In vitro and *in vivo* impairment of embryo implantation by commonly used fungicide Mancozeb

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ABSTRACT

The fungicide Mancozeb is an endocrine disrupting chemical that targets mammalian reproductive functions. Whether Mancozeb affects embryo implantation and stromal cell decidualization in mice is largely unknown. Mancozeb (1 and 3 μg/mL) significantly reduced Jeg-3 trophoblastic spheroid (embryo surrogate) attachment to human endometrial epithelial Ishikawa cells. In pregnant mice, Mancozeb treatment from gestation day (GD) 1 to GD8 or from GD4 to GD8 significantly lowered the number of implantation sites on GD9, resulting in a higher incidence of morphological abnormalities in the reproductive tissues when compared to the controls. However, these results were not seen in the treatment from GD1 to GD4. Mancozeb at 30 mg/kg BW/d did not alter the expressions of p53, COX-2, or PGFS transcript in the uterus, but did downregulate PGES transcript and protein expressions. *In vitro* Mancozeb treatment in human primary endometrial stromal cells did not alter the decidualization response, but did affect the morphological transformation of the endometrial stromal cells. Taken together, exposure to a high concentration of Mancozeb in GD1 to GD8 or GD4 to GD8 affected embryo implantation probably through the modulation of stromal cell decidualization *in vitro*. However, the effects of lower/physiological doses of Mancozeb and the effects on subsequent generations need further investigation.

Keywords: Mancozeb, Endocrine disruptor, Embryo implantation, Decidualization, Gene expression.

1. Introduction

In recent decades, the ever growing use endocrine-disrupting compounds (EDCs) found in a wide variety of anthropogenic, industrial, agricultural, and domestic sources has meant that humans are increasingly exposed to these chemicals in the environment [1]. Exposure to EDCs from food, water, and/or inhalation can lead to disruption of the production, release, transport, metabolism, binding, action, and elimination of natural hormones that are responsible for maintaining homeostasis and regulating developmental processes [2, 3]. These EDCs are linked to adverse effects on development, reproduction, immunity, and other biological processes in humans and animal species [3, 4].

Many pesticides including Mancozeb have been identified as EDCs [5]. Mancozeb is commonly used to protect fruits, nuts, vegetables, grains, turf, flowers, and stored seeds from fungal diseases such as blight, root rot, and leaf spot [6, 7, 8]. Mancozeb is an organometallic and polymeric complex with the IUPAC name manganese ethylenebis (dithiocarbamate) (polymeric) complexed with zinc salt. Although it has low mammalian toxicity, high or repeated exposure to Mancozeb may interfere with the reproductive system in both males and females [6, 9]. Mancozeb and its metabolite Ethylene thiourea (ETU) have been shown to have disruptive effects on various cell types including female germ cells [11]. Furthermore, Mancozeb at a dose of 500 mg/kg of body weight (BW) impaired ovulation and significantly reduced fertilization in mice [12], and affected pregnancy [13] and induced gonadal toxicity in female rats [14]. More importantly, Mancozeb affected embryo development by increasing apoptosis in embryos [13], as well as reduced the mean cell number in embryos [13]. Low concentrations of Mancozeb (<1 μg/mL) impaired oocyte maturation *in vitro* by altering meiotic spindle formation leading to low fertilization rates [11]. Mice treated with Mancozeb (36 mg/kg BW/day) orally for 5 days showed 100% embryo loss [9]. In addition, Mancozeb decreased the

number of healthy follicles, disrupted hormone balance, and increased the number of atretic follicles in buffalo oocytes *in vitro* [15]. Moreover, a recent study reported that Mancozeb caused morphological changes and affected p53 expression in human granulosa cells [16]. Treatment with Mancozeb at a concentration of 40 mg/kg BW/day in male Wister rats for 18 consecutive days was found to be genotoxic by affecting the DNA integrity of cells [17]. Furthermore, Mancozeb at low doses affected thyroid homeostasis and reproduction in wild birds [18].

In the past few decades, human fertility has shown an overall decreasing trend [19]. The global mean prevalence of various fertility problems is 9%, which is believed to be linked with many factors including EDCs [19]. Endometrial receptivity, embryo implantation and related complications are believed to be among the main contributors of female infertility [20]. Endometrial receptivity and embryo-maternal communication play important roles in pregnancy success. Any substances that alter the endocrine system will affect the menstrual cycle and in turn the endometrial receptivity for embryo implantation. Previous studies have shown that excessive or high serum E_2 and/or progesterone hormone levels are detrimental to endometrial receptivity resulting in implantation failure [21, 22]. Furthermore, decidualization failure of endometrial stromal cells is also associated with implantation failure [21, 22]. Some molecules that play vital roles in mammalian decidualization and implantation include steroid hormones, p53 (Protein 53 (*Trp53*)) [25], COX-2 (*Cyclo-oxygenase 2*) [26], PGES (*Prostaglandin E synthase*) [27], and PGFS (*Prostaglandin F2-alpha* synthase) [27].

Mancozeb is commonly used in many agricultural and horticultural industries. Many studies suggest that Mancozeb and its metabolites can accumulate in our food chain, water supply, and air [10, 28, 29, 30]. Women of reproductive age are at particularly high risk from exposure to Mancozeb [31]. Various studies have already been conducted to investigate the effects of several endocrine disruptors on endometrial receptivity and

127 embryo implantation [32, 33,34], but there is limited data on the effects of Mancozeb in
128 humans or in animals [6]. Although the mechanism(s) by which Mancozeb affects female
129 reproductive organs is largely unknown, data from *in vivo* animals studies has
130 demonstrated that it can induce dysregulation of the estrous cycle [11]. A few studies
131 have shown that Mancozeb has anti-implantation effects in murine models and can
132 potentially mimic estrogen activity [16]. However, the exact modes of action of
133 Mancozeb on endometrial receptivity, embryo implantation, or on general fertility are
134 not fully understood. In the present study, we hypothesized that exposure to Mancozeb
135 affects endometrial receptivity and embryo implantation. We used an *in vitro* spheroid-
136 endometrial cell co-culture model, *in vivo* mouse implantation model, and *in vitro*
137 primary endometrial stromal cell culture model to study the effects of Mancozeb on
138 embryo implantation, especially during the decidualization period, to reveal the
139 underlying mechanism of its effects on female reproductive processes.
140

2. Materials and Methods

2.1. Ethics statement

The study protocol was approved by the Animal Ethical Review Committee of the University of Peradeniya, Peradeniya Sri Lanka (VER14-004). The collection of human endometrial biopsies for this study was approved by the ethics committee of the Institutional Review Board of Hospital Authority Hong Kong West Cluster and the University of Hong Kong (UW16-204), Hong Kong.

2.2. Chemicals and Mancozeb preparation

All the chemicals were purchased from Sigma-Aldrich Co., St Louis, USA unless otherwise specified. Analytical grade Mancozeb was dissolved in dimethyl sulfoxide (DMSO) at a range of concentrations (0.3, 1, 3, 16, 30 and 32 mg/kg BW/day) for use in the *in vivo* experiments.

2.3. Experimental model

Three separate studies were conducted to evaluate the effects of Mancozeb exposure on embryo implantation, endometrial receptivity, and mice fecundity using a minimum of five animals per group. Animals were allocated to the experimental groups randomly and all experiments were repeated at least twice. All animals in each group were healthy prior to the treatments and at sacrificing, and all data were included in the analyses.

2.4. *In vitro* embryo attachment in the trophoblastic spheroid-endometrial cells co-culture assay

Human endometrial epithelial Ishikawa cells (ECACC 99040201) and human trophoblastic Jeg-3 cells (ATCC, HTB-36) were used in the co-culture model as

described previously [35]. Receptive human endometrial epithelial Ishikawa cells and human trophoblastic Jeg-3 cells were plated in 6-well plates at a density of 2.5×10^5/well and 2.0×10^5/well, respectively. All cells were cultured in phenol red-free medium containing 10% charcoal-stripped FBS (csFBS) for 24 h followed by Mancozeb or DMSO treatment for a further 24 h. Trophoblastic spheroids were prepared by incubating trypsinized Jeg-3 cells overnight at 37°C on an orbital shaker rotating at 88 rpm. A fixed number of spheroids with approximate diameters between 60 and 200 µm with or without Mancozeb treatment were transferred onto the confluent monolayer of Mancozeb- (0.01, 0.1, 1, or 3 µg/mL) or DMSO-treated Ishikawa cells in culture media containing DMEM/F12 in 10% csFBS and incubated at 37°C for 1 h. After co-culture, the plates were shaken on a rotary shaker at 145 rpm for 10 min. The media was removed and new media was added. Spheroids attached onto the Ishikawa monolayer were counted with an inverted microscope (Nikon, Japan). The attachment rate was expressed as the percentage of attached spheroids out of the total spheroids added.

2.5. Effect of Mancozeb exposure on embryo implantation in mice *in vivo*

Female and male ICR mice (25-35 g) at 10-12 weeks of age were obtained from the Medical Research Institute, Colombo, Sri Lanka. The mice were reared in the animal holding facility at the Faculty of Medicine, University of Peradeniya, Sri Lanka. Male and female mice were kept separately in bedded cages under controlled conditions (12h light: 12h dark cycle) with access to standard feed and water ad libitum. Mice were acclimatized to the above conditions for 2 weeks.

The stage of the estrous cycle was determined by vaginal swab following the protocol of Parimala and Kaliwal [36]. Female mice (n=5) at proestrus phase were caged with mature fertile adult males in the afternoon. Mating was confirmed by the presence of a vaginal plug after examination on the next morning. The day of the confirmed mating

was marked as the first day of pregnancy, designated as gestation day 1 (GD1). Different doses of Mancozeb (1, 16 and 32 mg/kg BW/day) were administered by oral gavage from GD1 to GD8. These doses were below the acute LD_{50} level based on previous studies [9]. Mice in the control group were administered DMSO dissolved in olive oil. On GD9, mice were sacrificed and the uterus, oviduct, and ovary were dissected and freed from fat and connective tissues under a dissection microscope. The number of implantation sites in each mouse was then counted. The uterus, oviduct and one ovary were fixed in Bouin's solution for the histological examination.

2.6. Effect of Mancozeb on the morphology of the reproductive tissues in mice

Histological examination of tissues was performed as described previously [37, 38]. Briefly, paraffin-embedded ovaries, oviducts and uteri were sectioned (5 µm thick) and mounted on treated glass slides, and then stained with hematoxylin and eosin (HE). A total of five sections from each uterus, oviduct, and ovary were analyzed. For the morphometric measurements, micrographs were taken using an image capturing system (Infinity 2, Lumenera, Ottawa, Canada) mounted on an upright microscope (Meiji Techno, Japan). Morphometric parameters including epithelial thickness were measured using Image-J software (version 1.46r). Two independent investigators examined the micrographs under a microscopic to rank the tissues according to their appearance and structural abnormalities: disrupted, slightly disrupted, and normal.

2.7. Effect of Mancozeb on the expression of stromal cell decidualization markers

Female ICR mice (n= 5) at 12-15 weeks of age were randomly divided into four groups and mated with mature fertile adult males as described above. After confirmation of mating, female mice were administered Mancozeb (0.3, 3 and 30 mg/kg BW/day) orally from GD4 to GD8, coinciding with the period of decidualization. Mice in the

control group were administered DMSO in olive oil. At the end of the treatment period, mice were sacrificed and their reproductive tissues were collected, and the number of implantation sites was counted.

Total RNA from the uterine tissue was extracted with TRIZOL reagent (Life Technologies, USA) following the manufacturer's protocol. The concentration and purity of the total RNA were determined by measuring the optical density at 260 and 280 nm using a spectrophotometer (OPTIZEN POP, Mecasys Co., Ltd, Korea). Total RNA (1 µg) was reverse-transcribed using Improm-11 Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Next, PCR was performed to detect the expressions of p53, COX-2, PGES, and PGFS transcripts. The PCR reaction mix contained 10 µL Gotaq® 2× PCR master mix, 1 µL of 10 µM forward and reverse primers, 1 µL cDNA template, and nuclease-free water in a total volume of 20 µL. The primers used for each target are listed in supplementary Table 1. The PCR conditions were as follows: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 40 s; annealing for 35 s at 55.4°C for p53, 52.6ºC for COX-2, 54ºC for PGES, 56.7ºC for PGFS, or 59.2ºC for GAPDH; and extension at 72°C for 45 s; and a final extension at 72°C for 10 min. Normalization was performed using GAPDH as the housekeeping gene. The PCR products were resolved by gel electrophoresis on a 2% agarose gel containing ethidium bromide. The gels were visualized and documented using a gel documentation system (FUSION SL, Vilber Lourmat, France) and semi-quantification of mRNA expression was performed using ImageJ® software (version 1.46r).

2.8. Mancozeb downregulates PGES expression in the uterus of treated mice

The expression and localization of COX-2, p53, and PGES proteins were performed by immunohistochemical staining [39]. Briefly, tissue sections were de-paraffinized,

rehydrated, and then subjected to antigen retrieval using the Target Retrieval Solution (Dako Cytomation, Carpinteria, CA, USA) as described previously [40]. The sections were incubated overnight with anti-COX-2 (1:500; ab15191, Abcam), anti-p53 (1:50; ab31333, Abcam), or anti-PGES (1:1000, Ab62050, Abcam) antibodies at 4^{o}C. The sections were incubated in 3,3'-diaminobenzidine (DAB substrate chromogen, Dako Cytomation) and the nuclei were counterstained with Hematoxylin. Images were captured under a light microscope with a digital camera (Axioscope, Zeiss, Göttingen, Germany). Micrographs (magnification ×200) were captured for the histological scoring using ImageJ® software (version 1.46r). Digital histological scoring method (D-HSCORE) was employed to quantify the differential expression of proteins in the treatment and control groups as described previously [39].

2.9. Effect of preimplantation Mancozeb exposure on fecundity in F_1 mice

After confirmation of mating, mice were administered Mancozeb (0.3, 3 and 30 mg/kg BW/day) through oral gavage from GD1 to GD4. DMSO dissolved in olive oil was administered to mice in the control group. The lengths of the gestation period and litter sizes were determined. Pups from all litters within the treatment group were pooled and separated by sex before sexual maturation. At 12 weeks after birth, a group of female mice (F_1) was sacrificed and their reproductive tracts were weighed. Another group of F_1 female mice were allowed to sexually mature before mating with mature fertile adult males (avoiding siblings), and the lengths of the gestation period and litter sizes were recorded.

2.10. Isolation and identification of human primary endometrial stromal cells

Human endometrial biopsies were obtained from patients receiving IVF treatment at Queen Mary Hospital, Pokfulam, Hong Kong SAR. This study protocol was approved

by the ethics committee of the Institutional Review Board of Hospital Authority Hong Kong West Cluster and The University of Hong Kong, Hong Kong (UW16-204). The biopsies were taken 2 days after the luteinizing hormone surge (LH+2) and endometrial stromal cells (ESCs) were isolated using a method reported previously with modifications [41, 42]. The purity of the stromal cells was checked by vimentin and cytokeratin immunofluorescence staining. Primary endometrial stromal cells were cultured in a 48-well plate until confluency. Cells were washed with phosphate buffered saline (PBS), fixed with 4% cold paraformaldehyde in PBS (pH 7.4) on ice for 20 min, permeabilized with 0.1% Triton X-100 for 15 min, blocked with 10% donkey serum (D9663, Sigma) for 1 hr at room temperature (RT), and then incubated overnight with the primary antibodies at 4°C. Primary anti-human cytokeratin (1:100, M0821, Dako, Cambridge, UK) and anti-human vimentin (1:100, M0725, Dako) antibodies were used. The cells were washed and incubated with secondary antibody, Alexa Fluor 488 donkey anti-mouse IgG (1:200, A21202, Invitrogen) for 1 hr at RT. The nuclei of cells were counterstained with 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, D1306, Invitrogen) in PBS for 15 min. Images were captured under a fluorescence microscope (Nikon Eclipse Ti-S, Japan).

2.11. *In vitro* decidualization and Mancozeb treatment of human primary endometrial stromal cells

Isolated human endometrial stromal cells were seeded on a 24-well plate at a density of 1×10^5 cells per well and cultured in phenol-free DMEM/F12 supplemented with 10% csFBS until confluency. The cells were induced to decidualize with 0.5 mM cAMP (Sigma), 10 nM 17β-estradiol (Sigma), and 1μM progesterone (Sigma). The stromal cells were then co-treated with either 0.1% DMSO (vehicle control) or Mancozeb (3 μg/mL). The medium was collected every 3 days and changed with fresh medium on days 3, 6,

297 and 9 after decidualization. Images were captured with a phase-contrast microscope
298 (Nikon Eclipse Ti-S, Japan) and morphological changes were evaluated. The expressions
299 of decidualization markers including insulin-like growth factor binding protein 1
300 (IGFBP-1) and prolactin (PRL) were determined by qPCR. Briefly, total RNA extracted
301 from cells was reverse transcribed and qPCR was performed using TaqMan 2× Universal
302 PCR Master Mix (Life Technologies, USA) using an Applied Biosystems 7500 Detection
303 System (Applied Biosystems, USA). The expression of 18S RNA was used as the internal
304 control for RNA normalization. The TaqMan probes for human IGFBP1 (Hs
305 00236877_m1), PRL (Hs00168730_m1), and 18S (4318839) were purchased from
306 Applied Biosystems, USA. The cycle conditions for the PCR were 50°C for 2 min, 95°C
307 for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The formula of
308 $2^{-\Delta\Delta}$Ct was applied to quantify the relative expressions of mRNA.
309
310 **2.12. Statistical analysis**
311 The *in vitro* embryo attachment data were analyzed using one-way analysis of
312 variance (ANOVA) followed by mean separation using Turkey's multiple comparison
313 test. The results were expressed as mean ± SEM. The *in vivo* data were subjected to
314 ANOVA followed by a comparison of means using Dunnett's test (Minitab 17) to
315 establish the validity of the results. The values were expressed as mean ± SD. The *in*
316 *vitro* decidualization data were analyzed using nonparametric analysis by Kruskal-Wallis
317 test. The results were expressed as mean ± SEM. (*$*p<0.05$, **$p<0.01$, ***$p<0.001$*).
318

3. Results

3.1. Effects of Mancozeb on spheroid (blastocyst surrogate) attachment onto endometrial epithelial Ishikawa cells

In the co-culture model, Human endometrial epithelial Ishikawa cells were used as the receptive endometrium for trophoblastic Jeg-3 spheroid attachment (Fig. 1A). The co-culture experiments were performed with cells individually or both treated with Mancozeb. When only Ishikawa cells were treated with 3 μg/mL Mancozeb for 24 h, the spheroid attachment rate was significantly reduced ($p<0.05$) compared with the DMSO control; whereas none of the other concentrations showed significant effects. Interestingly, when both Jeg-3 and Ishikawa cells were co-treated with 1 and 3 μg/mL Mancozeb for 24 h, the attachment rate was significantly reduced ($p<0.05$) (Fig. 1B). Methotrexate (MTX) was included in the study as a positive control for the spheroid attachment assay.

3.2. Effects of periimplantation Mancozeb exposure on embryo implantation in mice

Female pregnant mice were treated with Mancozeb (1, 16 or 32 mg/kg BW/d) or DMSO control from GD1 to GD8, and the number of implantation sites was determined on GD9 (Fig. 2A). Mancozeb at 1 and 16 mg/kg BW/day had no significant effects on the number of implantation sites compared to the control group (Table 1). However, 32 mg/kg BW/day Mancozeb significantly reduced ($p<0.05$) the number of implantation sites compared to the controls.

Mancozeb at 1 and 16 mg/kg BW/day did not significantly change the morphology (data not shown) compared to the control group; whereas treatment with 32 mg/kg BW/day Mancozeb significantly altered the morphology of the ovaries, oviducts, and

uteri of mice (Supplementary table 2). There was a significant ($p<0.05$) reduction in the number of follicles in the ovaries and a significant ($p<0.05$) reduction in the number of folds in ampulla regions of the oviducts. In the Mancozeb-treated animals, the epithelial lining of the endometrium in the uterus showed marked abnormal histological changes, suggesting altered endometrial growth and development (Fig. 2B).

3.3. Effects of postimplantation Mancozeb exposure on stromal cell decidualization in mice

To investigate the effect of Mancozeb on endometrial stromal cell decidualization during embryo implantation, mice were treated with Mancozeb from GD4 to GD8 of the pregnancy and uterus tissues were collected on GD9. Mancozeb at 0.3 and 3 mg/kg BW/day did not significantly change the number of implantation sites (Table 2). However, Mancozeb at 30 mg/kg BW/day resulted in a complete loss ($p<0.05$) of implantation sites in treated mice compared with the DMSO controls. Furthermore, analysis of the expressions of genes related to uterine decidualization revealed that Mancozeb significantly ($p<0.05$) suppressed *PGES* mRNA expression in a dose-dependent manner, but not *COX-2, PGFS,* and *p53* mRNA expressions in the uterus of treated mice (Fig. 3). It should be noted that the expression of *PGFS* transcripts was relatively low after PCR amplification.

Immunohistochemical staining and DHSCORE were used to evaluate the expression and level of COX-2, p53 and PGES proteins in the uterus. Interestingly, the expressions of these proteins were mainly localized in the epithelial cells of the mouse uterus with higher expression in glandular epithelium compared to surface epithelium (Fig. 4A). The expression of COX-2 was high in all Mancozeb treated groups, but a significantly *($p<0.05$)* higher expression was found with 3 mg/kg BW/day Mancozeb compared to the control group (Fig. 4B). The p53 protein expression did not significantly change in any

of the Mancozeb treated animals compared to the controls. Interestingly, the expression of PGES protein was mainly localized at the apical region of the luminal epithelium and decreased significantly *($p<0.05$)* with 30 mg/kg BW/day Mancozeb compared to the control group (Fig. 4B).

3.4. Effect of prenatal Mancozeb exposure versus non-exposure on the subsequent generation of offspring

To investigate the effect of prenatal Mancozeb exposure on the development of reproductive tissues in offspring, adult mice were treated with Mancozeb (0.3, 3 and 30 mg/kg BW/day) from GD1 to GD4. The whole reproductive tract including ovary, oviduct, and uterus of the offspring (F_1) was collected on week 12. Mancozeb treatments (0.3, 3 and 30 mg/kg BW/day) did not affect the number of pups produced per litter or length of gestation period in both the F_0 and F_1 generations compared to the controls. Furthermore, Mancozeb treatments did not alter the weight of the female reproductive tract including ovary, oviduct, and uterus of F_1 mice compared with the controls (Table 3).

3.5. Effects of Mancozeb on the *in vitro* decidualization of human endometrial stromal cells

Stromal cells were isolated from human endometrial aspirate for the decidualization experiment. Immunofluorescent staining of stromal cells were positive for vimentin and negative for cytokeratin indicating pure stromal cells in the culture. More than 90% of cells were positive for vimentin (Fig. 5A), suggesting enrichment of stromal cells in the preparations. Decidualization was induced by adding a decidualization cocktail (containing cAMP, estrogen, and progesterone) into the culture media for 9 days. The morphology of the stromal cells changed from an elongated fibroblast to a round shape

(Fig. 5B). Mancozeb did not affect the expression of decidualization markers *IGFBP1* and *PRL* during the 9 days of decidualization (Fig. 5C-D). However, Mancozeb blocked the morphological changes induced by decidualization (Fig. 5B).

4. Discussion

The present study using *in vivo* mice models and two *in vitro* cell culture models investigated the effects of Mancozeb on embryo implantation, spheroid attachment of blastocyst surrogates, and stromal cells decidualization.

The spheroid-endometrial cells co-culture model using Ishikawa cells and trophoblastic Jeg-3 spheroids mimics the early attachment of embryos onto the endometrial epithelium. This *in vitro* model showed that Mancozeb could affect early embryo attachment during implantation. Results from the *in vivo* study suggest that Mancozeb exposure, particularly during the decidualization period, disrupts embryo implantation and suppresses the morphological transformation of endometrial stromal cells, although the expressions of decidualization markers were not altered.

Environmental concerns over the use of pesticides on human/animal reproductive health have been raised in many countries [43]. In particular, overuse of pesticides beyond recommended levels or concentrations is very common in agriculture-based developing countries. Embryo implantation and endometrial stromal cell decidualization are critical processes in the establishment of pregnancy in eutherian mammals. Pregnancy involves fine regulation of the uterine microenvironment via the coordinated efforts of the hypothalamic-pituitary-ovarian axis [44]. However, most of the EDCs in use nowadays have been found to interfere with the reproductive process by disrupting embryo implantation and endometrial cell decidualization [45].

Several studies found levels of Mancozeb in fruits and vegetables that exceeded the maximum residue limits [46, 47, 28]. Furthermore, studies have shown that females are exposed to high doses of Mancozeb through elevated levels found in the environment [31, 10]. Therefore, we selected doses of Mancozeb below the acute LD_{50} level based on these previous findings [9].

Implantation of the embryo is tightly controlled by steroid hormones that modulate endometrial receptivity and stromal cell decidualization [48]. Results from our *in vitro* embryo attachment study suggests that Mancozeb at 3 μg/mL suppresses the receptivity of endometrial cells. However, no such suppressive effects were observed when only Jeg-3 cells were treated with Mancozeb. Interestingly, when both cell types were treated with a lower dose of Mancozeb (1 μg/mL), the *in vitro* attachment rate of Jeg-3 spheroid was suppressed suggesting a synergistic effect of Mancozeb on both cells in suppressing the implantation process.

In mice, exposure to Mancozeb from GD1 to GD8 resulted in morphological changes in the uterus, ovary, and oviduct, as well as significantly reducing the number of implantation sites. However, Mancozeb exposure from GD1 to GD4 did not affect embryo implantation, the number of pups produced per litter, or the reproductive function of offspring, indicating that GD4 to GD8 is the critical period for Mancozeb exposure to have a detrimental effect on embryo implantation and subsequent pregnancy outcome. In line with this, direct exposure to Mancozeb leads to ovarian hypertrophy and disruption of the estrous cycle in hemi-ovariectomized albino rat, which was attributed to the direct effect of Mancozeb on the ovary or the hypothalamic-hypophysial-ovarian axis [44]. In the present study, acute high dose of Mancozeb (32 mg/kg BW/day) administered from GD1 to GD8 induced structural abnormalities in the uterus, oviduct, and ovarian tissues. The data suggests that Mancozeb induces pregnancy loss possibly through the direct disruption of endometrial receptivity, and indirect impairment of ovarian function. However, further investigations are needed to verify these findings. A recent study reported that bisphenol A affected reproduction through disruption of uterine decidualization and embryo implantation in mice [45].

Mancozeb treatment (32 mg/kg BW/day) from GD1 to GD8 could affect various biological processes during this critical period including embryonic development,

window of implantation, and embryo attachment to the uterine wall following the decidualization responses [48]. In mice, implantation occurs on GD 4.5 [49] and the attachment of the blastocyst to the uterine epithelium then triggers the process of decidualization, which initiates differentiation of the stromal cells into decidual cells.

Therefore, to delineate whether the effect of Mancozeb depends on the preimplantation or postimplantation period, mice were treated only from GD4 to GD8, which is the period of decidualization in the endometrium during implantation [48]. Our results indicated that Mancozeb treatment (30 mg/kg BW/day) from GD4 to GD8 impaired implantation, partly through modulating the decidualization process *in vivo*. There was a significant downregulation of PGES transcript and protein expression in the treated mice. It was reported that Mancozeb at 36 mg/kg BW/day from GD1 to GD3 or from GD1 to GD5 also induced significant implantation loss in mice [9]. The differences observed in the present study compared to these two studies may be due to the lower treatment doses used (30 mg/kg BW/day in our study) as well as the Mancozeb treatment period (GD1 to GD4 in our study). Interestingly, Mancozeb at other concentrations (24, 30 and 36 mg/kg BW/day) also caused significant embryo loss [9], but such effects were not observed in the present study.

Prostaglandins (PGs), especially $PGF_{2\alpha}$ and PGE_2 play a very important role in implantation as they are directly involved in decidualization [27]. Cyclo-oxygenase (COX) is the rate-limiting enzyme that converts arachidonic acid to PGs [50]. Uterine COX-2 is not directly regulated by progesterone and/or estrogen [50], instead, it is induced by the activated blastocyst at the time of attachment producing PGs needed for implantation and decidualization [26]. Furthermore, COX-2 is expressed in the luminal epithelium and sub-epithelial stromal cells at the anti-mesometrial pole surrounding the blastocyst at the time of attachment on GD4 and persists through the morning of GD5 [50]. Therefore, the proper expression and regulation of COX-2 in the uterine gland is

essential for embryo implantation and the establishment of endometrial receptivity [50]. Our data revealed that the expression of *COX-2* mRNA was not affected by the Mancozeb treatment (3 mg/kg BW/day) in the endometrium. However, COX-2 protein was significantly increased and localized in the endometrial surface epithelium and glandular epithelium as well as in stromal cells. Similar observations of a significant increase in COX-2 protein expression and activation of the ERK1/2 pathway were found when human skin HaCaT cells were exposed to Mancozeb at 0.015–30 mg/mL for 24 h [51].

Among the key enzymes involved in PG synthesis, PGES converts PGH_2 to PGE_2, whereas PGFS generates $PGF_{2\alpha}$ [26]. Both PGE_2 and $PGF_{2\alpha}$ are important for implantation and decidualization in rodent uterus [27]. In mice, PGE_2 exerts multiple effects during the preimplantation period [52] and is involved in uterine angiogenesis from GD6 to GD8, which is required for the establishment of the placenta [52]. Our data showed that the expression of *PGFS* mRNA did not change in the uterus in mice treated with Mancozeb (0.3, 3 and 30 mg/kg BW/day) from GD4 to GD8. However, PGES transcript was significantly downregulated at the end of the treatment period. This was in agreement with the significantly lower protein expression in the uterus of mice treated with Mancozeb (30 mg/kg BW/day) as observed in the immunohistochemical analysis using PGES antibody. In line with this, higher expression of PGES in the luminal epithelium surrounding the implanting blastocyst was observed at the implantation site in rat uterus [53]. The present data suggest that the downregulation of PGES has more of a local effect on embryo implantation than an indirect effect through ovarian steroid action. Furthermore, the observed morphological changes in the uterine epithelium of treated mice could also lead to a reduction in PGES expression.

Our *in vitro* decidualization model using human primary endometrial stromal cells revealed that Mancozeb suppressed the morphological changes of stromal cells induced

505 by decidualization. Interestingly, the mRNA expression of key markers related to
506 decidualization were not altered by any of the doses of Mancozeb. It would be
507 worthwhile to check the protein expression levels of these markers to confirm this
508 finding, as posttranscriptional modifications of mRNA can contribute to a reduction of
509 translation inside cells.

510 Transformation-related protein 53 (*Trp53*) gene, is a tumor suppressor gene whose
511 mutations are strongly associated with cancer [54]. This protein also plays important
512 roles in reproduction, especially in embryo implantation and pregnancy [25]. It has been
513 suggested that upregulation of p53 improves implantation in mice [42]. A study by Paro
514 et al [16] on human and mouse granulosa cells treated with Mancozeb (0.3, 3and 30
515 mg/kg BW/day) from GD4 to GD8 found that p53 expression was downregulated, but
516 uterine p53 expression level was not affected. A very recent study on murine granulosa
517 cells confirmed the downregulation of p53 together with ultrastructural changes even at
518 a very low concentration of Mancozeb [55]. Our data showed no significant changes in
519 p53 protein expression, and p53 was mainly expressed in endometrial surface epithelium,
520 glandular epithelium, and stromal cells of mice uteri at GD8 irrespective of the treatment,
521 which suggests Mancozeb exposure may cause possible differential expressions of p53
522 in different reproductive tract tissues.

523 The F_1 offspring of the treated mice were evaluated to study any generational imprint
524 effects of Mancozeb exposure during the preimplantation period (GD1 to GD4). Our data
525 suggested no such imprinting effect was present, as the litter size, length of the gestation
526 period, and weight of female reproductive organs in F_1 female mice were not altered
527 compared with the controls. Similar findings have been reported elsewhere, which
528 showed Mancozeb (18, 24, 30 and 36 mg/kg BW/day) did not affect the length of the
529 gestation period, litter size, or weight of female reproductive organs in F_1 generation rats
530 [9].

In vitro studies reported that the endocrine-disrupting potential of Mancozeb could be mediated via androgen receptor activities [56]. However, we did not evaluate androgen receptor expression in the present study, which would be worthwhile to test in future studies. Interestingly, a recent report indicated that Mancozeb could decrease androgen production in rats by reducing the expression of a key enzyme responsible for steroidogenesis [57].

In humans, Mancozeb is metabolized in the mucus membranes or respiratory and gastrointestinal tract to ethylenethiourea (ETU) [58]. Elevated concentrations of urinary ETU were detected in pregnant women after exposure to aerial spraying of Mancozeb [31]. Several studies have reported that Mancozeb and its known metabolite ETU can cause thyrotoxic effects in rats and mice [51, 52], and Mancozeb was shown to produce marked structural and functional changes in the thyroid of rats [61], indicating that thyroid toxicity is one possible mechanism of Mancozeb effects. Therefore, Mancozeb and its metabolite ETU could be responsible for the observed effects in the present study, which opens up a window for further investigations.

In summary, our *in vitro* and *in vivo* studies provide evidence that high dose Mancozeb can affect embryo implantation. The suppressive effects of Mancozeb exposure was more prominent from GD4 to GD8, but not from GD1 to GD4, which could be due to the direct suppression of PGES expression in the uterine microenvironment during the decidualization process. Whether these changes are associated with alterations in steroid receptor expression and responses, and/or induction of other signaling pathways remain to be investigated.

Competing interest

The authors declare they have no competing interests.

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Authors' Contribution

All authors were involved in planning and designing the experiments. The *in vitro* cell culture experiments were carried out by ZW and KFL. IA, MPBW, ES, CJR, and SPK carried out the *in vivo* mice experiments, histopathological studies, gene expression studies, and data analysis. IA and ES conducted the histopathological studies. All authors contributed to writing and revising the manuscript.

References

[1] P.A. Fowler, M. Bellingham, K.D. Sinclair, N.P. Evans, P. Pocar, B. Fischer, K. Schaedlich, J.-S. Schmidt, M.R. Amezaga, S. Bhattacharya, S.M. Rhind, P.J. O'Shaughnessy, Impact of endocrine-disrupting compounds (EDCs) on female reproductive health, Mol. Cell. Endocrinol. 355 (2012) 231–239. doi:10.1016/j.mce.2011.10.021.

[2] R.J. Kavlock, G.P. Daston, C. DeRosa, P. Fenner-Crisp, L.E. Gray, S. Kaatari, G. Lucier, M. Luster, M.J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D.M. Sheehan, T. Sinks, H.A. Tilson, Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop, Environ. Heatlh Perspect. 104 (1996) Supplement 4. doi:10.1289/ehp.96104s4715.

[3] E. Diamanti-Kandarakis, J.-P. Bourguignon, L.C. Giudice, R. Hauser, G.S. Prins, A.M. Soto, R.T. Zoeller, A.C. Gore, Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement, Endocr. Rev. 30 (2009) 293–342. doi:10.1210/er.2009-0002.

[4] T.K. Woodruff, C.L. Walker, Fetal and early postnatal environmental exposures and reproductive health effects in the female, Fertil. Steril. 89 (2008) e47–e51. doi:10.1016/j.fertnstert.2007.12.029.

[5] A.M. Vinggaard, C. Hnida, V. Breinholt, J.C. Larsen, Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro, Toxicol. Vitr. 14 (2000) 227–234. doi:10.1016/S0887-2333(00)00018-7.

[6] V.K. Malik, M. Singh, K.S. Hooda, N.K. Yadav, Efficacy of Newer Molecules , Bioagents and Botanicals against Maydis Leaf Blight and Banded Leaf and Sheath Blight of Maize, Plant Pathol. J. 34 (2018) 121–125. doi:10.5423/PPJ.OA.11.2017.0251.

[7] J. Shin, T. Fu, K.H. Park, K.S. Kim, The Effect of Fungicides on Mycelial Growth and Conidial Germination of the Ginseng Root Rot Fungus, Cylindrocarpon destructans, Mycobiology. 45 (2017) 220. doi:10.5941/MYCO.2017.45.3.220.

[8] G.P. Munkvold, C. a Martinson, J.M. Shriver, P.M. Dixon, Probabilities for profitable fungicide use against gray leaf spot in hybrid maize., Phytopathology. 91 (2001) 477–84. doi:10.1094/PHYTO.2001.91.5.477.

[9] B.B. Bindali, B.B. Kaliwal, Anti-implantation Effect of a Carbamate Fungicide Mancozeb in Albino Mice, Ind. Health. 40 (2002) 191–197.

[10] J. Runkle, J. Flocks, J. Economos, A.L. Dunlop, A systematic review of Mancozeb as a reproductive and developmental hazard, Environ. Int. 99 (2017) 29–42. doi:10.1016/j.envint.2016.11.006.

[11] S. Cecconi, R. Paro, G. Rossi, G. Macchiarelli, The effects of the endocrine disruptors dithiocarbamates on the mammalian ovary with particular regard to mancozeb., Curr. Pharm. Des. 13 (2007) 2989–3004. http://www.ncbi.nlm.nih.gov/pubmed/17979742.

[12] G. Rossi, R. Buccione, M. Baldassarre, G. Macchiarelli, M.G. Palmerini, S. Cecconi, Mancozeb exposure in vivo impairs mouse oocyte fertilizability, Reprod Toxicol. 21 (2006) 216–219. doi:10.1016/j.reprotox.2005.08.004.

[13] A.R. Greenlae, T.M. Ellis, R.L. Berg, Low-dose agrochemicals and lawn-care pesticides induce developmental toxicity in murine preimplantation embryos, Environ. Health Perspect. 112 (2004) 703–709. doi:10.1289/ehp.6774.

[14] P.N. Baligar, B.B. Kaliwal, Induction of Gonadal Toxicity to Female Rats after Chronic Exposure to Mancozeb, Ind. Health. 39 (2001) 235–243. doi:10.2486/indhealth.39.235.

[15] O.M. Kandil, R.M. Sabraa, A.A. Said, Effect of Direct Exposure to Mancozeb Fungicide on the Developmental Competnce of Buffalo Oocytes In vitro, Glob. Vet. 7 (2011) 242–248.
[16] R. Paro, G.M. Tiboni, R. Buccione, G. Rossi, V. Cellini, R. Canipari, S. Cecconi, The fungicide mancozeb induces toxic effects on mammalian granulosa cells., Toxicol. Appl. Pharmacol. 260 (2012) 155–61. doi:10.1016/j.taap.2012.02.005.
[17] A. Goldoni, C.R. Klauck, S.T. Da Silva, M.D. Da Silva, P.G. Ardenghi, L.B. Da Silva, DNA damage in Wistar rats exposed to dithiocarbamate pesticide mancozeb, Folia Biol. (Czech Republic). 60 (2014) 202–204.
[18] S.P. Pandey, B. Mohanty, The neonicotinoid pesticide imidacloprid and the dithiocarbamate fungicide mancozeb disrupt the pituitary-thyroid axis of a wildlife bird, Chemosphere. 122 (2015) 227–234. doi:10.1016/j.chemosphere.2014.11.061.
[19] J. Boivin, L. Bunting, J. a. Collins, K.G. Nygren, International estimates of infertility prevalence and treatment-seeking: Potential need and demand for infertility medical care, Hum. Reprod. 22 (2007) 1506–1512. doi:10.1093/humrep/dem046.
[20] N. Cui, C. Wang, Z. Zhao, J. Zhang, Y. Xu, Y. Yang, G. Hao, The Roles of Anandamide, Fatty Acid Amide Hydrolase, and Leukemia Inhibitory Factor on the Endometrium during the Implantation Window, Front. Endocrinol. (Lausanne). 8 (2017) 1–8. doi:10.3389/fendo.2017.00268.
[21] Y. Liu, S.P. Kodithuwakku, P.Y. Ng, J. Chai, E.H.Y. Ng, W.S.B. Yeung, P.C. Ho, K.F. Lee, Excessive ovarian stimulation up-regulates the Wnt-signaling molecule DKK1 in human endometrium and may affect implantation: An in vitro co-culture study, Hum. Reprod. 25 (2010) 479–490. doi:10.1093/humrep/dep429.
[22] E.H. Yu Ng, High serum oestradiol concentrations in fresh IVF cycles do not impair implantation and pregnancy rates in subsequent frozen-thawed embryo transfer cycles, Hum. Reprod. 15 (2000) 250–255. doi:10.1093/humrep/15.2.250.
[23] B. Gellersen, J.J. Brosens, Cyclic decidualization of the human endometrium in reproductive health and failure, Endocr. Rev. 35 (2014) 851–905. doi:10.1210/er.2014-1045.
[24] S.A. Robertson, L.M. Moldenhauer, Immunological determinants of implantation success, Int. J. Dev. Biol. 58 (2014) 205–217. doi:10.1387/ijdb.140096sr.
[25] W. Hu, Z. Feng, A.K. Teresky, A.J. Levine, p53 regulates maternal reproduction through LIF., Nature. 450 (2007) 721–724. doi:10.1038/nature05993.
[26] H. Lim, B.C. Paria, S.K. Das, J.E. Dinchuk, R. Langenbach, J.M. Trzaskos, S.K. Dey, Multiple female reproductive failures in cyclooxygenase 2-deficient mice, Cell. 91 (1997) 197–208. doi:10.1016/S0092-8674(00)80402-X.
[27] H. Ni, T. Sun, X.-H. Ma, Z.-M. Yang, Expression and regulation of cytosolic prostaglandin E synthase in mouse uterus during the peri-implantation period., Biol. Reprod. 68 (2003) 744–750. doi:10.1007/s10735-013-9501-z.
[28] A. Atuhaire, E. Kaye, I. Mutambuze, G. Matthews, T. Friedrich, E. Jors, Assessment of Dithiocarbamate Residues on Tomatoes Conventionally Grown in Uganda and the Effect of Simple Washing to Reduce Exposure Risk to Consumers, Environ. Health Insights. 11 (2017) 1–8. doi:10.1177/1178630217712218.
[29] M. Zizza, M. Di Lorenzo, V. Laforgia, E. Furia, G. Sindona, M. Canonaco, R.M. Facciolo, HSP90 and pCREB alterations are linked to mancozeb-dependent behavioral and neurodegenerative effects in a marine teleost, Toxicol. Appl. Pharmacol. 323 (2017) 26–35. doi:10.1016/j.taap.2017.03.018.
[30] O. López-Fernández, R. Yáñez, R. Rial-Otero, J. Simal-Gándara, Kinetic

modelling of mancozeb hydrolysis and photolysis to ethylenethiourea and other by-products in water, Water Res. 102 (2016) 561–571. doi:10.1016/j.watres.2016.07.006.
[31] B. van Wendel de Joode, A.M. Mora, L. Córdoba, J.C. Cano, R. Quesada, M. Faniband, C. Wesseling, C. Ruepert, M. Öberg, B. Eskenazi, D. Mergler, C.H. Lindh, Aerial Application of Mancozeb and Urinary Ethylene Thiourea (ETU) Concentrations among Pregnant Women in Costa Rica: The Infants' Environmental Health Study (ISA), Environ. Health Perspect. 122 (2014) 1321–1328. doi:10.1289/ehp.1307679.
[32] R. Li, C. Yu, R. Gao, X. Liu, J. Lu, L. Zhao, X. Chen, Y. Ding, Y. Wang, J. He, Effects of DEHP on endometrial receptivity and embryo implantation in pregnant mice, J. Hazard. Mater. 241–242 (2012) 231–240. doi:10.1016/j.jhazmat.2012.09.038.
[33] M. Yuan, M. Bai, X. Huang, Y. Zhang, J. Liu, M. Hu, W. Zheng, F. Jin, Preimplantation Exposure to Bisphenol A and Triclosan May Lead to Implantation Failure in Humans, Biomed Res. Int. 2015 (2015) 1–9. doi:10.1155/2015/184845.
[34] C. Mannelli, A.Z. Szóstek, K. Lukasik, C. Carotenuto, F. Ietta, R. Romagnoli, C. Ferretti, L. Paulesu, S. Wołczynski, D.J. Skarzynski, Bisphenol A modulates receptivity and secretory function of human decidual cells: An in vitro study, Reproduction. 150 (2015) 115–125. doi:10.1530/REP-14-0601.
[35] K.S.A. Kottawatta, K.-H. So, S.P. Kodithuwakku, E.H.Y. Ng, W.S.B. Yeung, K.-F. Lee, MicroRNA-212 Regulates the Expression of Olfactomedin 1 and C-Terminal Binding Protein 1 in Human Endometrial Epithelial Cells to Enhance Spheroid Attachment In Vitro1, Biol. Reprod. 93 (2015) 1–10. doi:10.1095/biolreprod.115.131334.
[36] M.D.P. and B.B. Kaliwal, Dose-Dependent Estrous Cycle, Ovarian Follicles and Biochemical Contents Reversal in Albino Mice after Exposure to Mancozeb., Casp. J. Env. Sci. 3 (2005) 118–131.
[37] M. Daniele, G. Fuhrich, B.A. Lessey, R.F. Savaris, Comparison of HSCORE Assessment of Endometrial β3 Integrin Subunit Expression with Digital HSCORE Using Computerized Image Analysis (ImageJ), Anal Quant Cytopathol Histpathol. 35 (2014) 210–216. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4090774/pdf/nihms596070.pdf.
[38] S.S.F. Yung, V.C.Y. Lee, P.C.N. Chiu, H.W.R. Li, E.H.Y. Ng, W.S.B. Yeung, P.C. Ho, The effect of 7 days of letrozole pretreatment combined with misoprostol on the expression of progesterone receptor and apoptotic factors of placental and decidual tissues from first-trimester abortion: A randomized controlled trial, Contraception. 93 (2016) 323–330. doi:10.1016/j.contraception.2015.12.005.
[39] S.P. Kodithuwakku, R.T.K. Pang, E.H.Y. Ng, A.N.Y. Cheung, A.W. Horne, P.-C. Ho, W.S.B. Yeung, K.-F. Lee, Wnt activation downregulates olfactomedin-1 in Fallopian tubal epithelial cells: a microenvironment predisposed to tubal ectopic pregnancy, Lab. Investig. 92 (2012) 256–264. doi:10.1038/labinvest.2011.148.
[40] K.F. Lee, J. Sen Xu, Y.L. Lee, W.S.B. Yeung, Demilune cell and parotid protein from murine oviductal epithelium stimulates preimplantation embryo development, Endocrinology. 147 (2006) 79–87. doi:10.1210/en.2005-0596.
[41] A. Masuda, N. Katoh, K. Nakabayashi, K. Kato, K. Sonoda, M. Kitade, S. Takeda, K. Hata, J. Tomikawa, An improved method for isolation of epithelial and stromal cells from the human endometrium, J. Reprod. Dev. 62 (2016) 213–218. doi:10.1262/jrd.2015-137.
[42] R.W.S. Chan, A.S.C. Mak, W.S.B. Yeung, K.-F. Lee, A.N.Y. Cheung, H.Y.S. Ngan, A.S.T. Wong, Human female reproductive tract epithelial cell culture.,

Methods Mol. Biol. 945 (2013) 347–63. doi:10.1007/978-1-62703-125-7_21.
[43] Y. Ma, X. He, K. Qi, T. Wang, Y. Qi, L. Cui, F. Wang, M. Song, Effects of environmental contaminants on fertility and reproductive health, J. Environ. Sci. (China). 77 (2019) 210–217. doi:10.1016/j.jes.2018.07.015.
[44] M.P. Mahadevaswami, U.C. Jadaramkunti, M.B. Hiremath, B.B. Kaliwal, Effect of mancozeb on ovarian compensatory hypertrophy and biochemical constituents in hemicastrated albino rat☆, Reprod. Toxicol. 14 (2000) 127–134. doi:10.1016/S0890-6238(00)00064-2.
[45] M. Yuan, M. Hu, Y. Lou, Q. Wang, L. Mao, Q. Zhan, F. Jin, Environmentally relevant levels of bisphenol A affect uterine decidualization and embryo implantation through the estrogen receptor/serum and glucocorticoid-regulated kinase 1/epithelial sodium ion channel α-subunit pathway in a mouse model, Fertil. Steril. 109 (2018) 735-744.e1. doi:10.1016/j.fertnstert.2017.12.003.
[46] S.M. Kavatsurwa, B. Kiremire, J. Wasswa, P.T. Mpiana, Dithiocarbamates Residues Level in Selected Vegetables from Bukavu , Democratic Republic of Congo, J Phys Chem Sci. (2014) 1–7.
[47] O. López-Fernández, R. Rial-Otero, C. González-Barreiro, J. Simal-Gándara, Surveillance of fungicidal dithiocarbamate residues in fruits and vegetables, Food Chem. 134 (2012) 366–374. doi:10.1016/j.foodchem.2012.02.178.
[48] H. Wang, S.K. Dey, Roadmap to embryo implantation: clues from mouse models, Nat. Rev. Genet. 7 (2006) 185–199. doi:10.1038/nrg1808.
[49] B.C. Paria, Y.M. Huet-Hudson, S.K. Dey, Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus., Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 10159–62. doi:10.1073/pnas.90.21.10159.
[50] I. Chakraborty, S.K. Das, J. Wang, S.K. Dey, Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids, J. Mol. Endocrinol. 16 (1996) 107–122. doi:10.1677/jme.0.0160107.
[51] S. Tyagi, J. George, R. Singh, K. Bhui, Y. Shukla, Neoplastic Alterations Induced in Mammalian Skin Following Mancozeb Exposure Using *In Vivo* and *In Vitro* Models, Omi. A J. Integr. Biol. 15 (2011) 155–167. doi:10.1089/omi.2010.0076.
[52] Z.M. Yang, S.K. Das, J. Wang, Y. Sugimoto, a Ichikawa, S.K. Dey, Potential sites of prostaglandin actions in the periimplantation mouse uterus: differential expression and regulation of prostaglandin receptor genes., Biol. Reprod. 56 (1997) 368–379. doi:10.1095/biolreprod56.2.368.
[53] J. Cong, H.-L. Diao, Y.-C. Zhao, H. Ni, Y.-Q. Yan, Z.-M. Yang, Differential expression and regulation of cylooxygenases, prostaglandin E synthases and prostacyclin synthase in rat uterus during the peri-implantation period., Reproduction. 131 (2006) 139–151. doi:10.1530/rep.1.00861.
[54] Y. Hirota, T. Daikoku, S. Tranguch, H. Xie, H.B. Bradshaw, S.K. Dey, Uterine-specific p53 deficiency confers premature uterine senescence and promotes preterm birth in mice, J Clin Invest. 120 (2010) 803–815. doi:10.1172/JCI40051.
[55] M.G. Palmerini, M. Belli, S.A. Nottola, S. Miglietta, S. Bianchi, S. Bernardi, S. Antonouli, S. Cecconi, G. Familiari, G. Macchiarelli, Mancozeb impairs the ultrastructure of mouse granulosa cells in a dose-dependent manner., J. Reprod. Dev. (2017). doi:10.1262/jrd.2017-143.
[56] L.S. Kjeldsen, M. Ghisari, E.C. Bonefeld-Jørgensen, Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity, Toxicol. Appl. Pharmacol. 272 (2013) 453–464. doi:10.1016/j.taap.2013.06.028.
[57] B.P. Girish, P.S. Reddy, Forskolin ameliorates mancozeb-induced testicular and

epididymal toxicity in Wistar rats by reducing oxidative toxicity and by stimulating steroidogenesis, J. Biochem. Mol. Toxicol. (2017) e22026. doi:10.1002/jbt.22026.
[58] P. Houeto, G. Bindoula, J.R. Hoffman, Ethylenebisdithiocarbamates and ethylenethiourea: possible human health hazards, Environ. Health Perspect. 103 (1995) 568–573. doi:10.1289/ehp.95103568.
[59] M. Axelstad, J. Boberg, C. Nellemann, M. Kiersgaard, P.R. Jacobsen, S. Christiansen, K.S. Hougaard, U. Hass, Exposure to the widely used fungicide mancozeb causes thyroid hormone disruption in rat dams but no behavioral effects in the offspring, Toxicol. Sci. 120 (2011) 439–446. doi:10.1093/toxsci/kfr006.
[60] P.M. Hurley, R.N. Hill, R.J. Whiting, Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents, Environ. Health Perspect. 106 (1998) 437–445. doi:10.1289/ehp.98106437.
[61] R. Kackar, M.K. Srivastava, R.B. Raizada, Studies on rat thyroid after oral administration of mancozeb: morphological and biochemical evaluations., J. Appl. Toxicol. 17 (1997) 369–75. doi:10.1002/(SICI)1099-1263(199711/12)17:6<369::AID-JAT449>3.3.CO;2-H.

802 **Figure legends**

803

804 **Fig. 1.** Effect of Mancozeb on the spheroid attachment rate on human
805 endometrial epithelial cells. **(A)** The morphology of Ishikawa cells, Jeg-3 spheroids and
806 co-culture conditions (left to right) (n=6). Magnification x100. **(B)** The effect of
807 Mancozeb on the attachment rate in treated Ishikawa and Jeg-3 cells. The number of
808 attached spheroids was expressed as a percentage of the total number of spheroids added.
809 There was a significant reduction in the attachment rate when both cells were treated
810 with 1 and 3 µg/mL Mancozeb. Methotrexate (MTX) was used as a positive control for
811 the co-culture assay.

812

813 **Fig. 2**. Effect of Mancozeb on the morphology of reproductive tissues in mice.
814 **(A)** Morphology of mouse uterus on day 9 of pregnancy. Mice were fed with 1, 16 or 32
815 mg/kg BW/day Mancozeb for 8 days during the gestation period. The implantation sites
816 were clearly observable in the control and Mancozeb (1 and 16 mg/kg BW/d) groups.
817 Control mice (n=5) were fed with DMSO in olive oil. **(B)** Micrographs of reproductive
818 tract tissue sections including ovary, oviduct, and uterus from control and Mancozeb (32
819 mg/kg BW/d) groups. Insert photos show higher magnifications of the sections.
820 Arrowheads indicate morphological changes in the treated samples. Magnification x100
821 and x200, respectively.

822

823 **Fig. 3.** Semi-quantitative RT-PCR of the expression of uterine COX-2, p53,
824 PGES, and PGFS in mice treated with Mancozeb from GD4 to GD8. GAPDH mRNA
825 levels in uterine tissues of mice (n=6) were used as loading controls. The expression of
826 PGES was significantly reduced for all concentrations of Mancozeb tested. *$p<0.05$
827 compared with the control (one-way ANOVA, Dunnett's test).

Fig. 4. Expression of uterine COX-2, p53, and PGES proteins in mice treated with Mancozeb. **(A)** The expression of uterine COX-2, p53, and PGES proteins were determined by immunohistochemical staining in the uterus of mice (n=5) on day 9 of pregnancy. Insert photos show higher magnifications of the sections. Magnification x100 and x400. **(B)** The relative expression levels of COX-2, p53, and PGES proteins in the uterus of the female mice expressed as Mean±SD. *p<0.05 compared with the control (one-way ANOVA, Dunnett's test).

Fig. 5. The expression of decidualization transcripts in human primary endometrial stromal cells (ESCs) with or without decidualization and Mancozeb co-treatment. ESCs isolated from the endometrial aspirate were cultured. Non-decidualized or decidualized cells were treated with Mancozeb. **(A)** The cells were stained with stromal marker vimentin (top) or epithelial marker cytokeratin (bottom) to evaluate the purity of the stromal cell population. DAPI (blue) was used for nuclei staining. The merged images are shown on the right. Magnification x200. **(B)** The morphology of ESCs with or without 9 days of decidualization (cAMP, progesterone and estrogen treatment) and 3 μg/mL Mancozeb co-treatment were evaluated under a light microscope. MZ, Mancozeb. Magnification x100. **(C)** PRL and **(D)** IGFBP1 transcript expressions in ESCs cells on day 3, 6, and 9 (left, middle, and right, respectively) were analyzed by qPCR. All the results were expressed as mean ± SEM. Nonparametric analysis by Kruskal-Wallis test was used to analyze the difference. (n=4, *p<0.05, **p<0.01, ***p<0.001).

851 **Table 1.** Effect of Mancozeb exposure from GD1 to GD8 on embryo
852 implantation.

Mancozeb (mg/kg BW/day)	Number of mice		Number of implantation sites	
	Mated	Pregnant	Total	Mean± SD (n=5)
Control	5	5	56	11.2 ± 2.6
1	5	5	61	12.2 ± 2.2
16	5	5	51	10.2 ± 1.1
32	5	3	6	1.2 ± 1.1*

853 * $p<0.05$ compared with the control (one-way ANOVA, Dunnett's test).

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Table 2. Effect of Mancozeb exposure from GD4 to GD8 on embryo implantation.

Mancozeb (mg/kg BW/day)	Number of mice		Number of implantation sites	
	Mated	Pregnant	Total	Mean ± SD (n=5)
Control	5	5	55	11.0 ± 1.7
0.3	5	5	57	11.4 ± 2.3
3	5	5	55	11.0 ± 1.9
30	5	0	0	0*

* $p<0.05$ compared with the control (one-way ANOVA, Dunnett's test)

860 **Table 3.** Effect Mancozeb exposure from GD1 to GD4 on litter size, gestation period, and female reproductive organ weights of F_0 and F_1 mice

861 generations.

		Mancozeb (mg/kg BW/day)			
	Number of mice	Control	0.3	3	30
No. of pups per litter (F_0)	5	8.6 ± 2.2	8.4 ± 2.5	8.8 ± 0.8	8.4 ± 0.9
Gestation period (days) (F_0)	5	18.6 ± 0.9	18.4 ± 0.5	19 ± 0.7	18.8 ± 0.8
Weight of reproductive (F_1) tract (mg)	5	134.5 ± 24.8	135.8 ± 24.1	132.7 ± 32.1	138.6 ± 9.1
No. of pups per litter (F_1)	5	8.3 ± 0.6	8.7 ± 0.6	8.7 ± 1.2	8.7 ± 0.6
Gestation period (days) (F_1)	5	18.7 ± 0.6	18.7 ± 1.2	19 ± 1.0	18.3 ± 0.6

862 Data are presented as means ± SD.

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