiosis could be stimulated or maturation facilitated once gvbd had occurred.

The present results, in conjunction with the general picture of follicular fluid steroid dynamics around the time of ovulation described in the literature of several species, lead us to suggest that steroids could be involved in the physiological regulation of the resumption of meiosis. This concept is offered as a possible alternative to the notion that there is an inhibitor of oocyte maturation present in the follicular fluid of many species which disappears after LH stimulation (for a review, see Tsafriri, 1978). Other equally attractive alternatives have been proposed by Zeilmaker & Verhamme (1974) and Dekel & Beers (1978). It seems especially important to consider alternative mechanisms or the interaction of more than one system for the regulation of the resumption of meiosis, as well as for the later stages in the maturation process, because various investigators have been unable to demonstrate that porcine or bovine (Hunter, Lawson & Rowson, 1972; Cho & Lim, 1975; Leibfried, 1976; M. V. Nekola, personal communication) or human (D. M. Smith, K. P. McNatty & D. Y. Tenney, unpublished observations) follicular fluid is inhibitory to the resumption of meiosis *in vitro* by oocytes of several species.

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