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Hydroxychloroquine attenuates cigarette smoke induced autophagic signaling in the mouse ovary

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ABSTRACT

We previously demonstrated that Cigarette Smoke (CS) induces autophagy in the ovary. Therefore we aimed to determine if chloroquine (CQ) could inhibit CS-induced autophagy in the ovary. Eight week old mice were implanted with CQ pellets; 0, 25, and 50 mg CQ/kg. Half of the animals in each group were exposed to room air and the other half were exposed to CS twice daily for 8 weeks. Ovaries were harvested for electron microscopy, gene and protein expression analysis. There was a significant increase in the production of autophagosomes in granulosa cells of mice exposed to CS ($p = 0.0297$). However 25 and 50 mg/kg CQ treatment significantly decreased the CS-induced autophagosomes ($p = 0.0505$; $p = 0.0065$) and attenuated the effects of CS on LC3B and BECN1 expression. In summary, this suggests that CQ attenuates CS-induced autophagy in the ovary and that ovarian protection from toxic insult is potentially feasible.

1. Introduction

Approximately 48.5 million couples worldwide struggle with infertility [1]. Of the many contributing factors investigated, exposure to environmental contaminants is thought to be a potentially important cause of infertility. Furthermore, exposure to the environmental toxicants contained in cigarette smoke has been documented to lower circulating concentrations of estradiol, decrease the rate of ovarian follicle development, increase follicle loss [2–7], and increase the frequency of reproductive failure in women who smoke compared to non-smokers [4,8]. Cigarette smoke (CS) exposure has been shown to attenuate the response to ovulation induction in women undergoing assisted reproductive therapies [9–13], and in utero exposure to CS has been shown to significantly affect human fetal development [14]. Sidestream-smoking (SS) is a type of second hand smoke that comes directly from a lit cigarette and thus exposes the non-smoker to harmful carcinogens. SS is also known to be equally as harmful to fertility as mainstream CS [15] and CS toxicants have been quantified in reproductive tissues and fluids of women who smoke at concentrations greater than in the serum [16,17].

Contrary to the existing dogma that ovarian follicle loss is mediated via apoptosis, we have shown that follicle loss in the ovary is in fact mediated via autophagy. Our previous work highlighted that CS-exposure increased the loss of ovarian follicles and initiated autophagic signaling in ovarian granulosa cells [18,19]. More recently we demonstrated that CS exposure-induced autophagy arose from both the activation of the pro-autophagic AMPK pathway combined with the inhibition of two anti-autophagic markers, AKT and mTOR [20]. Moreover, two positive regulators of autophagy Beclin 1 (BECN1) and microtubule-associated protein 1A/1B-light chain 3 (LC3) [21,22], were significantly over expressed in CS-exposed ovaries compared to control mice [19].

Having shown that CS-induced follicle loss is primarily mediated via activation of autophagy we postulate that follicle loss can thus be mitigated by inhibiting autophagy. Autophagy can be mediated through (1) the application of pharmacological inhibitors or (2) genetic intervention. In particular, anti-autophagic pharmacological interventions are currently being assessed for their efficacy as an adjunctive treatment to chemotherapy and radiotherapy. Autophagic inhibitors include; (1) Balfilomycin A1, which inhibits maturation of autophagosomes [23], (2) Chloroquine or Lys05, which impairs autophagy through alkalization of the lysosomes, (3) Pepstatin A and E64d, which suppress lysosomal proteases, and (4) Leupeptin, which blocks the degradation steps of autophagy [24]. However, of these, chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) are the most clinically relevant and widely used

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autophagic inhibitors. As CQ and HCQ are safe and effective anti-malarial and anti-inflammatory therapies [25], HCQ was therefore chosen as the inhibitor for the current investigation. CQ was discovered in 1934, but not utilised in medicine for many years as it was believed to be toxic to humans. However since its discovery, we now know that CQ is of low-toxicity to humans and widely employed as an anti-malarial drug. Additionally, it is an effective anti-inflammatory agent used to treat rheumatoid arthritis [26]. CQ accumulates in the lysosomes of cells, increasing the lysosomal pH and subsequently interfering with autophagosome degradation [26] during the late stages of the autophagic signaling pathway.

To evaluate the effects of CQ treatment on CS-induced ovarian autophagy, we used our well-established CS-exposed mouse model [20,27]. The goals of this study were as follows; (1) investigate autophagic markers in the ovary of mice exposed to CS, (2) investigate the effect of CQ alone or in combination with CS on autophagic signaling in the mouse ovary and (3) determine whether CQ is capable of inhibiting autophagic signaling in mice exposed to co-treatment of CS + CQ and determine which dose of CQ had the greater overall protective effect, if any.

2. Materials and method

2.1. Animals and ethics

All animal work in the present study was conducted using protocols approved by the McMaster Animal Research Ethics Board and was in accordance with the Canadian Council for Animal Care guidelines for the use of animals in research. AUP: 14-07-24.

Briefly, female C57BL/6 mice (8 weeks of age at the start of exposure) were obtained from Charles River Laboratories. Mice were maintained in polycarbonate cages at 22 °C ± 2 °C and 50% ± 10% relative humidity on a 12 h light:12 h dark photoperiod and were provided with normal rodent chow (LabDiet; PMI Nutrition International) and tap water ad libitum throughout the experiment. Mice were divided into 6 treatment groups of 10 animals each as follows; CS ± CQ (0, 25 and 50 mg/kg).

2.2. Hydroxychloroquine pellet design and subcutaneous administration

Treatment pellets were prepared using Elvax 40P resin beads (provided by Dupont, Canada, #MSDS 130000022269) as previously described [28,29]. The Elvax resin beads were dissolved in CH_2Cl_2 (dichloromethane), to give a 10%w/v solution during pellet preparation. Chloroquine diphosphate salt (Sigma Aldrich, Oakville Ontario, Canada Cat No.: C6628) was dispersed in the solution and mixed on a magnetic stirrer until the entire drug was uniformly dissolved. The mixture was poured into plastic molds, and marked precisely for cutting. The molds were initially stored at −20 °C and after 24 h stored at −80 °C. The plastic polymer mix was cut according to concentration required (1 mm^3 in size). Individual implants were placed into labelled sterile tubes prior to surgical implantation into mice. The CQ pellets were surgically implanted in the nape of the neck so that each animal received a pellet containing 0 (control), 25 or 50 mg/kg of CQ. The half-life of CQ is approximately 40 days in humans [30].

All surgical procedures were carried out in compliance with the survival surgery guidelines outlined by the central animal facility at McMaster University, permitted by The Ontario Ministry of Agriculture and Food and the Canadian Council of Animal Care. Briefly, the animals were deemed healthy and fully anaesthetized by isoflurane. They were subsequently prepared for surgery using an aseptic scrub. The analgesic, Anafen (10 mg/ml) was subcutaneously administered to the mice to control pain. Elvax pellets containing the HCQ or vehicle (95% ethanol) were implanted subcutaneously in the scapular region of the back through a small surgical incision under isoflurane anesthesia. The incisions were sutured closed and monitored post-surgery. All animals survived the surgery and there were no wound infections or other complications reported.

2.3. Cigarette smoke exposure

Cigarette smoke exposure was initiated one week post-surgical implantation of CQ pellets. Mice were exposed to cigarette smoke twice daily, 5 days a week for 8 weeks using a whole-body smoke exposure system (SIU48; Promech Lab AB). Details of the smoke exposure protocol have been described previously in detail [20,27,31]. Mice were euthanized at the end of the exposure period with CO_2, and ovaries were collected and weighed before processing.

2.4. Transmission electron microscopy

Ovaries were collected and processed as follows; one from each mouse for electron microscopy and one for RNA/Protein analysis, as described previously [18–20]. Briefly, ovaries were excised and fixed with 2% glutaraldehyde buffered in 0.1 M sodium cacodylate buffer containing 0.05% calcium chloride (pH7.4) at 4 °C. Tissue blocks from 10 mice per treatment group were sectioned (section thickness, 75 μm) with a Sorvall TC-2 mictotome and postfixed in 1.5% ferrrocyanide reduced osmium tetroxide, followed by dehydration in ethanol and infiltration in propylene oxide, and then embedded in Epon (Miller-Stephenson Chemical Co., Inc.). Ovarian tissue samples were randomly selected from each treatment group and autophagosomes in granulosa cells were counted in seven different fields of view per ovary at ×7500 magnification and the average number of autophagosomes per mouse per treatment group were calculated. Only granulosa cells with a visible nucleus were counted. Autophagosomes were counted independently by three observers blinded to treatment.

2.5. Quantitative real-time PCR

Total RNA and Protein were isolated from one ovary per mouse using a total RNA/Protein purification kit (NORGEN, Biotek Corp, Thorold Ontario, Canada) as per manufacturer's instructions and quantified by spectrophotometric analysis (Nanodrop). cDNA was then reverse transcribed using an iScript kit (Bio-Rad). Primers were designed using the online tools Primer3, IDTDNA and OligoAnalyzer3.0 and primer products between 50 and 130 bp were selected for analysis. Murine-specific primers were designed (Mobix Lab, Hamilton Ontario, Canada) and primer pairs (SA Biosciences) were prepared (Table 1). PCR amplification was carried out in a 10 μl reaction volume (7 μl master mix) containing 1 μg/100 ng of cDNA (2.5 μl), 1 μl of forward and 1 μl reverse primers (Mobix Lab, Hamilton Ontario, Canada) or 10 μM of forward and reverse primers (SA Biosciences, Toronto Ontario, Canada) with 5 μl of SYBR Green Master Mix (Roche, Mississauga Ontario, Canada) and 2 μl of ddH2O. Real-time PCR reactions were carried out using the Roche LC480 instrument and the program was set as follows: denaturation at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. Samples were run in duplicate and results were averaged. Relative quantification analysis was used to compare the levels of mRNA expression across the controls (room-air and either 0, 25 and 50 mg/kg CQ pellets) and between controls and smoke (CS and either 0, 25 and 50 mg/kg CQ pellets) mice using a housekeeper (reference) gene. Multiple reference genes were considered for normalization but the reference gene *Actb* was selected for analysis as it was constitu-