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In Vitro Protective Effect of Betaine on Peroxidative Injury Caused By Ethanol and Aspirin Exposure on Rat Brain Synaptosomes

ORIGINAL INVESTIGATION

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ABSTRACT

Objective: Aspirin intake of specific daily doses are advised by doctors to postmenapausal women and men above 40 years of age to prevent heart attack and even cancer in recent times. In vitro cytototoxic effects of different doses of ethanol (50 mM, 100 mM ve 200 mM) alone and together with 100 µg/mL aspirin, and possible protective role of 1 mM betaine on rat brain synaptosomes were investigated.

Materials and Methods: Fifteen male Sprague Dawley rat forebrains were divided into equal pieces and pooled to form ten study groups. Synaptosomal fractions extracted from pooled rat brains were incubated with different doses of ethanol, aspirin and betaine, and malondialdehyde (MDA) levels, an important indicator of cellular damage, were measured.

Results: A significant increase ($p<0.05$) was observed in MDA level of 200 mM ethanol group compared to control group. Different doses of ethanol (50 mM, 100 mM ve 200 mM) + aspirin exposure significantly increased ($p<0.001$) MDA levels compared to controls, whereas betaine administration significantly decreased ($p<0.001$) lipid peroxidation caused by ethanol+aspirin treatment.

Conclusion: We conclude that ethanol and ethanol+aspirin administration increases lipid peroxidation in rat brain synaptosomes while betaine helps prevent this peroxidative membrane injury.

Keywords: Aspirin, betaine, ethanol, malondialdehyde

INTRODUCTION

Patients with an intense allergic reaction or at a possible risk of hemorrhage owing to aspirin intake would not be prescribed this treatment. However, daily doses of aspirin are advised by doctors to postmenopausal women and men aged over 40 years to prevent the risk of heart attacks and even some types of cancers in recent times (1). Aspirin may be taken with acute or chronic alcohol intake in daily life, and previous experimental studies reported that increased toxicity of salicylic acid was observed, particularly in the rodent brain (2, 3). Furthermore, there are studies suggesting a synergistic effect of aspirin and alcohol (4, 5). On the contrary, using a mouse model of prenatal mortality and malformation, Randal and Anton showed that aspirin has a preventive effect on prenatal mortality against the detrimental effects of alcohol (6). Therefore, the primary aim of this study is to show if aspirin would protect the synaptosomes against the detrimental effects of alcohol or if it would increase the alcohol-induced peroxidative damage. The secondary aim is to determine if betaine would decrease the alcohol- or alcohol–aspirin-induced peroxidative damage.

Synapses are the main components of electrical and chemical communication between neurons (7). Synaptosomal fractions include all structures and properties of synapses. Previous studies have shown that brain synapses and synaptosomes are similar in structure and that synaptosomes can be considered as little copies of synapses (3, 8, 9).

Alcohol consumption has a direct and an indirect effect on the brain; the direct effect is on the cell membranes. Alcohol dissolves cell membranes and increases their fluidity, thereby altering the structures of ion channels and transmembrane proteins. The indirect effects include the formation of the oxidative and non-oxidative metabolites of alcohol. Alcohol is metabolized into acetaldehyde by alcohol dehydrogenase, the microsomal cytochrome p450 ethanol oxidation system, and the catalase–H_2O_2 system. During this process, functions of repair enzymes and the mitochondria are impaired. Non-oxidative metabolism of alcohol results in the production of fatty acid ethyl esters. This prevents esterification on neuronal cell membranes and also disrupts the mitochondrial and myelin metabolism (10). Oxidative damage occurs owing to decreased levels of repair enzymes and increased generation of free radicals. An important indicator of oxidative damage is MDA, an end product of lipid peroxidation (11).

Aspirin (acetylsalicylic acid; ASA) is a well-known and widely used anti-inflammatory, antipyretic, and analgesic drug that is mainly used in the treatment of moderate pain, inflammation, and fever. It is a prostaglandin that inhibits thromboxane and causes lipid peroxidation owing to its disruptive effect on the mitochondrial membrane

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potential (12). It may cause apoptosis because of the release of cytochrome c from the mitochondria, resulting in the loss of the mitochondrial membrane potential (13, 14). Long-term aspirin usage results in gastric mucosal damage (15). Goddard et al. (16) reported that aspirin decreased surface hydrophobicity of gastric mucosa by deteriorating the phosphatidylcholine inner mucus layer.

Betaine (trimethylglycine) is a molecular guide that protects cells against oxidative stress and mitochondrial damage and lowers S-adenosylhomocysteine (SAH) levels. It is the methyl source for transmethylation reactions and provides carbon units for the formation of methionine and choline (17). Choline can be generated from phosphatidylcholine, which is the most abundant phospholipid in tissues (18). Betaine is an organic osmolyte that protects cells under stress. Betaine is necessary for the conversion of homocysteine to methionine, keeping the methionine levels stable, and detoxification of homocysteine and S-adenosylmethionine (SAM) production (19). Betaine is known for its role in diminishing ethanol-dependent damage by increasing reduced glutathione with trans-sulfuration (20).

The aim of this study is to investigate the relationship between the in vitro administration of ethanol and aspirin and subsequent lipid peroxidation. The possible protective effects of betaine against ethanol and aspirin-induced lipid peroxidation were also investigated.

MATERIALS and METHODS

Sprague–Dawley strain albino rats (250±10 g) were used in experiments. The rats had access to an unlimited supply of food and water. They were kept at laboratory conditions at a temperature of 22°C±3°C with 55%±5% humidity in a 12-hour day/night cycle. This study was approved by the Ethical Committee of Eskisehir Osmangazi University.

Synaptosomal fractions were obtained using a method reported by Whittaker et al. (21) with some modifications. Fifteen rats were killed by cervical dislocation and their forebrains were removed. After weighing and washing in saline solution, each forebrain was equally divided into four parts and the samples were them put together (the specimen pool). Ten study groups were formed by randomly selecting forebrain parts from the specimen pool (Table 1). Each group contained 6 forebrain parts. Forebrain parts were homogenized in ice-cold HEPES (10 mmoL/L) and sucrose (0.32 mmoL/L) solution. Homogenates were centrifuged at 3.000×g for 10 minutes (Jouan MR 22 centrifuge). Supernatants were collected and recentrifuged at 15.000×g for 20 minutes. After the removal of supernatants, remaining pellets enriched with synaptosomal fractions were resuspended in artificial cerebro spinal liquid (116 mM NaCl, 5.4 mM KCl, 0.9 mM $MgCl_2$, 0.9 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 10 mM glucose; pH 7.2). Obtained synaptosomal fractions were incubated with either only ethanol (50, 100, or 200 mM) or 100 µg/mL aspirin with ethanol (50, 100, or 200 mM) or 100 µg/mL aspirin+ethanol (50, 100, or 200 mM) with 1 mM betaine at 37°C for 30 minutes according to the study group they belonged to (Table 1).

Synaptosomal fractions were combined with ethanol at the concentration of 50, 100, or 200 mM (equivalent to a human blood alcohol level of 23 g/dL, 46 g/dL, and 92 g/dL, respectively) (22). 50 mM ethanol dose was used as the low ethanol concentration, which was considered to be its level in the blood of social drinkers (23). Furthermore, 100 mM ethanol was used as the moderate ethanol level, which was considered to be its level in the blood of binge drinkers (24). Moreover, 200 mM ethanol was used as the heavy ethanol level, which was considered to be its level in the blood of heavy drinkers (25). Overall, 100 µg/mL aspirin corresponds to a value of between 0.5 and 1 mM aspirin, which is within the therapeutic dose range of the drug (0.1–2 mM) (26). In addition, according to pharmacology experiments, 100 µg/mL aspirin is considered as a low level, and approximately 90%, which is good a percentage, of plasma salicylate is bound to albumin (27). Overall, 1 mM betaine is used for the methyl donor substrate supplementation (28).

Table 1. Study groups

Group	Control
1. Group	50 mM Ethanol
2. Group	100 mM Ethanol
3. Group	200 mM Ethanol
4. Group	50 mM Ethanol + 100 µg/mL Aspirin
5. Group	100 mM Ethanol + 100 µg/mL Aspirin
6. Group	200 mM Ethanol + 100 µg/mL Aspirin
7. Group	50 mM Ethanol + 100 µg/mL Aspirin + 1 mM Betaine
8. Group	100 mM Ethanol + 100 µg/mL Aspirin + 1 mM Betaine
9. Group	200 mM Ethanol + 100 µg/mL Aspirin + 1 mM Betaine

After the incubation period, lipid peroxidation levels were measured by a method reported by Ohkawa et al. (29) based on the spectrophotometric measurement of color production due to the reaction of MDA with thiobarbituric acid. An aliquot of 0.4 mL synaptosomal fraction was added to 0.2 mL of sodium dodecyl sulphate (8.1%). This was followed by the addition of 1.5 mL of acetic acid (20%, pH 3.5) and 1.5 mL of aqueous solution of thiobarbituric acid (0.8%, pH 6). This mixture was finally diluted up to 4 mL with distilled water and then vortexed and heated in a water bath at 95°C for 60 minutes. After cooling to room temperature (24°C) using ice-cold water, it was revortexed and then centrifugated at 4000 rpm for 10 minutes. The absorbance of the supernatant layer was read at 532 nm using a Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) against a blank containing 0.1 mL distilled water. Results in the samples were expressed nmol/mg protein using a standard curve made by preparing serial dilution of 1,1,3,3, and 3 tetramethoxypropane (Sigma, USA).

Protein contents were measured by the biuret method (30). This method is used for determining the presence of peptide bonds in a sample. The formation of a Cu^{2+}–protein complex requires two peptide bonds and produces a violet-colored chelate product, which is measured by absorption spectroscopy at 540 nm. First, the biuret reagent was prepared by dissolving 0.3% (w/v) of copper sulphate ($CuSO_4.5H_2O$) and 0.9% (w/v) of sodium potassium tartarate ($KNaC_4H_4O_6$) in 500 mL of 0.2 moL/L sodium hydroxide (NaOH), and 0.5% (w/v) g of potassium iodide was added. The solution was diluted up to 1 litre with 0.2 moL/L NaOH. A standard curve was determined by preparing a serial dilution of bovine serum albumin (Sigma). An aliquot of 0.1 mL synaptosomal fraction was added to 3 mL of

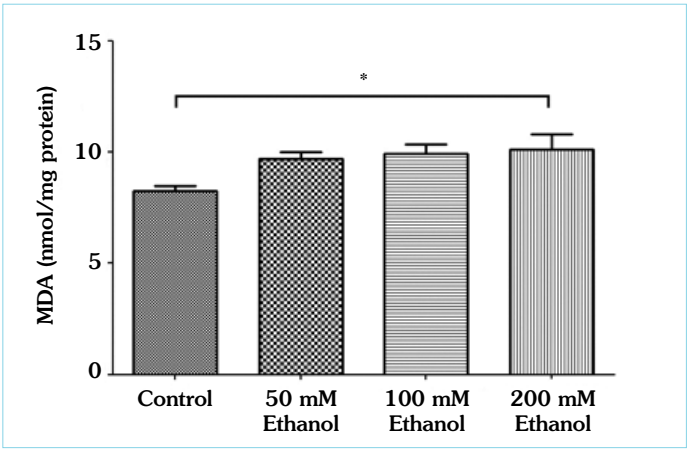

Figure 1. Comparison of MDA (nmol/mg protein) levels in control and different dose ethanol administration groups (50, 100 and 200 mM) *p<0.05

biuret reagent. This mixture was finally diluted up to 4 ml with distilled water, vortexed, and then heated to 37°C for 10 minutes or incubated at room temperature for 30 minutes. The net absorbance of the synaptosomal fraction and calibration standards were measured at 540 nm using a Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) against a blank 1 mL biuret solution. The results were calculated to give a standard curve corresponding to the concentration of protein in the synaptosomal fraction.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences 21.0 (SPSS, Inc.; Chicago, IL, USA) program. One-way ANOVA and Tukey's multiple comparison tests were used to assess the differences between groups. Results were expressed as mean±SD, and p<0.05 was considered significant.

RESULTS

Figure 1 shows the measured MDA levels after different doses of ethanol were applied to synaptosomes. MDA levels of the 200 mM ethanol group (10.12±0.68) were significantly increased (p<0.05) compared with those of the control group (8.25±0.22). No significant difference was observed between the 50 mM (9.71±0.28) and 100 mM (9.93±0.41) ethanol groups and the control group and between the former two groups (p>0.05).

Figure 2a shows MDA levels (nmol/mg protein) of synaptosomes treated with 50 mM ethanol alone, 50 mM ethanol+100 µg/mL aspirin, and 50 mM ethanol+100 µg/mL aspirin+1 mM betaine. MDA levels of the 50 mM ethanol group (9.71±0.28) significantly increased compared with those of the control group (8.25±0.22; p<0.05). MDA levels of the 50 mM ethanol+100 µg/mL aspirin group (12.20±0.44) also significantly increased compared with those of both the control and 50 mM ethanol groups (p<0.001). MDA levels of the 50 mM ethanol+100 µg/mL aspirin+1 mM betaine (9.66±0.46) group significantly decreased compared with those of the 50 mM ethanol+100 µg/mL aspirin group (p<0.001).

Figure 2b shows MDA levels (nmol/mg protein) of synaptosomes treated with 100 mM ethanol alone, 100 mM ethanol+100 µg/mL aspirin, and 100 mM ethanol+100 µg/mL aspirin+1 mM betaine. MDA levels of both the 100 mM ethanol (9.93±0.41) and 100 mM

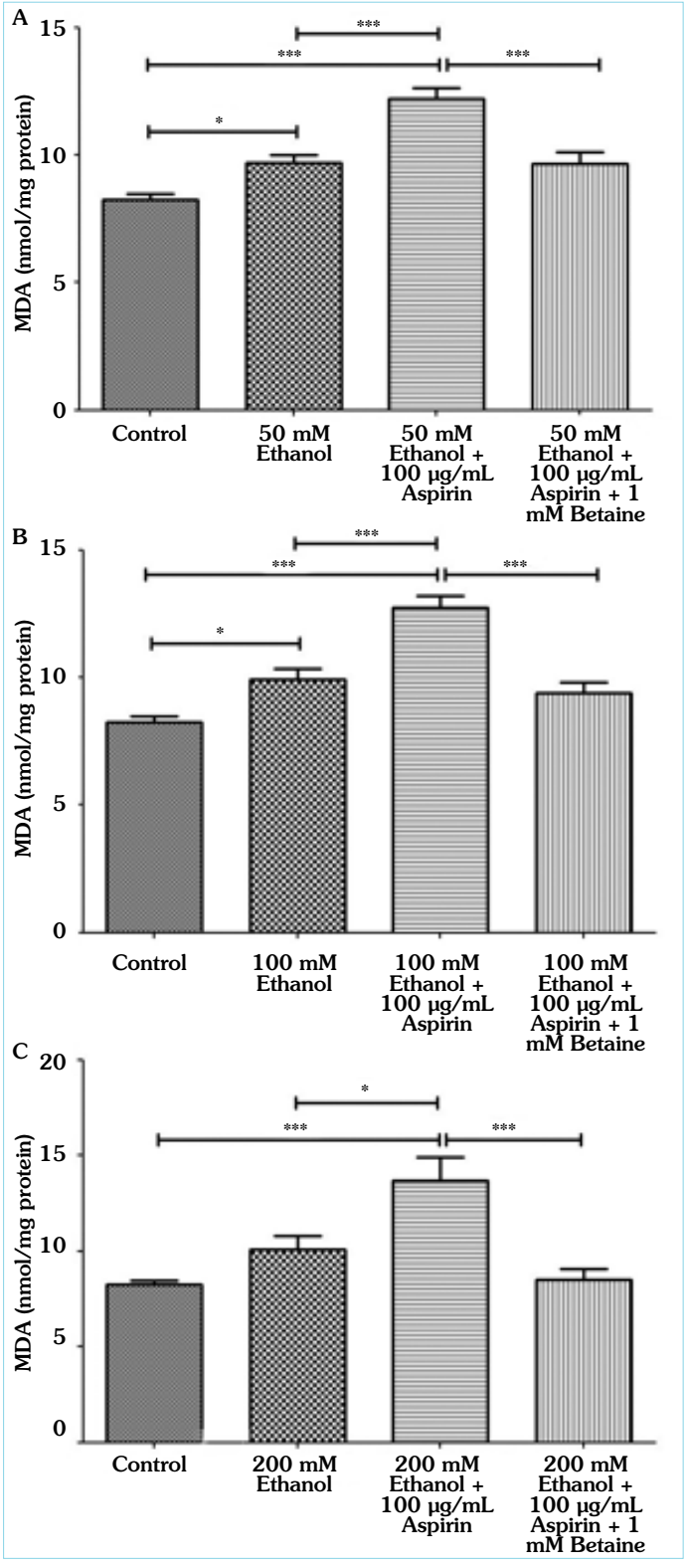

Figure 2. Comparison of MDA (nmoL/mg protein) levels in study groups of different doses of ethanol (50, 100 and 200 mM) together with 100 µg/mL aspirin and 1 mM betaine

ethanol+100 µg/mL aspirin groups (12.75±0.45) significantly increased (p<0.05 and p<0.001, respectively) compared with those of the control group. Furthermore, the 100 mM ethanol+100 µg/mL aspirin group showed significantly increased MDA levels com-

pared with the 100 mM ethanol group ($p<0.001$). However, MDA levels were significantly decreased in the 100 mM ethanol+100 µg/mL aspirin+1 mM betaine group (9.41±0.39) compared with those in the 100 mM ethanol+100 µg/mL aspirin group ($p<0.001$).

Figure 2c shows MDA levels (nmol/mg protein) of synaptosomal fractions treated with 200 mM ethanol alone, 200 mM ethanol+100 µg/mL aspirin, and 200 mM ethanol+100 µg/mL aspirin+1 mM betaine. MDA levels of the 200 mM ethanol group (10.12±0.68) increased compared with those of the control group (8.25±0.22), but the increase was not statistically significant ($p>0.05$), whereas those of the 200 mM ethanol+100 µg/mL aspirin group (13.68±1.25) significantly increased compared with those of both the control and 200 mM ethanol groups ($p<0.001$ and $P<0.05$, respectively). MDA levels of the 200 mM ethanol+100 µg/mL aspirin+1 mM betaine group (8.50±0.56) significantly decreased compared with those of the 200 mM ethanol+100 µg/mL aspirin group $p<0.001$).

DISCUSSION

In this study, we investigated the effect of different doses of ethanol alone or those of ethanol together with aspirin and betaine supplements on the MDA levels of isolated rat brain synaptosomes.

Oxidative stress is described as a disruption in balance between the prooxidant and antioxidant elements of an organism. Human body has an oxidative stress defense system, which is both enzymatic (superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic (glutathione, thiol, tocopherols, etc.) (31). The nervous tissue is more sensitive to oxidative stress than other tissues because of its low antioxidant activity (32). Besides, the nervous tissue has a high concentration of polyunsaturated fatty acids (after adipose tissue), which are easy targets for oxidative damage by free radicals due to unsaturated bonds (33, 34). Lipid peroxidation increased in our study, as indicated by MDA levels having an indirect relation with ethanol doses. Lipid peroxidation has an important role in cellular damage and the prognosis of many diseases. It is a reaction in which polyunsaturated fatty acids of cell membrane are broken down into peroxides, alcohols, and especially biologically active aldehydes by free oxygen radicals. These end products cause cellular damage in organisms (35). In a previous study, excessive ethanol consumption increased the MDA levels of rat brain synaptosomes (9). Montoliu et al. (31) showed that ethanol augments reactive oxygen species and lipid peroxidation in synaptosomes. Also, in another study, chronic ethanol consumption increased the MDA levels of rat brain synaptosomes (5). Furthermore, acute and chronic ethanol administration was found to decrease antioxidant activity in previous studies (36). Results of our study are also consistent with those of the previous studies (34, 36, 37).

The increased lipid peroxidation with aspirin administration observed in our study may be related to benzene ring of aspirin molecule via organic anion transporters or distruption in the acid–base balance. The first possible mechanism is that this highly lipophilic structure of ASA may disrupt the lipid structure of the cell membrane by increasing fluidity, as previously shown (3, 38). The second possible mechanism is that ASA has an acid property, and because of this, the combined effect of aspirin and alcohol may result in lipid peroxidation (4, 5). The final possible mechanism may be the disruption of synaptosomal membrane integrity by ASA because ASA has been shown to decrease the surface hydrophobicity of the mucosal membrane (16). Apart from this, it has been reported that aspirin increases lipid peroxidation by decreasing the levels of antioxidant enzymes (39). Aspirin is transported by organic anion transporters. These results increase the possibility that ethanol increases the transitional effect of aspirin in the brain via organic anion transporters (2). In addition, aspirin alone also could disrupt the mitochondrial permeability transition and membrane surface hydrophobicity (14, 16). We assume that lipid peroxidation is increased by the combined effects of aspirin and ethanol through increased acidity and disruption of the synaptosomal membrane permeability and hydrophobicity via organic anion transporters.

Betaine is an organic osmolyte that protects cells against osmotic stress. It is also a methyl donor in transmembrane reactions. Furthermore, it decreases the alcohol-related increase in SAH levels and increases SAM levels, thereby increasing the SAM/SAH ratio in the cell (17). Ethanol consumption could impair the methionine synthase and consequently increase the levels of homocysteine, which plays an important role in oxidative stress and activation of mitochondrial dysfunction and apoptosis (40). Many studies have revealed associations between the choline–betaine–methionine metabolism and peroxidative damage. Choline is valuable for cell membrane structure and neurodevelopmental differentiation (41). Ethanol consumption decreases methionine and phosphatidylcholine levels as well as the levels of homocysteine. Betaine has an important a role in the formation of phosphatidylcholine (42). In our study, betaine decreased the increased lipid peroxidation levels caused by ethanol and aspirin administration. This is caused by its biochemical and physiological functions as a methyl donor and osmolytic property (43). Previous studies have demonstrated that the protective effect of betaine is related to its membrane-stabilizing action and its ability to prevent lipid peroxidation (3, 40, 44). A possible function of betaine against peroxidative injury may be through reversing the increased membrane fluidity and normalizing the SAM/SAH ratio by elevating SAM levels. Also, peroxidative damage could be minimized by the phospholipid-enhancing effect of betaine.

CONCLUSION

In conclusion, the results presented in this report demonstrate that the administration of ethanol alone and ethanol together with aspirin increases lipid peroxidation in rat brain synaptosomes, and this increase in peroxidation levels is prevented by betaine. This may be an indicator of the neuroprotective properties of betaine, although additional histological and clinical studies are needed to support this data.

Ethics Committee Approval: Ethics committee approval was received for this study from Eskişehir Osmangazi University.

Informed Consent: The informed consent was not required because the study was performed on animals.

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Authors' Contributions: Conceived and designed the experiments or case: İS, GK. Performed the experiments or case: İS. Analyzed the data: İS. Wrote the paper: İS. All authors have read and approved the final manuscript.

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