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Prevention of dabigatran ınduced cytotoxicity by N-acetyl cysteine: An *in vitro* study

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Dabigatran (DBG) is an oral direct thrombin inhibitor used for prevention of systemic embolism and venous thromboembolism. The major side effect of DBG is gastrointestinal upset. In the present study, we have investigated whether N-acetyl cysteine (NAC) showed a protective effect on dabigatran-induced cytotoxicity in the *in vitro* setting. The medium not containing DBG but containing NAC were served to assay the effect of NAC on cell proliferation and apoptosis. Comparing DAB and all other groups, the cell viability was the lowest in the D group. However, there was no statistically significant difference between the NAC I and DBG-NAC I group, while the difference was statistically significant compared to all other groups. The cells in the DBG group showed a degenerative and round-shaped morphology with nuclear condensation. In other dilutions, the cell morphology was healthy with a fibroblastic morphology. Based on our study results, NAC at high concentrations exerts cytoprotective effects against DBG, while moderate or low concentrations have no favorable effect on cell viability of NAC. Although using concomitant NAC at appropriate doses appears to be effective agent against dabigatran cytotoxicity in the current study, further experimental and clinical studies are needed to confirm our findings.

Keywords: Antioxidant agent, Apoptosis, Direct thrombin inhibitor, Gastrointestinal upset

Dabigatran (DBG) is an oral direct thrombin inhibitor used for the prevention of systemic embolism and venous thromboembolism due to atrial fibrillation[1]. In recent years, DBG has become used widely due to its predictable pharmacokinetic properties precluding routine monitorization and the lack of drug-food interaction[2]. Dabigatran etexilate is an oral prodrug which is metabolized by intestinal carboxylesterases to form its active metabolite DBG[3]. The most common side effect of DBG is gastrointestinal (GI) upset which is comparable with warfarin, a vitamin K inhibitor. Direct cytotoxic effect of anticoagulants is one of the underlying GI side effect mechanisms[5]. Such a direct cytotoxic effect damages the mucosal cells, thereby, leading to formation of ulcer in different sites of the GI tract[3]. With increasing use of DBG in recent years, GI side effects have become more apparent which pave the way for ongoing efficacy and safety studies in the long-term.

N-acetyl cysteine (NAC), which was originally used in the management of acetaminophen poisoning, is a mucolytic and antioxidant agent[6]. It is a glutathione precursor of the amino acid L-cysteine and stimulates glutathione synthesis, inhibiting free radicals including reactive oxygen species (ROS)[7]. Due to these properties, it is a useful antioxidant and can be used in the treatment of various diseases. Previous studies have demonstrated that NAC plays a key role in the cell proliferation and apoptosis, redox-related gene expression, inflammatory response production, and angiogenesis[6]. Therefore, NAC can be used to correct the direct cellular damage mechanisms.

In the literature, there are several reports showing GI side effects of DBG. In our previous report, we also demonstrated that DGB was more cytotoxic agent than other anticoagulants[8]. In view of the protective nature of NAC against the cellular damage, hypothetically it can reduce the cytotoxic effects of DBG. Therefore, in this *in vitro* study, we investigated whether NAC shows any curative effect on DBG-induced cytotoxicity.

Materials and Methods

Cell cultures

L929 mouse fibroblast cells were cultured in a 96-well plate (Greiner Bio-One, Germany) and 50,000 cells/mL in each six replicate plates were seeded. The

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cells were incubated in the Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (Biowest Inc., Nuaillé, France) containing 10% fetal bovine serum (FBS) (Biowest Inc., Nuaillé, France) at a humid environment and 95% air and −5% CO_2 and 37°C for 12 h and then, the cells were treated with 3 µM of DBG. The DBG concentration was prepared according to our previous study protocol and minimum cytotoxic dose of DBG was used[8]. The cells were, then, treated five dilutions of the NAC (ASİST^{TM}, 300 mg/3 mL, Hüsnü Arsan İlaç Sanayi, Turkey). NAC concentrations were determined according to literature[9,10]. A wide concentration range of NAC was used in these studies. Considering these studies, we determined 10 mM as optimal among these different concentrations and applied its lower and upper dilutions. Dilutions I to V @ 43.75, 21.875, 10.9375, 5.4675 and 2.7343 mM, respectively were prepared in the cell culture medium. The DMEM/F12 not containing DBG but containing five dilutions of NAC were served to assay the effect of NAC on cell proliferation. In the DBG group, the cells were incubated in 3 µM DBG in the cell culture medium and the cells were incubated in only culture medium in the control group. Concentration of the test materials are shown in Table 1.

Assessment of cell viability

We described assessment of cell morphology and viability our previous studies[8,11,12]. Briefly, the cell viability was analyzed through (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. At 24 and 48 h of incubation, the media were removed and 12.5 µL MTT solution (Sigma-Aldrich, Germany) was added into 100 µL FBS-free DMEM/F12 for the each well. The cell culture plates were wrapped with aluminum foil and incubated for four h. The MTT solution was, then, removed and 100 µL isopropyl alcohol (Amresco Inc., USA) was added to discontinue reaction. The cell viability was measured through an ultraviolet (UV)-visible spectrophotometer (EZ Reac 400 Microplate Reader, Biochrom, UK) at an absorbance of 560 nm wavelength.

Table 1 — Concentration of test materials

Groups	Dilutions
Control	Culture medium without DAB and NAC
DBG	3 µM DAB in culture medium
NAC I	43,75 mM
NAC II	21,875 mM
NAC III	10,9375 mM
NAC IV	54675 mM
NAC V	2,7343 mM
DBG+NAC I	3 µM+43,75 mM
DBG+NAC II	3 µM+21,875 mM
DBG+NAC III	3 µM+10.9375 mM
DBG+NAC IV	3 µM+5,4675 mM
DBG+NAC V	3 µM+2,7343 mM

[DBG: Dabigatran, NAC: N-acetyl cysteine]

Assessment of cell morphology and viabilty

Acridine orange/propidium iodide staining was made for staining dead cells with degenerated nucleus to support cell viability[11]. At 24 hours of incubation, the media on the cells were removed and AO/PI (Sigma-Aldrich, Germany) was added without fixation at a v/v ratio of 1:1 and incubated for 20 s. Subsequently, the cells were washed with phosphate buffer saline (PBS) (Sigma-Aldrich, Germany) for 10 s and covered with a PBS: glycerol (v/v: 1:1) mounting medium. Then the cells were examined under a florescence microscope. Dead cells were evaluated by counting red cells with fragmented nuclei. The AO/PI-stained cells were observed under a narrow band fluorescein (FITC) filter (520-560 nm) in green colour, and PI-stained cells were observed under rhodamine filter (510–560 nm) as stained red. The percentage of apoptotic cells was given by cell counting.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Descriptive statistics were expressed in mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used for the normality test. The analysis of variance (ANOVA) was used to compare the means of more than two groups. A *post-hoc* tests (Tukey and Tamhane's tests) were used to examine significant differences between the groups. A P value less than 0.05 was considered statistically significant.

Results

Assessment of cell viability

As shown in Table 2, the cell proliferation ratio was the highest at 24 h of incubation in the NAC I group, indicating a statistically significant difference compared to the control group (P <0.001). In the DBG group alone, the cell viability ratio was the lowest, indicating a statistically significant difference compared to the control group (P <0.001). Comparing DBG and all other groups, the cell viability was the lowest in the DAB group, indicating a statistically significant difference (P <0.05). However, there was no statistically significant difference between the NAC I and DBG-NAC I group (P >0.05), while the

difference was statistically significant compared to all other groups (P <0.05).

As shown in Table 2, the cell proliferation ratio was the highest at 48 h of incubation in the NAC I group; however, the difference between the NAC I and control groups was not statistically significant (P=1.000). In the DBG group alone, the cell viability ratio was the lowest, indicating a statistically significant difference compared to the control group (P=0.007). The cell viability was statistically significantly lower in the DBG group alone compared to NAC I, NAC II, NAC III, NAC IV, NAC V, and control group (P <0.05). In addition, the cell viability was lower in the DBG group alone compared to DBG+NAC I, DBG+NAC II, DBG+NAC III, DBG+NAC IV, and DBG+NAC V groups, indicating no statistically significant difference (P >0.05). There was no statistically significant difference in the cell viability between the NAC I and NAC II, NAC III, NAC IV, and NAC V groups (P >0.05). However, the cell viability was lower in the dilutions containing DBG, indicating a statistically significant difference between the NAC I and DBG+NAC II, DBG+NAC III, DBG+NAC IV, and DBG+NAC V and control groups (P <0.05).

Table 2 — MTT results24 and 48 h in each dilution compared to control group

	Dilutions		Mean		SD	
	24	48	24	48	24	48
Control	0.865	0.871	0.210	0.153	-	-
DBG	0.552	0.473	0.034	0.038	<0.001	0.007
NAC I	1.242	0.868	0.101	0.141	<0.001	1.000
NAC II	1.100	0.663	0.099	0.056	<0.001	0.328
NAC III	0.863	0.646	0.056	0.044	0.035	0.227
NAC IV	0.813	0.645	0.041	0.020	0.489	0.240
NAC V	0.725	0.651	0.032	0.056	1.000	0.248
DBG+ NAC I	1.165	0.591	0.081	0.080	<0.001	0.057
DBG+ NAC II	0.943	0.542	0.228	0.076	<0.001	0.018
DBG+ NAC III	0.813	0.485	0.082	0.043	0.987	0.008
DBG+ NAC IV	0.745	0.502	0.082	0.039	1.000	0.012
DBG+ NAC V	0.679	0.431	0.030	0.046	0.606	0.003

[DBG: Dabigatran, NAC: N-acetyl cysteine; SD: Standard deviation. Mean: Absorbance (OD). P values compared to control groups]

Assessment of cell morphology and apoptosis

Fig. 1 shows the cell morphology at 24 h of incubation. Accordingly, the cells in the DBG group showed a degenerative and round-shaped morphology with nuclear condensation (Fig. 1B). In other dilutions, the cell morphology was healthy with a fibroblastic morphology, but showing a higher density in the NAC I and DBG+NACI groups, as evidenced by MTT results (Fig. 1 A & F). Other groups showed a similar morphology with the control group with occasional round-shaped morphology (Fig. 1 A,D,E,G & H).

Apoptotic morphological alterations are shown in Fig. 2. Accordingly, the majority of the cells in the DAB group were apoptotic with a round-shaped morphology and fragmented nuclei. In addition, these cells showed no membrane blebbing (Fig. 2B). In the NAC I and DBG+NAC I groups, the apoptotic cell

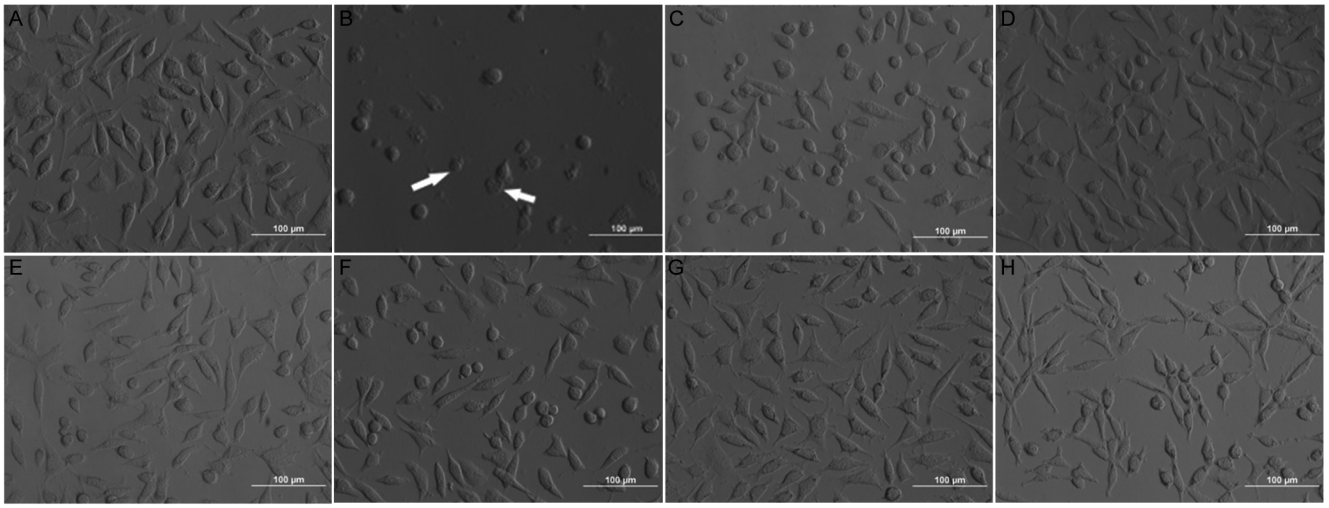

Fig. 1 — Morphological appearance of L929 mouse fibroblasts exposed to test materials at 24 h of incubation. (A) Control; (B) DBG; (C) NAC I; (D) NAC III; (E) NAC V; (F) DBG+NAC I ; (G) DBG+NAC III and (H) DBG+NAC V[Arrows indicate rounded and degenerated cells, 20X]

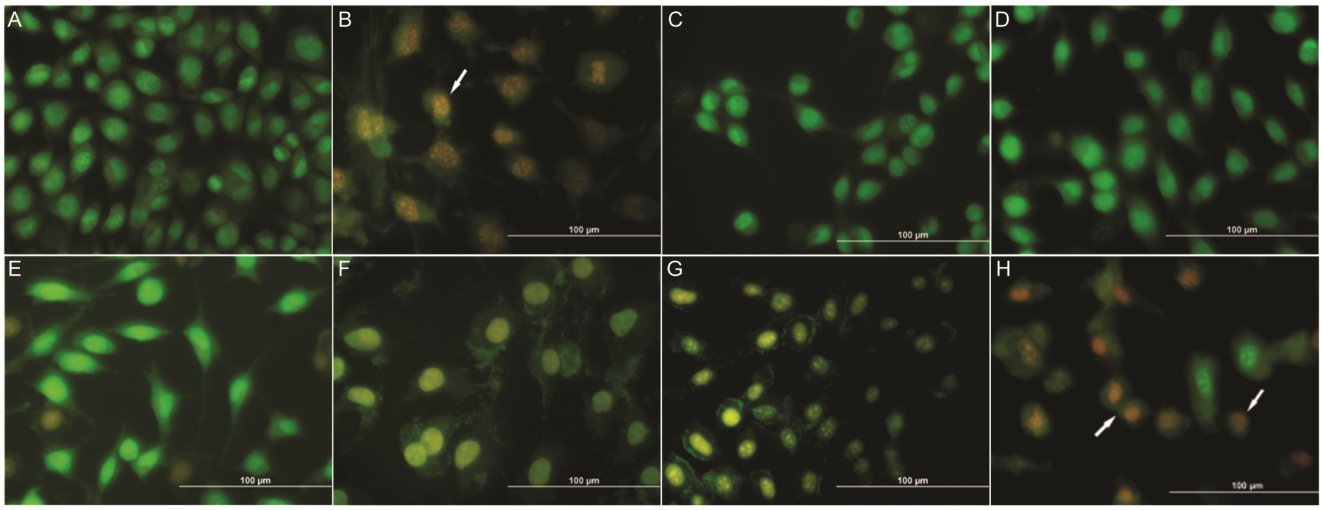

Fig. 2 — AO/PI staining of L929 mouse fibroblasts exposed to test materials at 24 h of incubation. (A) Control; (B) DBG; (C) NAC I; (D) NAC III; (E) NAC V; (F) DBG+NAC I ; (G) DBG+NAC III and (H) DBG+NAC V. [White arrows indicate round dead cells. 40X]

density was very low, showing a similar morphology with the control group (Fig. 2 A & F). With decreased NAC concentration, apoptotic cells as well as healthy cells were observed. The cell morphology of healthy cells was similar to the control group (Fig. 2 D,E,G & H). The ratio of apoptotic cells at 24 h in each dilution and control group was given in Table 3.

Table 3 — The ratio of apoptotic cells at 24 h in each dilution and control group

Dilutions	Apoptotic cells (%)	Normal cells (%)
Control	30	70
DBG	80	30
NAC I	2	98
NAC II	11	89
NAC III	31	69
NAC IV	35	65
NAC V	42	58
DBG+NAC I	6	94
DBG+NAC II	24	76
DBG+NAC III	35	65
DBG+NAC IV	40	60
DBG+NAC V	45	55

[DBG: Dabigatran, NAC: N-acetyl cysteine]

Discussion

The mucosal membrane of the GI system which shows regional variations starting at the mouth and ending at the anus protects the GI functions exerting secretory, absorptive, protective effects, or combination of these effects along the GI tract[9]. The GI system produces these functions through its enriched mucosal and submucosal network and effective blood circulation support[14]. Despite these properties, drug use and infections may lead to GI bleeding, disrupting the mucosal integrity[14].

The most common GI side effects of DBG include gastroesophageal reflux, upper abdominal pain, classic dyspepsia, dysmotility-related symptoms or gastritis of the gastroduodenal mucosa, duodenitis, and peptic ulcer[3]. In their study, Desai *et al*.[5] reported that anticoagulant-induced GI side effects might results from activation of one or more topical biological, direct cytotoxic, systemic, and topical anticoagulant effects[5]. In a database study in the real-time setting, DBG was associated with GI bleeding comparable with other anticoagulants[15]. However, discontinuation rate of DBG was higher due to dyspepsia compared to other anticoagulants[4]. In our previous *in vitro* study investigating the cytotoxicity of anticoagulants, DBG was found to be more cytotoxic than other anticoagulants[8]. Dabigatran is an oral prodrug which is absorbed in the proximal small intestines with a bioavailability of ~6%. Despite this, inactive DBG can be converted to its active form through intraluminal bacterial esterases[9]. The GI system epithelium is exposed to long-term cytotoxic effect of DBG, leading to more apparent cell damage. Therefore, it should be kept in mind that DBG-induced GI side effects can be reduced using agents which prevent cell damage concomitantly.

In the literature, there are preclinical and clinical studies showing the protective effects of NAC against GI injury due to miscellaneous causes. Shirazi *et al*.[16] have shown that NAC has positive curative effects on ulcerative colitis patients. Administration of amlodipine with NAC is reported to have reduced gastric inflammation in indomethacin-induced

gastritis in rats[17]. In addition, Hegab *et al*.[18] investigated the possible contribution of NAC to the gastric mucosal healing in a rat model with indomethacin-induced gastric mucosal damage and showed that NAC decreased the malondialdehyde tumor necrosis factor α, myeloperoxidase, and matrix metaloproteinase-9 levels, suggesting its potential cytoprotective, antioxidant, and anti-apoptotic effects. Also, Soliman *et al*.[19] examined the curative effect of NAC in a rat model with indomethacin-induced peptic ulcer. The interleukin 1β, interferon γ, and cytokine-induced chemoattractant-2α levels decreased, while myeloperoxidase, glucose-6-phospate dehydrogenase, and Bcl-2 levels increased, showing anti-ulcerative, anti-inflammatory, and anti-apoptotic effects. Similarly, Atalay *et al*.[20] examined the anti-inflammatory and anti-ulcerative effects of NAC in a rat model with carrageenan-induced inflammation and indomethacin-induced gastric ulcer and showed that NAC regulated the antioxidant enzyme activity with a cytoprotective effect on the gastric mucosa. This finding suggests that NAC may have cytoprotective effects against DBG which has a carrageenan content in the Pradaxa™ (Boehringer Ingelheim Pharma GmbH & Co. KG, Germany), oral DBG formulation, inducing inflammation. Pradaxa is an acidic drug because it contains carrageenan and tartaric acid in its galenic structure, and therefore its side effects on the gastrointestinal tract are known[21,22]. Carrageenans are sulfated polysaccharides obtained from red marine algae. The position and number of these sulfate groups in the molecule determine the properties and function of the molecule[23,24]. Although carrageenans are used in the food industry and have anti-coagulant, anti-thrombotic, antiviral and anti-inflammatory effects, the shows cytotoxic and anti-cancer effects[25]. Studies on different cell lines in the literature, these effects of carrageenan are described[26]. In a study with MDA-MB-231 and T98G cell lines, direct anti-proliferatif effect of carrageenan was showed[27]. The cytotoxic effects of karregan on breast cancer cell lines were studied in different studies[28,29]. Based on these studies, it is concluded that carrageenan has a direct cytotoxic effect and therefore, the toxicity of Pradaxa increases with the effect of carreganan in its content.

In the present study, the MTT results showed that the cell viability ratio was the highest at 24 and 48 h of incubation in the NAC I group. This finding suggests the beneficial effect of NAC on cell viability at high concentrations. The cell viability was also significantly higher in the dilutions containing NAC at 24 h of incubation compared to DBG alone, DBG+NAC I, DBG+NAC II, DBG+NAC III, DBG+NAC IV, and DBG+NAC V groups. The NAC I group containing high concentration of NAC showed the highest cell viability. This finding suggests that NAC at all doses prevented DBG-induced cytotoxicity, although the effect is more evident at high concentrations. In addition, the cell viability was lower in the DBG group alone compared to DBG+NAC I, DBG+NAC II, DBG+NAC III, DBG+NAC IV, and DBG+NAC V groups, indicating no statistically significant difference. This finding shows that NAC yields inadequate protection even at high concentrations in dilutions containing DBG at 48 h of incubation. This can be explained by the fact that NAC, which is a water-soluble molecule, losses its efficacy with prolonged incubation period. However, this conclusion can be only confirmed by identifying the NAC concentration in the medium. Also, NAC should be continued during DBG treatment to maintain the GI system protection.

The MTT assay is a common cytotoxicity test for the evaluation of cell viability depending on the metabolic activity and mitochondrial enzyme activity[30]. The bioenergetic capacity decreases due to mitochondrial damage, leading to altered oxidative proliferation, oxidative stress, and programmed cell death eventually[30]. Certain enzymes such as mitochondrial dehydrogenase and succinate dehydrogenase play a key role in the MTT assay. Increased cell viability and mitochondrial activity result in formation of formazan crystals from MTT, forming more purple-colored crystals[31]. As a result, the cell viability is identified at an absorbance value of 560 nm wavelength. Previous studies highlighted the regulatory role of NAC in the mitochondrial enzyme activity[30,31]. In addition, NAC was reported to increase the mitochondrial function in certain neurodegenerative disorders[32]. In their study, Xiao et al. examined the antioxidant effect of NAC on H_2O_2-induced intestinal epithelial cells[33]. The IPEC-J2 cells were treated with H_2O_2 and incubated at different concentrations of NAC. Two days after incubation, cell proliferation ratio, antioxidant capacity, mitochondrial respiration, and apoptosis were evaluated. The results showed that H_2O_2 significantly reduced the cell proliferation ratio, antioxidant capacity, and mitochondrial respiration and induced

apoptosis. However, in the NAC groups, the cell proliferation ratio, antioxidant capacity, and mitochondrial respiration increased with decreased apoptosis. Increased mitochondrial activity with NAC indicates an increase in the other key enzyme activities in the MTT assay. Similarly, our study results showing higher MTT absorbance values in NAC groups is consistent with these findings. In our study, the majority of the cells in the DAB group were apoptotic and the apoptotic cell density was very low in the NAC I and DAB+NAC I groups. However, with decreased NAC concentration, apoptotic cells as well as healthy cells were observed. This can be attributed to the fact that antioxidant effect of NAC with increased cell proliferation and mitochondrial activity is useful in the prevention of apoptosis.

Nonetheless, there are some limitations to this study. The *in vitro* design of the study is not sufficient to draw definitive conclusions about the cytoprotective effects of NAC *in vivo* setting. In addition, GI epithelial cells, the first cells exposed to the drug, could be used for this study in order to better evaluate the in vivo setting. Caco-2 and HT-29 cell lines are the most common used cells in the GI system research fields[34]. However, the origin of these cells lines is human neoplastic tissue. Therefore, these cells do not exhibit typical characteristics of intestinal epithelial cell biology. Additionally, they show an extremely high variability of experimental outcomes between different laboratories and this problem is particularly prominent in CaCo-2 cells. Therefore, we used L929 mouse fibroblast cells which are widely used in the cytotoxicity tests with reliable and consistent results.

Conclusion

The above results have shown that N-acetyl cysteine (NAC) at high concentrations exerts cytoprotective effects against Dabigatran (DAB), while moderate or low concentrations have no favourable effect on cell viability of NAC. Therefore, this study suggests that NAC may be beneficial for gastrointestinal side effects related to DBG. Although using concomitant NAC at appropriate doses appears to be effective agent against dabigatran cytotoxicity in the current study, further experimental and clinical studies are needed to confirm our findings.

Conflict of interest

Authors declare no competing interests.

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