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# Possible Cytoprotective Potential of Ruthenium Red in Evaluation of the Rapid Apoptotic Model Induced by $H_2O_2$

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In the present paper, hydrogen peroxide ($H_2O_2$) induced an apoptotic process in 1 h and over the doses of 10 mM, while cisplatin did not, in cortical neuronal homogenates of rats. It was proved in DNA fragmentation, MTT and WST-1 assays. Especially, according to WST-1 assay results, apoptotic effect was decided to be very obvious in all tests in the doses of 20 mM of the rapid model of apoptosis of $H_2O_2$. Ruthenium red, as a mitochondrial $Ca^{2+}$ modulator, was tested alone and co-application with $H_2O_2$. Without $H_2O_2$, at low doses of it, ruthenium red seems to have a slight viability inducing effect in respect to controls in MTT and WST-1, despite that this effect was not observed clearly in DNA fragmentation test. Another point is that the highest dose of ruthenium red ($10^{-4}$ M), induces apoptosis, even stronger than the 20 mM $H_2O_2$, controversy to the general believing.

**Key Words: Apoptosis, Mitochondria, $H_2O_2$, Cisplatin, Ruthenium Red, DNA fragmentation.**

## INTRODUCTION

Apoptosis is a genetically regulated and finely tuned process of cell elimination essential for the embryogenesis, development and tissue homeostasis of multicellular organisms[1]. Cell death by apoptosis is accompanied by a stereotyped and interconnected series of events among which cell collapse, formation of membrane blebs, chromatin condensation and DNA degradation is well recognised[2]. Caspases selectively cleave a set of about 200 targets[3]. Cleavage by caspases usually results in the degradation and inactivation of their substrates, *e.g.*, the enzymes involved in DNA cleavage and repair (DNA-PK, PARP, topoisomerase-1, MCM3, DFF, CAD) with the consequent impairment of DNA repairing machinery[4].

Mitochondria plays a crucial role in the amplification of the apoptotic process by releasing proapoptotic factors such as cytochrome-c, apoptosis inducing factor (AIF) and Smac/DIABLO. During the apoptotic process, cytochrome-c translocates from the mitochondrial intermembrane space to the cytoplasm, forming a complex with Apaf-1 and activates procaspases[5,6]. Apoptosis inducing factor was reported to release from mitochondria during apoptosis and concurs to nuclear modifications. It was reported to induce chromatin condensation and large scale DNA fragmentation[7]. The proapoptotic factor Smac/DIABLO is also located in the mitochondrial intermembrane space and is released upon induction of apoptosis[8]. Moreover, several different pro- or antiapoptotic proteins belonging to the Bcl-2 family reside in or translocate to the mitochondria[9]. According to Bernardi *et al.*[10], the apoptotic process is preceded by collapse of the mitochondrial potential, opening of a multiprotein structure named the permeability transition pore (PTP). The permeability transition pore assembles at sites of contacts between the inner and outer mitochondrial membranes, swelling the matrix, rupturing and change in the permeability the outer membrane and finally release of apoptotic factors from mitochondria[11].

Ruthenium red (RuR) has been shown to inhibit several mechanisms involved in intracellular calcium regulation including the mitochondrial uniporter[12], $Ca^{2+}$ release channel/ryanodine receptor[13], interacting with the N- and P-type $Ca^{2+}$ channels[14] and prevents mitochondrial permeability transition pore opening[15]. It was also reported to inhibit cytochrome-C release, activation of caspase-3 and apoptosis in U-937 cell line[16].

Despite being not a free radical by itself, hydrogen peroxide ($H_2O_2$) as a major ROS, can alter the intracellular redox state of cells, inducing oxidative damage by its conversion into a highly reactive hydroxyl radical and by interaction with transition metal ions, of which the most important is probably iron[17]. In addition, the level of $H_2O_2$ and hydroxyl radical in mitochondria is elevated during neurodegenerative diseases[18]. Therefore, $H_2O_2$ has been extensively used to furnish a cell

culture model of oxidative stress for studying neurotoxicity and neuroprotection in central nervous system[19]. Hydrogen peroxide has been employed by now for several studies as a reference apoptotic agent. It was mostly used in up to 4 mM dose for 24, 48 and even 96 h periods[20]. On the other hand, it was believed that some chemotherapy drugs such as cisplatin, cause apoptosis by inducing formation of intracellular oxidants[21]. But it was also reported that the induction of apoptosis by chemotherapeutics occurs without generation of reactive oxygen species and distinct apoptotic pathways of cisplatin and $H_2O_2$ was dedicated[22].

In the present study, by using the increased doses of $H_2O_2$ and cisplatin, short term cytotoxic effects were investigated, as well as possible protective potential of RuR was tested by visualizing DNA fragmentation and measuring mitochondrial activity.

## EXPERIMENTAL

Twenty-five male Spraque-Dawley rats, 8-12 weeks old and weighing 200 ± 20 g bred at Art and Science Faculty of Abant Izzet Baysal University, Experimental Animal Laboratory, were used for the present experiment. All procedures involving rats were carried out to adhere to principles of the use of animal research. The rats were housed in seven per group to a cage in a windowless laboratory room with automatic temperature (22 ± 2 ºC) and lighting controls (12 h light/12 h dark) and fed standard chow and water ad libitum.

**General procedure:** The day of experiment, each rat was anaesthetized with ether and then an intracardiac withdrawal of blood was performed for the sacrification of rat. Cranium was opened, both temporal cortical tissue was dissected out and transferred into the freshly prepared and warmed DMEM (Dulbecco's Modified Eagle's Medium) solution.

Hydrogen peroxide and cisplatin were prepared daily and very short-period toxicity experiments were carried out first. It was aimed to optimize the ideal dose of DNA fragmentation for very short-period. Hydrogen peroxide at doses of 1, 2, 5, 10, 20 and 40 mM and cisplatin at doses of 25, 50, 100 and 250 µM concentration was added into the cortical brain tissue homogenates for 1 h. Following the determination of ideal toxic dose of 20 mM for $H_2O_2$ and 25 µM for cisplatin, ruthenium red was tested alone or 15 min prior to $H_2O_2$ and cisplatin at $10^{-10}$, $10^{-8}$, $10^{-6}$ and $10^{-4}$ M concentrations. The dishes were kept in a 37 ºC chamber contained 95 % air and 5 % $CO_2$ during the experiment.

**DNA fragmentation assay:** To measure oligonucleosome-sized fragments resulting from cleavage of nuclear DNA, the DNA fragmentation assay was modified and performed as previously described[23]. In brief, control/treated cells were collected by centrifugation at 3500 rpm for 3 min and lysed in 0.1 M NaCl, 10 mM tris (pH: 8.0), 10 mM EDTA (pH:8.0), 1 % SDS and 2 µL of proteinase K, overnight. The soluble fractions in the lysates were collected after centrifugation and contained the fragmented DNA only. DNA was extracted sequentially with phenol/chloroform/isoamyl alcohol (25:24:1) twice and precipitated in absolute chilled ethanol contained 0.3 M of sodium acetate for 10 min and centrifuged at 11,000 rpm for 10 min. Supernatant was removed and pellet was collected. Then, to remove sodium acetate into the pellet, 100 µL of 70 % cold ethanol was applied for 5 min and centrifuged at 11,000 rpm for 2 min. Supernatant was removed and pellets let to dry. Precipitant was dissolved in 100 µL of milli-Q water at 50 ºC for 1 h on a heating block. The DNA was electrophoresed on 1 % agarose gel at 90 V, for 50 min.

**MTT assay:** Cytotoxicity of $H_2O_2$, cisplatin or ruthenium red on parietal cortical cells was assessed by an MTT assay kit following the manufacturer's instructions. Briefly, cells were plated at a density of $10^4$ cells/well in a 96-well plate. The cells were incubated in 5 % $CO_2$ and 95 % air for desired period and treated with various concentrations of ruthenium red, with or without $H_2O_2$ or cisplatin. After the period of incubation, 10 µL MTT labelling reagent was added in each well and the plates were further incubated for 4 h. Afterwards, 100 µL solubilization solutions were added into each well, The plate was incubated overnight at 37 ºC. The optical density of the wells was measured at a wavelength of 570 nm with reference of 690 nm, using an enzyme-linked immunosorbent assay (ELISA) plate reader. Results were calibrated with optical density measured without cells in wells. The results were converted to the percentage of the control measurements.

**WST-1 assay:** At the end of the incubation period with various concentrations of ruthenium red, with or without $H_2O_2$ or cisplatin, the medium was removed after centrifugation and 10 µL of WST-1 reagent (final volume of 1/10) was added and the plates were further incubated for 4 h at 37 ºC. The optical densities of the wells were measured at a wavelength of 480 nm with reference of 690 nm using an ELISA plate reader. Results were calibrated with optical density measured without cells in wells. The results were converted to the percentage of the control measurements.

**Statistics:** The groups were compared by one-way ANOVA and then post-hoc Tukey tests between subgroups were conducted. The results lower than 0.05 were accepted as statistically significant.

## RESULTS AND DISCUSSION

This is a pioneer study to present a rapid model of apoptosis induced by $H_2O_2$. Hydrogen peroxide obviously induces apoptosis in the short time interval rapid model. DNA fragmentation assay dose-response results were confirmed by MTT and WST-1 tests (Figs. 1 and 2). On the other hand, testing the other well known anticancer and apoptotic agent cisplatin, did not show any beneficial result in a very short time interval (Figs. 3 and 4). Despite the fact that almost all doses tested seem to induce apoptosis in DNA fragmentation and MTT assays, according to the WST-1 assay results, apoptotic effect was decided to be very obvious in all tests in the doses of 20 mM of the express model of apoptosis of $H_2O_2$.

Ruthenium red, as a mitochondrial $Ca^{2+}$ modulator, was tested to sort out the possible apoptotic or antiapoptotic effects with or without 20 mM of $H_2O_2$. Without $H_2O_2$, at the low doses of ruthenium red, it seems to have a slight viability inducing effect in respect to controls, while it has no effect on $H_2O_2$ induced neurotoxicity in MTT and WST-1 tests (Fig. 5).

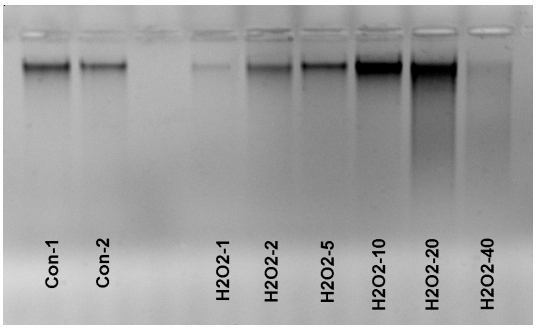


Fig. 1. Dose-response (1, 2, 5, 10, 20, 40 mM concentrations) graph of hydrogen peroxide ($H_2O_2$) induced DNA-fragmentation assay. Con: control

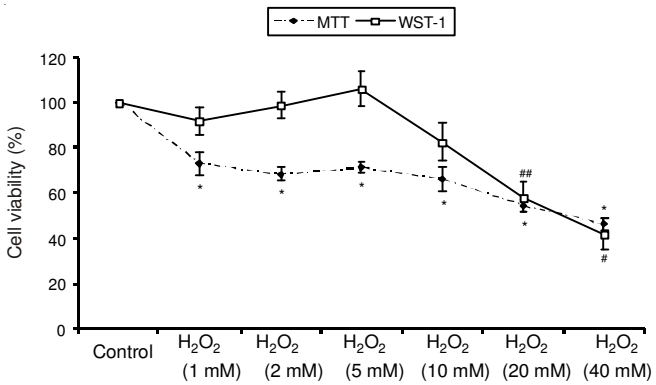


Fig. 2. Dose-response (1, 2, 5, 10, 20, 40 mM concentrations) curves of hydrogen peroxide ($H_2O_2$) induced MTT and WST-1 assays. Con:control group. * and # mean $p < 0.01$, ## means $p < 0.05$ in respect to control

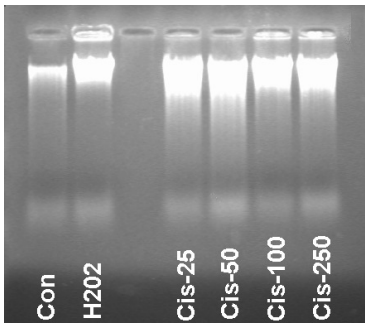


Fig. 3. Dose-response (25, 50, 100 and 250 μM concentrations) graph of cisplatin (Cis) induced DNA-fragmentation assay. Con: control. $H_2O_2$: hydrogen peroxide in 20 mM concentration

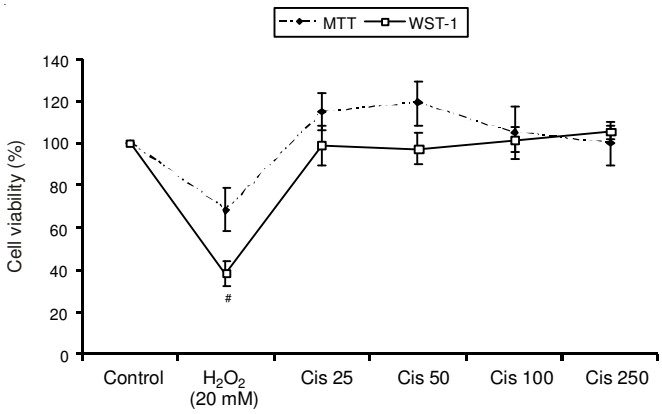


Fig. 4. Dose response (25, 50, 100 and 250 μM concentrations) curves of cisplatin (Cis) induced MTT and WST-1 assays. Con: control group. *mean $p < 0.05$ in respect to control

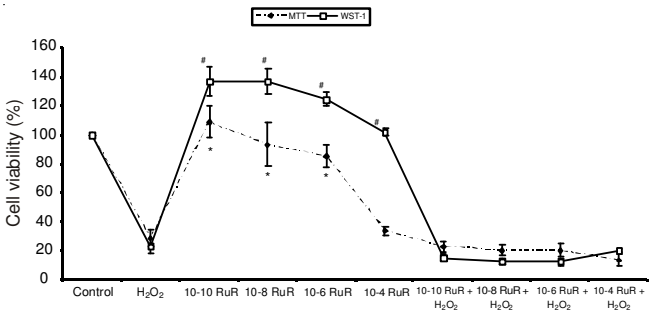


Fig. 5. Dose response (0.1 μM, 10 μM, 1 mM and 0.1 M concentrations) curves of ruthenium red (RuR) and $H_2O_2$ induced MTT and WST-1 assays. Con: control group. * and # means $p < 0.05$ in respect to control

Despite that this effect was not observed clearly in DNA fragmentation (Fig. 6). Highest dose of ruthenium red ($10^{-4}$ M) found to induce apoptosis, even stronger than the 20 mM $H_2O_2$. Co-application with $H_2O_2$, ruthenium red in all doses tested, potentialised the apoptotic effect of $H_2O_2$ in MTT while did not make any remarkable effect in WST-1 and DNA fragmentation (Fig. 6).

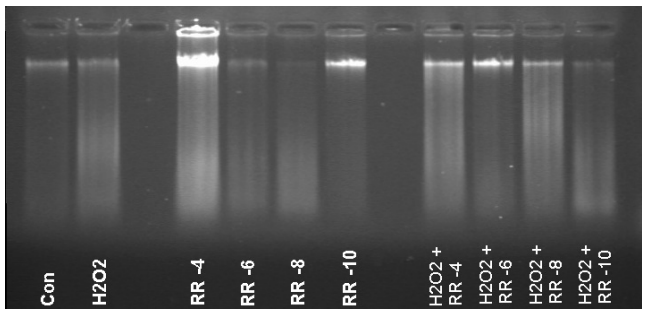


Fig. 6. Dose response (0.1, 10 μM, 1 mM and 0.1 M concentrations) graph of ruthenium red (RR) alone or co-existance with $H_2O_2$ in 20 mM concentration in DNA fragmentation assay. Con: control

In present study, it was documented the first time that $H_2O_2$ obviously induced DNA fragmentation and mitochondrial disfunction in 10 mM and higher doses for 1 h period, in cortical cell homogenates of rats. Surprisingly, cisplatin did not show any remarkable changes on DNA fragmentation or mitochondrial assays in short time intervals. Hydrogen peroxide was reported to block apoptosis by consuming intracellular ATP in low doses[24]. It was also confirmed by present results. We did not get any alterations of apoptotic or mitochondrial activity of the lower doses of $H_2O_2$. The strategic location of mitochondria close to the source of the $Ca^{2+}$ rise allows them to be exposed to $Ca^{2+}$ that meet the affinity of their transporters and allows the rapid and large accumulation of the cation in the matrix. This accumulation, stimulating intramitochondrial effectors such as $Ca^{2+}$-dependent dehydrogenases of the Krebs cycle, allows the prompt tuning of organelle metabolism and hence ATP production, to the increased needs of an activated cell[25]. Mitochondria that undergo the mitochondrial permeability transition may cause cytochrome-C release and thus initiate apoptosis[26]. Increased mitochondrial $Ca^{2+}$ accumulation triggers the release of cytochrome-C from the mitochondrial intermembrane space into the cytosol. It was reported that $Ca^{2+}$ and the proapoptotic protein Bax induce cytochrome-C

release from mitochondria[27]. $Ca^{2+}$ and Bax induce the release of both cytochrome-C and procaspase-9 which are crucial for the inducing of apoptosis[28]. Accordingly to the results obtained from the present study, it is obvious that DNA damage was found highly correlated with the lowered mitochondrial activation, probably the co-existance with increased mitochondrial $Ca^{2+}$ accumulation activated Bax protein levels and induced cytochrome-C levels.

Since the mitochondria are considered a potential source of intracellular reactive oxygen species (ROS), cisplatin and $H_2O_2$ were tested to induce apoptosis by causing formation of intracellular oxidants. It was reported that cisplatin did not induce any reproducible increases in protein or lipid oxidation. In contrast, all assays detected oxidation induced by the hydrogen peroxide. It was also reported that, as much as 10 mM $H_2O_2$ was required to induce detectable intracellular changes in short time (0.5 h) intervals[22]. Those results were much correlated with our mitochondrial assays, as well as DNA fragmentation tests in the present study. According to this, we may speculate that cisplatin activates a distinct pathway for inducing apoptosis from $H_2O_2$ and that pathway for cisplatin does not work for intracellular oxidant generation. It may also report that only $H_2O_2$ at 10 mM and higher doses is able to induce apoptotic process *via* inducing protein and lipid oxidation.

One of the two pore domain potassium channels, TASK-3, was selectively inhibited by ruthenium red[29]. It was as well indicated a direct link between TASK $K^+$ channel activity and apoptosis[30]. Ruthenium red was also clearly reported[31] that it protects against cell death *via* a direct interaction with voltage-dependent anion channel-1 to inhibit cytochrome-C release and subsequent cell death. In this study, ruthenium red was expected to show some antiapoptotic effect, according to these reports. Viability inducing effects of low doses of ruthenium red can be explained by TASK-3 inhibition or interaction with voltage-dependent anion channels, in the present study, despite this effect was disappeared in higher doses. In one of the previous studies, ruthenium red was reported to have a neurotoxic effect in cerebellar granular cell cultures, while it was found neuroprotective in NMDA-induced neurotoxicity and this effect was dedicated to the partial agonistic effect, in the same study[32]. Ruthenium red was also tested in caffeine induced neurotoxicity. Caffeine and ruthenium red was found to induce cell death in granular cell culture of rats. On the other hand, co-application of ruthenium red with caffeine exerted protective profile and this effect was dedicated to partial agonistic effect of ruthenium reds, just like another previous paper[33]. Controversially, we have not enough clues to speculate that ruthenium red has partial agonistic effect, in the present study. It was previously reported that, ruthenium red induced neurotoxicity in primary cortical cultures by alteration of intracellular calcium homeostasis and mitochondrial function[34]. In present study, ruthenium red has not been found as a neuroprotective compound in $H_2O_2$ induced intervals, as speculated before.

## Conclusion

As a result, $H_2O_2$ may be referred as a very rapid neurodegenerative/apoptotic compound over the doses of 10 mM, cisplatin cannot be an alternative for that application and finally, ruthenium red has no effect on $H_2O_2$-induced short term periods. Despite it was found to induce viability slightly in low doses, it causes neurodegeneration in relatively higher concentrations, controversy to the general believing. It will be useful to test calcium channel blockers in $H_2O_2$-induced fast neurotoxic model to sort out the exact molecular mechanism of that model.

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