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Investigation of radioprotective effects of WR-1065 and acetylsalicylic acid by micronucleus assay

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ABSTRACT

Background: The current study aimed to investigate the ability of acetylsalicylic acid (ASA) to prevent ionizing radiation-induced DNA damage in human lymphocytes via the micronucleus (MN) assay and compare it with the efficacy of the agent WR-1065 (2-[(3-Aminopropyl) amino] ethanethiol dihydrochloride), which exhibits radioprotective activity. ***Materials and Methods:*** Peripheral blood samples were obtained from four participants, and nine experimental groups were established. Blood samples were treated with 25 µg/mL ASA and 40 µg/mL WR-1065 for 30 min before irradiation. After treatment, the samples were irradiated with 6 MV X-rays in the linear accelerator (LINAC) unit at doses of 2 Gy and 4 Gy. All blood samples were then cultured for the micronucleus assay. ***Results:*** In vitro treatments with ASA and WR-1065 at the indicated doses did not result in statistically significant changes in MN frequencies (p>0.05). ***Conclusion:*** Both drugs were did not exhibit radioprotective effects in the experimental model used.

INTRODUCTION

Acetylsalicylic acid (ASA) is a nonsteroidal anti-inflammatory (NSAI) drug that is used as analgesic, antipyretic, and anticoagulant. It has been used as a traditional medicine for more than 3500 years. It was developed as a drug in the late 19th century. During the influenza outbreak of World War I, aspirin was widely used to treat influenza symptoms; however, it was ineffective in reducing mortality. Aspirin has long been recognized as an effective antipyretic and analgesic drug with few side effects when used at standard doses. In the 1970s, ASA was classified as a cardioprotector against myocardial infarction and a primary prophylactic drug [1].

Nowadays, ASA has lost its ground in cardiovascular care. Population-based studies, reviewed by Patrono and Baigent [2] and Brown et al. [3], show that the role of ASA in cardiovascular prevention is weakening. However, ASA is recommended in several preeclampsia prophylaxis guidelines [4] and, meta-analysis results showed that ASA reduces the hepatocellular carcinoma risk [5, 6] and colorectal carcinoma [7-9].

Based on this information, we aimed to investigate whether ASA inhibits DNA damage induced by ionizing radiation using the MN assay in human lymphocyte culture and to compare its radioprotective effect to that of WR-1065. Literature review did not reveal any similar study investigating the radioprotective effectiveness of ASA by MN analysis in human lymphocytes, as in our study, and the other difference was the doses at which the drugs were tested. Both drugs were administered at a therapeutic dose range (Cmax, 29.62 mg/L for ASA; Cmax, 47.5 µM -96 µM for WR-1065) [20- 22].

If ASA is proven to have radioprotective activity, it may be viewed as a low-cost drug that can be taken orally in medically suitable patients as an alternative to amifostine, which is costly and requires iv administration. Its prophylactic use in radiation workers may also be considered.

MATERIALS AND METHODS

Main chemicals and reagents

The drugs, chemicals and reagents used were as follows: WR-1065 (2-[(3-Aminopropyl) amino] ethanethiol dihydrochloride, Sigma W2020), ASA (Sigma A5376-USA), RPMI-1640 (With L-glutamine,

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Sigma R8758-USA), FBS (Fetal Bovine Serum, Capricorn FBS-HI-11b- Germany), Penicillin-Streptomycin (Sigma, P4333-USA), PHA-L (Phytohaemagglutinin-L, Sigma L2769-USA), Cytochalasin B (Cyt-B, Sigma C6762-USA).

Drug preparation

Each experiment used freshly prepared ASA, which was dissolved in a complete medium at a stock concentration of 2.5 mg/mL. The final concentration of ASA was determined based on pharmacokinetic parameters. To simulate a single oral dose of 500 mg, a dose of 25 µg/mL was used, which corresponds to the approximate peak concentration of aspirin in the plasma. Furthermore, the incubation time of 30 minutes was chosen to represent the time required for aspirin to be metabolized [20].

WR-1065 was dissolved in phosphate-buffered saline (PBS) (Stock concentration: 50 mM). The stock solution was aliquoted and stored at -80°C for 1 month. The final concentration of WR-1065 was 40 µM. This concentration was selected because it is non -toxic and in the peak concentration range of WR-1065 in the plasma [21, 22].

Participants and blood sampling

Blood samples were obtained from four male participants aged 20-30 years who were non-smokers, without chronic and systemic diseases, had not used drugs in the previous 15 days, and did not work in radiation-related jobs. The participants signed informed consent forms.

Blood samples were acquired from cubital veins into tubes containing lithium heparin (Li-heparin). Then, samples were transferred to treatment tubes. For each volunteer, the following 9 groups were set up: Control (1), WR-1065 (2), ASA (3), 2 Gy (4), 4 Gy (5), 2 Gy + WR-1065 (6), 2 Gy + ASA (7), 4 Gy + WR-1065 (8), 4 Gy + ASA (9).

Drug treatment

Blood samples were treated with ASA and WR-1065 at final concentrations of 25 µg/mL and 40 µg/mL respectively, 30 min before irradiation. Following the treatment, the cells were washed twice with RPMI-1640 media before beginning the irradiation procedure.

Irradiation

Blood samples were irradiated with 6 MV X-rays in the LINAC device (Elekta Synergy Platform, Stockholm, Sweden) at the 2 Gy and 4 Gy doses and at the dose rate of 400 MU/min. The 2 Gy dose in our study corresponds to the daily fractional dose used in curative locoregional radiotherapy (conventional fractionation). However, 4 Gy is the dose that is expected to kill 50 % of an exposed population within 30 days (LD 50/30) when the whole body is exposed to radiation for a short length of time [23].

Cell cultures

Following the irradiation and/or treatment, all blood samples were cultured in the whole medium, including 10% Fetal Bovine Serum and 1% Pen-Strep in RPMI 1640 and PHA-L (5 µg/ml). The blood medium ratio was adjusted to 1:9.

The flasks were placed in an incubator at 5 % CO_2 and 37°C. At the 44th hour of cultures, Cyt-B was added to the flasks at a final concentration of 6 µg/ml. Cell cultures were stopped after 68h of incubation. The experimental protocol was set up using Fenech's standard MN protocol [24].

Microscopy and scoring

After the harvest, the slides were stained with Giemsa. The slides were then examined using a light microscope at 400x magnification. Approximately 1,000 binucleated (BN) cells were scored for each slide following Fenech's recommendations [24].

Equation 1 calculates the nuclear division index (NDI) using Eastmond and Tucker's formula [25] M1-M4 indicates the number of cells containing 1-4 nuclei and N represents the total number of viable cells scored. The results are shown in table 1.

$$NDI = \left(\frac{M1 + (2xM2) + (3xM3) + (4xM4)}{N}\right) \quad (1)$$

Table 1. Mean frequencies of MN scored in blood samples obtained from participants.

Participants	Cont.	ASA	WR-1065	2 Gy	2 Gy+ ASA	2 Gy+ WR-1065	4 Gy	4 Gy+ ASA	4 Gy+ WR-1065
I	3	2	2	93	-	-	331	181	250
II	4	3	4	116	135	113	471	332	388
III	2	6	1	102	109	100	341	365	350
IV	1	1	1	73	62	83	232	170	238
Mean of MN± SD	2.5± 1.29	3± 2.16	2.5± 1.29	96± 18.02[a]	102± 37	98.67± 15.04	343.75± 98.07 [b,c]	262± 100. 89	306.5± 73.98
Mean of NDI± SD	1.72± 0.07	1.70± 0.04	1.73± 0.05	1.40± 0.11	1.33± 0.17	1.35± 0.15	1.22± 0.12	1.26± 0.11	1.19± 0.06

ASA (Acetylsalicylic acid), MN (Micronucleus), BN (Binucleated), NDI (Nuclear Division Index). * MN/BN at 1,000 cells. a There is a statistically significant difference between the control and 2 Gy groups (p = 0.002). b There is a statistically significant difference between the control and 4 Gy groups (p = 0.006). c There is a statistically significant difference between the 2 Gy and 4 Gy groups (p = 0.009).

In addition to group statistics, equation 2 was used to calculate how ASA and WR-1065 applications influenced the frequency of MN at 2 and 4 Gy. The results are shown in table 2.

$$MN\ exchange = \frac{MN\ Frequency(R) - MN\ Frequency(R+T)}{MN\ Frequency\ (R)} * 100 \quad (2)$$

R: Radiation Dose, T: Drug Treatment

Table 2. Individual reduction/induction levels of MNs at irradiation doses with/without ASA and WR-1065 treatments.

Participants	2 Gy+ASA (%)	2 Gy+WR-1065 (%)	4 Gy+ASA (%)	4 Gy+WR-1065 (%)
I	-	↓ 7.5	↓ 45.3	↓ 24.5
II	↑ 16.1	↓ 2.9	↓ 29.5	↓ 17.6
III	↑ 6.9	↓ 2.0	↑ 7.1	↑ 2.7
IV	↓ 15.1	↑ 14.0	↓ 26.7	↑ 2.4

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Statistical analyses

Statistical analyses of this study were performed using General Public License (GPL) statistics software R 4.1.0. Friedman Test, Analysis of Variance (ANOVA), Spearman Correlation analysis, and Kruskal-Wallis test were employed. Data are presented as mean ± standard deviation (SD). $p<0.05$ was considered statistically significant.

RESULTS

The Friedman test was used to compare the MN frequencies of the control, ASA, and WR-1065 groups to assess how ASA and WR-1065 affected the natural MN frequency. There was no statistically significant difference between the groups (n = 4; p = 0.497; table 1 and figure 1).

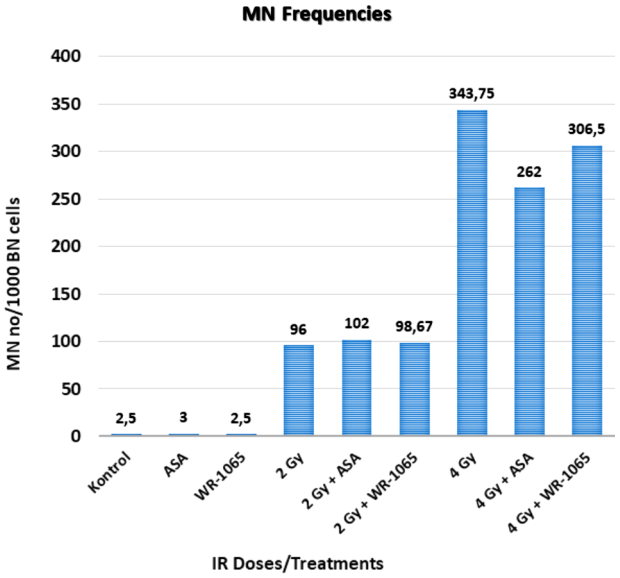

Figure 1. MN frequencies of all experimental groups. While ASA and WR-1065 had no statistically significant effect on natural MN frequencies (p = 0.497), 2 Gy and 4 Gy radiation doses increased natural MN frequencies (p < 0.001). ASA and WR-1065 treatment also did not cause statistically significant changes in MN numbers in 2 and 4 Gy irradiation groups (p = 0.864).

The dependent groups (Control, 2 Gy, and 4 Gy) were compared using ANOVA for repeated measures. The relationship between doses and MN frequencies was investigated using Spearman correlation analysis. Descriptive data are presented as mean ± SD. There was a significant correlation coefficient ($r=0.948$; $p<0.001$) between dose levels (Control, 2 Gy, and 4 Gy) and MN frequencies (n=4; table 1 and figure 1).

Three participants (n=4 for all other treatments) were evaluated for the ASA and WR-1065 treatments and 2 Gy. Because the 2 Gy+ASA and 2 Gy+WR-1065 slides of one participant could not be examined, statistics were based on the results of three participants. The results are shown in table 1. In 1,000 BN cells, the frequency of MN with a 2 Gy dose was 97±21.93 (n=3, sample 1 is excluded). MN frequencies in ASA and WR-1065 treatments with 2 Gy were 102±37 and 98.67±15.04, respectively. There was no statistically significant difference between the groups (p=0.864).

The MN frequencies for the 4 Gy, 4 Gy + ASA, and 4 Gy+WR-1065 groups were 343.75±98.07, 262±100.89, and 306±74.02, respectively, per 1,000 BN cells (table 1). There was no statistically significant difference between the groups (p=0.089). Table 2 shows changes in MN levels individually.

NDI results showed no significant difference between groups excluding the WR-1065 group and 4 Gy+WR-1065 group (p=0.047). The results of NDI can be considered, irradiation and other treatments did not affect the cell division patterns, but it is clearly seen that irradiation reduced the cell proliferation rate or induced the cell death rate.

DISCUSSION

In this study, we investigated the radioprotective effectiveness of ASA and compared it with the efficacy of the reference molecule WR-1065 agent. To achieve this, we investigated the effects of ASA and WR-1065 treatments on the frequency of MN in blood samples collected from four participants exposed to 2 Gy and 4 Gy radiation.

At first, the effects of ASA and WR-1065 on natural MN frequencies were evaluated, and there was no statistically significant difference (p=0.497; table 1). Similarly, Dandah *et al.* [26] and Guma *et al.* [27] found that ASA did not affect the frequency of natural MN.

Then we examined the effects of 2 Gy and 4 Gy radiation treatments on natural MN frequencies and found that both doses increased the natural MN frequencies as predicted (p=0.006; table 1).

The interesting fact is that the frequency of MN for 2 Gy was lower than in some previous studies. The current study demonstrated that the frequency of MN was 96±18.02 (n=4) at 2 Gy. Other studies have reported MN frequencies of 153±45 [28], 192.4±31.0 [29], 212.08±6.9 [30], 270±19.8 [31], 274±53.03 [32], 285 [33], 397 [43]. Kopjar [35] found the most similar results (MN frequency of 89).

MN frequencies at 4 Gy were also lower than in other studies. In our study, the mean frequency of MN after a 4 Gy dose was 343.75±98.07 (Mean±SD) (table 1). Previously reported results are as follows: 449 [28], 417 [29], 760 [31], 790 [32], 899 [34]. These changes may be attributed to multiple factors, such as biological diversity among sample groups, radiation type, and irradiation conditions. Our findings for each radiation dose revealed the need to develop a dose-response curve for the LINAC device we used during irradiation.

With regard to the primary aim of our study, we found some studies that investigated the effect of ASA

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on radiation-induced damage using various approaches and in different organisms and cell types [10-13, 35,36]. However, no study has investigated the radioprotective effect of ASA in human lymphocytes using MN analysis at the plasma concentrations of the drugs.

Our findings revealed that concomitant ASA treatment did not result in a statistically significant change in MN numbers at 2 or 4 Gy in the experimental series. Although there is no statistically significant difference, when individual data are examined one by one, it is seen that ASA causes a remarkable decrease in the number of MNs in most of the samples at 4 Gy, although not at 2 Gy. While WR-1065 also caused a low decrease at 2 Gy, the rate of decrease in the number of MNs observed in two samples at 4 Gy was thought to be a more likely result (table 2).

Unlike our findings, several studies conducted on animals, proved the radioprotective effect of ASA with different methodologies such as chromosomal aberrations in bone marrow cells of rats [10], sperm morphology [11], antioxidant enzymes, and lung tissue in rats [12], before and after treatment of ASA on mice [13], DNA damage in macrophages [36].

Recently, Jiang *et al*. [37] conducted a comprehensive in vivo animal study that included genome repair mechanisms, such as Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and cytosolic DNA sensors (CDS). They presented several findings from various experiments demonstrating how ASA reduces radiation-induced DNA damage.

When all the results are evaluated together, it is clear that ASA has a radioprotective effect. However, it is necessary to investigate whether this effect is also valid for the human organism and at what doses it occurs. In our study, we selected an accessible, non-toxic dose for the human organism and tested it with MN, one of the most valid methods in dosimetry. However, we could not provide sufficient evidence due to our small number and the biological diversity of samples. In addition, individual differences within the group led to the conclusion that we could reach a more realistic result by increasing the number of samples.

WR-1065 is a drug considered the gold standard approved for use in humans. In our study, we also assessed the protection efficacy of WR-1065 against radiation-induced DNA damage. When we reviewed previous studies that used similar approaches, it was evident that WR-1065 has this protective effect. In our study, it was not possible to demonstrate this effect with the chosen methodology and dose.

Littlefield *et al*. [38] investigated the effects of WR-1065 on human lymphocytes using the MN and chromosome aberration method. They treated lymphocytes from participants with different doses of WR-1065 (from 1 mM to 12 mM) for 30 min before exposing them to 3.1 Gy X-ray irradiation. The study found that concentrations of 1 and 2 mM resulted in a 50% reduction in MN formation, whereas concentrations of 4, 8, and 12 mM resulted in an 80% decrease. It should be noted that the high doses of WR -1065 used in this study differ significantly from amifostine concentrations found in pharmacokinetic studies [21, 22]. Inconsistencies between our results and those of Littlefield *et al*. may be due to the significantly lower dosage we used (one in 50 of the lowest doses in the study by Littlefield *et al*.) [38].

Lee *et al*. [39] evaluated the radioprotective effects of WR-1065 and North American Ginseng extract (NAGE) on 12 participants. They administered NAGE and WR-1065 at the onset of the cell culture and 90 min after irradiation. Notably, WR-1065 caused a significant decrease in MN levels, with reductions of 57.5% and 61.2% reported at concentrations of 1 mM and 3 mM, respectively. Similarly, 2-Gy WR-1065 treatment resulted in reductions of 36.2% and 54.4% at the same concentrations, even when administered 90 min after irradiation. The same researchers [30], expanded their study to include 40 participants. In the subsequent study, they observed MN levels that were roughly but consistently lower, with the following results: 42.6% at 1 Gy with 1 mmol/L, 52% at 1 Gy with 3 mmol/L, 38.3% at 2 Gy with 1 mmol/L, 33.4% at 2 Gy with 3 mmol/L.

The WR-1065 doses used in the compared studies ranged from 50- to 600-times higher than that used in our study. As a result, it was determined that the dose we used could not show a statistically significant protective effect (Please, recall the low values observed in two samples at 4 Gy+WR-1065) because it was significantly lower than the dose used in the studies of Littlefield [38] and Lee [39]. When we consider the high concentrations (1-12 mM for WR-1065) used in previous studies, achieving such levels in humans without inducing toxicity, particularly given the amifostine metabolic rate, presents a significant challenge. Consequently, we found that the WR-1065 dose used in our study is more realistic and reflective of probable biological effects than earlier research findings.

Finally, WR-1065 results were comparable with ASA results. Even though it is not statistically significant, when we evaluate them individually, the decrease values observed in the number of MNs in ASA at 4 Gy, although not at 2 Gy, seem more effective than WR-1065.

Because the primary goal of the study was to reveal the radiation response of healthy cells, studies involving cancer cells were excluded from the evaluation because the DNA damage response may have differed.

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CONCLUSION

This study is the first to investigate the potential radioprotective effects of ASA on human lymphocytes in in vitro settings using the MN assay. Based on the results, in vitro treatments with ASA and WR-1065 did not produce statistically significant changes in MN frequencies. Therefore, both drugs were shown to have no radioprotective effects in the specified experimental model.

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***Conflicts of interest*:** None to declare. (No potential competing interest was reported by the authors).

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***Ethical consideration*:** This study was performed in accordance with ethical standards and was approved by the Trakya University Faculty of Medicine Scientific Research Ethics Committee. (Protocol Code: TÜTF–BAE 2019/80).

***Author contribution*:** All authors contributed to the study equally and approved final version of the manuscript for publication.

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