Alleviation Effects of Diosmetin on H₂O₂-Induced Oxidative Damage in Human Erythrocytes

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Abstract: Free radicals (FRs) are formed in the high amounts result of the metabolic imbalance in cells and tissue. These radicals-induced oxidative damages constitute the basis of many diseases. Organisms have antioxidant defence systems (ADS) to eliminate the destructive effects of the oxidative damage. In addition to these antioxidant systems, dietary flavonoids have the antioxidant effect and the protective role against oxidative damage. In the present study, it was investigated whether a flavonoid derived diosmetin (10, 50, and 100 μM) have the elimination potential on hydrogen peroxide (H_2O_2)-induced oxidative damage in erythrocyte culture by using biomarkers such as lipid peroxidation (LP) level, catalase (CAT), total superoxide dismutase (SOD) activity and changes of SOD isozymes containing the manganese SOD (Mn SOD) and the cupper-zinc SOD (CuZn SOD). CAT, total SOD, Mn SOD and CuZn SOD activities showed a serious decline with H_2O_2 treatment, but diosmetin addition significantly increased their activities. While the H_2O_2 application critically increased LP products in erythrocytes, diosmetin considerably reduced these oxidative damage products. In conclusion, it has been determined that diosmetin can moderate oxidative damage in human erythrocytes by activating or protecting the ADS.

1. INTRODUCTION

Erythrocytes carry oxygen to tissues throughout their lives, therefore the survival of other cells in the body depends on their health and number in blood. They do not contain nuclei and mitochondria and have a very limited life span. These cells are in constant contact with many oxidants factors which cause oxidative stress. Therefore, their metabolism and structural components could easily be disrupted by oxidative damage, thus their life spans could reduce further. Even most oxidant drugs facilitate the conversion of oxyhemoglobin in erythrocytes to hydrogen peroxide which induces LP and protein denaturation (Smith, 1987). As a result of the reduction in the number of erythrocytes occur anemia and oxygen deficiency, and all tissues are negatively affected by this situation (Libregts et al., 2011).

Under normal conditions, antioxidant systems in the cells ensure a balance between the FRs formation and their elimination. In this situation, these systems containing antioxidant substances and antioxidant enzymes have enough strength for scavenging the reactive oxygen

species (ROS). However, due to various reasons, an excessive increase in ROS might render insufficient the antioxidant systems and causes pathological phenomena called oxidative stress or oxidative damage (Memişoğulları, 2005; Atmaca & Aksoy, 2009). Many studies have shown that the ADS in cells could insufficient alone in protection from oxidative damage. It has been suggested that dietary antioxidant ingredients such as vitamin E, ascorbic acid and flavonoids could support antioxidant systems against oxidative damage. Therefore, these antioxidants could be effective in protecting against many diseases and delaying the ageing period (Özşahin et al., 2011). Flavonoids, natural antioxidant compounds found in abundance in plants, are known to reduce damages result from oxidative stress in cell and tissues. The antioxidant substances are lipophilic such as vitamin E and ascorbic acid. They have the ability to suppress LP by eliminating FRs in subcellular fractions such as liposomes, mitochondria, microsomes and erythrocyte membrane (Yang et al., 2001; Sánchez-Gallego et al., 2010). Recently, diosmetin (Figure 1), a flavonoid derivative, stands out with reducing oxidative damage in many tissues. It shows a cardio protective effect by reducing oxidative damage and mitochondrial apoptosis (Mo et al., 2020). Also, diosmetin attenuates ischemia and reperfusion-induced kidney damage (Yang et al., 2017). Moreover, it has been suggested that it can be used in the treatment of asthma (Ge et al., 2015). However, the role of diosmetin in protecting oxidative damage in erythrocytes is still not completely understood. In the present study, it was investigated whether the diosmetin effect on the alleviation of oxidative damage in human erythrocyte cells.

Figure 1. Chemical structure of diosmetin.
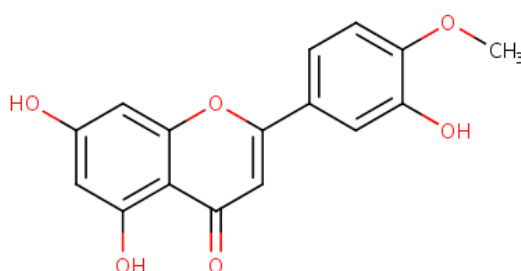
2. MATERIAL and METHODS

2.1. Preparation of Erythrocyte Samples

Erythrocytes used in the present study were obtained from Agri State Hospital Blood Center. Care was taken to ensure that erythrocyte samples are vital unexpired and their human sources were people who did a healthful, non-smoker, non-alcoholic between ages 20-30. The blood samples were centrifuged at 4500 rpm for 10 minutes, and the separated plasma was discarded. Afterwards, erythrocytes were washed with physiological saline. These processes were repeated 3 times without haemolysis of erythrocytes. The cells were transferred to Roswell Park Memorial Institute (RPMI)1640 medium to keep to them live during the experiment. This medium was purchased from Sigma Aldrich. It has been developed at RPMI, and is used as a growth medium in many different cell cultures, including mammalian cells (Moore, Gerner & Franklin, 1967). This research was conducted with the approval of the Scientific Research Ethics Committee of Agri Ibrahim Cecen University, dated 03.04.2020 and numbered 67.

2.2. Preparation of Oxidative Damage Model for Erythrocytes

All materials and devices used in the experiment were sterilized. To determine the concentration of H_2O_2 used as an oxidant agent were conducted preliminary studies with 25, 50, 100 and 200 µM H_2O_2. Considering the LP findings obtained from these studies and literature data, the H_2O_2 concentration was decided to be 100 µM.

2.3. Diosmetin Treatment Model to Erythrocytes

Diosmetin and other chemicals were purchased from Sigma Aldrich. Diosmetin was dissolved in the least amount of dimethyl sulfoxide (DMSO) as proposed by the vendor. DMSO was added to the H_2O_2 group as much as the amount of DMSO used in the diosmetin groups. This experiment was designed as five experimental groups, including control, H_2O_2, diosmetin (10, 50, and 100 µM), respectively. Diosmetin concentrations were decided based on previous study (Wang et al., 2020). The changes in oxidative damage parameters were determined after the erythrocytes were incubated at 37°C for 24 hours after the treatments.

2.4. Determination of Total SOD Activity and The Changes in SOD Isoenzymes

The total SOD activity was measured by the method of the Beauchamp and Fridovich (1971). This method is fundamentally based on the measurement of amount of formazan, which a blue-coloured complex. This blue complex is formed by nitro blue tetrazolium chloride reacting with superoxide anions produced on illumination of riboflavin in the presence of methionine as an electron donor. The total SOD amount is directly proportional with the decrease in formation of formazan, and one-unit total SOD is accepted as the amount of SOD that reduces the formazan formation 50% ratio.

Erythrocyte samples were subjected to polyacrylamide gel electrophoresis (Native-PAGE) not contain sodium dodecyl sulphate according to Laemmli method (1970). Electrophoretic separation of SOD isoenzymes was performed in 10% polyacrylamide gel (PAGE) at 120 V by Beuchamp and Fridovich methods (1971). Band densities of Mn SOD and CuZn SOD were calculated using the Gel Analyser Program.

2.5. Determination of Catalase Activity

Catalase activity was measured with methods described by Aebi (1984). The erythrocyte lysates were suspended in 50 mM potassium phosphate buffer (pH 7.0) for 30 min at 25°C. Then, 30 mM hydrogen peroxide was added to the lysates. The decomposition of hydrogen peroxide was recorded at 240 nm for 3 min. Catalase activity was calculated using an extinction coefficient of $0.0436 \, mmol^{-1} \, cm^{-1}$.

2.6. Determination of Lipid Peroxidation in Erythrocyte Cultures

LP levels were determined with the method described by Chang et al. (2013). At the end of 24-hour incubation, erythrocytes were lysed and then centrifuged for 5 minutes at 4°C and 3500 *g*. Samples were taken from the supernatant part, and HCl solution containing thiobarbituric acid (TBA) and trichloroacetic acid (TCA) was added. The samples were left to incubate for 30 minutes in boiling water, and then the reaction was stopped by standing in an ice bath. After centrifugation for 5 minutes at 4°C at 5000 *g*, absorbance at 532 nm were recorded. Thiobarbituric acid reactive substances (TBARS) values were calculated and expressed as $µM.g^{-1}Hb$ according to the haemoglobin amount of the blood samples.

2.7. Statistical Analysis

The study results are presented by taking the average of the values obtained after 3 repetitions of each sample. The results were compared with one-way analysis of variance (ANOVA) using SPSS 20 package program and Duncan's Multiple Comparison Test at *p<0.05* significance level.

3. RESULTS and DISCUSSION

FRs are formed in organisms as a result of various metabolic events and disorders. These radicals with low molecular weight and unstable structure are highly reactive substances due to their suitability for electron exchange (Alugoju et al., 2015). Erythrocytes are highly susceptible to ROS-induced oxidative stress due to the high content of unsaturated fat in the membrane

membranes and the excess of iron groups in haemoglobin. The decrease in the number of erythrocytes as a result of oxidative damage could cause anaemia and thus oxygen deficiency in tissues (Libregts et al., 2011). Although hydrogen peroxide is a non-radical, it can be the precursor of FRs such as hydroxyl radical, therefore it could be used to create an experimental of oxidative stress in vitro studies. In the study carried out by Becker (2003), it was shown that H_2O_2 caused oxidative stress by forming hydroxyl radicals through Fenton type reactions that formation of radicals by the H_2O_2 breakdown catalysed by metals such as iron and copper. Also Morabito et al. (2016) reported that H_2O_2 causes oxidative stress by increasing the LP level in erythrocyte cultures. Consistent with these studies, the results of present study also revealed that the LP level showed a serious increase with H_2O_2 treatment to the erythrocytes (Figure 2). The probable reason of this increase might be triggered oxidative damage by forming ROS through Fenton-type reactions of H_2O_2. Meanwhile, the high iron content in erythrocytes makes more likely to acceleration of the Fenton reactions (Yee & Liu, 1997). However, the H_2O_2 concentration used in this experiment is much lower than the H_2O_2 concentration used to cause oxidative damage in erythrocytes in previous studies (An et al., 2016). On the other hand, in previous studies, the exposure of erythrocytes to H_2O_2 has been kept considerably short (about 90 mins). In the present study, the oxidative damage model could be created with a lower concentration of H_2O_2 due to their longer exposure to oxidative stress (24 hours).

Figure 2. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 µM (100 D)) treatments on LP level. Different letters in the graph mean statistically significant differences (*p< 0.05*).
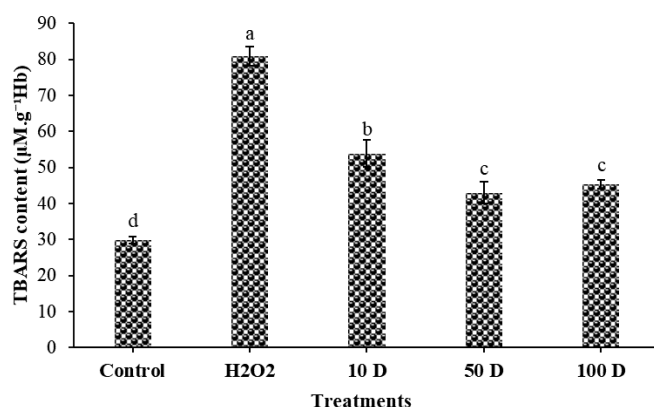

FRs naturally formed in cells or exogenously originated are attempted to be scavenged by ADS containing antioxidant enzymes and substances. However, when the radicals reach high amounts for whatever reason, the strength of these systems insufficient to scavenge the molecules. In this instance, the ROS cause irreversible oxidative damages in the cell structural components such as proteins, lipids and nucleic acids (Schieber & Chandel, 2014). Also, this damage might be ended the life of cells and is among the main reasons for diseases such as cancer, diabetes and autoimmune disorders (Ratnam et al., 2006; Cemeli et al., 2009; Pellegrini et al., 2009).

The balance between oxidants and antioxidants in cells is essential for health, but sometimes this balance might be broken in favour of oxidants. Recent studies showed that the dietary antioxidant supplement is extremely important to reduce oxidative damage. Also, it has been argued that vitamin E constitutes one of important defence factor that protects from the harmful effects of FRs (Brigelius-Flohe & Traber, 1999). Dietary antioxidants might play a role in protecting the body against radicals, and preventing many diseases and even cancer (Noroozi & Angerson, 1998). At this point, it is of great importance to take antioxidant substances from outside before being exposed to oxidant substances. It is well known that flavonoids, plant secondary metabolites in polyphenolic structure, have strong antioxidant effects, and are among

the important dietary antioxidants with their non-toxic properties for organisms (Yang et al., 2001). In the present study, it was also revealed that diosmetin, a flavonoid derivate, considerably mitigated the H_2O_2-induced oxidative damage by decreasing the LP level. However, there was no significant difference between the values of the 50 and 100 μM diosmetin groups (Figure 2).

The SOD converting superoxide radicals into hydrogen peroxide and molecular oxygen constitutes the first step among cellular antioxidant enzymes that have an important role in scavenging reactive oxygen species. Besides this effect of SOD, catalase (CAT) converts hydrogen peroxide into water and oxygen. As a conclusion, two toxic oxygen species, hydrogen peroxide and superoxide radical, are eliminated by CAT and SOD. (Weydert & Cullen, 2010). The total SOD enzyme in the eukaryotic cells presence three isomers containing Mn SOD in 88 kDa, Cu SOD in 36 kDa, and Zn SOD in 36 kDa (Wong et al., 1989). These isoenzymes can be determined as two bands Mn SOD and CuZn SOD in natural electrophoresis (Beauchamp & Fridovich, 1971). Although it is considered to be present in mitochondria, Mn SOD has been reported to be present in erythrocytes that do not contain mitochondria (Adžić et al., 2004). Cu SOD and Mn SOD show antioxidant effect in the cytoplasm, intercellular areas, and another cell component. In the present study, in addition to determining the total SOD and CAT activities with spectrophotometrically methods, changes in the isomers of the SOD enzyme were determined by electrophoretic methods. The total SOD activity of erythrocytes treated with only H_2O_2 decreased seriously compared to the control group. However, especially 10 and 50 μM diosmetin applications significantly increased the SOD activity compared to the H_2O_2 group (Figure 3).

Figure 3. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 μM (100 D)) treatments on superoxide dismutase activities in human erythrocytes. Different letters in the graph mean statistically significant differences (*p< 0.05*).
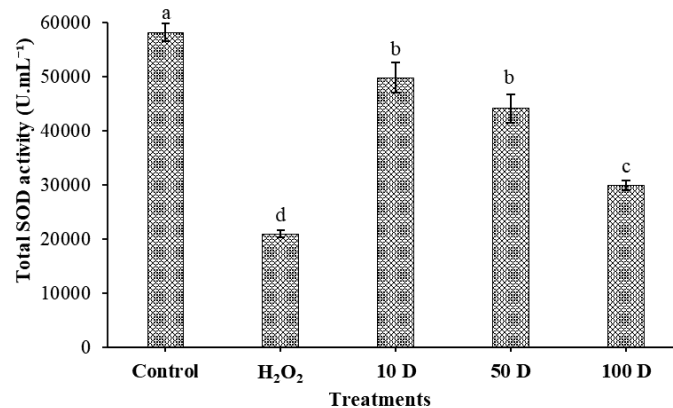

The change in SOD isoenzymes can be clearly seen in native gel electrophoresis. Moreover, band densities of Mn SOD and CuZn SOD were calculated using the Gel Analyser program and shown in Figure 4. Images and calculations obtained from the gel were shown that the application of H_2O_2 caused a critical decrease in the amount of Mn SOD and CuZn SOD protein. On the other hand, diosmetin applications compared to H_2O_2 application significantly increased the band density in SOD isoenzymes. As in total SOD activity, highest density SOD isoenzyme bands in erythrocytes exposed to oxidative stress were observed in 10 and 50 μM diosmetin applications.

Figure 4. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 μM (100 D)) treatments on relative band intensity of different types of superoxide dismutase isoenzymes in human erythrocytes. (*) The band intensity of control was accepted as one hundred percent and the other groups were compared to the control.

	Control	H_2O_2	10 D	50 D	100 D
Mn SOD (%)	100 *	74	86	86	80
CuZn SOD (%)	100	73	78	80	76
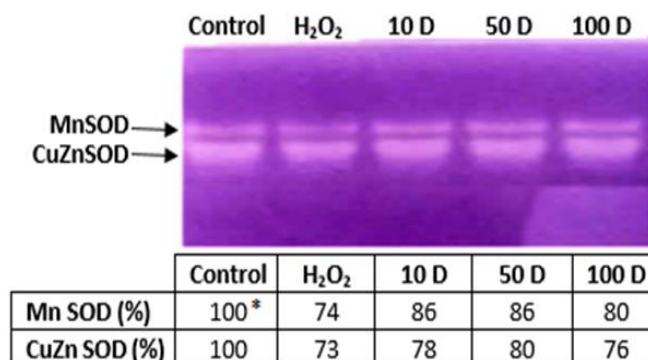
Similar trends to the changes in SOD activity were also detected in CAT activity. While CAT activity is declined by H_2O_2, diosmetin treatments considerably increased as compared to H_2O_2 group. In all of diosmetin treatments, CAT showed higher activity than H_2O_2 groups but there were no statistical differences among the diosmetin groups (Figure 5) ($p<0.05$).

Figure 5. Effects of alone H_2O_2 and H_2O_2 plus diosmetin (10 (10 D), 50 (50 D), and 100 μM (100 D)) treatments on catalase activities in human erythrocytes. Different letters in the graph mean statistically significant differences ($p< 0.05$).
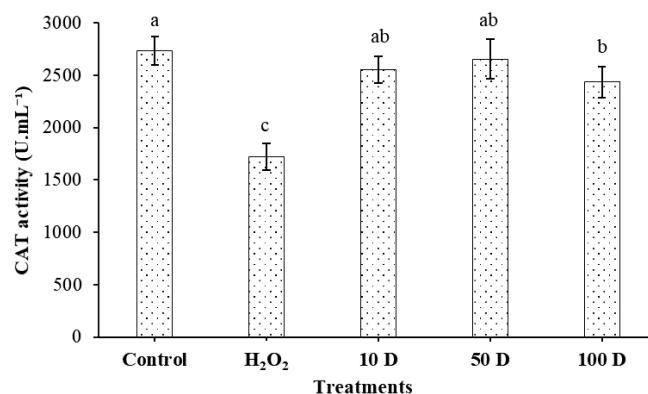
The high ADS power resulted from diosmetin can be explained by the fact that it directly removes reactive oxygen species or indirectly contributes to antioxidant enzyme activities. The diosmetin-induced upward trend in total SOD activity was compatible with the increases of band densities in SOD isoenzymes. It is well known that erythrocytes do not contain organelles such as nuclei and ribosomes, therefore the diosmetin-induced increases in these isoenzyme bands cannot be explained with newly synthesized SOD proteins. The possible reason for these increases might be a protective effect of diosmetin on the activity of existing SOD isoenzymes. Moreover, the lowest LP levels were detected in the diosmetin applications where total SOD, CAT activities and SOD isoenzyme bands were highest except for control groups. In a recent study, it has been reported to be low oxidative damage in erythrocytes with high SOD activities as compatible with our results (Schieber & Chandel, 2014).

4. CONCLUSION

In this study, the mitigating effects of diosmetin on human erythrocytes exposed to oxidative damage were revealed with changes in antioxidant enzyme activities and lipid

peroxidation. Also, for the first time, the changes in SOD isoenzymes of damaged erythrocytes were detected. These results indicated that diosmetin, a flavonoid derivate, has a considerable effect on mitigating oxidative damage by reducing lipid peroxidation and, by increasing antioxidant enzyme activities in erythrocyte cells. Further studies of diosmetin is recommended being done and, investigated its possible drug active substance potential.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Mucip Genisel: Resources, Investigation, Formal Analysis, Methodology, Writing-original draft, Supervision, Validation. **Fatma Kubra Yildizoglu:** Investigation, Methodology, Validation.

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