Mancozeb impairs the ultrastructure of mouse granulosa cells in a dose-dependent manner

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Abstract. Mancozeb, an ethylene bis-dithiocarbamate, is widely used as a fungicide and exerts reproductive toxicity in vivo and in vitro in mouse oocytes by altering spindle morphology and impairing the ability to fertilize. Mancozeb also induces a premalignant status in mouse granulosa cells (GCs) cultured in vitro, as indicated by decreased p53 expression and tenuous oxidative stress. However, the presence and extent of ultrastructural alterations induced by mancozeb on GCs in vitro have not yet been reported. Using an in vitro model of reproductive toxicity, comprising parietal GCs from mouse antral follicles cultured with increasing concentrations of mancozeb (0.001–1 µg/ml), we sought to ascertain the in vitro ultrastructural cell toxicity by means of transmission (TEM) and scanning (SEM) electron microscopy. The results showed a dose-dependent toxicity of mancozeb on mouse GCs. Ultrastructural data showed intercellular contact alterations, nuclear membrane irregularities, and chromatin marginalization at lower concentrations, and showed chromatin condensation, membrane blebbing, and cytoplasmic vacuolization at higher concentrations. Morphometric analysis evidenced a reduction of mitochondrial length and chromatin marginalization at lower concentrations, and showed chromatin condensation, membrane blebbing, and cytoplasmic vacuolization at higher concentrations. In conclusion, mancozeb induced dose-dependent toxicity against GCs in vitro, including ultrastructural signs of cell degeneration compatible with apoptosis, likely due to the toxic breakdown product ethylenethiourea. These alterations may represent a major cause of reduced/delayed/missed oocyte maturation in cases of infertility associated with exposure to pesticides.

Keywords: Granulosa cells, Mancozeb, Scanning electron microscopy, Transmission electron microscopy, Ultrastructure

Mancozeb, an ethylene bis-dithiocarbamate (manganese/zinc ethylene bis-dithiocarbamate), is widely used as a broad-spectrum organometallic fungicide for field crops, fruits, nuts, vegetables, and ornamental plants. Introduced in 1962, mancozeb continues to play a significant role in the global fungicide market, mostly because of its low acute toxicity and long persistence on leaves. The toxicity of mancozeb is related to metabolic processes, which generate ethylenethiourea, and to its subsequent bioaccumulation in tissues and biological fluids, even at very low concentrations [1].

The toxic effects associated with the routine use of mancozeb have been studied in vivo and in vitro in various animal models and in humans (see for review [2–4]). Available data have provided evidence of the endocrine-disrupting activity of this pesticide, including impairment of the hypothalamic-pituitary-gonadal axis [5, 6]. Animal studies have provided high-to-moderate confidence of reproductive and developmental toxicity for mancozeb, while human observational studies have indicated a moderate-to-low level of confidence [2]. Risk assessment procedures should also consider adverse cumulative effects associated with the use of mixtures of pesticides, including mancozeb, responsible for persistent developmental and reproductive toxicity [7, 8].

Data from in vitro experiments have revealed dose-dependent alteration of the spindle in mouse oocyte-cumulus complexes (OCCs) exposed to increasing concentrations of the fungicide (0.001, 0.01, 0.1, and 1 µg/ml) [9]. Although 98% of control oocytes showed normally assembled spindles, located at the periphery of the cell and with well-aligned chromosomes on the metaphase plate, mancozeb induced a significant increase in abnormal spindle configurations. The fungicide induced microtubule elongation and spindle length reduction, especially at the highest concentrations (0.1 and 1 µg/ml) [9]. Cytoskeletal alterations were also found in mouse granulosa cells (GCs) exposed to increasing concentrations of mancozeb. After 24 h of in vitro culture (IVC), cell reshaping and changes in the actin cortical cytoskeleton, evaluated by immunofluorescence, were visible with 0.1 and 1 µg/ml concentrations. A marked rearrangement in stress fibers was also detected following prolonged culture (36 h), even under exposure to 0.01 µg/ml of mancozeb [10]. A similar pattern of cytoskeletal alterations was observed in human GCs exposed to concentrations higher than 0.01 µg/ml for 36 h. These changes were accompanied by dose- and time-dependent modifications in GC
morphology, which changed from an epithelial-like to a spindle-like/elongated shape. Furthermore, mancozeb treatment of both mouse and human GCs led to a marked decrease in p53 mRNA and protein levels, which was likely connected to the observed cell reshaping [10].

Mancozeb targets the mitochondria, as shown by ΔΨm impairment with a subsequent increase in ROS generation, alterations in the cellular redox state, and decreased ATP levels in mouse GCs, detected using cytofluorimetric assays [11]. Interestingly, at the concentration tested (0.01 μg/ml), these alterations were not related to the initiation of apoptosis or DNA damage response but to AKT activation and the maintenance of energy homeostasis [11].

We therefore aimed to ascertain whether the biomolecular alterations and cell reshaping observed following mancozeb exposure were connected to ultrastructural damages and cell-to-cell uncoupling, with subsequent isolation and cell death. We used a model of reproductive toxicity previously described in a study of the hexachlorocyclohexane lindane [12], comprising mouse GCs cultured in vitro in the presence or absence of increasing concentrations of mancozeb. Ultrastructural analysis of GCs was performed by transmission (TEM) and scanning (SEM) electron microscopy. Morphometric evaluation of cytoplasmic organelles was also performed.

Materials and Methods

Chemicals

All materials were purchased from Sigma Chemical (St. Louis, MO, USA), unless stated otherwise.

Animals

Swiss CD1 mice (Harlan Italy, Udine, Italy) were housed in individual cages with a 12:12 h light:dark cycle, controlled temperature (21 ± 1°C), and free access to food and water. Prepubertal females (21–23 days old) were intraperitoneally administered 5 IU of PMSG (pregnant mare serum gonadotropin) (Intervet, Milan, Italy) and euthanized 48 h later. Animals were maintained in accordance with the Italian Department of Health Guide for Care and Use of Laboratory Animals. The local committee on animal care and use approved the experimental protocols (Univaq 2012-2015) [10, 11], which were also compliant with international standards of animal care and veterinary medical practice.

GC isolation and experimental protocol

Mouse ovaries were collected, washed in PBS (phosphate buffered saline, pH = 7–7.4) at 37°C, and transferred to culture dishes (Becton Dickinson and Company, Franklin Lakes, NJ, USA) containing MEM Hepes (Life Technologies Italy, Monza MB, Italy). The ovaries were punctured with insulin syringe needles to release the mural GCs. GCs were then transferred into culture dishes for IVC. To assess the reproductive toxicity of mancozeb, GCs were cultured in DMEM (Dulbecco's modified Eagle's medium, GE Healthcare, Little Chalfont, Buckinghamshire, UK) containing 5% FBS (fetal bovine serum) supplemented with 2 mM L-glutamine and antibiotics (100 mM penicillin and 100 μg/ml streptomycin) in 35-mm tissue culture-treated dishes for 12 h to allow adhesion [10, 12]. Mancozeb (PESTANAL®, 45553) was dissolved in DMEM to obtain the desired concentrations. GCs were, then, cultured in vitro with fresh medium containing or not (Control) increasing concentrations of mancozeb at 37°C and 5% CO2 for 36 h.

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

Following IVC, GCs were aspirated, gently washed in PBS, centrifuged at 290 rcf (1200 rpm) for 5 min at 37°C, and immediately fixed in 2.5% glutaraldehyde (Agar Scientific, Cambridge Road Stansted Essex, UK)/PBS. The pellets were maintained at 4°C for 2–5 days until the next preparative for TEM [13–18]. Briefly, GCs were embedded in small blocks of 1% agar of about 5 × 5 × 1 mm in size, dehydrated in an ascending series of ethanol, immersed in propylene oxide for solvent substitution, embedded in epoxy resin EMBed-812 (Electron Microscopy Sciences, 1560 Industry Road, Hatfield, PA, USA), and sectioned using a Reichert-Jung Ultracut E ultramicrotome (Reichert Technologies, Munich, Germany). Semi-thin sections (1 mm thick) were stained with Toluidine Blue, examined by LM (Zeiss Axioskop), and photographed using a digital camera (Leica DFC230). Ultra-thin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids, and contrasted with saturated uranyl acetate and lead citrate (SIC, Rome, Italy) before being examined and photographed using Zeiss EM10 and Philips TEM CM100 electron microscopes operating at 80 KV.

The following parameters were evaluated by LM and TEM and used in the qualitative assessment of GCs: general features; cell membrane integrity; type and quality of organelles and inclusions; characteristics of the nucleus, chromatin, and nuclear envelope; and presence and extent of cytoplasmic vacuolization, intercellular projections, blebbing, and cellular debris [19, 20].

Scanning electron microscopy

For SEM observations, IVC was performed on 18-mm sterile polysilanized coverslips placed in culture dishes. Next, adherent GCs were washed in PBS, fixed in 2.5% glutaraldehyde/PBS at 4°C for at least 48 h, and then subjected to SEM specimen preparation [21–26]. Briefly, fixed GCs were post-fixed with osmium tetroxide, washed in PBS, and dehydrated with increasing concentrations of alcohol. Samples were then subjected to critical point drying, mounted onto aluminum stubs, and sputtered with platinum or gold particles before being observed with a scanning electron microscope (Philips XL – 30 CP) under low voltage (3–10 kV).

Morphometric analysis

ImageJ software (http://rsweb.nih.gov/ij/) was used to measure the number and dimensions of mitochondria and vacuoles observed on low-magnification TEM micrographs of control and mancozeb-treated GCs. For each experimental group, at least 15 GCs from three different experiments were selected for morphometric analysis.

Statistical analysis

All data are expressed as means ± standard deviation (SD). Statistical comparisons were performed using one-way ANOVA
Results

Control

Under LM, GCs showed a round-to-ovoid shape with a high nucleus:cytoplasm ratio. Nuclei, delimited by an intensely stained nuclear membrane, presented a variable number of nucleoli (Fig. 1A, inset). TEM of GCs showed that nuclei were delimited by a roundish, continuous, and electron-dense nuclear membrane (Fig. 1A). Chromatin was uniformly distributed or sometimes clustered in central clumps. In the cytoplasm, numerous groups of round/elongated mitochondria and tubular elements of the smooth endoplasmic reticulum (SER) were observed (Fig. 1A, 1B). Vesicles of the Golgi apparatus and lipid droplets, which are highly electron-dense, were occasionally found. Secondary lysosomes were also occasionally found (Fig. 1B). The plasma membrane presented numerous microvilli and cytoplasmic projections (not shown).

SEM showed GC stratification on the culture support, with a first layer of irregular and flattened cells and outer layers comprising rounder cells (Fig. 1C). The GC surface was densely covered with microvilli, even if adherent GCs, with a reduced length and distribution of microvilli and pseudopodia, were occasionally found. Numerous long and thin cytoplasmic protrusions (pseudopodia) connected adjacent GCs (Fig. 1C). The main results are summarized in Table 1.

Mancozeb 0.001 µg/ml

GCs cultured in vitro with the lowest concentration of mancozeb (0.001 µg/ml) presented morphological features similar to the controls under LM (Fig. 1D, inset).

Under TEM, GCs showed a round-to-ovoid shape with large roundish nuclei, delimited by an intact electron-dense nuclear membrane (Fig. 1D). Chromatin distribution did not differ from that of controls, with spots of heterochromatin normally located in the center of the cell or more rarely eccentric. The GC cytoplasm showed numerous round/ovoid mitochondria delimited by a double electron-dense mitochondrial membrane, with numerous intensely stained mitochondrial cristae (Fig. 1E). Lipid droplets and tubular elements of the endoplasmic reticulum (ER) were also found. Numerous intercellular contacts were observed to have been established by intercellular junctions and projections (Fig. 1D).

SEM analysis showed that the distribution of GCs and surface morphology were similar to those of controls. A retraction of intercellular connections and reduction in length and number of microvilli was occasionally observed. Cytoplasmic blebs were also occasionally found (Fig. 1F). The main results are summarized in Table 1.

Mancozeb 0.01 µg/ml

GCs cultured in the presence of 0.01 µg/ml mancozeb occasionally showed smaller cells among normal-sized elements, and intensely stained intracellular spots (Fig. 2A, inset).

TEM showed the presence of alterations such as chromatin marginalization, nuclear membrane invaginations, vacuolization, and membrane blebbing (Fig. 2A). No alterations were observed in organelles such as mitochondria, ER, and lipid droplets.

SEM showed smooth GCs with short microvilli and occasional blebbing (Fig. 2B), and cytoplasmic projections were reduced in number and length (Fig. 2B). The main results are summarized in Table 1.

Mancozeb 0.1 µg/ml

Cells cultured in the presence of mancozeb 0.1 µg/ml showed an irregular shape under LM, as well as varying dimensions and the occasional presence of dark cytoplasmic bodies (Fig. 2C, upper inset).

Under TEM, GCs did not show evident alterations in mitochondria, lipid droplets, and ER compared with previous groups (Fig. 2C). However, a pattern of alterations to the nucleus was frequently observed, as well as nuclear membrane invagination, chromatin condensation, and marginalization. An evident increase in vacuolization and blebbing, as well as a reduction in intercellular contacts, was also noticed. Degenerating GCs, characterized by cytoplasmic fragments often in proximity to integral nuclei or organelles, were frequently observed (lower inset of Fig. 2C).

By SEM, the presence of blebbing (Fig. 2D, inset), the retraction of intercellular communications, and a diminution of microvilli was frequently found (Fig. 2D). The main results are summarized in Table 1.

Mancozeb 1 µg/ml

At the highest concentration tested, large vacuoles were frequently observed in the cytoplasm of GCs, and numerous cell fragments were dispersed among the cells, as seen under LM (Fig. 2E, inset).

Ultrastructural TEM analysis showed the increased presence of peculiar alterations to the nucleus, as observed in the previous group (0.1 µg/ml) but more frequent, and/or to the cytoplasm, such as vacuolization and membrane blebbing (Fig. 2E). Mitochondrial cristae appeared less electron-dense. Degenerating cells, recognizable by the presence of membrane-bound cell fragments dispersed in cell debris, were commonly observed (Fig. 2E).

Under SEM, numerous blebs were observed on the surface of GCs, often characterized by a smooth appearance with rare short microvilli (Fig. 2F, inset). A reduction in pseudopodia and retraction of intercellular contacts was also observed (Fig. 2F). The main results are summarized in Table 1.

Morphometric analysis

Mitochondrial length did not differ between the control and mancozeb 0.001 µg/ml groups (0.550 ± 0.074 µm vs. 0.539 ± 0.127 µm, respectively; P > 0.05). However, a significant reduction in mitochondrial length was observed in GCs exposed to 0.01 µg/ml (0.364 ± 0.091 µm), 0.1 µg/ml (0.366 ± 0.074 µm), and 1 µg/ml (0.437 ± 0.087 µm) mancozeb compared to that of control and mancozeb 0.001 µg/ml groups (P < 0.01) (Table 2). The mean mitochondrial count was significantly increased only in GCs exposed to mancozeb 0.1 µg/ml (40 ± 6) compared to that of the other groups (approximately 25) (P < 0.05).

Vacuole length increased significantly in a dose-dependent manner, ranging from 0.336 ± 0.141 µm in the control group to 1.059 ± 0.170 µm with mancozeb 1 µg/ml (P < 0.01). The number of vacuoles doubled in GCs exposed to concentrations of mancozeb higher than
Fig. 1. Ultrastructural analysis of GCs in control and mancozeb 0.001 µg/ml groups. A–C. Control. A. Ultrastructure of a granulosa cell (GC) with a large nucleus (N) delimited by a continuous nuclear membrane (nm), numerous round/ovoid mitochondria (m) with extensive cristae, and elements of the endoplasmic reticulum (ER). A vacuole (V) with electron-lucent content is also shown (TEM. Bar: 400 nm). Inset in A: A representative image of a semithin section of GCs (LM. Mag: 40 ×). B. Cytoplasmic content of a GC showing a lysosome (Ly), lipid droplets (ld), tubular elements of the endoplasmic reticulum (ER), and mitochondria (m) surrounded by a double membrane (TEM. Bar: 200 nm). C. Adjacent cells (GC) are connected by numerous long, thin pseudopodia (arrows). The surface of the outer layer of GCs shows densely packed microvilli (mv) (SEM. Bar: 40 µm). D–F. Mancozeb 0.001 µg/ml. D. Three granulosa cells (GC) with roundish nuclei (N) and small spots of marginalized chromatin under the double layer of the nuclear membrane (nm). Numerous mitochondria (m) and extensive mitochondrial cristae are visible in the cytoplasm. Arrow indicates intercellular connections among adjacent cells; mv: short microvilli (TEM. Bar: 1 µm). Inset in D. A representative image of a semithin section of GCs (LM. Mag: 40 ×). E. At high magnification, a transversal section evidences the arrangement of numerous mitochondrial cristae of mitochondria (m) and parallel tubular elements of the endoplasmic reticulum (ER). V: vacuoles; He: heterochromatin; Eu: euchromatin (TEM. Bar: 1 µm). F. Adherent granulosa cells (GCs), with a smooth appearance, are covered by roundish cells covered by numerous microvilli (mv). GCs are connected by means of numerous long, thin pseudopodia (arrows) (SEM. Bar: 40 µm).
0.01 μg/ml (~16) compared to that of control and mancozeb 0.01 μg/ml of mancozeb for 96 h induced apoptosis in blastomeres exposed to increasing concentrations of mancozeb evidenced high preservation of both nucleus and cytoplasmic organelles at the lowest concentration. However, presumptive indicators of cell death were observed already at the lowest doses, and ranged from 1) early signs such as chromatin condensation and marginalization under the nuclear membrane (only occasionally seen in GCs treated with 0.001 μg/ml); 2) intermediate signs such as nuclear membrane indentation, vacuolization, and reduction of the microvillar-covered surface; and 3) late signs such as membrane blebbing and the presence of cellular debris. Morphological findings obtained by electron microscopy showed signs of cell death compatible with apoptosis (as previously reviewed [32]), but probably associated with secondary necrosis due to in vitro conditions (see the “apoptosis-necrosis continuum” described previously [33] for further details).

Other studies have described ultrastructural changes in apoptotic cells comparable with those found in the present study, such as chromatin marginalization, nuclear alteration, cytoplasmic reorganization, and loss of membrane integrity [34, 35]. Apoptotic GCs showed progressive alterations in the nuclear envelope form, the presence of condensed chromatin around the inner margins of the nuclear membrane, and cytoplasmic vacuolization [36], in accordance with our findings.

However, vacuolization observed at the highest concentrations, both as increased numbers (significantly higher compared with 0.01 μg/ml of mancozeb) and dimension (significantly higher at a concentration as low as 0.001 μg/ml mancozeb) may be the result of autophagic phenomena, with degradative autophagic structures (autolysosomes) encircled by a single membrane and containing partially degraded materials with a high electron density [37]. It is noteworthy that the mitochondrial ultrastructure appeared to

### Table 1. Summary of main results obtained by TEM and SEM observations –Comparisons of ultrastructural characteristics were performed with respect to the less-exposed group

| Mancozeb | 0.001 μg/ml | 0.01 μg/ml | 0.1 μg/ml | 1 μg/ml |
|----------|-------------|------------|----------|--------|
| Nuclear membrane shape | roundish | invaginated | invaginated or interrupted |
| Chromatin | uniformly distributed; occasionally clustered | condensed and marginalized | condensed and marginalized | less visible, more opalescent and with less electron-dense cristae |
| Mitochondria | numerous, rich of electron-dense cristae | numerous, rich of electron-dense cristae | numerous, rich of electron-dense cristae | less visible, more opalescent and with less electron-dense cristae |
| Vacuoles | few | numerous | abundant |
| Cytoplasmic projection | numerous, long and thin | reduced in number and length | less numerous, shorter and thicker |
| Microvilli | numerous, long and thin | less numerous, shorter and thicker | rare and short |
| Blebbing | occasional | frequent |
| Cell debris/fragments | rare | numerous |

In this study, we aimed to investigate the effects of mancozeb—a fungicide commonly used for its low toxicity—on cell ultrastructure in a model of reproductive toxicity previously used to study the hexachlorocyclohexane lindane [12], and comprising mouse GCs cultured in vitro in the presence or absence of increasing concentrations of mancozeb.

Results obtained by TEM and SEM indicated the harmful action of this fungicide on the somatic compartment of the mouse ovarian follicle, with dose-related effects. The gonadal toxicity of mancozeb has previously been shown in vivo to impair fertilization [27], disrupt the estrous cycle, significantly decrease the number of healthy follicles, and increase the number of atretic follicles [28]. Our previous in vitro studies in GCs, which used a biomolecular approach, revealed that concentrations of mancozeb ranging from 0.001 to 1 μg/ml evidenced high preservation of both nucleus and cytoplasmic organelles at the lowest concentration, but probably associated with secondary necrosis due to in vitro conditions (see the “apoptosis-necrosis continuum” described previously [33] for further details).

### Discussion

The status of GCs exposed in vitro to increasing concentrations of mancozeb, we used electron microscopy, considered the gold-standard for the determination of cell death and identification of apoptosis [31, 32]. Our morphological analysis of mouse GCs exposed to increasing concentrations of mancozeb evidenced high preservation of both nucleus and cytoplasmic organelles at the lowest concentration, but probably associated with secondary necrosis due to in vitro conditions (see the “apoptosis-necrosis continuum” described previously [33] for further details).

Other studies have described ultrastructural changes in apoptotic cells comparable with those found in the present study, such as chromatin marginalization, nuclear alteration, cytoplasmic reorganization, and loss of membrane integrity [34, 35]. Apoptotic GCs showed progressive alterations in the nuclear envelope form, the presence of condensed chromatin around the inner margins of the nuclear membrane, and cytoplasmic vacuolization [36], in accordance with our findings.

However, vacuolization observed at the highest concentrations, both as increased numbers (significantly higher compared with 0.01 μg/ml of mancozeb) and dimension (significantly higher at a concentration as low as 0.001 μg/ml mancozeb) may be the result of autophagic phenomena, with degradative autophagic structures (autolysosomes) encircled by a single membrane and containing partially degraded materials with a high electron density [37]. It is noteworthy that the mitochondrial ultrastructure appeared to
Fig. 2. Ultrastructural analysis of GCs in mancozeb 0.01–1 µg/ml groups. A–B. Mancozeb 0.01 µg/ml. A. Two adjacent granulosa cells (GCs) with different phenotypes: the one on the right, with a roundish shape and nucleus (N), shows good preservation of the cytoplasm with numerous mitochondria (m), vacuoles (V), and endoplasmic reticulum (ER); the other on the left, with high preservation of mitochondria (m), vacuoles (V), and other organelles, shows significant blebbing (b). He: heterochromatin; Eu: euchromatin (TEM. Bar: 2 µm). Inset in A: Representative image of a semithin section of GCs (LM. Mag: 40 ×). B. Smooth, adherent GCs covered with irregular cells with highly blebbed surfaces (b). Connecting pseudopodia (arrows) are reduced in number and extension (SEM. Bar: 40 µm). C–D. Mancozeb 0.1 µg/ml. C. Granulosa cell (GC) showing high preservation with numerous ovoid mitochondria (m), surrounding a nucleus (N) delimited by a nuclear membrane (nm). Upper inset in C: Representative image of a semithin section of GCs (LM. Mag: 40 ×). Lower inset in C: Altered GCs with cytoplasmic fragments (cf) released in proximity to the nucleus (N); nm: nuclear membrane. D. Smooth adherent GCs are covered by small and roundish blebs; superficial GCs appear rich of blebs. Arrows: detail of pseudopodia, short and rare (SEM. Bar: 40 µm). Inset in D: High magnification of a cytoplasmic bleb (b) (SEM. Bar: 8 µm). E–F. Mancozeb 1 µg/ml. E. Altered, irregularly-shaped cells, highly vacuolated (V) and with extensive blebbing (b), interspersed in cell fragments (cf), containing numerous membrane-bound organelle remnants, and debris (*) (TEM. Bar: 4 µm). N: nucleus. Inset in E: Representative image of a semithin section of GCs (LM, Mag: 40 ×). F. Abundant blebbing (b) is visible on the GC surface. Note the scarcity of cytoplasmic projections (arrow), with a quasi-absence of pseudopodia (SEM. Bar: 40 µm). Inset in F: High magnification of degenerating GCs, completely covered by cytoplasmic blebs (b) (SEM. Bar: 8 µm).
be properly maintained in integral cells, including at the highest concentrations. However, the reduction in mitochondrial size observed in groups treated with mancozeb 0.01–1 µg/ml, associated with the absence of electron-dense cristae and a homogeneous matrix, may be indicative of pre-apoptotic changes [38].

The inhibition of intercellular junctions followed by the disconnection of adjacent cells has been shown to severely disrupt communication between oocytes and GCs, thus inhibiting the supply with consequent GC atresia and loss of oocyte development capacity [39, 40]. Therefore, our findings on the dose-dependent reduction in the microvillar layer, increase in membrane blebbing, and decrease in pseudopodia and intercellular junctions exerted by mancozeb may represent major causes of reduced/delayed/missed oocyte maturation observed in cases of infertility associated with exposure to pesticides such as mancozeb.

The results obtained from this study confirm that mancozeb may be a possible cause of infertility in exposed human populations [3, 4]. Since GCs actively participate in the development and maturation of the oocyte and cooperate with theca cells in the production of steroids [41], alterations in their normal physiological functions may affect the capacity of oocytes to complete meiosis and become fertilized [42]. This observation should consider that endocrine-disrupting pesticides can interfere with the development and maintenance of secondary sexual characters and sexual behaviors. In fact, it has recently been reported that in vivo exposure to mancozeb induces a significant reduction in plasma levels of luteinizing hormone (LH) and testosterone, a slight reduction in follicle stimulating hormone (FSH), and an increase in estradiol and prolactin levels [43].

In conclusion, the results of the present study are the first to indicate that mancozeb exerts dose-dependent toxic effects against the ultrastructure of GCs in vitro, with associated indications of cell death. Given that the toxic effects of mancozeb are also exerted in oocytes, as previously demonstrated [9], a study on the ultrastructural analysis of exposed cumulus-oocyte complexes is warranted, as these effects have not yet been demonstrated to our knowledge. Moreover, GC alterations may be associated with reduced oocyte and sterility capacity [44], and therefore ultrastructural analysis of oocytes from exposed OCCs may highlight how impairments in somatic compartments can affect oocyte ultrastructure. These results are likely to be of significant interest in the study of fertility preservation, in both animals and humans.

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### Table 2. Dimension and number (expressed as mean ± standard deviation) of mitochondria and vacuoles in granulosa cells (GCs) exposed or not (control) to mancozeb 0.001–1 µg/ml

|                  | Control  | 0.001 µg/ml | 0.01 µg/ml | 0.1 µg/ml | 1 µg/ml |
|------------------|----------|-------------|------------|-----------|---------|
| Mitochondria (µm) | 0.550 ± 0.074 a | 0.539 ± 0.127 a | 0.364 ± 0.091 b | 0.366 ± 0.074 b | 0.437 ± 0.087 b |
| N. of mitochondria | 25 ± 8 a | 24 ± 8 a | 29 ± 9 a | 40 ± 6 b | 24 ± 11 a |
| Vacuoles (µm)    | 0.336 ± 0.141 a | 0.546 ± 0.186 b | 0.689 ± 0.314 b | 0.804 ± 0.289 bc | 1.059 ± 0.170 c |
| N. of vacuoles   | 8 ± 4 a | 9 ± 4 a | 15 ± 4 b | 16 ± 5 b | 16 ± 4 b |

Approximately 10–20 mitochondria and 5–10 vacuoles were measured in low magnification TEM micrographs from at least 15 GCs from three different experiments. Morphometry was performed using one-way ANOVA with Tukey’s HSD post-hoc analysis.
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