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ORIGINAL PAPER

High diluted and dynamised follicle stimulating hormone modulates the 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) ■, 1–6.

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]

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