

THE EFFECT OF QUERCETIN ON RAT THYMOCYTE MITOCHONDRIA TREATED WITH MANCOZEB

Voja Pavlović, Snežana Cekić

Quercetin is one of the most commonly used flavonoids, which people continuously consume through food. This flavonoid has potent antioxidant and anti-inflammatory effect. In the current study, we evaluated the effect of Mancozeb (0.2, 2 i 5 µg/ml), Mancozeb and quercetin, only quercetin (10mM) on viability, apoptosis, ROS production and mitochondrial membrane potential (MMP) in rat thymocytes, in vitro conditions. The application of Mancozeb resulted in dose-dependent reduction of cell viability, apoptosis induction, which was followed by increased ROS production and MMP reduction. Quercetin significantly reduced the cytotoxicity in cell cultures with 0.2 and 2µg/ml of Mancozeb, together with the reduction of ROS and MMP increase. Quercetin in cell cultures treated with 5µg/ml of Mancozeb failed to reduce toxicity but increased the total number of apoptotic cells. The obtained results show that ROS production, together with mitochondrial dysfunction, may represent a key factor in toxicity induced by Mancozeb. The application of quercetin reduces cell toxicity which is induced by lower Mancozeb concentrations, with a possibility to induce apoptosis and prevent necrosis, with final reduction of the development of secondary immunological consequences. *Acta Medica Medianae* 2015;54(4):5-11.

Key words: mancozeb, toxicity, thymocytes, quercetin, mitochondria

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Introduction

Quercetin is one of the most distributed flavonoids in plants (1), which is, in huge amounts, present in plants as well as in different food products commonly used in human nutrition (2). Daily intake of flavonoids, in human nutrition, is about 23mg, 60-75% of which belong to quercetin (3). On the other hand, different studies have shown that quercetin has antioxidant potential (4, 5), different pharmacologic function, including anti-inflammatory and immunostimulating effects (5, 6). Previous studies have documented the protective effect of quercetin on toxicity induced by different drugs and initiating of oxidative stress in in vivo conditions (7, 8). Recent study has shown the protective effect of quercetin in red blood cells on toxicity induced by contact fungicide-Mancozeb (9).

Mancozeb is the one of the most commonly

used fungicides in the world. Human population is not only exposed to Mancozeb by direct contact but through different food products (10). Various studies have shown a toxic effect of Mancozeb in different cells (11, 12). Also, it has been shown that one of the toxic mechanisms of Mancozeb is forming the oxygen reactive species (ROS) (13). Even though the protective effect of quercetin has been studied in different cells, so far it has not been evaluated in thymocytes or in the cells of the primary lymphoid organs. Therefore, in the current study, we tried to evaluate the potential protective effect of quercetin on Mancozeb-induced toxicity in rat thymocytes as well as potential mechanisms involved in this process.

Material and methods

Experimental animals

Experiments were performed on 11 adult male Wistar rats (180–200 g), 9–11 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Faculty of Medicine in Niš, under conventional laboratory conditions and in accordance with the national animal protection guidelines.

Materials

Culture medium (CM) was prepared using RPMI 1640 (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. CM con-

tained 25 mM HEPES, 2 mM glutamine, peni-cillin (100 U/ml), streptomycin (100 µg/ml) and 10% foetal calf serum (FCS).

2',7'-dichlorofluorescein diacetate (H2DCF-DA), Cell Counting Kit (CCK-8), Rhodamine 123 were purchased from Sigma - Aldrich.

Propidium iodide (PI) was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Quercetin was purchased from Pure Bulk, Rosen-burg, OH, USA while Mancozeb was purchased from Galenika-Fitofarmacija a.d., Belgrade, Ser-bia.

Preparation of thymocytes and Mancozeb solution

Rat thymocytes were isolated as described earlier (14). The viability of the isolated cells, as determined by trypan blue dye exclusion test, was always over 93 %. Thymocytes were counted and adjusted to a density of 1×10^6 cells/ml.

Mancozeb and quercetin solutions were prepared immediately before use in dimethyl sulphoxide and diluted in appropriate amount of CM. Control cells were treated with the same amount of vehicle alone. The final dimethyl sul-phoxide concentration never exceeded 0.5 % (v/v).

Cell culture

Isolated cells were cultivated in 96-well round-bottom plates (NUNC, Aarhus, Denmark), containing 100µl of cell suspension (5×10^5 cells)

in each well. Thymocytes were treated with increasing concentrations of Mancozeb (0.2, 2 and 5 µg/ml) and quercetin (10mM), only Mancozeb (0.2, 2 and 5 µg/ml), only quercetin (10mM) or left in CM alone. All cell cultures were done in triplicates and incubated in an incubator (Galaxy, Wolf Laboratories, Edison, NJ) at 37 °C for 24 h in an atmosphere of 95% air and 5% carbon dioxide.

The dose response study was conducted by exposing the rat thymocytes to different concentrations of Mancozeb (0.2–10 µg/ml) for 24 h. The results revealed a dose-dependent decrease in cell viability, with an IC50 value of 1.6µg/ml, and therefore we selected 0.5, 2 and 5 µg/ml doses of Mancozeb for 24 h exposure for further studies. The concentration of quercetin used in the current study was based on a previous report (15).

Analysis of cell viability

Cell viability of rat thymocytes after the cultivation period was estimated by tripan blue exclusion test as was described earlier (16).

Apoptosis analysis

Thymocytes undergoing apoptosis were identified by their reduced relative nuclear DNA content as previously described (17). Single apoptotic cells were detected using an Epics®XL flow cytometer (Coulter, Krefeld, Germany) as a reduction in fluorescence of the DNA-binding dye

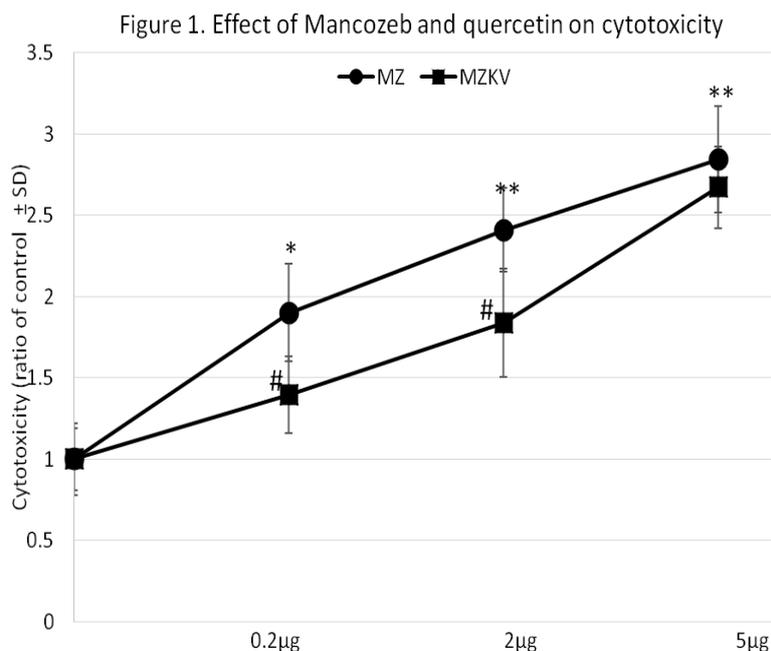


Figure 1. Effect of Mancozeb and quercetin on cytotoxicity

Rat thymocytes are cultivated with increasing concentrations of Mancozeb (0.2, 2 i 5 µg/ml) and/or quercetin (10mM) for 24 hours and cell toxicity was evaluated as was described in Material and method section. The values are presented as a ratio compared to the control group for further evaluation. MZ-cells treated with Mancozeb, MZKV-cells treated with Mancozeb and quercetin, * $p < 0.05$, ** $p < 0.01$ compared to the control cells, # $p < 0.05$ compared to the cells treated with Mancozeb.

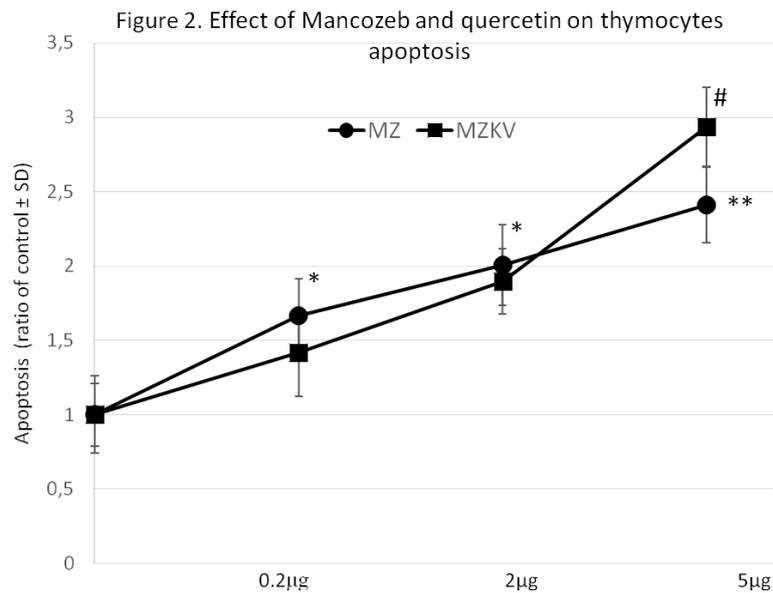


Figure 2. Effect of Mancozeb and quercetin on thymocytes apoptosis

Rat thymocytes were cultivated using increasing concentrations of Mancozeb (0.2, 2 i 5 μg/ml) and/or quercetin (10mM) for 24 hours and apoptosis was evaluated as was described in Material and method section. The values are presented as a ratio compared to the control group for further evaluation. MZ-cells treated with Mancozeb, MZKV-cells treated with Mancozeb and quercetin, * $p < 0.05$, ** $p < 0.01$ compared to the control cells, # $p < 0.05$ compared to the cells treated with Mancozeb.

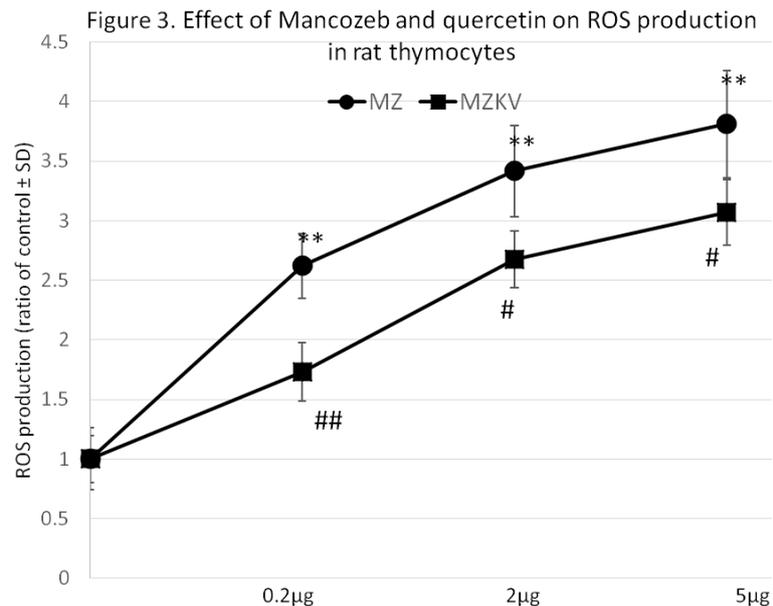


Figure 3. Effect of Mancozeb and quercetin on ROS production in rat thymocytes

Rat thymocytes are cultivated with increasing concentrations of Mancozeb (0.2, 2 i 5 μg/ml) and/or quercetin (10mM) for 24 hours and ROS production was evaluated as was described in Material and method section. Values are presented as ratio compared to the control group for further evaluation. MZ-cells treated with Mancozeb, MZKV-cells treated with Mancozeb and quercetin, ** $p < 0,01$ compared to the control cells, # $p < 0,05$, ## $p < 0,01$ compared to the cells treated with Mancozeb.

PI in apoptotic nuclei. The percentage of apoptotic cells (subdiploid DNA) was determined and presented as a ratio of control for further comparison.

Measurement of intracellular reactive oxygen species (ROS) production

A redox-sensitive probe (H2DCF-DA) was used to determine the changes in the overall cellular ROS levels, as described previously (18, 19). The change in fluorescence was measured sample, basal fluorescence intensity values were

using an Epics XL flow cytometer. For each subtracted from those obtained after different treatments and the results were presented as the mean fluorescence intensity (MFI) ratio of control for further comparison.

Determination of mitochondrial membrane potential

Changes in mitochondrial membrane potential (MMP) of thymocytes were evaluated by the uptake of lipophilic cation Rhodamine 123 into mitochondria, as previously described (20, 21). For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and the results were expressed as the MFI ratio compared to control for further comparison.

Statistical analysis

All values are expressed as mean \pm SD. The comparisons among groups were carried out using the analysis of variance (ANOVA) coupled to the Dunnett's post hoc test. P value <0.05 was considered significant.

Results

After cultivation period ended, cytotoxic potential of increasing Mancozeb concentrations on

rat thymocytes was evaluated. Obtained results showed that Mancozeb application resulted with dose dependent ($p<0.05$; $p<0.01$) cell viability reduction. As shown in Figure 1, the most prominent cytotoxicity was detected in cultures with highest Mancozeb concentrations. Simultaneously, cytotoxicity was detected together with significant increase ($p<0.05$; $p<0.01$) of apoptotic cells (Figure 2), showing proapoptotic potential of Mancozeb. Given results were followed with significant ($p<0.01$) ROS production, as well as with significant MMP decrease ($p<0.05$; $p<0.01$) in rat thymocytes, after Mancozeb application (Figure 3 and 4).

In next experiments, we evaluated protective potential of quercetin in Mancozeb-induced toxicity in rat thymocytes. Obtained results showed that quercetin application markedly reduced cytotoxicity in cell cultures with 2 ($p<0.05$) i 2 $\mu\text{g/ml}$ ($p<0.05$) of Mancozeb. On the other hand, quercetin application significantly increases ($p<0.05$) apoptotic cells in cell cultures with Mancozeb in concentrations of 5 $\mu\text{g/ml}$ (Figure 2). Application of quercetin resulted with significantly reduced (0.2 $\mu\text{g/ml}$, $p<0.01$; 2 $\mu\text{g/ml}$, $p<0.05$; 5 $\mu\text{g/ml}$, $p<0.05$) ROS production in cells (Figure 3). Simultaneously, given changes were followed with significant MMP increase (0.2 $\mu\text{g/ml}$, $p<0.05$; 2 $\mu\text{g/ml}$, $p<0.05$) in rat thymocytes (Figure 4).

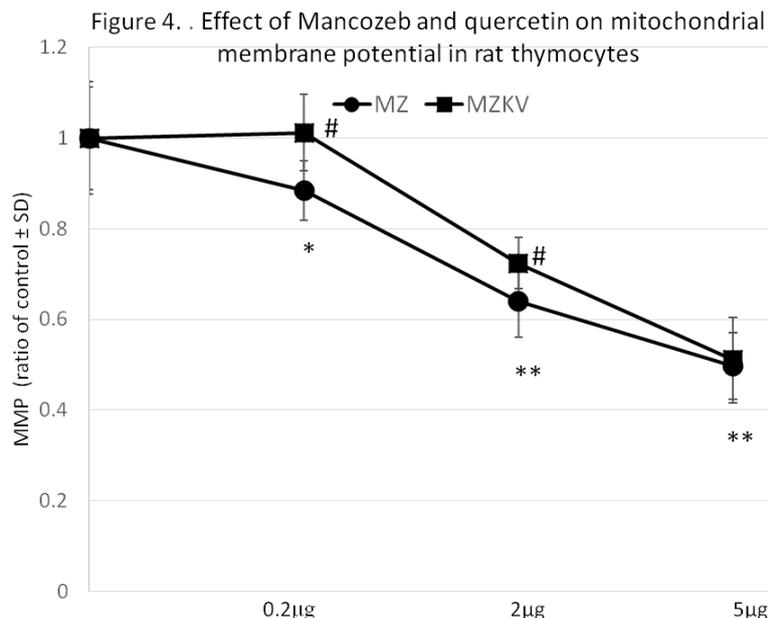


Figure 4. Effect of Mancozeb and quercetin on mitochondrial membrane potential in rat thymocytes

Rat thymocytes are cultivated with increasing concentrations of Mancozeb (0.2, 2 i 5 $\mu\text{g/ml}$) and/or quercetin (10mM) for 24 hours and MMP was evaluated as was described in Material and method section. The values are presented as a ratio compared to the control group for further evaluation. MZ-cells treated with Mancozeb, MZKV-cells treated with Mancozeb and quercetin, * $p<0,05$, ** $p<0,01$ compared to the control cells, # $p<0.05$ compared to the cells treated with Mancozeb.

Discussion

In the last few years, there has been a growing interest about the influence of different pesticides on health and human environment. Due to its short halftime, Mancozeb is intensively used worldwide even though it has been shown to possess a toxic effect (22). In our study, we evaluated the toxic effect of Mancozeb on rat thymocytes and potential protective effect of quercetin in this process. The results obtained in our study show that Mancozeb exerts toxic effects on rat thymocytes, which is dose-dependent. Also, cytofluorimetric analysis shows that thymocytes die by apoptotic mechanism. These findings are in line with earlier results which showed cytotoxic effect of Mancozeb in different cells (23, 24), including the cells of the immune system (11, 12). Our results also show that exposure of thymocytes to increasing concentrations of Mancozeb results in increased ROS production, together with decreased MMP, which corresponds to decreased cell viability. Due to high content of polyunsaturated fatty acids in their membrane, cells of the immune system are very sensitive to oxidative stress (25). Intensive and long-lasting ROS production may lead to the induction of apoptotic process, through oxidative stress or direct damaging of cell components (26). Cell death, in part, depends upon mitochondrial dysfunction which is often characterized by increased ROS production (27). Taken together with our results, it seems that increased ROS production and mitochondrial dysfunction may represent the key factors in Mancozeb-induced toxicity in thymocytes with secondary immunological consequences. Based on the previous findings, in the next experiments we evaluated the possible modulatory effect of quercetin on toxicity induced by Mancozeb. The application of quercetin significantly reduced the ROS production induced by Mancozeb, showing

its protective effect. Also, quercetin markedly reduced cytotoxicity in cultures treated with 0.2 and 2µg/ml. Protective effect of quercetin on Mancozeb-induced toxicity has been shown in red blood cells (9), while these are the first results about protective effect in thymocytes. In the last years, various studies have shown different antioxidative and anti-inflammatory effects of quercetin, as well as the effect on cell apoptosis (28, 29). Antioxidative capacity of quercetin develops as a consequence of its rapid diffusion into cell membrane (30), as well as due to its chemical structure (9). Our results are in line with previous studies which show that quercetin enhances antioxidative capacity of T cells and has a key role in cell apoptosis (29). Further, results show that in cell cultures treated with 5µg/ml of Mancozeb quercetin did not reduce toxicity, but increased the total number of cells undergoing apoptosis. Based on the previous findings, we may suggest that quercetin has a possibility to induce cell apoptosis and by doing that inhibits cell necrosis. In line with these findings, it has been shown earlier that cells undergoing apoptosis may be necrotic if ATP levels in cells are markedly reduced, showing that quercetin may have a possibility to maintain bioenergetic changes inside the cells preventing the immunologic dysfunction.

In summary, our results showed that Mancozeb application leads to rat thymocytes toxicity mainly by the induction of the oxidative stress and mitochondrial dysfunction. Quercetin showed a protective effect on rat thymocytes when lower concentrations on Mancozeb were used, whereas it led to apoptosis with higher concentrations of Mancozeb. Understanding the mechanisms involved in the protective effect of quercetin may lead to therapy improvement and disease prevention in humans who are exposed to pesticides, that should prevent secondary immunological consequences.

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Original article

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doi:10.5633/amm.2015.0401**EFEKAT KVERCETINA NA MITOHONDRIJE TIMOCITA
PACOVA TRETIRANIH MANKOZEBOM***Voja Pavlović, Snežana Cekić*

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Kvercetin je jedan od najrasprostranjenijih flavonoida koji se putem hrane kontinuirano unosi u ljudski organizam. Ovaj flavonoid poseduje snažno antioksidativno i antiinflamacijsko dejstvo. U studiji je evaluiran efekat Mankozebe (0,2, 2 i 5 µg/ml), Makozebe i kvercetina, samo kvercetin (10 mM) na vijabilnost, apoptozu, produkciju slobodnih kiseoničkih radikala (ROS) i mitohondrijalnog membranskog potencijala (MMP) u timocitima pacova u *in vitro* uslovima. Tretman ćelija sa Mankozebom doveo je do doznno zavisnog smanjenja vijabilnosti ćelija, indukcije apoptoze koja je praćena povećanom sintezom ROS-a i smanjenjem MMP-a. Aplikacija kvercetina značajno je smanjila citotoksičnost u kulturama sa 0,2 i 2 µg/ml Mankozebe, zajedno sa smanjenjem ROS produkcije i povećanjem MMP-a. Kvercetin u ćelijama tretiranim sa 5 µg/ml Mankozebe nije uspeo da redukuje toksičnost, već je indukovao povećanje broja apoptotičnih ćelija. Dobijeni rezultati pokazuju da produkcija ROS-a, zajedno sa mitohondrijalnom disfunkcijom, predstavlja značajan faktor u toksičnosti indukovanoj Mankozebom. Administracija kvercetina redukuje toksičnost ćelija koja je indukovana nižim koncentracijama Mankozebe, sa mogućom sposobnošću da indukuje apoptozu i spreči nekrozu ćelija, a samim tim i razvoj sekundarnih imunoloških konsekvenci. *Acta Medica Medianae 2015;54(4):5-11.*

Ključne reči: Mankozebe, toksičnost, timociti, kvercetin, mitohondrija

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