



## Effects of aspirin on oxidative and nitrosative stress in vascular endothelial cell cultures

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### ABSTRACT

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In this study, it was aimed to investigate that whether any change in activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), lipid peroxidase and nitric oxide (NO) over time, and whether any difference between the different doses by giving different doses of aspirin to endothelial cells. Endothelial cells (HUVEC) in 24 wells microplates used in this study and 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu$ M aspirin to 2 of 4 wells at each row were given, the other 2 wells were included as controls. Accordingly, while CAT, SOD, GSH-Px levels and lipid peroxidation were being measured, NO release from cell media was observed. The significant differences were not found between the baseline (0 hour) CAT, SOD, GSH-Px and lipid peroxidase levels measured from lisates that obtained from the cells that different drug doses given and controls ( $p > 0.05$ ). Also, CAT, SOD, GSH-Px and lipid peroxidase levels at 24 ( $p > 0.05$ ), 48 and 72 hours did not show any difference among different drug doses and control ( $p > 0.05$ ). In the control group significant differences were found between CAT, SOD and GSH-Px levels measured at 0, 24, 48, 72 hours ( $p < 0.05$ ,  $p < 0.05$  and  $p < 0.05$  respectively) but lipid peroxidase activity and NO levels showed no difference. Increase in antioxidant enzyme activity in the cells that aspirin was not given, caused by raised free radical formation due to increase in number of cells by time was observed. Aspirin prevented the increase in reactive enzyme activity which increases by time. These results suggest that nontoxic doses of aspirin might protect the cells.

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### Introduction

Free radicals are molecule or molecular portions that carry one or more unpaired electrons in their atomic

or molecular orbital (Valko et al., 2007). The most important free radicals in biological systems are derived from oxygen. High doses or redundant reactive oxygen

species cause oxidative stress that result in damage of biological macromolecules and metabolic dysfunction (Singal et al., 1983; Singal et al., 1998). There are protective mechanisms against the harmful effects of free radicals in the organism. Most studies used antioxidants to modulate side effects which results from free radicals production and inflammation (Yapıslar et al., 2016). Antioxidant mechanisms are assessed by measuring the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) like enzymes in practice. (Mates et al., 1999, Mates et al., 2000; Valko et al., 2007). Lipid peroxidase activity is used as a marker of membrane damage caused by free radicals.

Endothelium protects vascular homeostasis by providing local maintains that regulate the vascular tonus, platelet adhesion, inflammation, fibrinolysis, and vascular proliferation. In case of oxidative stress, endothelial cells lose their protective phenotype and synthesize pro-inflammatory molecules (Kunsch and Medford, 1999). Endothelial dysfunction forms the cardiovascular risk increases because of its negative effects on all of these events (Irani, 2000; Nedeljkovic et al., 2003).

Aspirin treatment is important in heart disease caused by vascular problems such as atherosclerosis. In addition to the known anti-clotting effects, it was shown that aspirin provides protection of the vascular endothelium, and therefore, the prevention of occlusive cardiovascular and cerebrovascular diseases, and also it is very effective on damages developed at the beginning of the atherosclerosis (Watala and Gwozdziński, 1993). Based on the clinical data showing that aspirin provided improvement of endothelial functions, we designed our study to determine mechanisms of this improvement at cellular level.

In this study, by giving different doses of aspirin to endothelial cell culture, whether there are any changes over time in CAT, SOD, GSH-Px, lipid peroxidase and nitric oxide (NO) activities and whether there are differences between the different doses are investigated.

## 2. Methods

### The cell culture phase

Human Umbilical Vascular Endothelial Cell (HUVEC) provided from the Istanbul University Istanbul Faculty of Medicine, Biophysics Department were used in the experiments. Cells were cultured in DMEM-F12 raw medium (Dulbecco's Modified Eagle Medium, nutrient mixture F12 Ham medium) containing 10% inactivated fetal calf serum (FCS), 0.2 mM glutamine, 100 mg/ml streptomycin, 100 IU/ml penicillin at 37°C, under 5% CO<sub>2</sub> and 1 atm pressure. The cells were routinely passaged 2 times per week. The cells were used in experiments when their densities occupy half of the flask surface.

1 ml DMEM-F12 raw medium was added to each well of the 24-well culture plates and there were two wells for each dose. One ml of medium without active substance was added to control wells and again there were two wells for each dose. HUVEC from the 100% living single cell suspension with calculated cell size as 1 ml containing 100.000 cells were seeded to each. As mentioned above, two wells were used for each concentration of active substance, and 0-1, 24, 48, 72-hour experimental groups were formed for each concentration in the experiments.

A stock solution of 1 M Aspirin was prepared. Final concentrations of 25 µM, 50 µM, 100 µM, 250 µM, 500 µM, 750 µM, 1000 µM and 1500 µM were prepared by diluting with culture medium.

### Evaluation of the cytotoxic doses

For each concentration of the active ingredient, at the end of all periods, cells were photographed under an inverted microscope and cytotoxic dose specified areas are defined on images. Cytotoxic effect was detected by measurement of 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT). 96-well microplates were used for this measurement. After waiting for 24 hours for attachment of the cells to the wells, 100 µl cell suspensions were transferred to each well. At the end of the test period, upper medium was withdrawn from wells and 100 µl MTT solution was added and incubated for 4 h at 37°C and at 5% CO<sub>2</sub>. MTT solution was prepared to be 5 mg/ml dissolved in PBS and transferred to a flask by sterile filtration. At the end of four hours incubation, MTT solution was withdrawn from the wells, 200 µl of dimethyl sulfoxide (DMSO) was added to solve formazon crystals formed with MTT on wells, immediately after, it was read by the 540 nm wavelength microplate reader. The level of cytotoxicity was determined by the absorbance value. Concentration having 50% cytotoxic effect compared to the control was accepted as cytotoxic dose.

### Determination of the enzyme activities

Media of experimental groups were removed at the end of the incubation period. These media were used as the supernatant for NO measurement. The cells of which media were removed were taken from the attached region. Homogenization of removed cells was provided by adding 2 ml lysis buffer and vigorous pipetage. The homogenized cells were collected in this mixture and centrifuged at 10.000 g for 15 minutes at +4°C. Supernatants were separated to measure enzyme activity. Supernatants stored at -80°C until measurement.

NO production was evaluated by measuring the levels of nitrite (NO<sub>2</sub>) which is a stable product of NO. For this purpose, 50 µl supernatant was taken and an equal amount of Griess reagent (1% sulfanilamide /

naftiletlen diamine dihydrochloride 0.1%/2.5%  $H_3PO_4$ ) was added and measured spectrophotometrically at a wavelength of 550 nm. Lipid peroxidation levels defined by Varshey and Kale (1990) with thiobarbituric acid (TBA) method were measured spectrophotometrically. Method based on malondialdehyde (MDA) and TBA reactivity which is the aldehyde product of lipid peroxidation. Results were expressed as the amount of MDA per mg protein. Determination of GSH-Px, (Cayman, GSH-Px measurement kit, U.S.A) CAT (Cayman, Catalase measurement kit, U.S.A) and SOD activities (Cayman, Superoxide Dismutase measurement kit, U.S.A) were performed according to the manufacturers' recommendations. The results obtained were compared with control.

### Statistical analysis

Continuous variables were expressed as mean  $\pm$  standard deviation. Differences in continuous variables between the study and control groups were investigated with the Kruskal-Wallis test. Differences in antioxidant enzyme activity values within the groups according to the time were measured using the Friedman test. p values  $<0.05$  were considered statistically significant. The "Statistical Package for Social Sciences (SPSS) version 15.0 program" was used for statistical analysis.

### 3. Results

In this study; 2, 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu M$  doses of aspirin were given to endothelial cells in 2 of 4 wells in each row of 24 well microplate, and the other two wells were used as controls. Accordingly, while the CAT, SOD, GSH-Px and lipid peroxidation measurements in cell lysates were being performed at basal conditions (0 hour), 24<sup>th</sup> hour, 48<sup>th</sup> and 72<sup>nd</sup> hours, the release of NO were measured from the cell medium. For the measurement of MTT; 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu M$  aspirin doses given to cells in three wells of 96 wells microplate 3 wells served as control wells. Absorbance measurements were done at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>th</sup> hours.

#### MTT measurements

The toxic dose level which is determined by decrease in absorbance by 50% compared to control group by MTT analysis was found as 1500  $\mu M$  in analysis at 0, 24<sup>th</sup> and 72<sup>nd</sup> hours and it was found as 1000  $\mu M$  in 48<sup>th</sup> hour analysis (Table 1, Fig. 1).

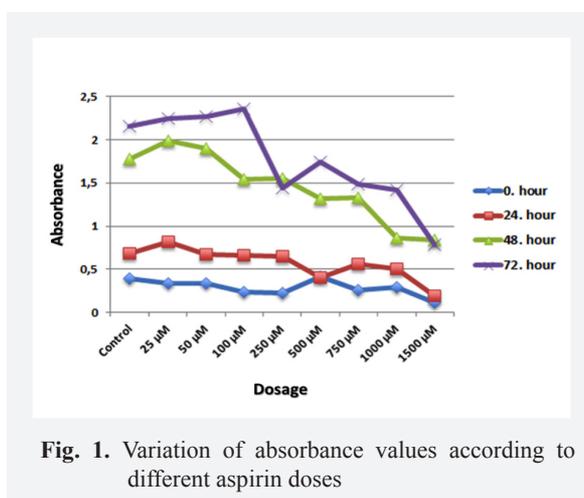
Morphologic evaluation of vascular endothelial cells to which 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu M$  aspirin were given did not show any visible toxic effect between drug groups and controls. The observed finding is the better opening of the cells especially in higher doses such as 1000  $\mu M$  and 1500  $\mu M$  compared to lower doses.

**Table 1.** Absorbance values measured in different aspirin doses and controls according to time

Dose	0. Hour	24 <sup>th</sup> Hour	48 <sup>th</sup> Hour	72 <sup>nd</sup> Hour
Control	0.394	0.685	1.776	2.157
25 $\mu M$	0.333	0.814	1.987	2.246
50 $\mu M$	0.332	0.669	1.897	2.266
100 $\mu M$	0.231	0.103	1.547	2.357
250 $\mu M$	0.222	0.644	1.549	1.437
500 $\mu M$	0.418	0.407	1.321	1.749
750 $\mu M$	0.257	0.558	1.327	1.484
1000 $\mu M$	0.289	0.508	0.857	1.42
1500 $\mu M$	0.117	0.187	0.841	0.779

#### Evaluation of the catalase activity

The catalase levels measured at basal (0 hour) conditions were not significantly different between the control wells and the wells that different dosages of drugs were given ( $p>0.05$ ). Additionally, the catalase levels measured at 24<sup>th</sup> hour ( $p>0.05$ ), 48<sup>th</sup> hour ( $p>0.05$ ) and 72<sup>nd</sup> hour ( $p>0.05$ ) were not different between the control and different dosages of drugs.

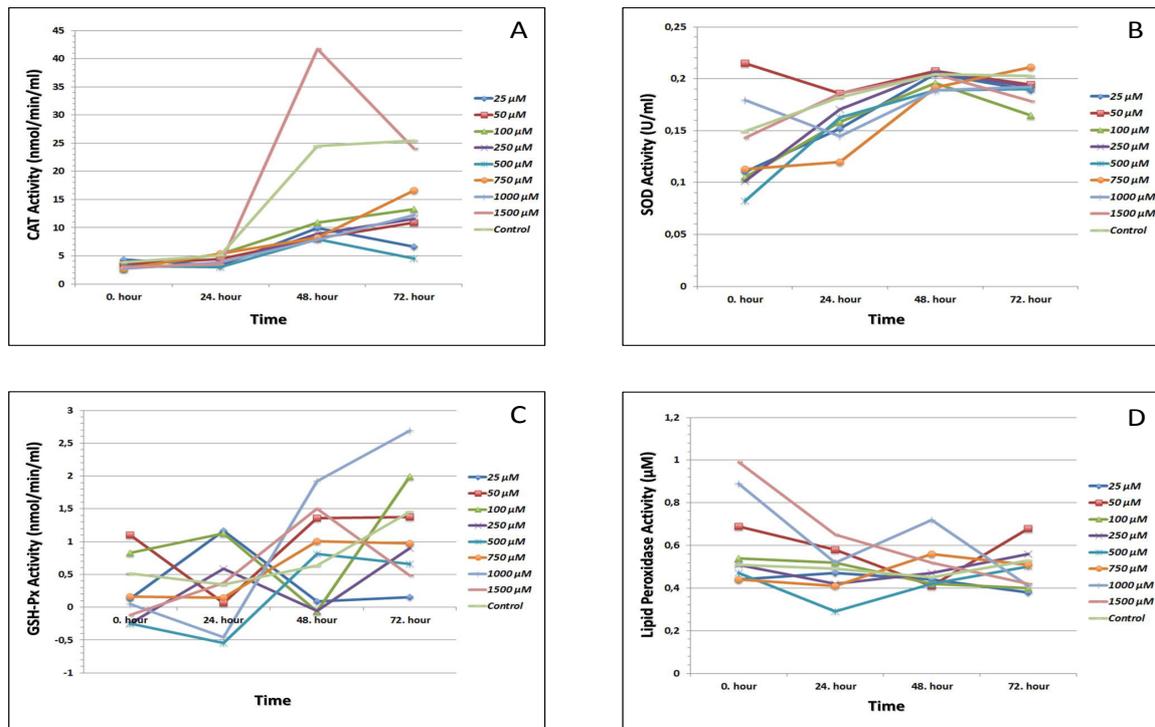


**Fig. 1.** Variation of absorbance values according to different aspirin doses

When the catalase levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours from each well to which 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu M$  drugs given were compared, they did not show difference according to time ( $p>0.05$ ). The significant difference was determined in catalase levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours in control groups to which aspirin was not given ( $p<0.05$ ). The data related to catalase activities measured at different time and dosages were presented in Table 2 and Figure 2.

#### Evaluation of the superoxide dismutase activity

SOD levels measured at basal (0 hour) conditions were not significantly different between the control wells and the wells that 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu M$  drugs were given ( $p=0.494$ ). Additionally, the SOD levels measured at 24<sup>th</sup> hour ( $p>0.05$ ), 48<sup>th</sup> hour ( $p>0.05$ ) and 72<sup>nd</sup> hour ( $p>0.05$ ) were not different between the control and different dosages of drugs.



**Fig. 2.** Change of antioxidant enzyme activity. **2A:** Change of catalase (CAT) activity measured at different drug doses and controls by time; **2B:** Change of superoxide dismutase (SOD) activity measured at different drug doses and controls by time; **2C:** Change of glutathione peroxidase (GSH-Px) activity measured at different drug doses and controls by time; **2D:** Change of lipid peroxidase activity measured at different drug doses and controls by time

When the SOD levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours from each well to which 25, 50, 100, 250, 500, 750, 1000 and 1500 µM drugs given were compared, they did not show difference according to time ( $p > 0.05$ ). The significant difference was determined in SOD levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours in control groups to which aspirin was not given ( $p < 0.05$ ). The data related to SOD activities measured at different time and dosages were presented in Table 3 and Figure 2B.

#### Evaluation of the glutathione peroxidase activity

The glutathione peroxidase (GSH-Px) levels measured at basal (0 hour) conditions were not significantly different between the control wells and the wells that

25, 50, 100, 250, 500, 750, 1000 and 1500 µM drugs were given ( $p > 0.05$ ). Additionally, the GSH-Px levels measured at 24<sup>th</sup> hour ( $p > 0.05$ ), 48<sup>th</sup> hour ( $p > 0.05$ ) and 72<sup>nd</sup> hour ( $p > 0.05$ ) were not different among to the control and different dose groups.

When the GSH-Px levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours from each well to which 25, 50, 100, 250, 500, 750, 1000 and 1500 µM drugs given were compared, they did not show difference according to time ( $p > 0.05$ ). The significant difference was determined in GSH-Px levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours in control groups to which Aspirin was not given ( $p < 0.05$ ) (Fig. 2C). The data related to GSH-Px activities measured at different time and dosages were presented in Table 4.

**Table 2.** The catalase activity measured at 0, 24<sup>th</sup>, 48<sup>th</sup>, and 72<sup>nd</sup> hours in different aspirin doses and controls (nmol/min/ml)

Dosages	0. hour	24 <sup>th</sup> hour	48 <sup>th</sup> hour	72 <sup>nd</sup> hour	p value
25 µM (n=2)	4.373 ± 2.295	3.291 ± 0.765	9.953 ± 5.595	6.594 ± 1.899	> 0.05
50 µM (n=2)	3.515 ± 0.073	4.478 ± 0.289	8.105 ± 1.780	10.893 ± 2.176	> 0.05
100 µM (n=2)	2.750 ± 0.000	5.307 ± 3.616	10.926 ± 4.120	13.240 ± 3.379	> 0.05
250 µM (n=2)	2.909 ± 0.615	3.468 ± 0.006	8.865 ± 0.709	11.523 ± 4.172	> 0.05
500 µM (n=2)	3.160 ± 0.000	3.030 ± 0.395	7.977 ± 3.327	4.504 ± 1.583	> 0.05
750 µM (n=2)	2.726 ± 0.350	5.380 ± 0.007	8.193 ± 0.767	16.599 ± 0.193	> 0.05
1000 µM (n=2)	2.750 ± 0.000	3.851 ± 1.557	7.826 ± 1.108	12.230 ± 1.739	> 0.05
1500 µM (n=2)	3.030 ± 0.395	3.477 ± 1.028	41.685 ± 11.504	24.025 ± 12.197	> 0.05
Control (n=16)	3.892 ± 2.409	5.145 ± 2.812	24.496 ± 10.023	25.489 ± 22.013	< 0.05
p value	0.571	0.696	0.249	0.118	

**Table 3.** The SOD activity measured at 0, 24<sup>th</sup>, 48<sup>th</sup>, and 72<sup>nd</sup> hours in different aspirin doses and controls (U/ml)

Dosages	0. hour	24 <sup>th</sup> hour	48 <sup>th</sup> hour	72 <sup>nd</sup> hour	p value
25 $\mu$ M (n=2)	0.11026 $\pm$ 0.06867	0.15190 $\pm$ 0.05561	0.20460 $\pm$ 0.15555	0.18910 $\pm$ 0.01711	> 0.05
50 $\mu$ M (n=2)	0.21464 $\pm$ 0.10142	0.18601 $\pm$ 0.01054	0.20755 $\pm$ 0.01441	0.19408 $\pm$ 0.01081	> 0.05
100 $\mu$ M (n=2)	0.10485 $\pm$ 0.08803	0.15838 $\pm$ 0.03368	0.19597 $\pm$ 0.14750	0.16485 $\pm$ 0.07177	> 0.05
250 $\mu$ M (n=2)	0.10149 $\pm$ 0.03956	0.17064 $\pm$ 0.06477	0.20619 $\pm$ 0.00642	0.19199 $\pm$ 0.00772	> 0.05
500 $\mu$ M (n=2)	0.08213 $\pm$ 0.01707	0.16235 $\pm$ 0.07035	0.18895 $\pm$ 0.03768	0.18990 $\pm$ 0.36345	> 0.05
750 $\mu$ M (n=2)	0.11296 $\pm$ 0.05007	0.11981 $\pm$ 0.00704	0.19185 $\pm$ 0.00777	0.21092 $\pm$ 0.00533	> 0.05
1000 $\mu$ M (n=2)	0.17940 $\pm$ 0.00339	0.14435 $\pm$ 0.04886	0.18885 $\pm$ 0.00063	0.19230 $\pm$ 0.03295	> 0.05
1500 $\mu$ M (n=2)	0.14318 $\pm$ 0.09604	0.18600 $\pm$ 0.03945	0.20408 $\pm$ 0.01482	0.17845 $\pm$ 0.00063	> 0.05
Control (n=16)	0.14913 $\pm$ 0.06912	0.14913 $\pm$ 0.06912	0.18193 $\pm$ 0.02845	0.20430 $\pm$ 0.01358	< 0.05
p value	0.494	0.565	0.566	0.659	

SOD: Superoxide dismutase

**Table 4.** The GSH-Px activity measured at 0, 24<sup>th</sup>, 48<sup>th</sup>, and 72<sup>nd</sup> hours in different aspirin doses and controls (nmol/min/ml)

Dosages	0. hour	24 <sup>th</sup> hour	48 <sup>th</sup> hour	72 <sup>nd</sup> hour	p value
25 $\mu$ M (n=2)	0.130285 $\pm$ 0.276797	1.164839 $\pm$ 4.044800	0.088341 $\pm$ 0.771087	0.158243 $\pm$ 0.434973	> 0.05
50 $\mu$ M (n=2)	1.100569 $\pm$ 1.055960	0.063526 $\pm$ 1.020622	1.354878 $\pm$ 0.553523	1.376815 $\pm$ 2.687833	> 0.05
100 $\mu$ M (n=2)	0.829307 $\pm$ 2.174858	1.122900 $\pm$ 0.810627	-0.065442 $\pm$ 0.395426	1.989691 $\pm$ 1.799198	> 0.05
250 $\mu$ M (n=2)	-0.250178 $\pm$ 0.686040	0.589871 $\pm$ 0.229931	-0.052255 $\pm$ 0.744080	0.900379 $\pm$ 0.105861	> 0.05
500 $\mu$ M (n=2)	-0.245208 $\pm$ 0.830400	-0.540780 $\pm$ 0.316345	0.815326 $\pm$ 1.324688	0.661543 $\pm$ 0.790856	> 0.05
750 $\mu$ M (n=2)	0.163651 $\pm$ 0.128836	0.148060 $\pm$ 1.093653	1.003614 $\pm$ 0.892688	0.972225 $\pm$ 0.701923	> 0.05
1000 $\mu$ M (n=2)	0.046401 $\pm$ 0.039542	-0.456900 $\pm$ 0.316345	1.919788 $\pm$ 2.609826	2.688716 $\pm$ 0.296571	> 0.05
1500 $\mu$ M (n=2)	-0.121365 $\pm$ 0.158170	0.367952 $\pm$ 0.217485	1.500373 $\pm$ 1.937598	0.479794 $\pm$ 1.680572	> 0.05
Control (n=16)	0.519243 $\pm$ 0.821678	0.346849 $\pm$ 0.781278	0.633676 $\pm$ 1.265019	1.451028 $\pm$ 0.89589	< 0.02
p value	0.624	0.513	0.590	0.375	

GSH-Px: Glutathione peroxidase

### Evaluation of the nitric oxide activity

In the analysis performed at the wells that drugs given and control wells in which drugs were not given, the quantitative NO activity measurement in significant proportion of the samples could not be done because of the low level of activity. The analysis of present measurements revealed that; The nitric oxide levels measured at basal (0 hour) the conditions were not significantly different between the control wells and the wells that 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu$ M drugs were given ( $p=0.406$ ). Additionally, the NO levels measured at 24<sup>th</sup> hour ( $p=0.392$ ), 48<sup>th</sup> hour ( $p=0.392$ ) and 72<sup>nd</sup> hour ( $p=0.416$ ) were not different between the control and different dosages of drugs.

**Table 5.** The NO activity measured at 0, 24<sup>th</sup>, 48<sup>th</sup>, and 72<sup>nd</sup> hours in different aspirin dosages and controls ( $\mu$ M)

Dosages	0. hour	24 <sup>th</sup> hour	48 <sup>th</sup> hour	72 <sup>nd</sup> hour
25 $\mu$ M (n=2)	2.350	4.138	3.021	3.759
50 $\mu$ M (n=2)	-	-	-	3.349
100 $\mu$ M (n=2)	3.691	3.244	3.244	3.485
250 $\mu$ M (n=2)	2.126	-	-	-
500 $\mu$ M (n=2)	3.468	3.691	3.468	3.622
750 $\mu$ M (n=2)	-	-	-	3.349
1000 $\mu$ M (n=2)	3.244	3.691	3.759	3.554
1500 $\mu$ M (n=2)	-	-	-	-
Control (n=16)	-	-	-	-
p value	> 0.05	> 0.05	> 0.05	> 0.05

NO: Nitric oxide

The analysis of NO activity measurements obtained from samples to which different drug dosages given and controls to find out whether there was any difference between different time intervals could not be done because of the lack of data. The data related to NO activity measured at different time and dosages were presented in Table 5.

### Evaluation of the lipid peroxidase activity

The lipid peroxidase levels measured at basal (0 hour) conditions were not significantly different between the control wells and the wells that 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu$ M drugs were given ( $p>0.05$ ). Additionally, the lipid peroxidase levels measured at 24<sup>th</sup> hour ( $p>0.05$ ), 48<sup>th</sup> hour ( $p>0.05$ ) and 72<sup>nd</sup> hour ( $p>0.05$ ) were not different between the control and different dosages of drugs.

When the lipid peroxidase levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours from each well to which 25, 50, 100, 250, 500, 750, 1000 and 1500  $\mu$ M drugs given were compared, they did not show difference according to time ( $p>0.05$ ). The data related to lipid peroxidase activity measured at different time and dosages were presented in Table 6 and Figure 2D.

## 4. Discussion

Oxidative stress that is derived from reactive oxygen species causes cardiovascular tissue damage associated with cardiac and vascular myocytes. Oxidative stress plays an important role in various cardiovascular diseases such as atherosclerosis, ischemic heart disease,

**Table 6.** The lipid peroxidase activity measured at 0, 24<sup>th</sup>, 48<sup>th</sup>, and 72<sup>nd</sup> hours in different aspirin dosages and controls ( $\mu\text{M}$ )

Dosages	0. hour	24 <sup>th</sup> hour	48 <sup>th</sup> hour	72 <sup>nd</sup> hour	p value
25 $\mu\text{M}$ (n=2)	0.44 $\pm$ 0.01	0.47 $\pm$ 0.01	0.44 $\pm$ 0.10	0.38 $\pm$ 0.01	> 0.05
50 $\mu\text{M}$ (n=2)	0.69 $\pm$ 0.32	0.58 $\pm$ 0.27	0.41 $\pm$ 0.12	0.68 $\pm$ 0.33	> 0.05
100 $\mu\text{M}$ (n=2)	0.54 $\pm$ 0.05	0.52 $\pm$ 0.08	0.42 $\pm$ 0.07	0.40 $\pm$ 0.02	> 0.05
250 $\mu\text{M}$ (n=2)	0.51 $\pm$ 0.18	0.42 $\pm$ 0.03	0.47 $\pm$ 0.15	0.56 $\pm$ 0.14	> 0.05
500 $\mu\text{M}$ (n=2)	0.47 $\pm$ 0.01	0.29 $\pm$ 0.10	0.42 $\pm$ 0.07	0.50 $\pm$ 0.02	> 0.05
750 $\mu\text{M}$ (n=2)	0.44 $\pm$ 0.07	0.41 $\pm$ 0.02	0.56 $\pm$ 0.03	0.51 $\pm$ 0.04	> 0.05
1000 $\mu\text{M}$ (n=2)	0.89 $\pm$ 0.63	0.52 $\pm$ 0.18	0.72 $\pm$ 0.07	0.41 $\pm$ 0.17	> 0.05
1500 $\mu\text{M}$ (n=2)	0.99 $\pm$ 0.30	0.65 $\pm$ 0.02	0.52 $\pm$ 0.27	0.42 $\pm$ 0.22	> 0.05
Control (n=16)	0.51 $\pm$ 0.10	0.49 $\pm$ 0.12	0.45 $\pm$ 0.11	0.53 $\pm$ 0.13	> 0.05
p value	0.383	0.362	0.25	0.477	

hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart disease (Valko et al., 2007). Singal et al. (1983) and Hess et al. (1983) showed the importance of increased oxygen free radicals in pathogenesis of many heart disease in vitro and in vivo studies.

Complications of atherosclerosis are the basis of cardiovascular diseases (Singh and Jialal, 2006). Endothelial dysfunction is seen in the early stages of atherogenesis as well. Damaged endothelial function produces even more dangerous results when combined with several risk factors. Setting up a ground for the long-term atherosclerotic lesions by endothelial dysfunction is one of these results, and this condition is important in the diagnosis of coronary syndromes. (Vogiatzi et al., 2009).

Aspirin treatment is important in heart diseases caused by vascular problems especially atherosclerosis. Aspirin treatment is effective in reducing recurrent events in patients with one or more of the risk factors like hyperlipidemia, hypertension, diabetes and smoking, as well as patients who had coronary or other vascular events (stroke, peripheral vascular disease).

Most of the risk factors associated with atherosclerosis and cardiovascular morbidity and mortality are also associated with endothelial dysfunction. Many of the risk factors including hyperlipidemia, hypertension, diabetes, and cigarette smoking are associated with excessive production of reactive oxygen species and increased oxidative stress (Hink et al, 2001; Channon and Guzik, 2002 ).

In this study, aspirin was given to endothelial cells in different doses and the changes in CAT, SOD, GSH-Px lipid peroxidase and NO activities with respect to time, and the differences between the doses were investigated. The doses of aspirin given to cells were determined by MTT assay (McGahon et al., 1995). In the MTT analysis, the dose of aspirin leading to 50% decrease in absorbance compared to the control group was determined as cytotoxic dose. The cytotoxic doses were determined as 1500  $\mu\text{M}$  at 0, 24<sup>th</sup> and 72<sup>nd</sup> hour analysis, 1000 and 1500  $\mu\text{M}$  at 48<sup>th</sup> hour analysis, respectively. In this way, the number of damaged cells was evaluated by MTT assay.

When the CAT, SOD and GSH-Px enzyme activities were evaluated; there was a significant increase in activity when the enzyme activity results of control group at 0 and 24<sup>th</sup> hours compared to enzyme activity 48<sup>th</sup> and/or 72<sup>nd</sup> hours. The changes in the level these three enzyme activities over time measured at the cells to which aspirin 25, 50, 100, 250, 500, 1000 and 1500  $\mu\text{M}$  given were not statistically significant. A remarkable, but not statistically significant increase in CAT activity at 48<sup>th</sup> and 72<sup>nd</sup> hours was observed only in the cells to which 1500  $\mu\text{M}$  aspirin given. Additionally, there was no difference between the control and groups to which different drug doses given regarding the enzyme activities measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours.

Endothelial cell proliferation in cell culture over time increases the amount of free radicals in the environment and at the same time increases the activities of CAT, SOD and GSH-Px enzymes which are enzymatic indicators of the reactive antioxidant defense. Increased enzyme activity at 72<sup>nd</sup> hour in the control group to which aspirin was not given can be explained by this hypothesis. Statistically insignificant increase in the CAT activity at the 48<sup>th</sup> and 72<sup>nd</sup> hours in the cells to which 1500  $\mu\text{M}$  aspirin given can be explained by toxic effect of this dose which was expected to be protective aspirin dose.

In control groups, the increase in enzyme activities over time suggests that the presence of protective effect of aspirin dose which is less than 1500  $\mu\text{M}$  given to endothelial cells.

The basic mechanism of aspirin is inactivation of a key enzyme cyclooxygenase resulting in inhibition of prostaglandin synthesis. This explains the analgesic, anti-inflammatory, antipyretic and inhibition of platelet aggregation (Vargaftig, 1978). Our study showing the potential beneficial effects of aspirin on oxidative processes at cellular level is supported by a large number of clinical investigations associated with this process. Grosser and Schröder (2003) reported that aspirin showed a cell protective effect by protecting the endothelial cells from oxidative damage in the presence of NO and also Taubert et al. (2004) stated the aspirin caused an increased NO secretion from endothelial cells and this effect increased the activity of SOD

enzyme which catalyzes the dismutation of superoxide radicals.

In addition to the classical platelet inhibition effect of aspirin, it provides the protection of vascular endothelium and therefore, it was shown that it is quite effective in the prevention of occlusive cardiovascular and cerebrovascular diseases and on the damages occurred in the beginning of atherosclerosis (Watala and Gwozdziński, 1993).

Podhasky et al. (1997) in their study showed that oxidative stress is a cardiovascular risk factor and an important source of endothelial damage during atherogenesis and oxygen radicals released by neutrophils and monocytes had direct damage to the endothelium. Also they stated that aspirin provides endothelial integrity and thus protecting the antithrombotic and anti-atherogenic functions of endothelium under oxidative stress. Clinical studies showed that low-dose aspirin caused endothelium-dependent arterial relaxation which is the basis of vascular homeostasis (Taubert et al., 2004).

Metal binding property of aspirin is also determined. With this feature, it can take the iron ions from the area where oxygen radicals formed. Therefore; compared to the hydrogen peroxide-dependent cell damage, aspirin had been suggested to pretend to be 10 times more potent cell protector (Podhasky et al., 1997).

There are evidences about the reduction in living endothelial cells which exposed to hydrogen peroxide. Studies performed with aspirin demonstrated that, aspirin (3-30 mol/L) protects cells from cytotoxicity in a concentration-dependent manner. This suggested that the aspirin has a capacity to protect the endothelial cells from harmful effect hydrogen peroxide and also has a property of free radical scavenger (Grosser and Schröder, 2003).

The oxygen radicals show reactions with unsaturated lipids and initiate the lipid peroxidation reactions on membranes. Beside this, free radicals cause oxidation of sulfhydryl groups of proteins and the nucleic acids strands. Interaction of membrane lipids by free oxygen radicals initiates lipid peroxidation chain reactions and causes deterioration of the membrane structure, increase in the permeability of cell, loss of cellular ion gradient and leads to tissue damage (Jackson et al., 2002). Lipid peroxidation starts as a chain reaction form and generates an uninterrupted source for the free radicals that further initiate peroxidation. This self-perpetuating chain reactions cause irreversible damage to the cell membrane. Lipid peroxidation in cell membranes causes membrane lipoproteins oxidation and a loss of structural integrity and with the entry of abnormal ion causes cell death. When this event cannot be controlled, chain reactions occur and it causes spread of cellular death (Srivastava et al., 1989). As a result, cell membranes losses semi-permeable properties. The

enzyme systems located in the cell membrane fail to do transport function. Thus, the intra-and extracellular densities of various compounds, inorganic substances and electrolytes which enter and exit the cell by using the active transport and other transport systems change. All these events bring about damage to the cells that are difficult to reverse back and this can result in the death of the cell (Srivastava et al., 1989).

The aspirin's antioxidant effects which reduce vascular tone and suppress lipid peroxidation were shown at the clinical level (Grosser and Schröder, 2003). Associated to this condition, according to the data of our study at the cellular level, there was no difference in lipid peroxidase activity measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours between control group and the cells to which various doses of aspirin given. Moreover, there was no change in lipid peroxidase activity over time in both the control group and cells to which the different doses of aspirin given.

NO and oxygen radicals production occur simultaneously, or a radical type production can be accelerated by the effects caused by other radical type in pathological conditions (Tabima et al., 2012). Immediate removal of H<sub>2</sub>O<sub>2</sub> produced in biological systems is required because of the oxidizing properties. This task is carried by CAT and GSH-Px which are important antioxidant enzymes in cells (Mohazzab-H et al., 1999).

NO synthesized by endothelial cells passes through the smooth muscle cells by diffusion and activates guanylate cyclase and, increases the level of cGMP, causes the smooth muscle relaxation. Because the activation of eNOS ends by reduction in the concentration of cytoplasmic calcium by calcium pumps, eNOS provides a low concentration of NO synthesis in a short period of time (Tabima et al., 2012). Endothelial cells in culture spontaneously release NO which is responsible for a physical and chemical stimulation. However, the NO-release is not against to acetylcholine, and it is thought that this is caused by either loss of acetylcholine receptors or loss of receptor-effector effect in cell culture (Calver et al., 1993).

Superoxide radicals in blood vessels are synthesized continuously as a constitutive. Thus, superoxide by controlling the concentration of NO in blood vessels regulates its vasodilator effect. In the case of inhibition of SOD enzyme by diethyldithiocarbamate in blood vessels, the concentration of superoxide increases ten times and NO-dependent relaxation is inhibited. In various vascular diseases, by the increased production of superoxide, the capacity of SOD enzyme can be exceeded and nitric oxide is inactivated by superoxide. Thus, the superoxide produced at normal levels regulates concentration of NO and its effect, the high concentration in pathological conditions both prevents functions of NO, and causes oxidative damage by

formation of an oxidative type (peroxynitrite) (Wolin, 1991).

Endothelium-derived NO plays a role in the physiological regulation of blood pressure, blood flow and vascular tone in different organs. NO-release can be induced by various receptor dependent (acetylcholine, bradykinin, histamine, and serotonin adenine nucleotides) and independent (free fatty acids) agonists. Endothelial cells convert physical pressure of circulating blood applied to endothelium to biochemical signals (Büssemaker et al., 2007).

The acetyl group of aspirin increases the eNOS synthesis and the usefulness (Bulckaen et al., 2008). This also indicates that it provides the protection of the cell caused by NO. NO like aspirin decreases the sensitivity of endothelial cells to H<sub>2</sub>O<sub>2</sub> and other oxidizing agents. Both aspirin and NO have long-term protective effect on endothelial cells (Grosser and Schröder, 2003).

In this study, the analysis of quantitative measurement of NO activity could not be performed in some samples from the control cells and cells to

which aspirin given due to the lack of activity. There was no significant difference between the NO levels measured at 0, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hours from the control wells and the wells to which different doses of aspirin given. The analysis to show the change in NO activity at different time intervals in the control samples and the samples to which different drug doses given could not be performed due to lack of data.

The most important limitation of this study is that especially the scarcity of examples in different doses of aspirin prevented the evaluation of the data such as NO and to reach the expected level of significance.

In conclusion, increase in the activity of antioxidant enzymes is caused by production of free radicals in the environment due to increased cell number over time (after 48 hours) in endothelial cells. This average result might lead us to think that administration of aspirin doses of 1000 µM or less might have a cell protective effect and administration of aspirin doses over 1000 µM would result in a toxic effect causing reduction in the number of cells.

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