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# Repeated hyperstimulation affects the ultrastructure of mouse fallopian tube epithelium

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**Short title:** Morphology of hyperstimulated ampullae

## ABSTRACT

Controlled ovarian hyperstimulation (COH) is routinary used in assisted reproductive technologies (ARTs) to increase the yields of mature oocytes. The possibility that patients with a history of failures or poor-responders may develop side-effects following these treatments is still debated. Epidemiological studies reported controversial results about pregnancy outcome and the risk of developing gynecological cancers. By using a mouse model, here we compared the ultrastructural features of Fallopian tubes (FTs) obtained from mice undergoing or not (control, CTR) four (4R) and eight (8R) rounds of gonadotropin stimulation.

Although the morphological characteristics of oviductal layers seemed unaffected by repeated treatments, dose-response ultrastructural alterations in the ampulla appeared in the 4R group and even more in the 8R group. The targets were oviductal ciliated (CCs) and non-ciliated (NCCs) cells, which showed damaged mitochondria and glycogen accumulations in the cytoplasm. The drastic reduction of CCs, evident after 4R, was supported by the absence of cilia. After 8R, glycogen granules were significantly reduced and massive degeneration of mitochondria, which appeared swollen and/or vacuolated, occurred in NCCs. Moreover, disintegrated mitochondria were found at the periphery of mitophagic vacuoles with evident signs of cristolysis. The morphometric analysis evidenced a significant increase in the density and frequency of damaged mitochondria after 4R and 8R. The absence of cilia, necessary to sustain oviductal transport of oocytes, spermatozoa and embryos, may originate from either mitochondrial dysfunction or glycogen consumption.

These results suggest that repeated COH treatments could induce alterations impairing fertilization and embryo transport toward the uterus.

**Keywords:** mouse, fallopian tubes, controlled ovarian hyperstimulation, columnar epithelial cells, electron microscopy.

## INTRODUCTION

Controlled ovarian hyperstimulation (COH) is routinely used in Assisted Reproductive Technologies (ARTs) to increase the number of retrieved mature oocytes and, therefore, to enhance the success rate of these protocols [1]. However, increased number of treatments (n>4), especially with gonadotropins, can induce complications in treated patients, including premature progesterone elevation throughout the follicular phase, defective endometrium receptivity, luteal phase insufficiency or severe ovarian hyperstimulation syndrome (OHSS) [2-4]. Although the relationship between hyperstimulation and cancer is still controversial [5-6], a major risk is that COH-induced supraphysiologic estrogen (E2) levels may promote, with time, the growth of estrogen-sensitive tumors, such as endometrial, ovarian and estrogen receptor-positive breast cancers [7-9].

An interesting question is how fallopian tubes (FTs) could respond to COH. Indeed, during the reproductive cycle, hormonal oscillations impact on the histophysiology of the FTs and, specifically, of the *tunica mucosa* [10, 11]. In mice, the relationship between the number of columnar epithelial ciliated cells and non-ciliated cells - also known as secretory, intercalar or peg cells - changes during the hormonal cycle with the predominance of ciliated cells during the follicular phase and of secretory cells during the luteal phase, even if with individual differences [12]. In this species, E2 stimulates epithelial cell hypertrophy, cilia beat frequency (CBF) and oviductal secretions [13], while progesterone (P) acts on *tunica mucosa* by inducing epithelial cell atrophy, deciliation and decrease of CBF [14, 15]. If P production is altered, a delay in the transportation timing of the ovum or zygotes occurs [16]. In our previous papers, we analyzed oocyte spindle morphology and the content of proteins involved in cell cycle control in ovaries and FTs retrieved from mice treated up to 8 times (8

Rounds, 8R) with a standardized protocol of gonadotropin [17, 18]. Despite after 4R the only modification recorded was an increase in cyclin D1 content in FT, after 8R also p53, phospho-p53, phospho-AKT, GSK3B and OCT3/4 contents were significantly higher than in controls. The over-expression of these proteins, all involved in the control of cell cycle progression, and the drastic decrease of oocyte number and spindle quality strongly supported an altered differentiation processes of FT epithelial cells and an impaired ovulation and oocytes spindle organization [17-19].

Physiological/pathological changes of FT epithelium have been found in different species as rat [20], hamster [21], pig [22], goat [23] and horse [24]. Also, the ultrastructural features of mucosa and epithelial cells, such as the presence, distribution, and location of secretory and ciliated cells in the various portions of the human and murine FTs during the hormonal cycle have been described [25, 26]. Interestingly, in mouse FTs the percentage of the ampullar epithelial cells and their proliferation rate were found to be estrus cycle-dependent [26]. Exogenous ovarian steroids, especially E2, accelerate the differentiation and maturation of secretory cells in mouse FTs [27].

However, the effects on FT ultrastructure exerted by COH are, to our knowledge, not yet investigated. Therefore, in the present study, we evaluated the ultrastructure of the ampulla, the third portion of mouse FTs, following 4R and 8R of gonadotropin administration by transmission (TEM) and scanning (SEM) electron microscopy.

## MATERIALS AND METHODS

### *Animals*

93 *Mus musculus* Swiss CD1 female adult mice (2-3-month-old; Harlan Italy, Udine,
94 Italy, *N* = 15) were housed in the animal facility under controlled temperature (21 ± 1°C),
95 with 12:12 h light: dark cycle and free access to food and water. All the experimental
96 procedures and the animals were maintained in accordance with national and international
97 law and policies (European Economic Community Council Directive 86/609, OJ 358, 1 Dec
98 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana n.
99 40, Feb 18, 1992; National Institutes of Health Guide for the Care and Use of Laboratory
100 Animals, NIH publication no. 85-23, 1985). The Italian Ministry of Health and the local
101 committee (University of L'Aquila) for the animal care and use approved the experimental
102 protocols, in compliance with the accepted veterinary medical practice. Animals were
103 euthanized by cervical dislocation after an inhalant overdose of carbon dioxide ($CO_2$, 10-
104 30%), followed by cervical dislocation. All efforts were made to minimize animal suffering.
105
106 ***Experimental protocols and hormonal treatments***
107 Animals in which the early luteal phase of the estrous cycle was evaluated by
108 examination of the vagina and vaginal smears (N = 4), and without any hyperstimulation,
109 were used as Control (Ctr). Repetitive cycles of ovarian stimulation were performed
110 according to previous studies [17, 18]. Briefly, mice (N = 11) were injected i.p. with 5 IU of
111 PMSG (pregnant mare serum gonadotropin) (Folligon, Milano, Italy) and after 48 h with 5
112 IU of hCG (human chorionic gonadotropin) (Corulon, Milano, Italy). The timing of four to
113 eight repeated rounds (4R, 8R) of stimulation was selected according to [17] and [18]. In
114 particular, the COH protocol (PMSG+hCG) was repeated four times in the mice of 4R group
115 (N=6) and eight times in those of 8R group (N=5). All the rounds were performed at intervals

of one week between each. After approximately 16 hours from the last hCG injection (in the 4th and 8th week respectively), mice were sacrificed as described above. From each animal, both oviducts were collected, washed in PBS and used for light and electron microscopy analysis.

***Light microscopy (LM) and transmission electron microscopy (TEM)***

Left FTs were isolated from their neighboring ovaries, gently washed in phosphate-buffered saline (PBS) solution and immediately fixed in 2.5% glutaraldehyde (Agar Scientific, Cambridge Road Stansted Essex, UK)/0.1M PBS. Fixed samples were maintained at 4°C for at least 48h until the next preparative for TEM [28-31]. Firstly, FTs from each experimental group (Ctr, 4R, 8R) were cut into small sections to isolate the third portion, i.e. the ampullae. Successively, the pieces were rinsed in PBS, post-fixed with 1% osmium tetroxide (Agar Scientific, Stansted, UK)/0.1 M PBS and rinsed again in 0.1 M PBS. Samples were then dehydrated in ascending series of ethanol (Carlo Erba Reagenti, Milan, Italy), immersed in propylene oxide (BDH Italia, Milan, Italy) for solvent substitution and embedded in epoxy resin EMbed-812 (Electron Microscopy Sciences, Hatfield, PA, USA). Semithin sections (1 mm thick) were stained with Methylene Blue, examined using a light microscope (LM) (Zeiss Axioskop) and photographed using a digital camera (Leica DFC230). Ultrathin sections (60–80 nm) were cut with a diamond knife, on a Reichert-Jung Ultracut E ultramicrotome (Reichert Technologies, Munich, Germany), mounted on copper grids and contrasted with saturated uranyl acetate followed by lead citrate (SIC, Rome, Italy). They were examined and photographed using Zeiss EM10 and Philips TEM CM100 Electron Microscopes operating at 80 kV.

We evaluated the following parameters for the qualitative assessment of the ultrastructural preservation of mouse oviducts: general features; morphology of the *tunicae mucosa*, *muscularis* and *serosa*; membrane integrity of the cells; morphology of the nucleus, chromatin, and nuclear envelope; type and quality of organelles and inclusions; configuration of mitochondria; type of granules, vesicles and vacuoles; intercellular projections; microvillus pattern and presence or extent of cilia [32-36].

***Scanning electron microscopy (SEM)***

Ampullar samples of the right FTs were processed for conventional [37-41] and variable pressure (VPSEM) SEM analysis. For each experimental group (Ctr, 4R, 8R), the isolated oviducts of their neighboring ovaries were fixed in 2.5% glutaraldehyde/0.1M PBS at 4°C for at least 48 h. Selected sections of ampulla were processed either for conventional or VPSEM preparation. Briefly, fixed samples were washed in 0.1M PBS, post-fixed in 1% osmium tetroxide (Agar Scientific, Stansted, UK)/0.1M PBS, washed again in 0.1M PBS and dehydrated in ascending ethanol series (Carlo Erba Reagenti, Milan, Italy). Following critical point drying in a $CO_2$ atmosphere (Emitech K850, Ashford, Kent, UK), samples processed for conventional SEM and VPSEM analysis were mounted onto aluminum stubs and coated with platinum (4 nm in thickness) (Emitech K550 sputter coater, Ashford, Kent, UK). The observations were performed at a low accelerating voltage (5-10kV in conventional SEM (Hitachi S-4000) and at higher accelerating voltage (15kV) in VPSEM (Hitachi SU 3500) [32, 35, 42, 43].

***Morphometric analysis***

The ImageJ software (http://rsbweb.nih.gov/ij/) was used to measure the numerical density and dimension of normal and damaged mitochondria from columnar epithelial cells (CECs) of the *tunica mucosa* on low-magnification TEM micrographs of Ctr, 4R and 8R groups. The numerical density of mitochondria was expressed as number of organelle per 100 $\mu m^2$ of epithelial area [44]. Mitochondria were classified, according to their ultrastructural appearance, in: i) normal, i.e. intact, with round/elongated shape, with electron-dense lamellar cristae; ii) damaged, with either swollen, irregular, or deficient cristae; patchy or degenerated matrix and evident breaks in the wall of mitochondrial membrane. The quantity/plethora of normal and damaged mitochondria per group was also expressed as percentage of total mitochondria numerical density. For each experimental group, at least three sections from three different experiments were selected for the morphometric analysis.

### *Statistical analysis*

Dimension and numerical density of normal and damaged mitochondria were expressed as mean ± standard deviation (SD). Statistical comparisons were performed using one-way ANOVA with Tukey's honest significant difference (HSD) tests for post-hoc analysis (GraphPad InStat. GraphPad Software, La Jolla, USA). Differences in values were considered significant if $P < 0.05$.

## RESULTS

### *Control group*

By LM, the *tunica mucosa* of the ampullar region of FTs was folded in many branches projecting into the lumen and constituted by columnar epithelial cells (CECs), ciliated (CCs) and non-ciliated (NCCs) (Fig. 1A). Cuboidal cells were also found. CCs showed less intense staining than the secretory NCs. The CECs presented integral nuclei delimited by an evident nuclear membrane. The *tunica muscularis* showed the characteristic orientation of smooth muscle fibers, arranged in two usually well-defined layers (Fig. 1A). In particular, the outer layer showed individual fiber bundles oriented in a longitudinal plane while the inner one was made of circular fibers. The *tunica serosa*, the external most layer, was made of a squamous mesothelium and an underlying subtle rim of connective tissue (Fig. 1A).

The TEM analysis of the FT epithelium showed that nuclei were roundish-to-amoeboid in shape, delimited with a continuous and electron-dense nuclear membrane often indented, (Fig. 1B). Inside the nucleus, the chromatin appeared abundant and uniformly distributed or, occasionally, with heterochromatin clustered in clumps or located as marginal patches under the nuclear membrane (Fig. 1B). In the cytoplasm, numerous round and elongated mitochondria with electron-dense lamellar cristae were visible and interspersed with those characterized by an electron-pale content. Mitochondria were often found in association with thin endoplasmic reticulum (ER) tubules and/or networks (Figs. 1B-1D). Highly electron-dense secondary lysosomes and multivesicular bodies were frequently detected (Fig. 1C). Lipid droplets (Fig. 1B) and Golgi apparatus were rarely found scattered throughout the CEC cytoplasm. The cytoplasm also contained moderate electron-dense aggregates of glycogen particles (Fig. 1C, 1E). Electron-negative vacuoles were rarely seen. CCs appeared electron-lucent with motile cilia protruding into the lumen. They consisted of an evident axoneme of nine peripheral doublet microtubules, surrounding a central complex with two central

207 microtubules and the central sheath (9 + 2 arrangement) (Figs. 1E). Among the cilia,
208 numerous long and thin microvilli were also evident (Figs. 1B-E). The luminal side of CCs
209 contained the typical cylindric basal bodies, located in rows immediately beneath the cell
210 membrane (Fig. 1E). NCs showed apical protrusions with numerous short and long
211 microvilli, usually continuous; occasionally, areas with rare microvilli were detected (Figs.
212 1C, 1D). It was also noticed the presence of dense secretory granules (Figs. 1C, 1D).
213 Junctional complexes between neighboring epithelial cells were well developed (1B).
214 Usually, adjacent epithelial cells were connected by apical *zonulae occludens*, followed by
215 *zonulae adhaerens* (Figs. 1B, 1D).
216
217 ***4 ROUNDS***
218 After 4R of COH, the *tunica mucosa* of FTs was folded and highly branched, as seen
219 by LM. The epithelium, as in the control group, was made by a single layer of cuboidal or
220 columnar cells, whose round/ovoid nuclei were intensely stained (Fig. 2A). Both the outer
221 and the inner layers of the *tunica muscularis* were visible in transversal sections (Fig. 2A).
222 The *tunica serosa* did not show any difference in respect to controls (Fig. 2A).
223 The ultrastructural analysis by TEM showed single-layered CECs with round-to-ovoidal
224 nuclei delimited by an uninterrupted electron-dense nuclear membrane. Irregularly shaped
225 nuclei showed a marginal organization of electron-dense heterochromatin, sometimes
226 organized in highly condensed patches, with occasionally dense nucleoli (Figs. 2B-2E). In
227 the cytoplasm, round/elongated and electron-dense mitochondria were found together with
228 abundant severely swollen mitochondria, characterized by a pale and marginalized content,
229 degenerated matrix and a few or damaged cristae (Figs. 2B-2F). Numerous electron-negative

vesicles were observed and, at high magnification, most of them were autophagosomes containing degraded mitochondria (Fig. 2E and *inset*). Prominent intracytoplasmic vacuoles lined by microvilli were occasionally detected, presumably corresponding to intracytoplasmic pouches of the plasma membrane (Fig. 2F). Multivesicular bodies with large vesicles, high electron-dense secondary lysosomes, ER tubular elements, and lipid droplets were also found (Figs. 2B, 2D). Small elements of the Golgi complex and glycogen accumulations were rarely detected (not shown). Among CECs, intercellular junctions were well preserved and organized as in controls (Figs. 2B, 2C). The microvillar distribution pattern in NCs did not show evident variations, respect to those seen in the control group. However, areas with rare microvilli were occasionally found (Fig. 2B, 2C). In the observed sections, CCs were not found.

***8 ROUNDS***

After 8R, the *tunica mucosa* and *tunica muscularis* did not appear different respect to the previous groups, as seen by LM (Fig. 3A). The nuclei of CECs from the highly folded *tunica mucosa* and the two layers of *tunica muscularis* were detected, as previously described (Fig. 3A). The *tunica serosa*, when visible in section, did not show any difference in respect to the previous groups (Fig. 3A).

TEM analysis showed that the general ultrastructure of nuclei, lipid droplets, ER tubules/networks, Golgi apparatus, secondary lysosomes, multivesicular bodies, microvilli and intercellular connections was similar between 4R and 8R (Figs. 3B-3F). Similarly to the 4R group, the most evident and peculiar morphological alterations affected the mitochondria. Mitochondrial damages were observed more frequently respect to 4R. They ranged from a

severely swollen appearance up to a disrupted aspect with electron-negative content and partial or complete cristolysis (Fig. 3C). The matrix of swollen mitochondria appeared patchy, and often, the mitochondrial membrane presented evident breaks in the wall (Figs. 3B-3D). Mitochondria showing a partly swollen aspect, with some area of still dense matrix, were also found together with a few rounds/elongated normal electron-dense mitochondria with lamellar cristae (Fig. 3B). Mitophagic vacuoles were often close to lysosomes or multivesicular bodies (3E). Secretory granules, with an electron-dense homogeneous content, appeared beneath the cell surface facing the lumen in NCs (Fig. 3F). Coated vesicles were occasionally detected as in the 4R group; CCs with cilia projecting into the lumen and glycogen accumulations were not found in the observed sections.

***Surface Ultrastructural Analysis by SEM***

Representative micrographs of CECs of *tunica mucosa* from the ampullar region of FTs taken by conventional SEM (Figs. 4A-4C) and VPSEM (Fig. 4D) were selected. A panoramic micrograph showed tufts of cilia nonuniformly distributed in the surface of CCs, protruding into the lumen and surrounded by short microvillar projections of NCs (Fig. 4A, control group). At higher magnification, a single tuft of cilia appeared well preserved (Fig. 4B, control group). Numerous microvilli densely covered the NCs surface. They were long and homogeneous (Fig. 4C, 4R group) or short and not homogeneously distributed (Fig. 4D, 4R group).

***Morphometric analysis***

Morphometric analysis revealed that the dimension (expressed as mean ± SD) of normal mitochondria per 100 $\mu m^2$ was 0.444 ± 0.096 μm in Ctr, 0.415 ± 0.060 μm in 4R and 0.472 ± 0.086 μm in 8R, without significant variations among groups ($P$>0.05) (table 1). When dimension was measured in damaged mitochondria, we found a significant increase after 4R (0.553 ± 0.118 μm *vs.* 0.659 ± 0.099 μm; $P$<0.05) and 8R (0.553 ± 0.118 μm *vs.* 0.817 ± 0.179 μm; $P$<0.001). This increase was significant also between treated groups (0.659 ± 0.099 μm *vs.* 0.817 ± 0.179 μm, 4R *vs.* 8R respectively; $P < 0.001$) (table 1).

The numerical density of normal mitochondria dramatically decreased after 4R and 8R, respect to Ctr group (5.3 ± 0.5 and 9.6 ± 4.1 *vs.* 40 ± 12.1, respectively; $P < 0.01$), with no significant variation among treated groups ($P$>0.05) (table 1).

Concomitantly, the density of damaged mitochondria significantly increased after 4R, respect to controls (57.6 ± 7.3 *vs.* 22.3 ± 2.3, respectively; $P$<0.01); however, after 8R the increase was not significant respect to controls (33 ± 7.8 *vs.* 22.3 ± 2.3, respectively; $P > 0.05$) (Table 1).

Quantification of the frequency of damaged mitochondria among groups revealed that their percentage significantly increased after 4R, by reaching the highest values, and less after 8R (36.665 ± 7.629 %, 91.45 ± 1.41 % , 78.103 ± 4.405 %, CTR *vs.* 4R *vs.* 8R, respectively; $P < 0.05$) (Table 1).

## DISCUSSION

Gonadotropins are used for COH but their effects after repeated cycles of hyperstimulation on the ampullar epithelium of FTs have not yet investigated from an ultrastructural point of view. We found that, after 4R and 8R, the *tunicae mucosa* and

298 *muscularis* of mouse FTs appeared well-preserved by LM with no evident signs of
299 morphological alterations, as in other mammals [45, 46]. The ultrastructural analysis of CECs
300 by TEM showed, in all groups, the presence of well-preserved nuclei and cell junctions, lipid
301 droplets, ER tubules/networks, Golgi apparatuses, secondary lysosomes, multivesicular
302 bodies and microvilli, in accordance with the literature describing the physiological
303 morphology of oviducts [23, 24, 27]. However, electron microscopy evidenced specific
304 changes affecting mitochondria and cilia of the CECs of the mucosa layer, especially after
305 8R.
306 Literature data show that repeated rounds of hyperstimulation may affect at a different extent
307 the female reproductive tracts by inducing a decrease in the pregnancy rates from 25.2% to
308 17.8% after the 4R, and up to 11.2 % after more than 12R [47]. This can be accounted for
309 significant changes in oocyte and follicular quality, as well as for the reduced developmental
310 potential of embryos [17, 19, 48, 49]. In particular, after 4R there were observed a decrease
311 in the percentage of mouse oocytes with a uniform distribution of mitochondria, an increase
312 in the aggregation of the Golgi apparatus and endoplasmic reticulum, and the induction of
313 spindle disruption [49]. However, in a recent study on women stimulated by a standard
314 antagonist protocol, no adverse effects were found after repeated cycles of COH. At 3R or
315 more the number of mature oocytes, zygotes and clinical pregnancy rates were even higher
316 than those obtained by 2R or less [50]. These contrasting results could be accounted for the
317 milder stimulation used and for a different specie-specific sensitivity that deserves to be
318 better addresses.
319 Repeated cycles of COH were also connected to oxidative stress. In fact, while ROS
320 production augmented after 4R of COH, the *Oct4* transcripts started to decrease [49]. This is

in agreement with our previous data, showing a progressive reduction of the protein OCT3/4 from 4 to 8R in mouse [18]. Moreover, oxidative damages were also detected in mouse ovaries. The oxidative damage, including an increased content of 8-OH-dG, lipid peroxides and protein carbonyls, positively correlated with the number of COH cycles [51]; it induced mitochondrial abnormalities and a decrease in antioxidants compatible with ovarian aging [52]. The effects of COH were determined also in mouse cumulus cells, where the mtDNA copy number decreased as the number of stimulation increased, together with altered DNA methylation in nuclear-encoded DNA polymerase gamma A (PolgA] and consequent changes in PolgA expression [53]. Moreover, mRNA expression levels of cytochrome b (CYTB), cytochrome c oxidase subunit 1 (COX1), NADH dehydrogenase subunit (ND4), and NADH dehydrogenase subunit 2 (ND2), all encoded by mtDNA, were altered following repeated superovulation in cumulus cells [53]. Detrimental effects of repeated cycles of COH on mitochondria were seen by the reduction of ATP production on GV-stage mouse oocytes matured *in vitro* after four rounds [54].

These adverse effects on the mitochondrial function after superovulation in oocytes, cumulus cells and embryos [44, 48, 49], prompted us to focus on the ultrastructure of mitochondria dispersed in the columnar epithelial cells of FTs. Interestingly, it was found the presence of numerous swelled or even disrupted elements, most of them undergoing to phenomena of mitophagy, evident already after 4 repeated cycles, in agreement to a previous study in rabbit oviducts after hCG injection [55]. These ultrastructural alterations of CEC mitochondria due to COH, in agreement with the above reported findings, can be probably responsible for a hampered energetic support of the oviductal epithelium [56, 57]. Moreover, the presence of reduced storage of glycogens after repeated cycles of COH, respect to controls, can further

sustain the hypothesized energetic imbalance at a time where it is expected the fertilized egg to be in the FTs and in need of the proper enzymes and nutrients for growth and survival [58-60].

Reactive oxigen species (ROS) are present in the FT fluids [61]. Raised levels of ROS are responsible for aberrations in the tubal environment which result in infertility [62]. We could, therefore, speculate that repeated cycles of COH in FTs could alter hormonal imbalance, inducing stressful condition with the increase of ROS and the reduction of endogenous antioxidants. ROS, as the nitric oxide (NO), have a relaxing effect on the tubal smooth musculature at physiological concentrations but displayed a cytotoxic action when their levels increase [62]. Some isoform of the NO synthase (NOS) is upregulated by hormones and gonadotropins (LH/hCG), thus increasing NO production [63]; this could probably occur after repeated cycles of COH. Furthermore, the mitochondrial manganese superoxide dismutase (Mn-SOD) of epithelial cells is involved in mitigating oxygen toxicity to ovulated oocyted and developing embryos in the FT. Its reduction induces a redox imbalance and subsequent mitochondrial damage, as evidenced by swallowing, vacuolization or barely visible cristae found here and by others [61].

The morphometric analysis evidenced that, after 4R onwards of COH, there was a significant increase in the frequency and density of damaged mitochondria from the oviductal epithelium, thus strenghtening the ultrastructural observations described [55-57]. The slight improvement observed in terms of density and frequency of damaged mitochondria at 8R, respect to 4R, could be probably due to cytoplasmic remodeling mechanisms activated by the oxidative damage, that may establish domains of autonomous regulation of mitochondrial function and activity, as previously reported in mammalian oocytes and embryos [44, 64].

The mitochondrial dysfunction here observed may, probably, be connected to the absence of ciliated epithelium in the sections observed, as the fibrils' tips during ciliogenesis are laterally connected to mitochondria [65]. Malfunction of the ciliary beat impairs the laminar fluid flow above the FT epithelia, thus likely reducing the clearance of oxidative stress caused by follicular fluid after ovulation [66].

In mouse oviducts, CCs can be rise from NCs under the direction of ovarian steroids [67, 68]. As recently seen in bovine, at least seven types of cells at different translational/transcriptional states of ciliogenesis are present in the oviductal epithelium, and their numbers are regulated by the estrous cycle [69]. This might provide the optimal environment for gamete transport, fertilization and embryo development [69]. The fine tuning of the histoarchitecture of FTs could be particularly sensitive to excessive estrogens induced by COH. After mild stimulation, an increased proliferation was, in fact, observed within the tubal epithelium, with pseudostratification of the epithelial cells and loss of their ciliated border [70]. Increased proliferation and multilayering of the tubal epithelium with the appearance of a micropapillary pattern were frequently observed at higher doses of gonadotropins [70].

FT epithelial cells expressed both estrogen and progesterone receptors, that were down-regulated by treatment with exogenous steroids [70, 71]. However, while membrane steroid-binding proteins (as membrane progestin receptors) are exclusively expressed in CCs of mice and women - to probably control the beating of the cilia or receptor expression via feedback systems [71] - the nuclear Progesteron Receptor (PGR) is immunolocalized also in smooth muscle cells [72]. Here, high levels of progesterone or progestagens promote in the luteal phase relaxation of the sphincter in the isthmus, allowing cilia to transport the pre-embryo

390 into the uterine cavity. Differently, the endogenous rise of estradiol (E2) during the
391 periovulatory period activates contractions of the myosalpinx, aiding the transport of
392 spermatozoa [73].
393 Gonadotropin receptors (FSHR and LHR) are also expressed in FTs and regulated by ovarian
394 steroids [74]. It is noteworthy that hyperstimulation with FSH downregulated FSHR present
395 in the blood and lympatic vessels of the *tunica* mucosa as well as in the smooth muscle cells,
396 thus affecting angiogenesis, vasculogenesis and oviductal contractions. High doses of
397 eCG/hCG induced a reduction in the protein levels of LHR in the oviductal ampulla with a
398 strong impact on ampullary physiology and function, as glycoprotein syntesis and secretion
399 [75].
400 The findings obtained from this study may corroborate to the harmful side-effects of the
401 ovarian hyperstimulation in regard to the maintenance of cellular integrity and strengthen the
402 aspect that increased rounds can contribute to the risk of developing gynecological cancers
403 [5, 76]. Endometrial biopsies retrieved from cycling IVF patients, with at least three repeated
404 IVF cycles with no pregnancy, revealed a lower expression of the tumor suppressor p27 and
405 a higher expression of its ligase S-phase kinase-interacting protein-2 (Skp2), compared to
406 non-stimulated normal cycling women [77]. Moreover, in the 25% of patients of the study
407 group, Skp2 expression reached concentrations demonstrated in endometrial carcinoma.
408 These findings suggested that repeated hormone stimulation cycles may disrupt endometrial
409 physiology, potentially towards abnormal proliferation [77]. In fact, it has been found that
410 gonadotropin stimulation could produce tubal and ovarian histopathologic abnormalities with
411 a dose-effect [78]. Moreover, the oviducts were reported to be the origin of OC as the high-
412 grade intraepithelial serous carcinoma and ovarian carcinosarcoma [79, 80], thus making the

tubal ligation and hysterectomy methods of choice in reducing the possibility for OC development [81, 82].

In conclusion, the ultrastructural alterations found in mitochondria and ciliar coverage of the mouse FT epithelium after repeated rounds of hyperstimulation, further demonstrate that COH has a detrimental impact on CECs morphology. Due to the role of FTs in ensuring fertilization and the subsequent transport of the embryo in the uterus, ultrastructural alterations here described may also contribute to infertility.

## Conflict of interest

The authors declare that they have no competing interests.

**Author contribution statement:** SBi, SC, MGP and GM conceived the study; SBi, GR and SBe treated the animals and collected the samples; SA, MGP and MB performed TEM and SEM experiments and analyzed data; SA and MGP wrote the paper; GM, SAN and SC analyzed data and revised the manuscript; GF, SBi and MAK critically revised the manuscript. All the authors accepted the final version of the manuscript.

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## LEGEND TO FIGURES

**Figure 1. Control group. A.** Representative LM picture of a transversal section of mouse ampulla showing the three layers of *tunica mucosa* (TM), *muscularis* (TMu) and *serosa* (TS). The TM appears regularly folded and characterized by adjacent columnar epithelial cells (CEC) with evident nuclei (*). In the *tunica muscularis* (TMu), the two well-defined outer and inner layers are evident. The former is organized in individual smooth muscle fiber bundles longitudinally oriented while the latter is organized in circular fibers. L: lumen (LM. Mag: 20 x. Bar: 5 μm). **B.** Ultrastructure of the columnar epithelium of TM, showing roundish-to-amoeboid shaped nuclei (N) delimited by a continuous nuclear membrane (nm). Heterochromatin (He) was clustered in clumps or located as marginal patches under the nuclear envelope among the dispersed euchromatin (Eu). The cytoplasm contains numerous round or elongated mitochondria (m), with electron-dense lamellar cristae or electron-pale content; lipid droplets (ld), short and long microvilli (mv), a few electron-negative vacuoles (V) and secretory granules with a slightly electron-dense content (asterisks). The junctional complexes between neighboring epithelial cells appear well-formed and constituted from the lumen by *zonulae occludens* (arrow) followed by *zonulae adherens* (arrowhead) (TEM. Bar: 2 μm). **C.** CECs of TM showing a large multivesicular body (mvb), an electron-dense secondary lysosome (Ly), tubular elements of the endoplasmic reticulum (ER), well-defined nuclei (N) and mitochondria (m). V: vacuoles; mv: microvilli; Gl: glycogen granules; asterisk: secretory granules (TEM. Bar: 1 μm). **D.** The luminal side (L) of TM shows numerous short or long microvilli (mv). m: mitochondria; arrows: zonulae occludens; arrowhead: *zonula adherens* (TEM. Bar: 1 μm). **E.** High magnification of motile cilia (Ci) showing an evident axoneme of nine peripheral doublet microtubules, surrounding a central

706 complex with two central microtubules and the central sheath (9 + 2 arrangement). Gl:
707 glycogen granule; arrows: *zonula occludens* (TEM. Bar: 2 μm).
708
709 **Figure 2. 4R group. A.** Representative picture of a transversal section of mouse ampulla
710 presenting a highly folded and branched *tunica mucosa* (TM), characterized by columnar
711 epithelial cells (CEC) with intensely stained prominent nuclei (asterisk). The outer and inner
712 layers of *tunica muscularis* (TMu) consist of longitudinal and circular fibers, respectively,
713 from the outer to the inner portion, similarly to the control group. L: lumen (LM. Mag: 20 x.
714 Bar: 5 μm). **B.** The surface of *tunica mucosa* shows round-to-ovoidal or irregularly shaped
715 nuclei (N) delimited by an uninterrupted electron-dense nuclear membrane (nm); the
716 chromatin was organized as in control group. The cytoplasmic membrane is folded in a
717 continuous layer of microvilli (mv). The cytoplasm contains multivesicular bodies (mvb),
718 well-defined junctional complexes (JC) and rare round/elongated electron-dense
719 mitochondria (m). Numerous damaged mitochondria with signs of swelling (sm) or
720 vacuolization (vm) are shown. He: heterochromatin; Eu: euchromatin (TEM. Bar: 2 μm). **C.**
721 Magnified micrograph of CECs with two evident nuclei containing patches of
722 heterochromatin (He) clustered under the nuclear membrane (nm). the microvillar coverage
723 (mv) shows numerous interruptions. Eu: euchromatin; nm: nuclear membrane; m:
724 mitochondria; sm: swollen mitochondria; vm: mitophagic vacuoles (TEM. Bar: 1 μm). **D.** a
725 detail of cytoplasm of an epithelial cell containing strongly indented nuclei, two
726 multivesicular bodies (mvb), an electron-dense granule (g) and lipid droplets (ld). Numerous
727 swelled mitochondria (sm) and mitophagic vacuoles (vm) are visible N: nucleus (TEM. Bar:
728 1 μm). **E.** High magnification of a nucleus (N) with a nucleolus (Nu) surrounded by

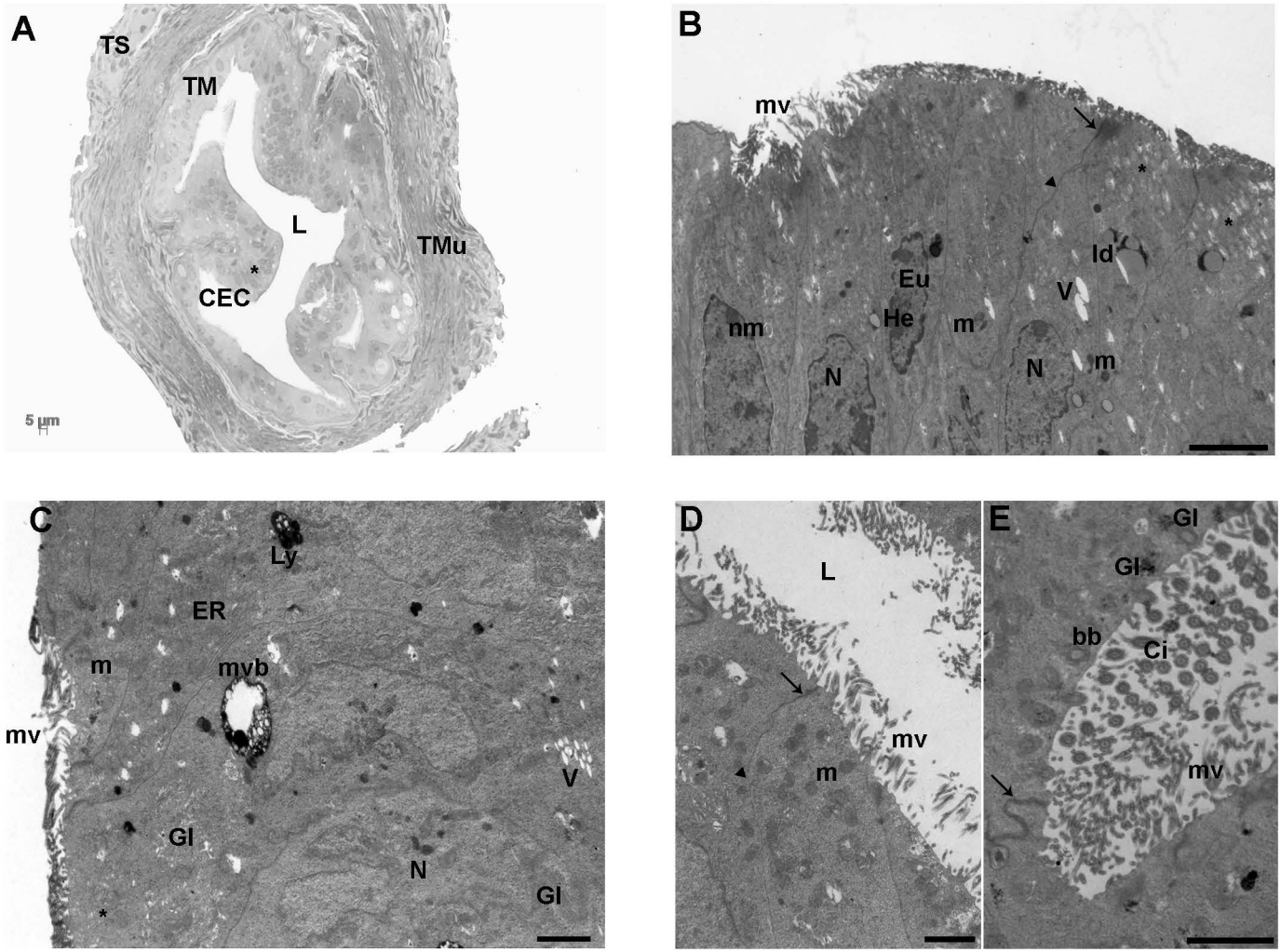
729 vacuolated mitochondria (vm) with marginalized content (TEM. Bar: 1 μm). *Inset.* Swollen
730 mitochondria (sm) with evident signs of matrix and cristae degeneration (TEM. Bar: 0,6 μm).
731 **F.** Detail of a big intracytoplasmic vacuole (*) lined by microvilli. N: nucleus; m:
732 mitochondria; sm: swollen mitochondria; vm: mitophagic vacuole (TEM. Bar: 1 μm).
733
734 **Figure 3. 8R group. A.** Representative image of a semithin section of the ampullar region
735 of mouse FTs showing the highly folded *tunica mucosa* (TM) with big nuclei (asterisk) and
736 the well-defined two layers of *tunica muscularis* (TMu) and the *tunica serosa* (TS). CECs:
737 columnar epithelial cells, L: lumen (LM. Mag: 20 x. Bar: 5 μm). **B.** Micrograph of the *tunica*
738 *mucosa* showing nuclei (N), round/elongated electron-dense mitochondria (m), endoplasmic
739 reticulum (ER) tubules/networks, secondary lysosomes (Ly), microvilli (mv) and junctional
740 complexes (JC). Eu: euchromatin; He: heterochromatin, sm: swollen mitochondria; vm:
741 vacuolated mitochondria (TEM. Bar: 1 μm). **C.** Detail of damaged mitochondria showing
742 numerous mitophagic vacuoles (vm) characterized by partial or complete cristolysis and
743 peripheric accumulation of the mitochondrial remains. mv: microvilli; arrow: *zonula*
744 *occludens*; arrowhead: *zonulae adherens* (TEM. Bar: 0,8 μm). **D.** Epithelial cells showing
745 interdigitated cell contacts (arrows). The cytoplasm contains irregularly shaped nuclei (N)
746 and numerous vacuolated mitochondria (vm). JC: junctional complexes (TEM. Bar: 1 μm).
747 **E.** Cytoplasmic content of columnar epithelial cells showing numerous mitophagic vacuoles
748 (vm) in proximity to multivesicular bodies (mvb) and lysosomes (Ly). ld: lipid droplet; mv:
749 microvilli; m: mitochondria; N: nucleus (TEM. Bar: 1 μm). **F.** High magnification of non-
750 ciliated cells rich in electron-dense secretory granules (Sg) with homogeneous content and
751 numerous long and thin microvilli (TEM. Bar: 1 μm).

**Figure 4. Surface analysis by SEM.** A-B. Control group. **A.** Numerous tufts of cilia (Ci) surrounded by short microvilli (mv) protrude into the lumen of the ampullar epithelium (SEM. Bar: 30 μm). **B.** High magnification of a well-preserved tuft of cilia (Ci) (SEM. Bar: 3 μm). C-D. 4R group. **C.** Numerous, long and homogenously distributed microvilli (mv) protrude from the globoid surface of epithelial cells (SEM. Bar: 3 μm). **D.** Detail of a heterogeneous carpet of short microvilli (mv) (SEM. Bar: 2.5 μm).

| Mitochondria | Normal | | | Damaged | | |
|---|---|---|---|---|---|---|
| | CTR | 4R | 8R | CTR | 4R | 8R |
| Dimension (μm) | 0.444±0.096 | 0.415±0.060 | 0.472±0.086 | 0.553±0.118[a] | 0.659±0.099[b] | 0.817±0.179[c] |
| Numerical density (N) | 40±12.1[a] | 5.3 ± 0.5[b] | 9.6±4.1[b] | 22.3±2.3[a] | 57.6±7.3[b] | 33±7.8[a] |
| Percentage (%) | 63.335±7.629[a] | 8.550±1.410[b] | 21.897±4.405[c] | 36.665±7.629[a] | 91.450±1.410[b] | 78.103±4.405[c] |

**Table 1. Morphometric analysis.** Dimension (μm), numerical density (N) and percentage (%) of normal and damaged mitochondria from columnar epithelial cells of mouse fallopian tube from control (CTR) and after four (4R) and eight (8R) rounds of repeated COH. The morphometric analysis was performed on a 100 $\mu m^2$ epithelial area in low-magnification TEM micrographs, from at least three sections from three different experiments per group. Approximately, 40 mitochondria were measured per each experimental group (9-11 mitochondria/animal for control group (N=4 animals); 6-8 mitochondria/animal for 4R (N=6 animals); 8-10 mitochondria/animal for 8R (N=5 animals). Values of each group were expressed as mean ± SD. The percentages of normal or damaged mitochondrial frequency were calculated over the total numerical density. Different superscripts indicate significant differences among the groups of normal or damaged mitochondria ($P < 0.05$).

# Figure 1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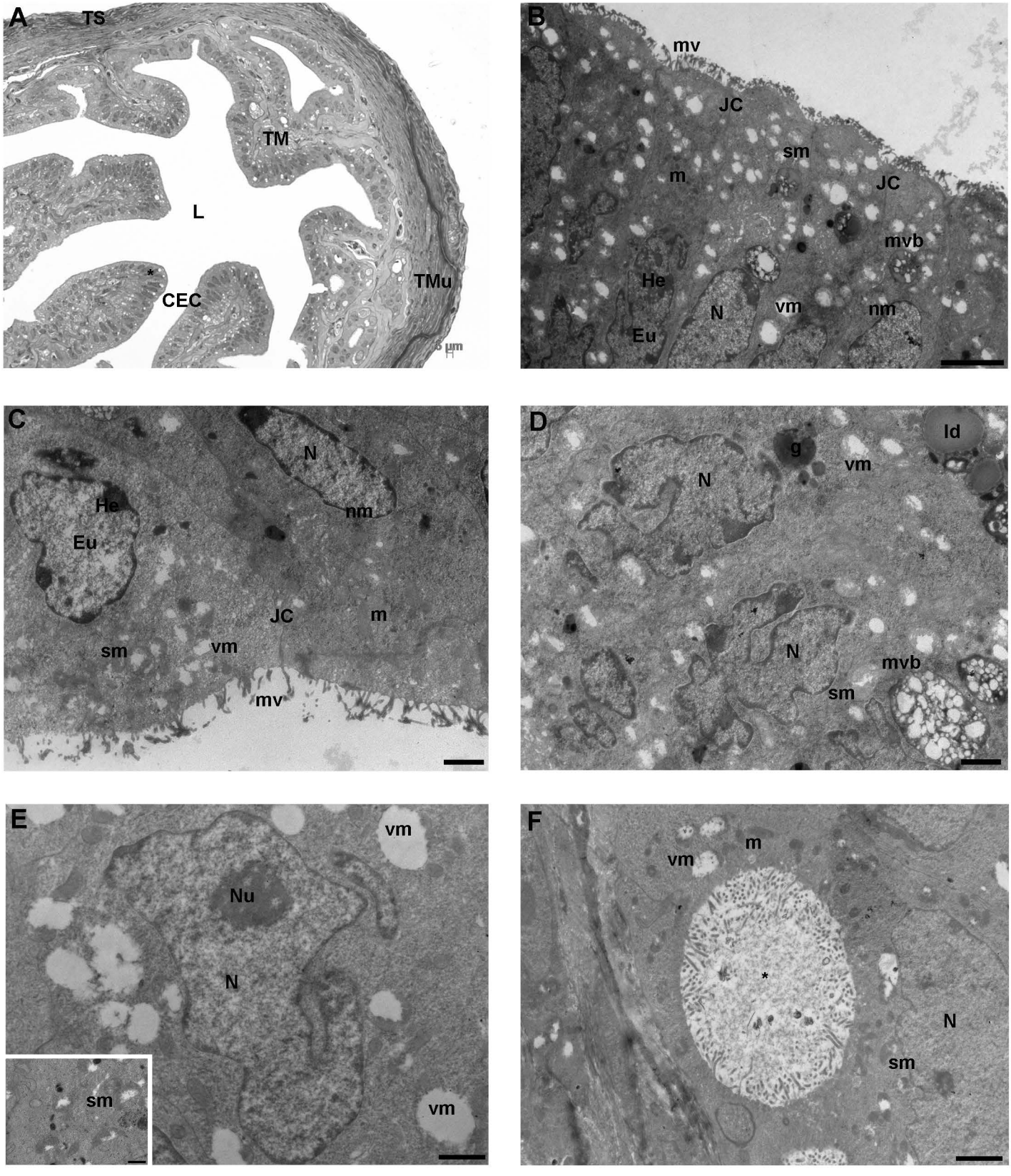
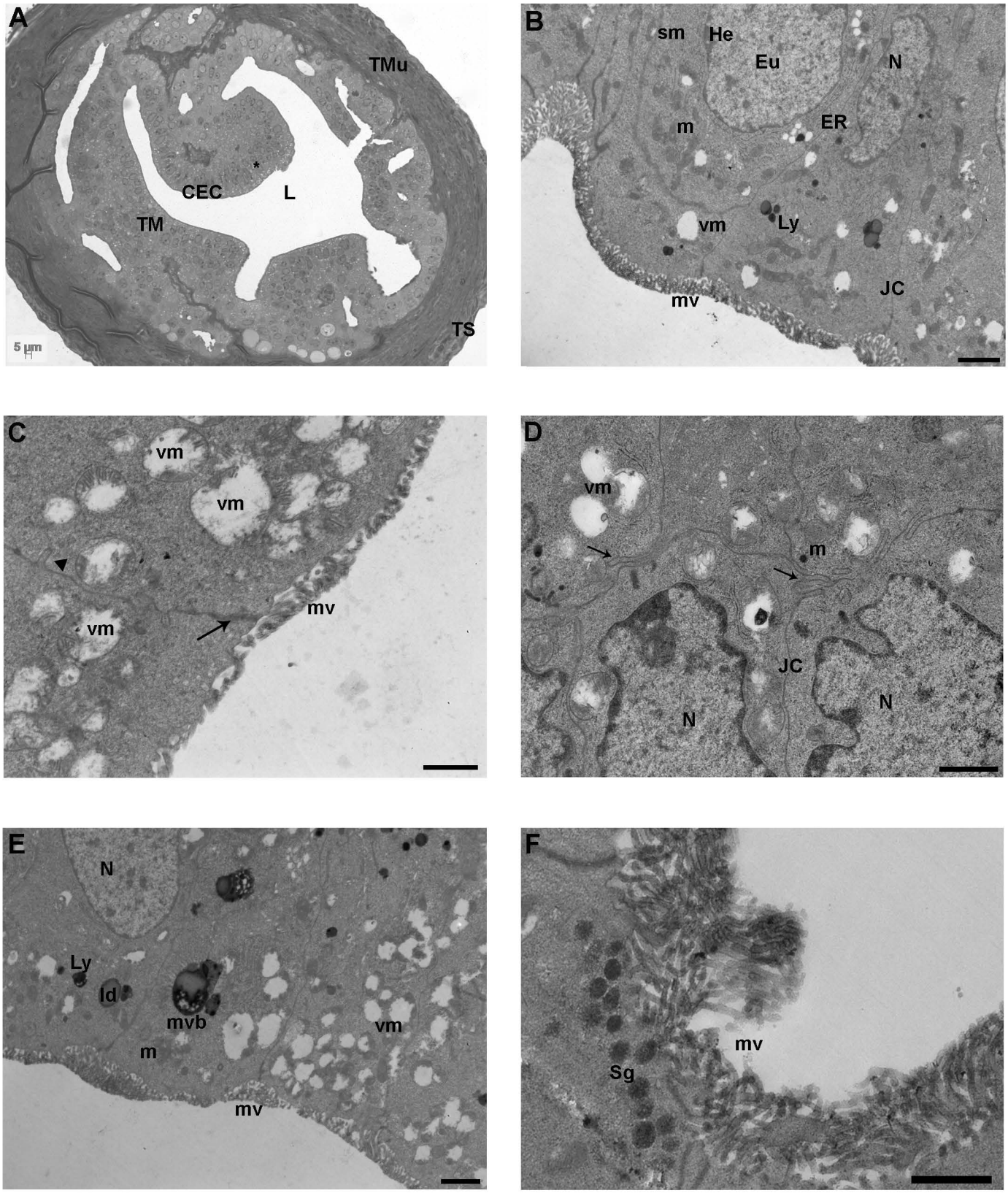
mv

# Figure 2


A
TS
TM
L
CEC
TMu
B
mv
JC
sm
m
JC
mvb
He
N
vm
nm
Eu
C
N
nm
He
Eu
JC
m
sm
vm
mv
D
Id
g
vm
N
N
mvb
sm
E
vm
Nu
N
sm
vm
F
m
vm
*
N
sm

## Figure 3


A
TMu
CEC
L
TM
TS
5 µm
B
sm
He
Eu
N
m
ER
vm
Ly
JC
mv
C
vm
vm
vm
mv
D
vm
m
JC
N
N
E
N
Ly
ld
mvb
vm
m
mv
F
mv
Sg

## Figure 4