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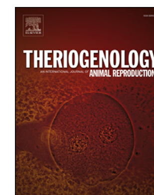

Rutin can replace the use of three other antioxidants in the culture medium, maintaining the viability of sheep isolated secondary follicles

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The present study evaluated the effect of addition of rutin alone or combined with other antioxidants (transferrin, selenium and ascorbic acid) present in the culture medium on the *in vitro* development of ovine isolated secondary follicles. After collection of the sheep ovaries, secondary follicles (200–230 μm) were isolated and cultured for 12 days in α-Minimal Essential Medium (α-MEM) supplemented with BSA, insulin, glutamine and hypoxanthine (α-MEM: antioxidant free-medium) or in this medium also added by transferrin, selenium and ascorbic acid (AO: base medium with antioxidants). Moreover, different concentrations of rutin (0.1; 1 or 10 μg/mL) were added to the different base media (α-MEM or AO). The parameters analyzed were morphology, antrum formation, extrusion rate, follicular diameter, growth and fully-grown oocytes (oocytes ≥ 110 μm) rates. In treatments that had the best results of morphology, follicular viability, apoptosis, glutathione (GSH), reactive oxygen species (ROS) levels and mitochondrial activity were also analyzed. After 12 days, the percentage of normal follicles was higher ($P < 0.05$) in α-MEM + 0.1 μg/mL rutin than the other treatments, except compared to AO medium ($P > 0.05$). There is no difference ($P > 0.05$) in the diameter and growth rate among treatments. Moreover, AO medium and α-MEM + 0.1 μg/mL rutin showed similar ($P > 0.05$) percentages of follicular viability, antrum formation, extruded follicles, fully-grown oocytes, levels of ROS and active mitochondria. However, α-MEM + 0.1 μg/mL rutin treatment showed higher ($P > 0.05$) GSH levels than AO medium. In conclusion, 0.1 μg/mL rutin can be used as the single antioxidant present in the base medium, replacing the addition of transferrin, selenium and ascorbic acid during *in vitro* culture of ovine secondary follicles, maintaining follicular viability and increasing GSH levels.

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1. Introduction

Production of embryos from oocytes derived from *in vitro* grown preantral follicles is still considered a challenge, since *in vitro* culture conditions may increase reactive oxygen species (ROS) exposure, decreasing antioxidant protection or causing mitochondrial damage, resulting in lower oocyte and embryo quality [1–3]. Therefore, the addition of antioxidants to the culture medium seems to be necessary to prevent or decrease the damage induced by *in vitro* oxidative stress [4,5]. Although different antioxidants have been routinely added to the base medium, such as transferrin, selenium and ascorbic acid [6,7], some studies have also tested the effects of other substances with antioxidant potential [8–10].

Recently the antioxidant effects of rutin have drawn increasing attention [11–13]. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is an important flavonoid, also known as vitamin P or quercetin-3-O-rutinoside, that is consumed in the daily diet, being found in fruits, vegetables or in medicinal plants [14,15]. Little is known about possible effects of rutin on reproduction. *In vitro* studies have shown that rutin decreases lipid peroxidation, ROS levels and DNA fragmentation in thawed deer spermatozoa after incubation at 37 °C [16] and preserves the viability and motility of human spermatozoa when used in the sperm selection by swim up procedure [11]. Moreover, rutin (5 μM) decreases oxidative stress in ovarian cells of Chinese hamster [17]. However, there is no information about the effect of rutin on the *in vitro* culture of ovarian follicles. Furthermore, as antioxidant substances may act as pro-oxidants when used in excess [18,19], we

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hypothesized that rutin could replace the other antioxidants present in the culture medium.

Therefore, the aim of this study was to evaluate the effect of different concentrations of rutin as the only antioxidant added to the base culture medium or combined with other antioxidants (transferrin, selenium and ascorbic acid) on *in vitro* morphology, viability, development, apoptosis, oxidative stress markers (ROS and GSH intracellular levels) and metabolically active mitochondria of isolated ovine secondary follicles.

2. Material and methods

Unless indicated, media, rutin, supplements and chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Source of ovarian tissue

Ovaries (n = 180) were collected at a local abattoir from 90 adult (1–3 years old) mixed-breed sheep (*Ovis aries*). Immediately post-mortem, pairs of ovaries were washed once in 70% alcohol and then twice in Minimum Essential Medium buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin). Next, the ovaries were transported within 1 h to the laboratory in tubes containing MEM-HEPES with antibiotics at 4 °C [20].

2.2. Isolation and selection of ovine secondary follicle

In the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries; large antral follicles and corpora lutea were removed. Ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in holding medium consisting of MEM-HEPES with antibiotics. Ovine secondary follicles, approximately 200–230 μm in diameter without antral cavities, were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and mechanically isolated by microdissection using 26-gauge (26 G) needles. These follicles were then transferred to 100 μL droplets containing base culture medium for the evaluation of quality. Only follicles that displayed the following characteristics were selected for culture: an intact basement membrane, two or more layers of granulosa cells and a visible and healthy oocyte that was round and centrally located within the follicle, without any dark cytoplasm. Isolated follicles were pooled and then randomly allocated to the treatment groups, with approximately 60 follicles per group.

2.3. In vitro culture of ovine secondary follicle (experimental protocol)

After selection, the follicles were randomly divided into eight study groups and individually cultured (one follicle per droplet) in 100 μL droplets of culture medium under mineral oil in petri dishes (60 × 15 mm, Corning, USA). The base control media consisted of α-MEM (pH 7.2–7.4) supplemented with 3.0 mg/mL BSA, 10 ng/mL insulin, 2 mM glutamine and 2 mM hypoxanthine (antioxidant free-medium, called α-MEM) or this medium also added by 5.5 μg/mL transferrin, 5.0 ng/mL selenium and 50 ng/mL ascorbic acid (medium containing antioxidants, called Antioxidant: AO). To verify the effects of rutin, both base media (α-MEM or AO) were supplemented with different concentrations of rutin (0.1, 1 or 10 μg/mL) (Fig. 1). The concentrations were chosen based on a previous study [21]. All follicles were cultured at 39 °C under 5% CO_2 for up to 12 days. Every 2 days, in all treatments, 60 μL of the culture media was replaced with fresh media in each droplet.

2.4. Morphological evaluation of follicle development

The morphological aspects of all follicles were assessed every 6 days using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon) at x 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 morphologically normal follicles. Follicular atresia was recognized when a darkening of the oocytes and surrounding cumulus cells or misshapen oocytes was noted. The rupture of the basement membrane was also observed and characterized as oocyte extrusion. The following characteristics were analyzed in the morphologically normal follicles: (i) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers, (ii) the diameter of healthy follicles, measured from the basement membrane, which included two perpendicular measurements of each follicle, and (iii) the daily growth rate, calculated as the diameter variation during the culture period (12 days).

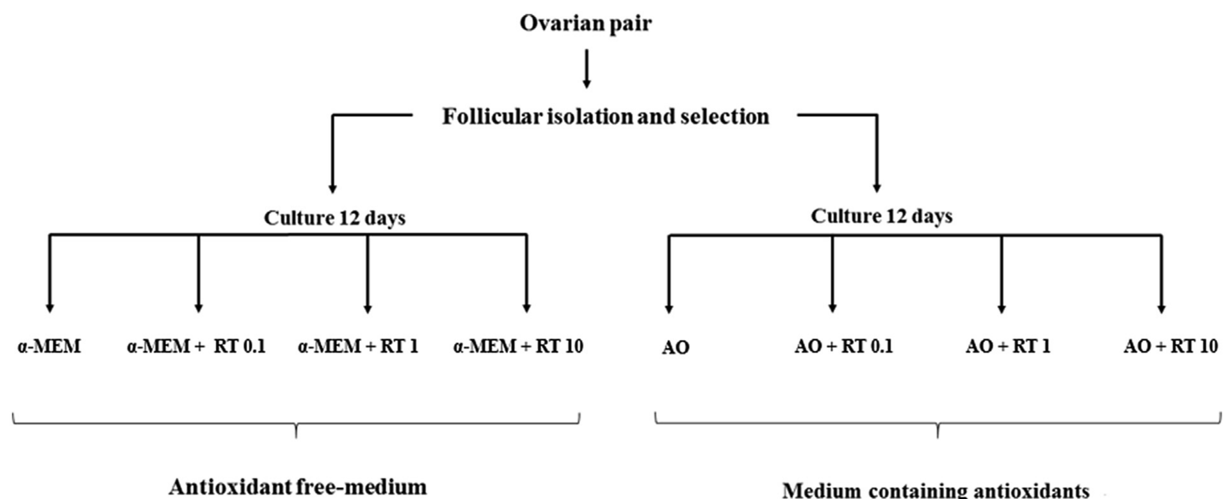

Fig. 1. General experimental protocol for *in vitro* culture of sheep secondary follicles in different media without or with rutin.

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