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ORIGINAL PAPER

High diluted and dynamised follicle stimulating hormone modulates steroid production in isolated porcine preantral follicles cultured *in vitro*

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Objective: **This study investigated the effect of two different follicle stimulating hormone (FSH) preparations (diluted/dynamised and diluted) on the *in vitro* development and steroid production (estradiol, progesterone and testosterone) of isolated porcine preantral follicle after *in vitro* culture.**
Methods: **Secondary follicles were cultured in Alpha Minimum Essential Medium (α-MEM$^+$) supplemented with grain ethanol (AL − 0.2%, v/v), diluted/dynamised FSH (rFSH 6cH − 0.05 fg/mL) or diluted-only FSH (1.5 ng/mL) for 4 days. Follicle development was evaluated on the basis of follicular growth, morphology and hormone production.**
Results: **The percentage of follicular integrity and extrusion were not affected by the treatments after culture. For all treatments, follicular diameter increased significantly from Day 0 to Day 4. On Day 2 of culture, the estradiol production was significantly higher in AL and diluted-only FSH treatments compared with diluted/dynamised FSH. However, diluted/dynamised FSH showed a significantly higher progesterone production on Day 2. Only on Day 4, the testosterone production was higher in the AL than diluted-only FSH treatments, but similar to diluted/dynamised FSH treatment. Except for diluted/dynamised FSH treatment, progesterone production increased ($P < 0.05$) from Day 2 to Day 4; only for AL treatment, a significant increase of testosterone production was observed during culture.**
Conclusion: **Compared to control the diluted/dynamised FSH addition increased progesterone production but decreased the estradiol production after *in vitro* culture of isolated porcine preantral follicles. Taken together the results suggest that at least for progesterone production the mechanism of action of diluted/dynamised FSH differs from its vehicle.** *Homeopathy* (2017) **106**, 87–92.

Keywords: Preantral follicle steroids; FSH; Homeopathy; Porcine

Introduction

Since the pioneer studies performed by Samuel Hahnemann in the 18th century[1] homeopathy, which is based on the use of high diluted and dynamised substances, has been proposed as a safe and low-cost therapy for numerous medical conditions.[2] However, many scientists are skeptical about the effectiveness of homeopathy, owing to possible placebo effects and lack of comprehensive studies

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in this field.[2,3] In this regard, *in vitro* culture studies represent an important tool to exclude the placebo effects and assess the efficacy of homeopathy. The *in vitro* culture of preantral follicles, for example, has been used to evaluate the effect of many drugs and chemicals during folliculogenesis, i.e., toxicity tests[4,5] for herbal medicine[6,7] and homeopathic medications.[8–10]

The viability of *in vitro* follicle culture is affected by many factors like: concentration and association of factors added to the culture medium; type of culture system (*in situ* or isolated), as well as animal species.[4,11] Among the factors that have been used in the culture medium, follicle stimulating hormone (FSH) has been extensively studied due to its crucial role in folliculogenesis ensuring both follicular survival and growth.[12–14] Among the types of culture systems, preantral follicles can be cultured in two ways, i.e., *in situ* (enclosed in ovarian tissue) or in the isolated form.[11] *In situ* culture has aimed at studying early preantral folliculogenesis (activation of primordial to primary follicle[11,15]), while the culture of isolated secondary follicles aims to study late preantral folliculogenesis (the transition from advanced secondary to early antral follicles[11,16]). *In vitro* culture has been used in human and veterinary medicine with the aim to study the early and late folliculogenesis.[11] Studies using animal ovaries from laboratory and farm (murine, caprine, ovine, bovine and porcine) species represent an important alternative to overcome the ethical problems with using human ovarian material.[17] Compared to the other species, swine seems to be a suitable animal model for humans, due to the ovarian similarities[18,19] between these two species. In addition, the advantage of using pig ovaries is that the ovaries can come from animals of similar age, breed and controlled nutrition.

In previous studies we have investigated the action of homeopathic preparations on folliculogenesis.[9,10] Our team was the first to demonstrate the *in vitro* effect of homeopathic substances on follicular development. In this study, we investigated the *in vitro* action of highly diluted and dynamised FSH on early preantral follicles (primordial to primary) enclosed in ovarian tissue fragments (*in situ* culture). It was observed that homeopathic FSH (FSH 6cH), added daily, maintains the follicular survival and promotes *in vitro* activation of ovine primordial follicles[9] and may be used as an alternative to rFSH for the *in vitro* culture of ovine preantral follicles enclosed in ovarian tissue.[10] However, there is no study that evaluates the effect of homeopathic medicine on development and steroid production of porcine preantral follicles. Therefore, we hypothesized that diluted and dynamised FSH (homeopathic preparation) improves follicular development and steroid production of porcine preantral follicles through a different mechanism of action from either its vehicle (alcohol) or a conventional FSH preparation.

The present study investigated the effect of two FSH preparations (diluted/dynamised or diluted-only) on the *in vitro* development and steroid production of isolated porcine preantral follicles cultured *in vitro*. In the present paper, we define diluted/dynamised FSH when the FSH is submitted successively to a series of dilution followed by succussion (dynamisation) to reach a final concentration of 0.05 fg/mL (equivalent to FSH 6cH) while "diluted-only" refers to a conventional dilution of FSH to reach a final concentration of 1.5 ng/mL.[*]

Materials and methods

Research ethics

Alternatives to animal testing are the development and implementation of test methods that avoid the use of live animals. One of the major alternatives to *in vivo* animal testing is *in vitro* cell culture. In line with this ethical issue, the present study aimed to evaluate the effects of the tested substances (alcohol and FSH preparations) on *in vitro* folliculogenesis using porcine follicles recovered from slaughterhouse ovaries. This source of ovarian material represents a by-product of the food industry and is more readily acceptable than euthanasia of animals specifically for scientific purposes.

Ovary collection

Ovaries (n = 90) from prepubertal gilts were collected at a local abattoir and transported to the laboratory in physiological saline at 30–35 °C. The ovaries were washed with 70% ethanol for 10 s, followed by a wash with saline solution supplemented with penicillin (100 μg/mL) and streptomycin (100 μg/mL).

Follicle isolation and *in vitro* culture of preantral follicles

The *in vitro* culture system used in the present study was performed according to the protocol of Wu *et al.*[20]; with slight modifications. Once in the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries. Ovarian cortical slices (1- to 2-mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in holding medium consisting of medium H-199 (Lonza 12-117F) with antibiotics and 3 mg/mL bovine serum albumin. Secondary follicles (250–330 μm) without antral cavities, containing two to three layers of granulosa cells, and a visible oocyte were mechanically isolated by microdissection using 26-gauge needles and transferred to 300-μl droplets containing base culture medium. The follicles were randomly distributed to the treatment groups with approximately 35–40 follicles per group. Then, follicles (3 per well) were placed in 280 μl of culture medium in 48-well plates and incubated at 39 °C and 5% CO_2 in air for 4 days. The base control medium consisted of α-MEM was supplemented with 3.5 μg/mL insulin, 10 μg/mL transferrin,

[*] The two different dilutions (dynamised; diluted-only) were prepared to two different concentrations because the concentration of 1.5 ng/mL is considered a gold standard for pig and the concentration of 0.05 fg/mL used in the homeopathic preparation was chosen because it was the best concentration determined in a previous study using sheep preantral follicle culture.[9,10]

100 μg/ml L-ascorbic acid, 7.5% porcine serum. The culture medium was partially replaced (140 μl) every 2 days with freshly prepared culture medium (α-MEM$^+$). The media obtained on Days 2 and 4 were stored at −20 °C in order to assess hormone content.

Experimental design

For the experimental conditions, isolated secondary follicles (n = 170) were randomly distributed in the following treatments: α-MEM$^+$ (control medium), α-MEM$^+$ supplemented with ovine FSH at 1.5 ng/mL (diluted-only FSH treatment), α-MEM$^+$ supplemented with grain ethanol at 0.2% (alcohol − AL treatment; 92.8 INPM; v/v), α-MEM supplemented with high diluted and dynamised FSH (diluted/dynamised FSH treatment; 0.05 fg/mL). A total of 40−45 follicles were cultured in each of the four conditions. Each condition was repeated nine times. The FSH and AL concentrations were chosen based on previous work performed by Wu *et al.*[20] and Lima *et al.*,[9] respectively for diluted-only FSH; diluted/dynamised FSH and AL.

Preparation of dynamised FSH

The preparation of dynamised rFSH (diluted/dynamised FSH) was performed in Naturalis Homeopathic Pharmacy (Fortaleza, CE, Brazil). One mL of rFSH at 5 mg/mL, constituted the mother tincture. This was diluted in 99 mL of water-alcohol (grain ethanol) at 20% (v/v) followed by 10 manual succussions to produce the first dynamization. Then, 1 mL of the resulting solution was added again to 99 mL water-ethanol, with the same procedure of agitation being completed to make the second dynamization. Continuing this process, we made a series of successive dilutions and succussions, until the 6th centesimal Hahnemannian dilutions (rFSH 6cH − 0.05 fg/mL).[9]

Morphological evaluation of follicle development

The morphological aspects of all preantral follicles were assessed every 2 days using a pre-calibrated ocular micrometer in a stereomicroscope (Nikon DIAPHOT 200) at 100× magnification. Only those follicles showing an intact basement membrane, with bright and homogeneous granulosa cells and an absence of morphological signs of degeneration, were classified as intact follicles. Every 2 days of culture (days 0 and 4), the following characteristics were analyzed in the morphologically normal follicles: (1) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers, and (2) the diameter of morphologically normal follicles, measured from the basement membrane, which included two perpendicular measures of each preantral follicle, with the aid of Carl Zeiss Microscopy BmbH 2011 and analyzed by Zen Lite program under 100× magnification. The growth rate was calculated as follows: the final diameter minus the initial diameter of viable follicles (at Day 0), divided by the days of *in vitro* culture.

Hormone assays

Spent medium was collected at Days 2 and 4 and stored at −80 °C for subsequent estradiol (E2), progesterone (P4) and testosterone (T) assays. Steroid concentrations were determined by Enzyme-Linked Immuno-Sorbent Assay (ELISA) kits: Arbor Assays K030-H1 (E2), K025-H1 (P4) and K032-H1 (T). The lower detection limits were, respectively: 26.5 pg/mL for E2, 52.9 pg/mL for P4, and 30.6 pg/mL for T.

Statistical analysis

Data were initially evaluated for homocedasticity and normal distribution of the residues, by Bartlett's and Shapiro−Wilk tests, respectively. Confirmed both requirements underlying analysis of variance, the effects of treatment, time, and treatment by time interaction were analyzed using DOC MIXED of SAS (2002), including *repeated* statement to account for autocorrelation between sequential measurements. The model was $Y_{ilk} = \mu + R_i + F_j + T_k + (RT)_{jk} + e_{ijk}$, were Y_{ilk} is the observation of the jth follicle in the ith treatment at the kth time of culture, μ is the overall mean, R_i is ith treatment, T_k is the kth time of culture, $(RT)_{jk}$ is the treatment by time interaction term and e_{ijk} is the random residual effect. Comparisons among treatments or times were further analyzed by Student−Newman−Keuls (SNK) test. Due to heterogeneity of variances, Kruskal−Wallis test was used to evaluate the results of hormone assay. The outlier data were excluded from the statistical analyses. A probability of $P < 0.05$ indicated a significant difference; results were expressed as mean ± S.E.M. (parametric analysis) or median with interquartile range (non-parametric analysis).

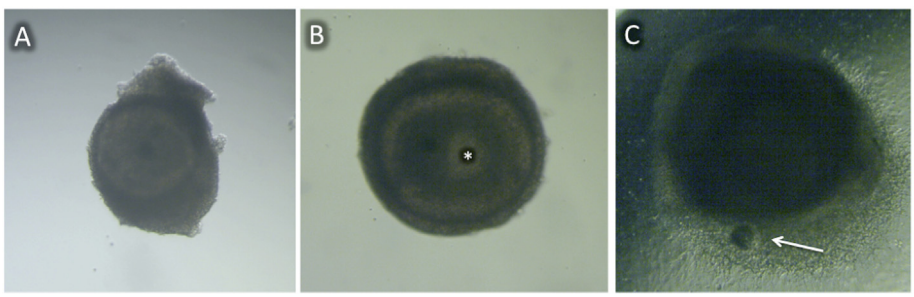

Figure 1 Isolated normal preantral (A), normal antral (B) and extruded follicle after 4 days of culture. * − Indicate the antrum cavity; White arrow − oocyte in a extrused follicle.

Results

Morphology and follicular growth after *in vitro* culture

Figure 1 shows a morphologically normal preantral follicle (Figure 1A), a normal antral follicle (Figure 1B) and an extruded follicle (Figure 1C), after *in vitro* culture of isolated porcine preantral follicles. The percentage of follicular integrity and extrusion were not affected by the treatments. However, for all treatments the percentage of follicular integrity decreased significantly from Day 0 to Day 4 (Table 1).

At Day 4 of culture, only follicles cultured in the presence of diluted/dynamised FSH had a higher rate of antral cavity formation than those follicles cultured in control medium (Table 1). For all treatments, follicular diameter increased significantly from Day 0 to Day 4. However, both follicular diameter and daily growth rate were not affected by treatments (Table 1).

Steroid production

The steroid production (estradiol, progesterone and testosterone) after *in vitro* culture of porcine preantral follicle is shown in Figure 2. Except for diluted/dynamised FSH treatment, progesterone production increased ($P < 0.05$) from Day 2 to Day 4, but only for AL treatment was there a significant increase of testosterone production observed during culture. Regardless of culture time, all treatments increased ($P < 0.05$) the progesterone production compared to control treatment. Higher levels of testosterone ($P < 0.05$) were observed in the AL treatment at Day 4 than in the diluted-only FSH treatment. The progesterone and estradiol production were significantly higher ($P < 0.05$) in the diluted-only FSH treatment compared to the diluted/dynamised FSH treatment. The progesterone levels were higher ($P < 0.05$ − Day 2) and the estradiol levels were lower ($P < 0.05$ − Day 4) in the diluted/dynamised FSH treatment compared to its vehicle, i.e., the AL treatment.

Discussion

Homeopathy is a controversial topic in complementary medical research.[2] The well-established *in vitro* models are appropriate methods to help tackle this problem.[21] The present study shows, for the first time, using an *in vitro* follicle culture model that the diluted/dynamised FSH positively affects the rates of antrum formation and steroid production.

In this study, all tested treatments were similar in relation to follicular integrity and extrusion. Moreover, in all treatments, the percentage of normal follicles decreased during culture. Such reduction in follicle viability represents common results reported by many authors and is due to the absence of an efficient culture medium.[22–24]

On the other hand, the addition of diluted/dynamised FSH increased the antrum formation when compared with control treatment, but it was similar to the other treatments. Antrum formation is a reliable and objective parameter commonly used in different studies to investigate the

Table 1 Percentage of follicle integrity, viability, antrum formation, follicular diameter (μm) and daily growth rate after 4 days of culture

Treatments	*Integrity (%)*		*Extrusion (%)*	*Degeneration (%)*	*Antrum formation (%)**	*Follicular diameter (μm)*		*Daily growth (μm)*
	Day 0	*Day 4*	*Day 4*	*Day 4*	*Day 4*	*Day 0*	*Day 4*	
α-MEM$^+$- control	100.00 (40/40)^{Aa}	82.50 (33/40)^{Ab}	5.00 (2/40)^{A}	12.50 (5/40)^{A}	52.5 (21/40)^{B}	280.81 ± 20.00^{Ab}	304.24 ± 38.09^{Aa}	3.41 ± 9.93^{A}
Diluted FSH	100.00 (40/40)^{Aa}	82.50 (33/40)^{Ab}	2.50 (1/40)^{A}	15.00 (6/40)^{A}	67.50 (27/40)^{AB}	284.58 ± 19.94^{Ab}	301.20 ± 34.31^{Aa}	3.88 ± 7.42^{A}
AL	100.00 (43/43)^{Aa}	76.74 (33/43)^{Ab}	6.98 (3/43)^{A}	16.27 (7/43)^{A}	67.44 (29/43)^{AB}	286.89 ± 19.34^{Ab}	303.29 ± 31.56^{Aa}	2.72 ± 6.19^{A}
Diluted/dynamised FSH	100.00 (42/42)^{Aa}	83.33 (35/42)^{Ab}	4.76 (2/42)^{A}	7.14 (3/42)^{A}	73.81 (31/42)^{A}	289.41 ± 19.79^{Ab}	313.80 ± 35.43^{Aa}	5.95 ± 7.95^{A}

*The % (ratio) of antrum formation data means the number of follicles that formed an antrum cavity at Day 4 of culture divided by the total number of follicles at Day 0 of culture.
^{A,B}Values within columns with different superscripts differ ($P < 0.05$).
^{a,b}Values within rows (days) with different superscripts differ ($P < 0.05$).

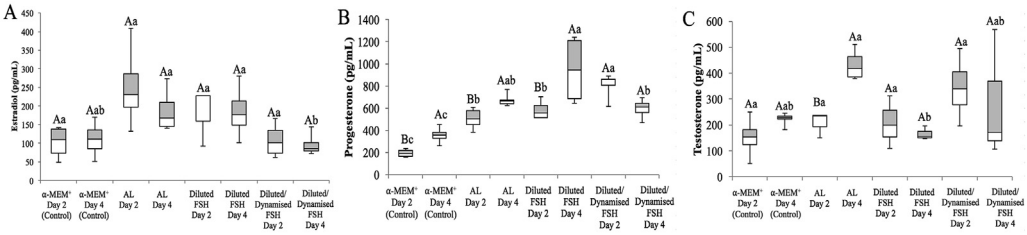

Figure 2 Steroid production by porcine preantral follicles cultured *in vitro* for 2 and 4 days in α-MEM supplemented with or without AL, diluted-only FSH or diluted/dynamised FSH. A, B Values with different superscripts differ between days within the same treatments ($P < 0.05$). a, b, c Values with different superscripts differ between treatments within the same day ($P < 0.05$).

efficiency of *in vitro* follicle culture systems in many species.[25–27] Taking into account that antrum formation may occur when follicles reach different sizes it is possible to observe *in vitro* differences among tested treatments in the antrum formation endpoint but not necessarily mean follicle size at the end of culture. In previous studies, the diluted/dynamised FSH acted in a similar way to the recombinant FSH, improving the follicular survival of ovine preantral follicles.[10] Moreover *in vivo*, diluted/dynamised FSH is used to improve the follicular development in women with fertility problems.[8] However, the mechanism of action of homeopathic medicines remains to be elucidated.

With regard to the steroid secretion at Day 4 of culture, the diluted-only FSH and AL (homeopathic vehicle) treatments increased the estradiol production when compared with diluted/dynamised FSH, but were similar to control. This suggests diluted/dynamised FSH and conventional FSH preparation seem to have different mechanism of action on steroid production. However, progesterone production (Day 2) was significantly higher in the diluted/dynamised FSH in relation to other treatments, including the AL treatment. In addition, just the treatment with diluted/dynamised FSH did not alter the progesterone production during the days of culture. Further, only the AL treatment increased the level of testosterone when compared Day 2 with Day 4.

These results from objective and reliable endpoints (hormone assays) showed that the effect of diluted/dynamised rFSH was not due to the vehicle (AL) alone suggesting a different mechanism of action may be occurring. Moderate alcohol consumption increased plasma estrogen levels in postmenopausal women.[28] During the culture of human granulosa cells, ethanol (10 and 20 mM) significantly increased basal estradiol secretion.[29] However, the mechanism of action of ethanol on steroid production is unknown and poorly examined. We suggest that the Ca_{2+} flushed into the granulosa and theca cells induced by AL could act as second messenger. The free cytoplasmic Ca_{2+} binds with substances like calmodulin, forming a complex that will activate metabolic enzymes, which regulate the gene expression, affect the adenylate cyclase and the membrane cell permeability.[30,31] The activation of adenylate cyclase increases the levels of intracellular cAMP, which activates the PKA and, consequently CYP19A1 expression, increasing the estradiol production.[32] Furthermore, Jayes *et al.*[33] demonstrated that the increase of intracellular calcium induced by FSH in swine granulosa cells raises the expression of P450ssc, which converts cholesterol into pregnenolone. These results are in agreement with a previous studies performed by our team.[9,10] Progesterone is an important substrate for the synthesis of androgens and estrogens.[34] In the present study, even though the treated groups increased progesterone production comparing the control treatment, this fact was not associated with significant increase in the testosterone and estradiol production. This event could be caused by the lack of appropriate expression of steroidogenic enzymes that precedes estradiol synthesis. Similar results were reported by Bridges *et al.*,[35] Mahalingam *et al.*,[36] and Paes *et al.*[37]

In conclusion, compared with the control treatment the diluted/dynamised FSH addition increased progesterone production but decreased the estradiol production after *in vitro* culture of isolated porcine preantral follicles. Taken together the results suggest that at least for progesterone production the mechanism of action of diluted/dynamised FSH differs from its vehicle. In addition, the data suggest that diluted/dynamised FSH and conventional FSH preparation seem to have different mechanism of action on follicular development and steroid production.

Conflict of interest statement

None of the authors has any conflict of interest to declare.

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