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Direct effect of curcumin on porcine ovarian cell functions

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ABSTRACT

Curcuma longa Linn (L.) is a plant widely used in cooking (in curry powder a.o.) and in folk medicine, but its action on reproductive processes and its possible mechanisms of action remain to be investigated. The objective of this study was to examine the direct effects of curcumin, the major *Curcuma longa* L. molecule, on basic ovarian cell functions such as proliferation, apoptosis, viability and steroidogenesis. Porcine ovarian granulosa cells were cultured with and without curcumin (at doses of 0, 1, 10 and 100 μg/ml of medium). Markers of proliferation (accumulation of PCNA) and apoptosis (accumulation of bax) were analyzed by immunocytochemistry. The expression of mRNA for PCNA and bax was detected by RT-PCR. Cell viability was detected by trypan blue exclusion test. Release of steroid hormones (progesterone and testosterone) was measured by enzyme immunoassay (EIA). It was observed that addition of curcumin reduced ovarian cell proliferation (expression of both PCNA and its mRNA), promoted apoptosis (accumulation of both bax and its mRNA), reduced cell viability, and stimulated both progesterone and testosterone release. These observations demonstrate the direct suppressive effect of *Curcuma longa* L./curcumin on female gonads via multiple mechanisms of action – suppression of ovarian cell proliferation and viability, promotion of their apoptosis (at the level of mRNA transcription and subsequent accumulation of promoters of genes regulating these activities) and release of anti-proliferative and pro-apoptotic progesterone and androgen. The potential anti-gonadal action of curcumin should be taken into account by consumers of *Curcuma longa* L.-containing products.

1. Introduction

For a long time, humans have used medicinal and spicy herbs for cooking, for the treatment of physiological disorders, and for the regulation of physiological processes, including reproduction (Nadkarni, 1976; Hardy, 2000; Nayak et al., 2016). However, the mechanisms of action of these herbs and their possible side effects are not yet fully understood, and they require rigorous investigation.

One of the plants often used in cooking and folk medicine is *Curcuma longa L.*— Indian Turmeric. This plant is a part of curry

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powder and other spices currently considered functional food. A major biologically active component of *Curcuma longa* L. is the phenolic compound curcumin, with the chemical name 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione (Aggarwal et al., 2007; Ak and Gülçin, 2008). Owing to its pro-apoptotic, anti-proliferative, anti-oxidant, and anti-angiogenic properties, curcumin has been studied as a potentially useful agent in the treatment of cancer and other disorders (Tiwari-Pandey and Ram Sairam, 2009; Terlikovska et al., 2014; Vallianou et al., 2015; Nayak et al., 2016). There is a growing body of evidence that curcumin can affect ovarian functions. Numerous in-vitro and in-vivo studies demonstrated its ability to directly suppress proliferation and promote apoptosis in ovarian cancer cells (Shehzad et al., 2010; Watson et al., 2010; Terlikowska et al., 2014; Vallianou et al., 2015; Seo et al., 2016), and to prevent the adverse effects of aging, ovarian insufficiency (Tiwari-Pandey and Ram Sairam, 2009; Voznesens'ka et al., 2010; Alekseyeva et al., 2011), ionizing radiation (Aktas et al., 2012), ischemia (Eser et al., 2015), oxidative stress (Qin et al., 2015) and mycotoxins (Qin et al., 2015) on ovarian function.

Despite its therapeutic action, curcuma/turmeric is currently consumed by healthy individuals mainly as a cooking additive and functional food for the prevention of various disorders and illnesses. Moreover, its beneficial effects could potentially be useful in agriculture for the maintenance of healthy farm animals. Nevertheless, in contrast to medical studies, the action of curcumin on function of healthy ovarian cells not exposed to any negative external factor is very poorly investigated. The available reports are scarce, contradictory, and involve mainly laboratory rodents. Only one study was performed on a farm animal, a pig (Nurcahyo and Soejono, 2007).

Some *in-vivo* studies have demonstrated the stimulatory effect of curcumin or its analogue on ovarian functions—it promoted proliferation, reduced apoptosis in murine ovarian cells (Voznesens'ka et al., 2010; Aktas et al., 2012), and supported murine ovarian oogenesis (Voznesens'ka et al., 2010; Alekseyeva et al., 2011), folliculogenesis and steroidogenesis (Tiwari-Pandey and Ram Sairam, 2009); on the contrary, other authors have reported the ability of curcumin to suppress reproductive functions—to prolong puberty, to reduce fecundity (Murphy et al., 2012), and to suppress proliferation and progesterone release in cultured rat ovarian luteal cells (Purwaningsih et al., 2012), as well as to inhibit proliferation, stimulate apoptosis, and suppress progesterone and estradiol release by porcine ovarian follicular granulosa cells (Nurcahyo and Soejono, 2007). The mechanisms of action of curcumin on healthy ovarian cells, especially in farm animals, require further elucidation. It remains unknown whether curcumin affects accumulation of proliferation and apoptosis regulators/markers via their gene transcription (accumulation of their mRNA), translation (peptide production) or metabolism, whether curcumin-induced changes in rate of proliferation- and apoptosis-related molecules affect ovarian cell viability, whether curcumin can affect ovarian androgen release, and what the hormone-based mechanisms behind reproductive effects of curcumin could be.

The aim of our *in-vitro* experiments was to examine the action of curcumin on basic ovarian cell functions—proliferation, apoptosis (accumulation of PCNA, bax and their mRNA), cell viability, and the release of progesterone and testosterone.

2. Materials and methods

2.1. Isolation and culture of granulosa cells

Granulosa cells were collected from the ovaries of prepubertal (100–120 days old, weight 105–130 kg) Slovakian White gilts following their slaughter at a local abattoir. Ovaries were transported to the laboratory at 4 °C and washed in sterile physiological solution. Follicular fluid was aspirated from 3 to 5 mm follicles and granulosa cells were isolated by centrifugation for 10 min at 200*g*. Cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittaker, Verviers, Belgium), and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 10^6 cells/mL medium. 1 ml/well of the granulosa cell suspension was dispensed in 24-well culture plates (Nunc, Roskilde, Denmark, for EIA, trypan blue test and RT-PCR, see below) and 200 µl/well in 16-well chamber slides (Nunc Inc., International, Naperville, USA) for immunocytochemistry. Both the plate wells and chamber slides were incubated at 37 °C and 5% CO_2 in humidified air until 60–75% confluent monolayer was formed (3–5 days), at which point medium was renewed. Further culture was performed in 2 ml culture medium in 24-well plates or 200 µl/medium in 16-well chamber slides as described previously.

After medium replacement experimental cells were cultured in the presence of curcumin (Changsha Sunfull Bio-tech. Co, Hunan, China, purity 98.9%) at concentrations of 0, 1, 10 and 100 µg/ml for immunocytochemistry and EIA; at 0 and 100 µg/ml for trypan blue extrusion test; 0, 10 and 100 µg/ml for RT-PCR. These doses correspond to the curcumin doses used in previous *in-vitro* experiments performed on healthy and malignant ovarian cells (Nurcahyo and Soejono, 2007; Shehzad et al., 2010; Watson et al., 2010; Purwaningsih et al., 2012). Curcumin was dissolved first in DMSO (concentration 10 mg/ml) and than in culture medium just before their addition to the cells. The maximal concentration of DMSO in culture was 0.1%. This amount of DMSO was added to the cells of control group.

After 2 days of culture with or without curcumin, the medium was removed. The cells in chamber slides were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2–7.4; 60 min), dehydrated in alcohols (70%, 80%, 96%; 10 min each) and held at 4 °C in preparation for immunocytochemistry. The medium from the 24-well plates was gently aspirated and frozen at −24 °C to await RT-PCR and RIA. Some of the granulosa cells cultured in these plates cultured both with (100 µg/ml) and without curcumin were subjected to trypan blue extrusion test.

2.2. Immunocytochemical analysis

After fixation and washing in PBS for 5 min, the cells were incubated in blocking solution (1% of goat serum in PBS) at room