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A protective role of cumulus cells after short-term exposure of rat cumulus cell-oocyte complexes to lifestyle or environmental contaminants

Kelly A. Campen [1], Kenneth P. McNatty, Janet L. Pitman *

School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington 6140, New Zealand

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A B S T R A C T

Ovarian follicular fluid provides a potential reservoir for exogenous compounds that may adversely affect oocyte quality. This study examined the effects of common lifestyle and environmental contaminants, namely bisphenol-A (BPA), caffeine, 3,4-methylenedioxymethamphetamine (MDMA), nicotine and Δ^9-tetrahydrocannabinol (THC) on gap junction genes (*Gja1, Gja4*) and proteins (GJA1), glucose metabolism genes (*Gfpt1, Pfkp*) and oocyte growth factor genes (*Bmp15, Gdf9*), as well as gap junction transfer rate, in rat cumulus-oocyte complexes (COCs). *In vitro* exposure to MDMA and THC accelerated the timing of meiotic resumption and all contaminants altered either gap junction gene expression (BPA, caffeine, MDMA and THC) or transfer rate (BPA and nicotine). *In vitro* exposure of COCs to MDMA also altered glucose metabolism genes. Overall, oocyte-derived genes were largely unaffected following exposure to any contaminant. In summary, the impact of short-term exposure to lifestyle and environmental contaminants on oocyte function may be diminished due to protective properties of cumulus cells.

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1. Introduction

The microenvironment within an ovarian follicle plays a key role in regulating the growth and maturation of the follicle, as well as the developmental competency of the associated oocyte [1–4]. Throughout follicular development, the oocyte and cumulus cells communicate via a bi-directional pathway [5]. The cumulus cells primarily have a nurturing role within the follicle, facilitating oocyte development through the transfer of nutrients [6]. The majority of small molecules including cyclic nucleotides, glucose metabolites, amino acids, RNA transcripts, and ions are transferred via gap junctions [6,7] and influence the timing of meiosis, ATP production, and pH within the oocyte [8–10]. In turn, the oocyte modulates cumulus cell function by secreting paracrine factors that include growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) [11]. These paracrine factors affect proliferation [12], apoptosis [13], metabolism [14,15], expansion [16], and luteinisation [17] of cumulus and granulosa cells by acting as transcriptional regulators. The oocyte-secreted factors also enhance glycolytic activity and expression of glycolytic enzymes, such as phosphofructokinase, in cumulus cells from large antral follicles. This subsequently allows the cumulus cells to provide the oocyte with pyruvate for oxidative phosphorylation [18]. Prior to ovulation, the oocyte secretes an unidentified factor that stimulates cumulus cell expansion [16], resulting in a larger proportion of glucose being metabolized through the hexosamine biosynthetic pathway to produce hyaluronic acid [19].

From the time of antrum formation, the cumulus cell-oocyte complex (COC) is bathed in follicular fluid, and this may provide a reservoir where harmful compounds accumulate and influence oocyte maturation. Indeed, plasticizers, phthalates, pesticides, caffeine, and components of cigarette and marijuana smoke have been detected in human follicular fluid [20–24]. Furthermore, several studies have demonstrated correlations between contaminant levels within the follicular fluid and reductions in female fertility or assisted reproductive technology (ART) outcomes [20,24,25]. For example, exposure to bisphenol-A (BPA) has been correlated with reduced antral follicle counts, and also fertilization and implantation rates [26–28]. The use of caffeine, cigarettes, and some illicit drugs has been implicated in reduced oocyte quality, increased time to pregnancy, and reduced likelihood of *in vitro* fertilization

Abbreviations: BMP15, bone morphogenetic protein 15; BPA, bisphenol-A; COC, cumulus cell-oocyte complex; GDF9, growth differentiation factor 9; MDMA, 3,4-methylenedioxymethamphetamine; THC, Δ^9-tetrahydrocannabinol.

* Corresponding author.
E-mail address: janet.pitman@vuw.ac.nz (J.L. Pitman).
[1] Present address: University of Tennessee, Animal Science Department, Knoxville, TN 37902, USA.

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(IVF) success [29–35]. Cigarette smokers require twice as many IVF cycles as non-smokers in order to conceive [32]. For caffeine and marijuana users, the association with infertility is still controversial and weak. However in some studies, caffeine consumption has been tied to delayed conception [30,33], reduced IVF success [36], and increased likelihood of miscarriage [20]. Acute marijuana exposure causes alterations in serum gonadotrophin levels, resulting in anovulation [37,38], however chronic exposure leads to restoration of normal pituitary hormone levels [38]. Although the link between some lifestyle choices and fertility is tenuous, there is a growing need for practical and accurate advice enabling women to minimise their intake of compounds that may compromise fertility.

Bi-directional communication between the cumulus cells and oocyte is critically important for oocyte quality however very little is known of the effects of common lifestyle and environmental factors on this pathway. Therefore, the aims of this study were to investigate the *in vitro* effects of five lifestyle and environmental factors on the (i) reagent transfer rate via gap junctions, (ii) expression levels of genes involved in key regulatory pathways, and (iii) protein levels of the gap junction protein connexin 43 (GJA1) in rat COCs. The lifestyle factors tested were nicotine, caffeine, Δ^9-tetrahydrocannabinol (THC), 3,4-methylenedioxymethamphetamine (MDMA) and the environmental factor tested was BPA. Furthermore, the *in vivo* effects of MDMA on COC function were also tested in rats. The key genes selected for this study included genes encoding: the cumulus cell-derived (namely connexin 43, *Gja1*) and oocyte-derived (namely connexin 37, *Gja*4) proteins composing the hexameric connexin complexes that form the gap junctions; oocyte-secreted growth factors responsible for regulating cumulus cell function (namely bone morphogenetic protein 15, *Bmp15* and growth differentiation factor 9, *Gdf*9); and metabolic enzymes key to glucose utilisation pathways employed by the cumulus cells for the provision of critical glucose substrates to the oocytes [14,39] (namely the rate limiting enzyme of the hexosamine biosynthesis (glutamine-fructose-6-phosphate aminotransferase 1, *Gfpt1*) and glycolysis (phosphofructokinase platelet, *Pfkp*) pathways).

2. Materials & methods

2.1. *Preparation of lifestyle and environmental factors and dosage justifications*

All reagents were purchased from Sigma-Aldrich (Auckland, New Zealand) unless otherwise stated. For the *in vitro* experiments, treatment reagents were dissolved in dimethyl sulfoxide (DMSO) at 200× final concentration and stored at −20 °C. Each reagent was then diluted 200-fold using incubation medium on the day of the experiment. To ensure that high physiological dosages were tested, the concentrations of all treatments were 10–100-fold higher than the mean values that previously measured in follicular fluid or plasma (see below). The final concentration of DMSO for all treatments was 0.5% (v/v).

BPA (kindly donated by Nuplex Industries, New Zealand) was tested at a final concentration of 20 ng/ml. Whilst this concentration is below the lowest observed adverse effect level (LOAEL) of 50 ng/ml for *in vitro* studies [40], BPA has been measured in follicular fluid at 2.4 ng/ml [22] and 338 pg/ml [41] in women undergoing IVF. Similar concentrations of BPA have been demonstrated to alter steroid production by rat and porcine granulosa cells [42,43] and to increase oocyte metaphase-II spindle abnormalities in humans and cows [44,45]. Caffeine was tested at a final concentration of 100 μg/ml. Caffeine has been measured in follicular fluid at average concentrations of 0.7 μg/ml (range <0.66–6.48 μg/ml) and in serum at average concentrations of 0.9 μg/ml (range <1.1–64.04 μg/ml) [20]. Nicotine was tested at a final concentration of 500 ng/ml. Nicotine has been measured in plasma of active smokers at concentrations between 30 and 50 ng/ml [46,47]. MDMA (kindly donated by Prof Susan Schenk, Victoria University of Wellington, New Zealand) was tested at a final concentration of 2 μg/ml. In a study to characterize the pharmacokinetics of MDMA, peak plasma concentrations were between 130 and 209 ng/ml when doses were used that reflected the average MDMA content of a single ecstasy pill (75–100 mg) [48]. THC (kindly donated by Dr Ryan Steel, Victoria University of Wellington, New Zealand) was tested at a final concentration of 500 ng/ml. THC has been measured at concentrations of 67–98 ng/ml in plasma of people who had smoked one marijuana cigarette [49]. For the *in vivo* experiments, MDMA was diluted in sterile saline to a concentration of 2.5 mg/ml and stored at 4 °C until use. A concentration of 5 mg/kg MDMA was administered by intraperitoneal route daily for three days. The treatment schedule was designed to produce maximum effect while still mimicking human exposures. A study of 329 ecstasy users found that 35% of users had 'binged' on ecstasy in the past six months, and that the median length of binging was 3 days [50]. This dose was chosen to further reflect an ecstasy binge [51], and has been demonstrated to alter reproductive endpoints in male rats [52]. Although ecstasy is primarily ingested orally, 10% of users report having injected ecstasy in the past six months [50]. Moreover, MDMA pharmacokinetic studies revealed the oral route in rats resulted in reduced absorption and/or significant first-pass metabolism in the liver and/or gut of rat compared to humans, suggesting the repeated intra-peritoneal injections of low-dose MDMA in the rat is an acceptable model for single oral doses in humans [53].

2.2. In vitro *exposure to lifestyle factors*

2.2.1. *Collection of cumulus cell-oocyte complexes*

In this study, the proportions of rat oocytes that spontaneously underwent meiotic resumption were 8, 33, 42 and 53% after 1, 5, 13 and 25 h in culture. Dipyridamole and rolipram are phosphodiesterase inhibitors that were included in all culture media to delay spontaneous meiotic resumption and consequent gap junction closure. The concentrations used in this study have previously been reported to maintain gap junction communication between the cumulus cells and oocyte for up to 19 h [54], which was necessary for the test reagents to have access to the oocyte. The timing of meiotic resumption, gap junction communication and expression of key regulatory genes of untreated rat oocytes using the culture system described herein has been reported previously [54].

Prepubescent (21–25 days old) female Sprague-Dawley rats were sacrificed by asphyxiation with carbon dioxide. Ovaries were extracted into Lebowitz L-15 medium containing phenol red and L-glutamine (Invitrogen, Auckland, New Zealand) and supplemented with 0.1% (w/v) bovine serum albumin, 100 IU/ml Penicillin-Streptomycin (Life Technologies NZ Ltd., Auckland, New Zealand), 15 nM HEPES, 50 μM dipyridamole, and 50 μM rolipram. Extraneous tissue surrounding the ovary was trimmed and antral follicles (≥2 mm) were punctured using a 20-gauge needle, releasing the COC into the medium. On each experimental day, COCs were pooled from multiple animals, typically between 2 and 7 rats depending on the experiment being performed. Each experiment was repeated independently 2–3 times. The COCs that contained at least three intact layers of cumulus cells were randomly transferred into wells of a 48-well plate containing 200 μl M199 medium containing L-glutamine, 2.2 g/l sodium bicarbonate and Earle's salt (Invitrogen) supplemented with 100 IU/ml Penicillin-Streptomycin and 0.1% (w/v) bovine serum albumin (wash medium). The COCs were washed twice in fresh wash medium.

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