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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-

Table 1
List of forward and reverse primer sequences used for qPCR analysis [18].

Gene	Left primer (Forward)	Right primer (Reverse)	Product size	% GC content	Melting point (°C)
Beclin-1	CCAGCCAGGATGATGTCTAC	CCCGATCAGAGTGAAGCTATT	51	55/47.2	58.1/58.42
Lc3b	CACTCCCATCTCCGAAGTGTA	TGCGAGGCATAAACCATGTA	72	52.38/45	60.12/60.1
Actb	CAT NUMBER: PPM02945B-200	CAT NUMBER: PPM02945B-200	N/A	N/A	N/A

tively expressed and did not change under all treatment conditions. Results are presented as the mean normalized ratios of the internal reference gene.

2.6. Western blot analysis

Protein expression was measured from the whole ovarian homogenates isolated from the mice using the total RNA/Protein purification kit as described above. Briefly, following SDS-PAGE and transfer to PVDF membrane (BioRad, Canada), membranes were blocked overnight with 5% (w/v) skim milk in Tris-buffered saline (TBS; 8 g/l [w/v] NaCl, 0.2 g/l [w/v] KCl, and 3 g/l [w/v] Tris base; pH 7.4) with Tween-20 (TBS-T; 1 × TBS and 0.5% [v/v] Tween-20) or a blocking solution of 5% BSA:TBS-T (depending on the manufacturer's instructions for the antibody) and incubated with primary antibody at 4 °C overnight. The following antibodies and dilutions were used for the present study: Beta-actin (ACTB; 1:5000; Abcam), Beclin-1 (BECN1; 1:1000; CST) and microtubule-associated protein 1 light chain 3 (LC3B; 1:2000; Novus). Following washing with TBS-T, blots were incubated with horseradish peroxidase-conjugated secondary anti-rabbit immunoglobulin (Ig) (1:4000; Amersham Biosciences) or anti-mouse IgG (1:4000; Amersham Biosciences) antibodies for 1 h at room temperature. Blots were thoroughly washed in TBS-T followed by TBS, whereupon reactive protein was detected using ECL Plus chemiluminescence substrate (Amersham Biosciences) and Bioflex X-ray film (Clonex Corporation). Densitometric analysis of immunoblots was performed using Image J 1.37v software (National Institutes of Health); all proteins were quantified relative to the loading control ACTB.

2.7. Statistical analysis

Statistical differences were assessed using GraphPad Prism software. Data were initially checked for normality by applying a D'Agostino & Pearson omnibus normality test, followed by either a one-way ANOVA for comparison across the three treatment groups (0, 25, or 50 mg/kg), followed by either a *T*-Test (normally distributed data) or a Mann-Whitney (non-normally distributed data) test. The qPCR results are presented as normalized mean ratios and ±SEM of the target:reference gene. Representative Western blot results are shown and ACTB was used as the loading control. A p value of 0.05 was considered significant for all statistical comparisons.

3. Results

3.1. Animal health

Treatment with CS had no effect on the general health of the mice, as shown by absence of stereotypical behaviors of smoking mice [19], hunched back and signs of lacrimation, porphyria, or ruffled coat. In mice exposed to 8 weeks of whole body CS exposure the body weight and relative ovarian weights were lower as compared with sham, room-air exposed controls. This was consistent with our previous investigation [18].

3.2. Cigarette smoke induces body-weight changes in mice

There was no effect of CQ (25 and 50 mg/kg), on body weight in control (room-air exposed) mice (Fig. 1A). However, in mice exposed to 8 weeks of CS the whole body weights were 16% and significantly lower than in the control group exposed to room air (0 mg/kg CQ; p <0.0001) (Fig. 1B). Additionally, smoke-exposed mice implanted with a 25 mg/kg CQ pellet revealed a 5% increase in body weight and the 50 mg/kg CQ pellet treated mice revealed a 7% significant increase in bodyweight compared to mice exposed to CS only (p = 0.0276; Fig. 1C).

The ovarian weights were measured relative to body weight. Relative ovarian weight was unaffected by CQ treatment (Fig. 2A). The data revealed a trend towards a decrease in relative ovarian

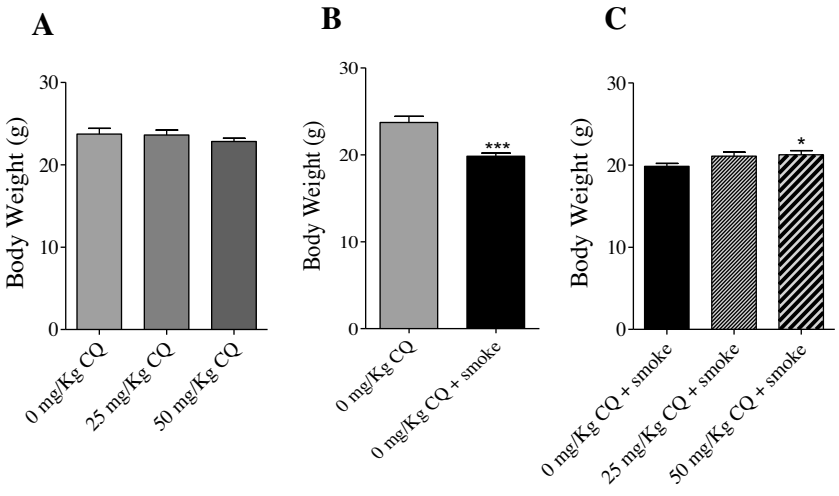

Fig. 1. Body weights (g) of mice at time of sacrifice. (A) Body weights of mice from the 0 mg/kg, 25 mg/kg and 50 mg/kg of chloroquine + no smoke treatment groups (exposed to room air only), (B) body weights of mice from the 0 mg/kg chloroquine + no smoke and mice from the 0 mg/kg chloroquine + smoke treatment groups. (C) Body weights of mice from the 0 mg/kg, 25 mg/kg and 50 mg/kg of chloroquine + smoke treatment groups. Each treatment group; n = 10. One way-ANOVA and Student's *T*-Tests were carried out and values p < 0.05 were deemed to be statistically significant. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

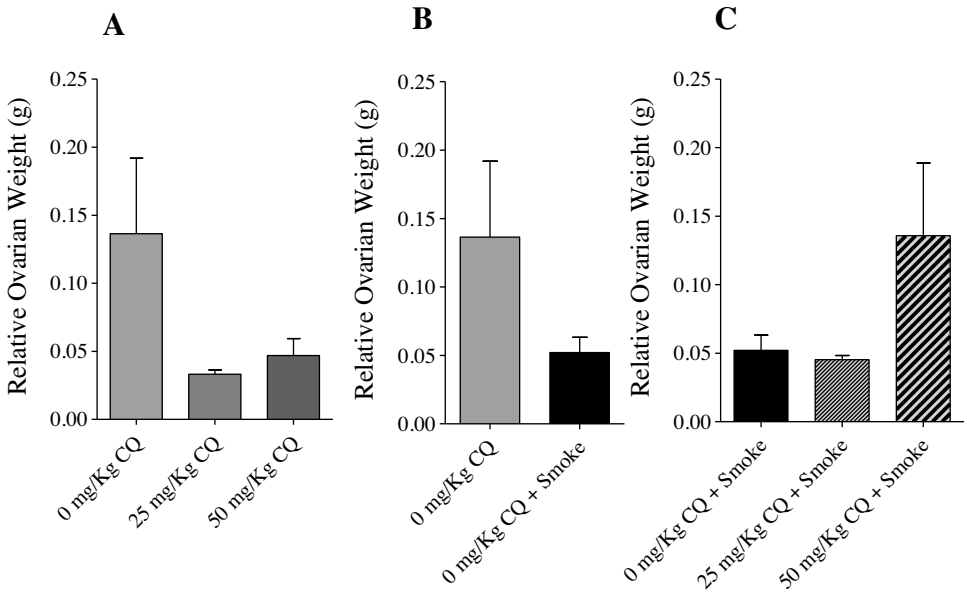

Fig. 2. Weight of ovary relative to body weight. Chloroquine containing pellets of either 0 mg/kg, 25 mg/kg or 50 mg/kg doses) were implanted in the nape of the mouse neck, and animals were exposed to cigarette smoke for 8 weeks. Mice were weighed at sacrifice to determine whether chloroquine and cigarette smoke treatment affected ovarian weight. (A) Ovary weights of mice from the 0 mg/kg, 25 mg/kg and 50 mg/kg of chloroquine + no smoke treatment groups, (B) ovary weights of mice from the 0 mg/kg chloroquine + no smoke and mice from the 0 mg/kg chloroquine + smoke treatment groups. (C) Ovary weights of mice from the 0 mg/kg, 25 mg/kg and 50 mg/kg of chloroquine + smoke treatment groups. Cigarette smoke exposure results in smaller ovaries. Relative ovarian weights of smoke exposed and control mice indicate that smoke exposed mice have smaller ovaries relative to body size. Each treatment group; $n = 10$. One way-ANOVA and Student's *T*-Tests were carried out and values $p < 0.05$ were deemed to be statistically significant. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

weight in the CS-exposed mice, although not statistically significant ($p = 0.0941$; Fig. 2B). Similarly, there was a 160% increase of relative ovarian weight in CS-exposed mice which were treated with 50 mg/kg CQ that verged on significance ($p = 0.0551$; Fig. 2C).

3.3. Cigarette smoke increases the quantity of autophagosome formation

Nuclei in the CS-exposed granulosa cells was displaced by autophagosomes (Fig. 3D). Additionally, autophagosomes were more abundant in granulosa cells of ovaries from the CS-exposed mice compared to room-air exposed controls. There was a low abundance of autophagosomes and no statistically significant difference between the 0, 25, and 50 mg/kg CQ groups which were exposed to room-air only (Fig. 3A–C and G). However, in mice exposed to CS, there was a statistically significant increase in autophagosomes as compared to the room-air exposed control ($p = 0.0297$; Fig. 3D and H). While the 25 mg/kg CQ treatment induced a partial decrease ($p = 0.0505$), mice treated with 50 mg/kg CQ had a significant decrease in the number of CS-induced autophagosomes ($p = 0.0065$) as compared to the 0 mg/kg treatment in CS-exposed mice, highlighting a protective function of CQ against CS-induced autophagosomes (Fig. 3E, 3F and I).

3.4. Cigarette smoke activates the autophagic cascade whereas chloroquine attenuates autophagy

3.4.1. BECN1

Real-time PCR and Western blot analysis were performed on the extracted RNA (cDNA) and protein from the ovaries of the mice to make comparisons between gene and protein expression of the autophagic markers *Beclin1* (BECN1) and *Lc3b* (LC3B). The changes in expression of each gene and protein were measured as a ratio, relative to the internal housekeeping gene *Actb* (ACTB). CQ treatment had no significant effect on *Beclin1* gene expression compared to the control group (Fig. 4A). CS-exposure had no effect on *Beclin1* mRNA expression ($p = 0.4657$; Fig. 4B). Similarly, *Beclin1* gene expression was comparable in CS-exposed groups treated with CQ as compared with the 0 mg/kg controls ($p = 0.3298$ $p = 0.3049$; Fig. 4C).

In the absence of CS, western blot analysis showed no significant changes in BECN1 expression between CQ treatment groups (Fig. 4D). However, BECN1 was significantly increased in the CS-exposed group in comparison to the control group exposed to room air ($p = 0.0015$; Fig. 4E). Interestingly, the CS-expose mice treated with 25 mg/kg CQ had a significant decrease in BECN1 expression ($p = 0.0334$) while those treated with 50 mg/kg CQ did not have a statistically significant decrease of BECN1 ($p = 0.0902$; Fig. 4F).

3.4.2. LC3B

In both CS-exposed and room air exposed mice, there was no effect of CQ treatment on *Lc3b* expression (Fig. 5A, B). However, further analysis of the CS-exposed groups revealed a significant decrease of *Lc3b* mRNA in response to 25 and 50 mg/kg CQ treatment; ($p = 0.0483$ and $p = 0.0347$; Fig. 5C).

LC3B protein expression was measured in CQ-treated groups in the absence of CS and no statistically significant changes were observed (Fig. 5D). While LC3B expression increased in the CS-exposed group as compared to mice exposed to room air and trended toward statistical significance ($p = 0.0583$; Fig. 5E), there was a significant decrease in LC3B expression in CS-exposed mice treated with 50 mg/Kg CQ ($p = 0.0413$; Fig. 5F) as compared to CS-exposed controls (0 mg/kg).

4. Discussion

In the current study, we examined the effects of the autophagic inhibitor chloroquine (CQ) on CS-induced autophagy in the ovary of adult mice. While mice exposed to CS showed an increase in the number of autophagosomes in the ovarian granulosa cells, which is harmonious with our previous work [19], CQ treatment attenuated CS-induced autophagy. Moreover, expression of autophagic markers were decreased in the mice exposed to CS and treated with 25

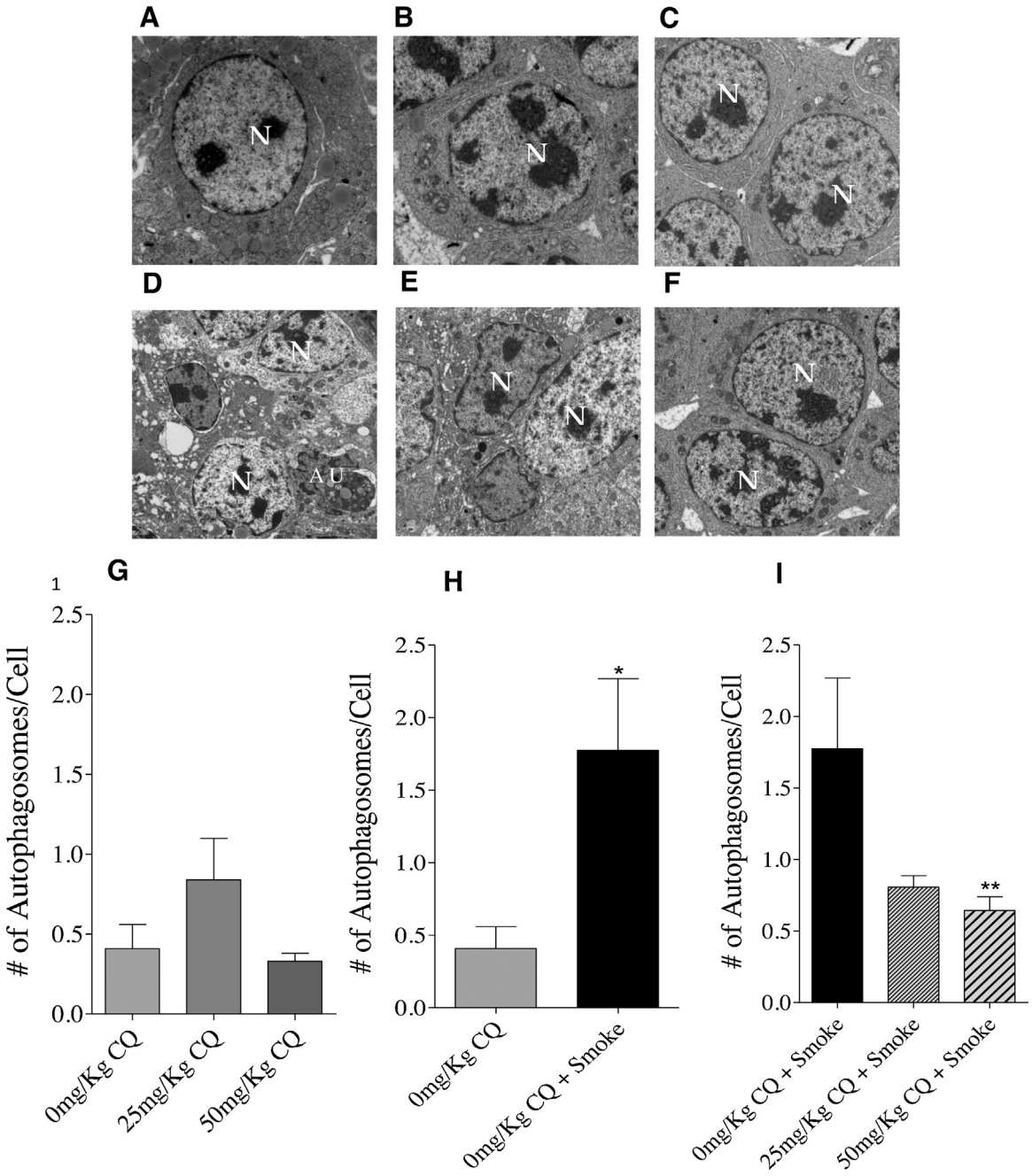

Fig. 3. Transmission electron microscopy revealed that autophagosomes are present in the granulosa cells of ovarian follicles exposed to cigarette smoke. Representative transmission-electron micrographs of granulosa cells from antral follicles are shown, (A) 0 mg/kg Chloroquine, (B) 25 mg/kg Chloroquine, (C) 50 mg/kg Chloroquine, (D) 0 mg/kg chloroquine + cigarette smoke, (E) 25 mg/kg chloroquine + cigarette smoke and (F) 50 mg/kg chloroquine + cigarette smoke exposed ovaries are shown. Nuclei (N) appear normal but are displaced by autophagosomes (Au) in smoke-exposed ovaries. Original magnification is ×7500. The incidence of autophagosomes in the granulosa cells of smoke-exposed ovaries are shown in the following groups (G) 0 mg/kg, 25 mg/kg and 50 mg/kg of chloroquine + no smoke treatment groups, (H) 0 mg/kg chloroquine + no smoke and 0 mg/kg chloroquine + smoke treatment groups (I) 0 mg/kg, 25 mg/kg and 50 mg/kg of chloroquine + smoke treatment groups. Each treatment group; $n = 10$. $^{*}P < 0.05$, and $^{**}P < 0.01$.

or 50 mg/kg CQ. Lower numbers of autophagosomes coupled with a greater body weight compared to the smoke exposed 0 mg/kg CQ group suggests that CQ attenuates CS-induced autophagy and offers the prospect of ovarian protection from toxic insult.

Herein we demonstrated that CS exposure alone induced an increase in the number of autophagosomes in ovarian granulosa cells compared to control animals that were only exposed to room air. These results are congruent with our prior studies [18,19]. However, in contrast to our previous studies, CS had no effect on *Beclin1* or *Lc3b* gene expression whilst BECN1 and LC3B protein expression was increased compared to room air + 0 mg/kg CQ control group. Divergent results in *Beclin1* and *Lc3b* mRNA expression between this and our prior studies are most likely the result of high variation in expression.

CS exposure activates oxidative stress measured by increased Hsp25 and decreased superoxide dismutase 2 protein expression [18]. More specifically, CS exposure triggers antral follicle destruction and increased oocyte dysfunction through oxidative stress [32] causing increased ovarian follicle loss in the mouse [18,19,33] coupled with activated autophagy. It was previously thought that

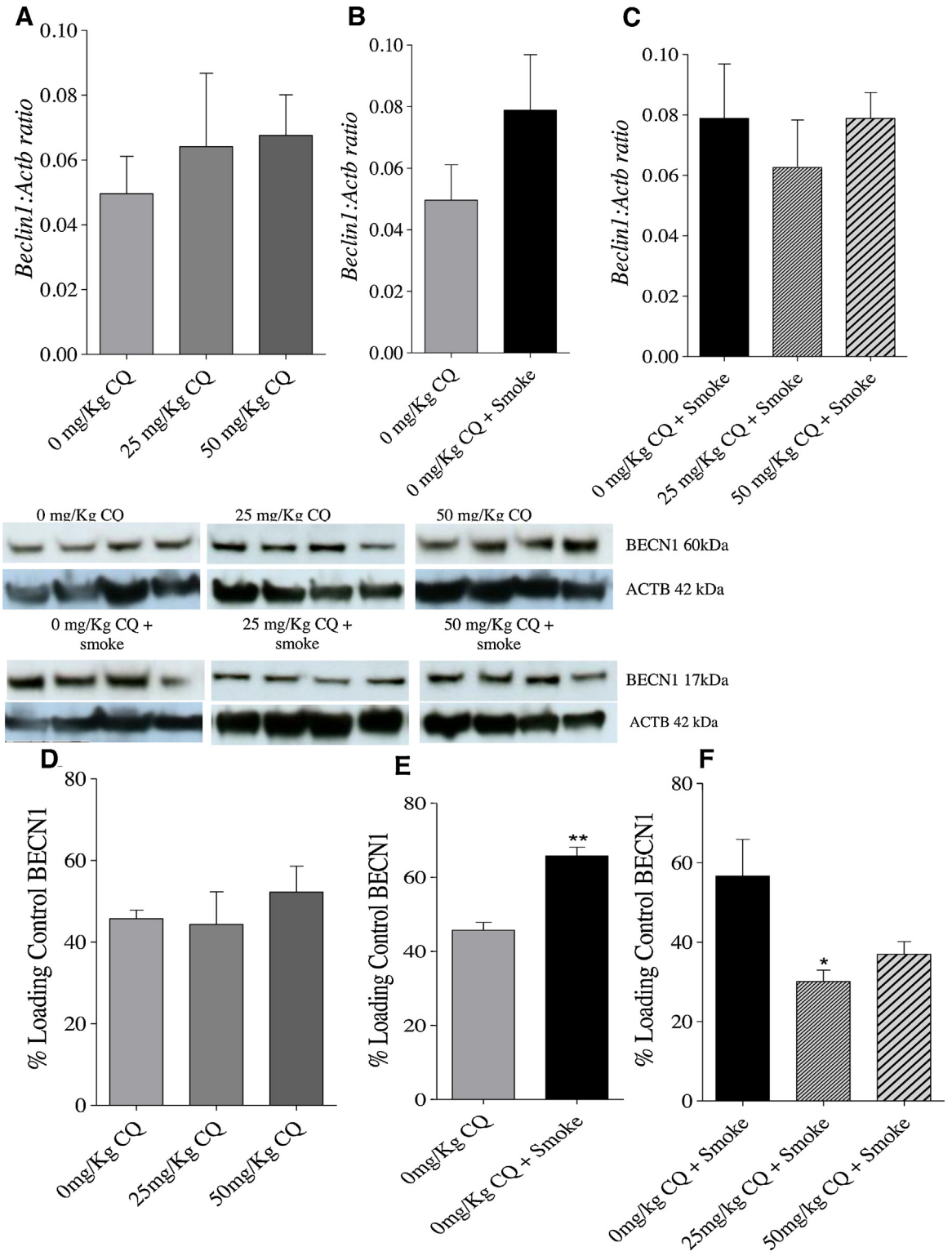

Fig. 4. Changes in *Beclin1* gene expression was performed on whole-ovarian homogenates from the cigarette smoke and chloroquine exposed mice. Panel (A–C) show qPCR gene expression changes relative to the *actb* control (n = 10 per group). Panel (D–F) show representative immunoblots and graphs demonstrating BECN1 protein expression changes relative to the ACTB loading control (n = 10 for each treatment group). Data were checked for normality and equal variance, and treatment effects were tested using *T*-test. Values are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

apoptosis played a role in CS-induced oxidative stress leading to ovarian follicle loss, however the pro-survival marker *Bcl-2* was shown to increase [33], and further investigations revealed a role for the alternative cell-death pathway, autophagy [19]. The same study revealed increases in autophagic markers, BECN1 and LC3B and increased production of autophagosomes. LC3B can have either an anti-apoptotic role in healthy cells or a pro-apoptotic role during CS exposure [34]. The activation of CS-induced autophagy in the ovary was recently shown to be activated through enhancement of AMPK [20]. Similarly, exposure to particulate matter (PM2.5) can

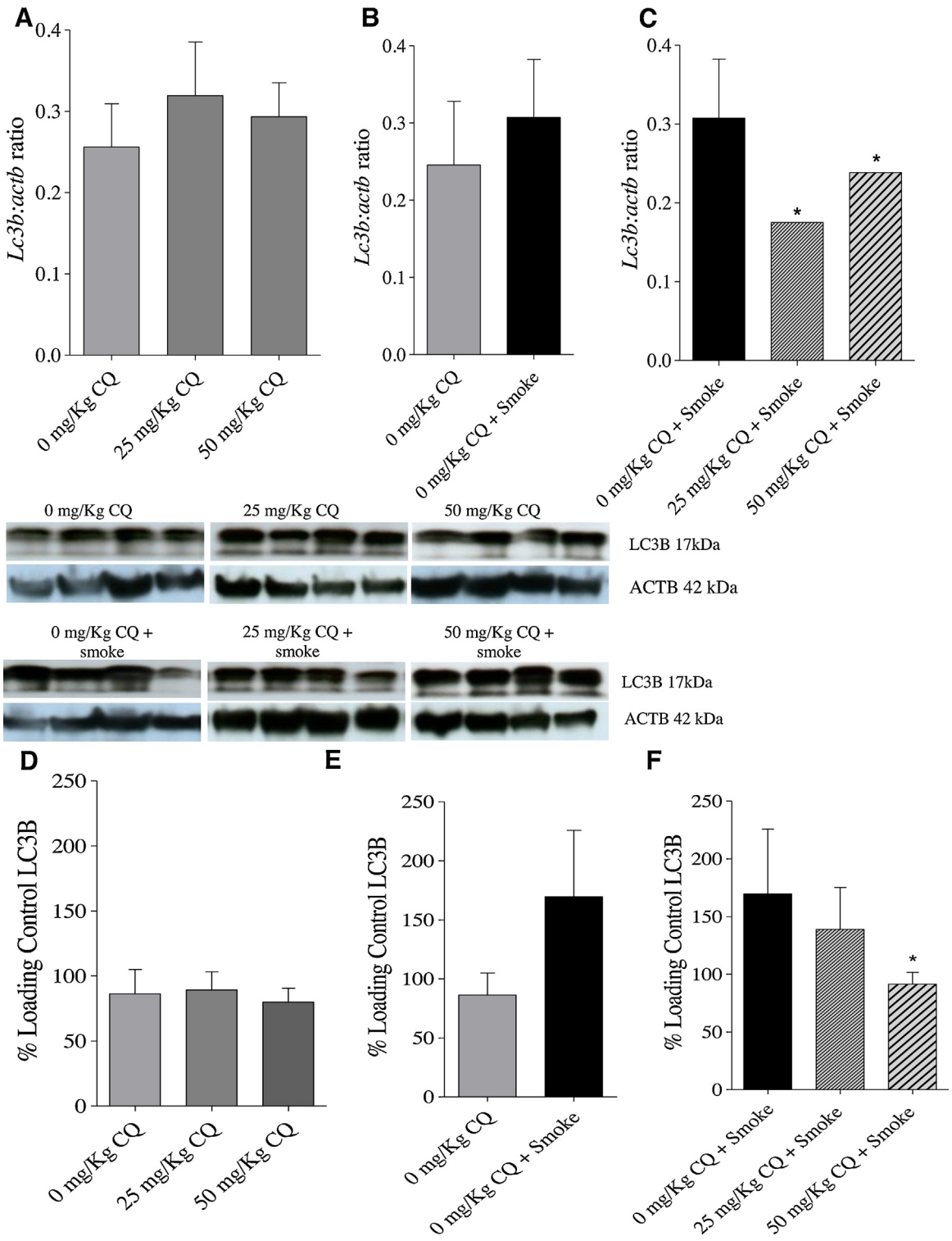

Fig. 5. Changes in *Lc3b* gene expression was performed on whole-ovarian homogenates from the cigarette smoke and chloroquine exposed mice. Panel (A–C) show qPCR gene expression changes relative to the *actb* control (n = 10 per group). Panel (D–F) show representative immunoblots and graphs demonstrating LC3B protein expression changes relative to the ACTB loading control (n = 10 for each treatment group). Data were checked for normality and equal variance, and treatment effects were tested using *T*-test. Values are expressed as mean ± SEM. $^{*}P < 0.05$.

instigate AMPK mediated autophagy in cell culture [35]. Thus, toxicant induced changes in autophagy are not unique to the ovary nor to CS exposure, suggesting that activation of autophagy is a common step in the pathway to toxic phenotypes. CS-induced autophagy has been shown in the lung and believed to contribute to the development of chronic obstructive pulmonary disease (COPD), [36–39] and thus is a potential target for disease therapy. Clinical studies investigating COPD have revealed a role for CS-induced oxidative damage coupled with mitochondrial associated autophagy [40]. CS-induced autophagy is believed to be selected over apoptosis as an adaptation to cellular stress, which is perhaps why the pathway is implicated in many cancers; as a result of apoptotic resistance [41,42]. Autophagy can contribute to the survival of dormant tumor cells instigating protection from chemotherapy

and radiotherapy and subsequent tumor resistance [43]. Chemical inhibition of autophagy may therefore inhibit the growth of the tumor in question yielding therapeutic benefit [44]. Therefore, we postulate that therapeutic disruption of autophagy is a potential target that may provide protection from CS or other chemical toxicant-induced damage.

In the present study, mice treated with CQ pellets implanted prior to CS exposure had a decreased number of autophagosomes in their ovarian granulosa cells as compared to mice treated with 0 mg/kg CQ which was also CS exposed. Furthermore, co-treatment with CQ and CS attenuated BECN1 and LC3B protein expression but not gene expression. CQ is a known inhibitor of autophagy and acts through its accumulation in the lysosomes, which increases the lysosomal pH and subsequently interferes with autophagosome degradation in the lysosomes [26]. Accordingly, inhibition of autophagy may either promote or inhibit cell death, which is ultimately dependent on the cell conditions at the time and the damaging agents a cell is exposed. CQ is able to inhibit cell proliferation and induce cell death in CT26 mouse colon fibroblast cells in vitro, results that were repeated in vivo indicative of the potential chemotherapeutic relevance of CQ [45]. Tissue uptake of CQ as a function of dosage is non-linear and CQ is metabolised through the kidneys (40–60%), excreted in feces (8–25%), sloughed off the skin (5%) and stored long term in lean body tissues (25–45%). Cells are known to utilise the process of autophagy to maintain cellular homeostasis, and so inhibition of autophagy through CQ sensitizes cells/tissues to treatment [46]. Therefore there are limitations associated with CQ therapeutic treatment including the potential adverse effects on normal cellular processes. Furthermore, the half-life of CQ is between 30 and 60 days [47], which substantiated the CS exposure period chosen for the current study. However, CQ could be metabolised at a faster rate in mice, and could therefore account for lack of effect at lower doses administered in the current study. Alternative methods for ovarian protection including targeted inhibition of aryl hydrocarbon receptor (AhR) activation, which occurs upstream of autophagy, have been considered. The effects of AhR ligand and CS constituent, benzo[a]pyrene was examined in isolated rat follicle cultures in vitro coupled with assessment of the effects of the AhR antagonist resveratrol, and revealed some promise for ovarian protection [3]. However, resveratrol functions at multiple molecular target sites and therefore lack of specificity for the autophagy pathway, was not considered further for the current study.

In summary, in the current investigation we demonstrated that CQ treatment attenuated some effects of CS on the autophagic markers LC3B and BECN1 which are upstream of autophagy, and could potentially protect the ovary from CS-induced autophagic signalling and ovarian follicle loss. The therapeutic benefits of CQ are well defined in other disease models; however, this study expands the literature elucidating beneficial effects of CQ in attenuating CS-induced autophagy in the ovary. Therefore, we suggest that ovarian protection from toxic insults may be achieved through interference with the autophagy cascade although the effects on fertility preservation have yet to be demonstrated.

Transparency document

The Transparency document associated with this article can be found in the online version.

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