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# Effect of Direct Exposure to Mancozeb Fungicide on the Developmental Competnce of Buffalo Oocytes *In vitro*

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**Abstract:** Fungicides are used to maximize the modern agriculture and to improve food production. It is extensively used in agriculture to protect many fruits, vegetables and field crops and seeds against large spectrum of fungal diseases. The present work was undertaken to investigate the effect of direct exposure to mancozeb fungicide on the developmental competence of buffalo oocytes *in vitro*. In experiment 1, buffalo cumulus-oocyte-complexes (COCs) were exposed during *in vitro* maturation (IVM, 22-24 h) to 0 (Control), 1.0, 2.5, 5.0 µg/ml of mancozeb. In experiment 2, after *in vitro* fertilization (IVF) the presumptive zygotes were *in vitro* cultured (IVC) in the presence of the same concentrations of mancozeb. Cumulus-cell expansion, nuclear maturation, cleavage rate and embryo development were determined. Mancozeb significantly ($P<0.01$) decrease cumulus cell expansion, nuclear maturation and increase the percentage of degenerated oocytes as compared to control in a dose dependent mannar. Mancozeb significantly ($P<0.01$) decrease cleavage rate and cleaved embryos were arrested at the 2 to 16-cell stage with fragmented cytoplasm. In conclusion, the direct effect of exposure to mancozeb has a detrimental effect on buffalo fertility through impairing oocytes maturation and development to the blastocyst.

**Key words:** Mancozeb · Buffalo · Oocytes · Maturation rate · Embryo development

## INTRODUCTION

Fungicides are used to maximize the modern agriculture and to improve food production. Mancozeb is an organometallic, polymeric complex of manganese zinc ethylenebisdithiocarbamate fungicide. It is extensively used in agriculture to protect many fruits, vegetables, field crops and seeds against large spectrum of fungal diseases [1]. During the last few decades, human fertility has decreased; it is estimated that one in every five couples is involuntarily sterile due to the adverse effect of chemicals on male sexual development [2], or through an increased incidence of gonadal tumors [3].

In female, it has been reported that direct exposure to mancozeb leads to ovarian hypertrophy and disruption of estrous cycle in hemiovarictomized albino rat and this may be due to a direct effect on the ovary or the hypothalamus-hypophysial-ovarian axis. Baligar and Kaliwal [4] found that mancozeb decrease the number of healthy follicles and increase of the number of atretic follicles due to hormonal imbalance or its direct toxic effect. In addition, mancozeb induced a significant decrease in the number of ovulated eggs and caused a significant decrease of fertilizability related to a reduction in the formation of male and female pronuclei [5]. In *in vitro* study, early cleaved embryos exposed to lindane showed a lower average number of blastomeres per morula, as well as a 40% reduction of the mitotic index [6]. Also, exposure of female hamster to carbendazim fungicide during meiosis increase aneuploide oocyte inducing early pregnancy loss and subsequent arrest embryonic cleavage and implantation [7]. Also, exposure to MBC during early pregnancy was shown to be embryotoxic, resulting in embryonic death, growth retardation and developmental abnormalities [8]. To date there is no information available on the effect of direct exposure to mancozeb on buffalo oocytes and embryos. The present work was undertaken to investigate the effect of direct exposure to mancozeb fungicide on the developmental competence of buffalo oocytes *in vitro*.

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## MATERIALS AND METHODS

All chemicals used in the present study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise mentioned.

**Experiment 1:** *The effect of direct exposure to mancozeb on buffalo oocyte maturation rates in vitro*

**Oocyte Collection and *in vitro* Maturation:** Buffalo ovaries were collected at a local slaughterhouse, transported to the laboratory within 1-2 h. Upon arrival ovaries were washed 3 time with warm physiological saline (0.9% NaCl) containing 100 IU penicillin sodium salt and 100 µg/ml sterptomycine. Cumulus-oocyte complexes (COCs) were aspirated from 3-8 mm follicles, after collection and evaluation under stereomicroscope at 20 x (based on the number of cumulus-cell layers and the homogenous cytoplasm), COCs with more than 3 layers of cumulus cells and homogenous cytoplasm were used in the present experiment. COCs were washed 3 times in maturation medium which consisted of TCM-199 supplemented 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulation hormone (FSH) + 10 IU/ml human chorionic gonadotropin (hCG, El Nile Comp, Egypt) + 50 µg/ml gentamycin. Mancozeb (Sino Harvest CO., Taichung, Taiwan) was added to maturation medium at concentrations of 0 (Control), 1.0, 2.5 or 5.0 µg/ml. COCs were cultured in 35 mm culture dish (Nunc, Denmark) at 38°C and under 5% $CO_2$ in humidified atmosphere for 24 hr.

**Assessment of Cytoplasmic and Nuclear Maturation of *in vitro* Mature Buffalo Oocytes:** After 24 h, oocytes were checked for maturation on the basis of the degree of cumulus cell expansion (grade 0, with no expansion; grade 1 with slight expansion; grade 2 with moderate expansion; and grade 3 with full expansion). After removal of cumulus cells by gentile pipetting, oocytes were fixed in acetic-ethanol (1:3 v/v) for 24 h then stained with 1% aceto-orcine stain for evaluation of nuclear configuration.

**Experiment 2:** *Effect of direct exposure to mancozeb on development of in vitro fertilized buffalo oocytes.*

***In vitro* Maturation:** COCs were washed 3 times in maturation medium which consisted of TCM-199 supplemented 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulation hormone (FSH) + 10 IU/ml human chorionic gonadotropin (hCG, El Nile Comp, Egypt) + 50 µg/ml gentamycin. COCs were cultured in 35 mm culture dish (Nunc, Denmark) at 38°C and under 5% $CO_2$ in humidified atmosphere for 24 h.

***In vitro* Fertilization:** Frozen semen straws from the same batch were thawed in water bath at 37°C for 45 second and then layered on the top of 2 layers of Percoll density gradient (2 ml of 45% Percoll at the top and 2 ml of 90% Percoll at the bottom). After that the tube was centrifuged at 500 g for 30 minutes. The supernatant was removed and the sperm pellet was washed by 5 ml Sp-TALP supplemented 4 mg/ml BSA + 50 µg/ml gentamycin and centrifuged again for 5 minutes. The supernatant was removed and the pellet was resuspended into 1 ml of Fertilization TALP (Fert-TALP) medium supplemented with 10 µg/ml heparin + 2.5 mM caffeine + 5 mg/ ml BSA-FAF (fatty acid free) plus 50 µg/ml gentamycin and the sperm concentration was adjusted to 1-2 x $10^6$ /ml.

For fertilization 300 µl of sperm suspension was allocated into 4-wells culture dish (Nunc, Denmark) and then covered with 200 µl of sterile paraffin oil. A total number of 15-20 matured buffalo oocytes were added to each well. Co-incubation of oocytes and spermatozoa was performed at 38.5°C under 5% $CO_2$ in humidified air for 18-20 h.

After fertilization, the presumptive zygotes were washed three times in *in vitro* culture (IVC) medium, which consisted of Christopher Rozenkran (CR1aa) medium supplement with 5% FCS + 50 µg/ml gentamycin and supplemented with 0 (Control), 1.0, 2.5 or 5.0 µg/ml mancozeb. Zygotes were cultured in $CO_2$ incubator at 38.5°C under 5% $CO_2$ in humidified air for 8 days. Cleavage rate was checked after 48 hr post fertilization and the subsequent embryo development to the morula and blastocyst stages were checked on Days 5, 7 and 8 of culture. IVC medium was changed every 48 h.

**Statistical Analysis:** Data were statistically computed using SPSS16. Results were pooled and analyzed by *Chi-square* test and analysis of variance (ANOVA) producer.

## RESULTS

**Experiment 1:** *Effect of direct exposure to mancozeb on maturation rate of buffalo oocytes*.

Data presented in table 1 demonstrate the effect of direct exposure to mancozeb on cumulus cell expansion of *In vitro* matured buffalo COCs. The percentage of buffalo COCs with slight expansion (G1) was significantly (P<0.01) higher in control group than in mancozeb treated groups (13.63, 28.84, 42.22 and 42.11% for 0, 1.0, 2.5 and 5.0 µg/ml for mancozeb, respectively). Also, COCs which has full cumulus cells expansion (G3) was significantly (P<0.01) higher in control compared to mancozeb treated COCs (63.63, 36.54, 22.22 and 14.03% for 0, 1.0, 2.5 and 5.0 µg/ml for mancozeb, respectively). Mancozeb at a concentration of 2.5 and 5.0 µg/ml significantly (P<0.01) increased the percentage of moderate (G2) cumulus cell expansion compared with control group.

The effect of addition of different concentrations of mancozeb to *in vitro* maturation medium on nuclear maturation of buffalo oocytes is illustrated in table 2. Data revealed that the percentage of matured buffalo oocytes was significantly (P<0.01) higher in control than all mancozeb treated groups. In addition, the percentages of degenerated oocytes were significantly (P<0.01) higher in all mancozeb exposed oocytes compared to control.

Moreover, the addition of 1.0, 2.5 or 5.0 µg/ml mancozeb to *in vitro* maturation medium of buffalo oocytes leads to degeneration of the cytoplasm and most of the exposed oocytes showed fragmented cytoplasm or brocken zona pellucida (Plate 1).

**Experiment 2:** *Effect of direct exposure to mancozeb during in vitro culture on cleavage rate and development of in vitro fertilized buffalo oocytes.*

Table 1: Effect of exposure to different concentrations of mancozeb fungicide on cumulus-cells expansion of *in vitro* matured buffalo oocytes

| | | Degree of cumulus-cell expansion (%) | | | |
|---|---|---|---|---|---|
| Mancozeb Conc. | No oocytes | G0 | G1 | G2 | G3 |
| 0.0 µg/mL | 124 | 22(4.50%) | 26(13.63%)$^{c}$ | 28(18.18%)$^{c}$ | 48(63.63%)$^{a}$ |
| 1.0 µg/mL | 132 | 26(11.53%) | 35(28.84%)$^{c}$ | 32(23.07%)$^{c}$ | 39(36.54%)$^{c}$ |
| 2.5 µg/mL | 125 | 22(4.40%) | 39(42.22%)$^{a}$ | 34(31.11%)$^{a}$ | 30(22.22%)$^{c}$ |
| 5.0 µg/mL | 177 | 24(7.01%) | 44(42.11%)$_{a}$ | 41(36.84%)$^{a}$ | 28(14.03%)$^{c}$ |

a,c within the same column differ significantly at P<0.01

Table 2: Effect of direct exposure to mancozeb on nuclear maturation of *In vitro* matured buffalo oocytes

| | | Immature oocytes (%) | | | Matured conc oocytes (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mancozeb | No. oocytes | GV | GVBD | Total | Ana | Tel | MII | Total | Degenerated |
| 0.0 | 56 | 0 | 6 | 6 (10.7)$^{a}$ | 8 | 4 | 34 | 46 (82.1)$^{a}$ | 4 (7.1)$^{a}$ |
| 1.0 µg/mL | 54 | 1 | 10 | 11 (20.4)$^{b}$ | 5 | 2 | 12 | 19 (35.2)$^{c}$ | 24 (44.4)$^{c}$ |
| 2.5 µg/mL | 49 | 0 | 11 | 11 (22.4)$^{b}$ | 0 | 8 | 9 | 17 (34.7)$^{c}$ | 21 (42.9)$^{c}$ |
| 5.0 µg/mL | 57 | 2 | 17 | 19 (33.3)$^{c}$ | 0 | 0 | 12 | 12 (21.1)$^{c}$ | 26 (45.6)$^{c}$ |

GV= Germinal Vesicle stage GVBD= Germinal Vesicle Break Down AN= Anaphase Tel= Telophase MII= Metaphase II

a,b superscript within the same column differ significantly at P <0.05

a,c superscript within the same column differ significantly at P <0.01

Table 3: Effect of direct exposure to mancozeb on the developmental competence of *In vitro* fertilized buffalo oocyte

| | | | Embryo development (%) | | | |
|---|---|---|---|---|---|---|
| Mancozeb Conc. | No. Fert. oocytes | Cleavage rate (%) | 2-4 cell | 8-16 cell | Morula | Blastocyst |
| 0.0 µg/mL | 74 | 63 (85.1)$^{a}$ | 5 (7.9)$^{a}$ | 13 (20.6)$^{a}$ | 28 (44.4)$^{a}$ | 17 (27.0)$^{a}$ |
| 1.0 µg/mL | 56 | 6 (10.7)$^{c}$ | 2 (33.3)$^{c}$ | 4 (66.7)$^{c}$ | 0$^{c}$ | 0$^{c}$ |
| 2.5 µg/mL | 53 | 4 (7.5)$^{c}$ | 1 (25.0)$^{c}$ | 3 (75.0)$^{c}$ | 0$^{c}$ | 0$^{c}$ |
| 5.0 µg/mL | 62 | 3 (4.8)$^{c}$ | 2 (66.7)$^{c}$ | 1 (33.3)$^{c}$ | 0$^{c}$ | 0$^{c}$ |

a,c superscript within the same column differ significantly at P<0.01

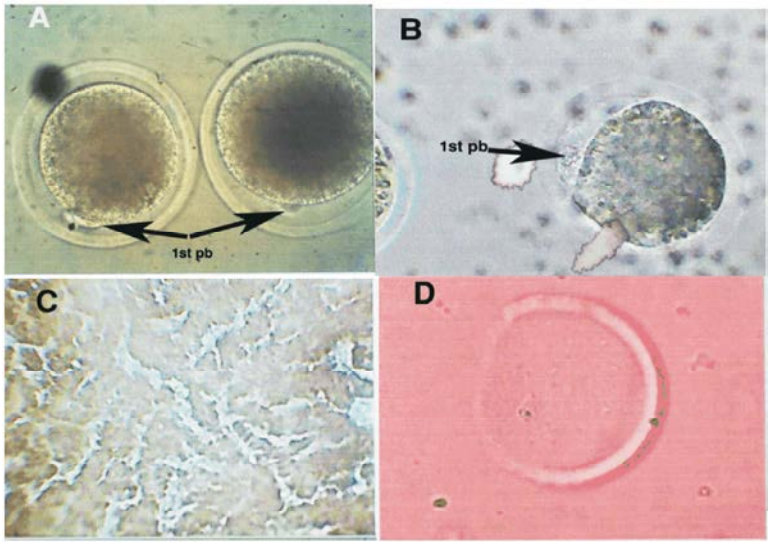


Plate 1: Photomicrograph for the effect of direct exposure to mancozeb on *In vitro* maturation of buffalo oocytes; (A) buffalo oocytes at M II stage and extruding first polar body (1st pb); (B) oocyte matured in the presence of mancozeb (1 µg/mL) domenestated degenrated cytoplasm and degenerated 1st pb; (C) cytoplasm of oocyte stained with orcein satin illustrated degenerated cytoplasm; (D) oocytes cultured in the presence of 5.0 µg/mL with brocken zona pellucida and empty cytoplasm.

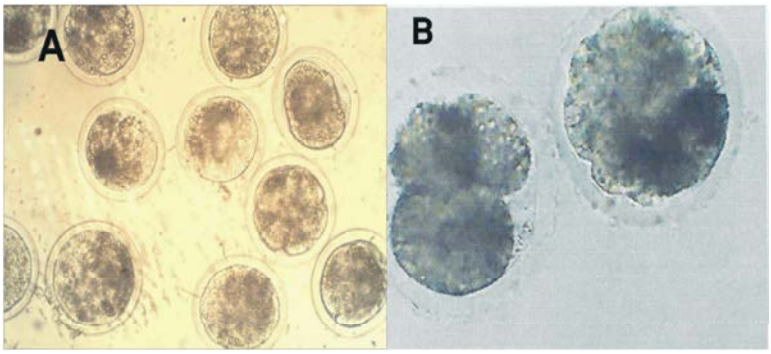


Plate 2: Photomicrograph showing *In vitro* produced buffalo embryos (A) control group, embryos developed to the morula and early blastocyst stage, (B) buffalo embryos stage cultured in the presence of mancozeb and developed to the 4 and 16-cell showing dark degenerated and fragmented cytoplasm

The effects of direct exposure to different concentrations of mancozeb during *in vitro* culture on cleavage rate and development of *in vitro* fertilized buffalo oocytes are demonstrated in table 3. Results showed that cleavage rate and embryo development to the morulae and blastocyst stages were significantly ($P<0.01$) lower in mancozeb groups than control. Addition of mancozeb to IVC medium arrested the development of buffalo embryo at the 2 to 16-cells stage. In mancozeb groups, cleaved embryos showed signs of degenerated and fragmented cytoplasm (Plate 2).

## DISCUSSION

Nowadays the fungicide problems become the focus of public interest because the application of fungicides is still the most effective and accepted means for protection of vegetables and seeds from fungus infection. On the other hand, they are dangerous for non target organisms such as animals and human begin.

The present study indicated that mancozeb possesses an adverse effect on female buffalo fertility. Concerning its deleterious effect on oocytes, mancozeb impair oocyte maturation rate which is a critical

prerequisite for the subsequent fertilization and development. In the present work, mancozeb was found to affect both cytoplasmic (cumulus cell expansion) and nuclear maturation and increased oocyte degeneration rates in a dose-dependent manner. Mancozeb significantly decrease cumulus cell expansion in buffalo COCs matured *in vitro*. A significantly higher percentage of slight (G1) and moderate (G2) cumulus cell expansion was detected in buffalo oocytes matured in the presence of 2.5 or 5.0 µg/ml mancozeb. Similarly Nandi *et al.* [9] reported that chlorpyrifos and endosulfan has a negative impact on buffalo oocytes at 0.02 and 0.1 mug/ml levels, respectively. These pesticides reduced the oocyte nuclear maturation by a direct effect on oocytes, cumulus cell-mediated action and by blocking the action of hormones. Chlorpyrifos was found to be more ovotoxic and embryotoxic than endosulfan. Other studies reported that cumulus expansion is influenced by the concentration of pesticides: the higher the pesticide concentration the weaker the cumulus expansion [10,11]. A relation between cumulus cell expansion and oocyte development was also observed, as cumulus cells play an important role in the nuclear and cytoplasmic changes associated with oocytes maturation [12,13]. The regulation of cAMP that contributes to meiotic arrest of porcine oocytes is mediated by the cumulus cell gap junctions [14-16]. Therefore, a defect in cumulus cell function and subsequent oocyte cAMP levels, might be an explanation among others for the maturation problem in oocytes exposed to the pesticides. It is possible that organochlorines perturb the gap junction communication of the cumulus cells, reducing the glutathione content of the treated oocytes and subsequently adversely affecting embryo quality [11].

In addition, the present work illustrated that exposure to mancozeb (1.0, 2.5 and 5.0 µg/ml) during oocyte maturation significantly decrease the percentage of nuclear maturation rate and increase the incidence of degenerated oocytes, as most of the exposed oocytes showed degenerated or fragmented cytoplasm or broken zona peludica. Similar results were reported that DDT, (gamma HCH) and (MXC) decrease the rate of normal bovine oocyte maturation and increase degeneration rate *in vitro* in a dose-dependent manner [11]. Oocytes exposed to polychlorinated biphenyls were arrested at metaphase I, caused by a blockage in the maturation process [17,18], or by interfere with normal spindle formation [5,19,20]. Impaired maturation can result from the direct toxic effect of pesticides on the oocytes, in some cases inducing destruction of the cell itself. Polychlorinated biphenyls decrease oocyte nuclear maturation by altering maternal RNA polyadenylation, modifying migration and exocytosis of cortical granules [19], or indirectly affect steroid production by granulosa cells, interfering with appropriate oocyte maturation [21,22]. Recently, Casas *et al.* [23] reported that pesticides showed more pronounced effect on maturation than viability, due to a blockage at germinal vesicle stage. Bonilla *et al.* [24] recorded a significant decrease in oocytes survival after 24-h exposure to 250 microM malathion or 900 nM diazinon and the effect of these insecticides on the regulation of genes encoding proteins involved in transcription (BP75), translation (ribosomal protein S5) and mitochondrial function (cytochrome oxidase subunits I and III), providing evidence for OP insecticides as toxicants for mammals oocytes during the early oogenesis. On the contrary, Rossi *et al.* [5], reported that exposure of mouse oocytes to mancozeb during IVM did not affected the percent of oocytes that reaching MII but it interfered, in a dose-dependent manner, with the formation of normal meiotic spindle. Also, Campagna *et al.* [25] reported that organochlorine mixture did not affect cumulus expansion, oocyte maturation, penetration, development to blastocyst, or the number of cells per blastocyst. This discrepancy could be attributed to species difference or the dose and type of fungicide used.

In addition, the present work demonstrated that all concentrations of mancozeb significantly decreased cleavage rate and arrested the embryo development at the 2 to 16-cell stage and almost all exposed embryos showed fragmented or degenerated cytoplasm. Our results are consistent with those obtained by Lindenau and Fischer [26] who found that exposure of rabbit embryos to polychlorinated biphenyls (PCB) led to complete degeneration of morula-stage embryos, associated with dense cytoplasm. A single oral dose of MBC at critical times, coincident with microtubule-dependent meiotic events, resulted in very early pregnancy loss in the hamster [27]. Genes related to mitochondrial metabolism as cytochrome subunits I and III, nuclear genes such as major histocompatibility complex I (MHC I) and a hypothetical protein related with a splicing factor were the target of malathion's deregulation effect [28]. In contrary, Greenlee *et al.* [29] found that mancozeb has no effect on blastocyst development or embryo cell numbers but there was an increase in the percentage of apoptosis in exposed embryos. Also, Graves *et al.* [30] indicated that low levels of atrazine do not have any effect on *in vitro* fertilization rates or the number of blastomeres per embryo produced.

In conclusion, the present study demonstrated that exposing of buffalo oocytes and embryos to mancozeb fungicide *in vitro* negatively affects oocyte maturation and impairs embryonic development. It decreases cumulus cell expansion and nuclear maturation an increase incidence of degenerated oocytes and embryos.

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