Correlation between Erythrocytes Echinocytic Transformation and Micronucleation in Rodents Exposed to Selected Toxicants: A Preliminary

Ahmad M. Khalil, Hasan M. Abo Siam, Heba R. Al Naimi, Heba F. Alnimer, and Amneh S. Alrabie

ABSTRACT

The genetic constitution and conformational state of living cells are characteristic of building, homeostasis, decay, and apoptosis. Genetic and morphological alterations of red blood cells (RBCs) may influence their survival and function. The purpose of this study was to determine the relation, if any, between the incidence of micronuclei (MN) formation in erythrocytes and their transformations from dissociates to echinocytes or echinocytosis. Two rodents models were exposed in vivo, at various doses and periods, to three environmental agents, physical (Radio Frequency Radiation, RFR), a phytochemical (plant extracts), and geological (Bentonite Nano-clay Particles, BNPs). Microscopic analysis of blood smears stained with Hematoxylin-Giemsaa, compared to erythrocytes from unexposed animals, confirmed that the three environmental agents, after long-time exposure, significantly induced MN and increased percentage micronucleated erythrocytes (MNE) as well as the rate of echinocytic transformation. Correlation analysis showed that there were good correlations between erythrocyte micronucleation and echinocytosis. The reason (s) behind this phenomenon is not fully clear at present. We believe that echinocytogenic factors may result in abnormal erythrocytes like echinocytes. The presented data could be an indication that cellular morphological changes may be related to alteration in the genome leading to a common pathway in apoptosis.

Keywords: Ajuga, Bentonite, Prasium, Radio frequency radiation.

I. INTRODUCTION

Various types of pollutants, physical, chemical, and biological exist in the environment. Radiofrequency radiation (RFR)-related technologies have been and will continue to be widely spread. Electromagnetic radiation (EMR) may be absorbed by different tissues according to the places the mobile is carried especially the brain, liver, kidney, and tests. Recently, Khalil (2021) reviewed the impact of RFR emitted from mobile phones and mobile phone towers on living organisms including modification of their genomes, behaviors, immune responses, growth, and reproduction. These effects are still debated, and further assessments are required.

Bentonite Nano-clay particles (Al₃H₂Na₂O₇Si₃) are platelet-like Aluminosilicates (or mixed Si-Al-oxides). Clay nanoparticles are among the most industrially popular Nano-sized materials that are widely used in the manufacturing of paint, ink, oil drilling, drug delivery, cosmetics, and pesticides (Hosseini et al., 2018). However, a general concern remains that these new nanomaterials ought to be evaluated for their health safety. Although the exposure of the general population to bentonite is low, occupational exposure to bentonite mining dust may be higher (Wiemann et al., 2020). Bentonite clay is a challenging group in risk assessment and the release of Al³⁺ ions raised serious health concerns. The potential biological hazards of Nano-clays have not been addressed and their effects on biological systems are poorly understood. More in vivo and in vitro studies are still needed to evaluate the genotoxic potential of Aluminosilicates found in this clay. To the best of our knowledge, only a little work has been carried out yet. Such research is essential before the advent of biological and pharmaceutical applications.

Two plant species, Ajuga orientalis L. and Prasium majus L., belong to the family Lamiaceae and are economic semishrub or perennial herbs with natural wide distribution in many countries. According to the available literature, P. magus (Chauouche et al., 2013), and A. orientalis (Luan et al., 2019) have long been used as traditional medicine all over the world. However, studies on their systematic toxicity and safety evaluations have been rare. Consequently, the two plants were chosen for the present investigation.

Using mammalian RBCs, cytotoxicity was evaluated by echinoctic transformation, while genotoxicity was assessed
by using cells in vivo MN assay. Erythrocytes are particularly well suited to investigate cytogenetic toxicity because blood circulates all over the body and reflects exposure to toxicants.

Echinocytosis (erythrocytes crenation) refers to morphological changes of erythrocytes, where the cell membranes display many small regular sharp spikes/projections that are evenly spaced on the membrane. Transformations from discocytes to echinocytes (more commonly known as burr cells) weaken blood flow through cellular interaction and increased the whole blood viscosity and erythrocytes aggregation. Even though their advantage of easy passage through narrow splenic sinusoids, they may contribute, among the factors, to anemia (Yunnam et al., 2017).

Echinocytosis may occur in normal blood samples as an artifact if the blood is overexposed to ambient air or pressurized. However, this phenomenon becomes more significant when echinocytes are observed at high incidence in many viewing fields and repeated specimens in various pathologic conditions (Agroyannis et al., 2001; Ahmed and Patel, 2015; Gallagagher, 2018). Among these conditions is abnormal lipid content of the plasma due to an accumulation of fatty acids and/or lyssolecithin, low RBC adenine triphosphate (ATP), pyruvate kinase deficiency, Vitamin E deficiency, heparinization, uremia related to kidney disease, and increased concentration of intracellular calcium. They also have been reported in acute blood loss, stomach cancer, as well as following exposure to environmental toxins, and induction of oxidative stress (Gallaagher, 2018).

The MN assay in immature erythrocytes (or polychromat erythrocytes, PCE) in peripheral blood has become one of the most popular methods to assess the genotoxicity of different chemical pollutants and physical factors resulting from environmental and occupational exposure (Sommer et al., 2020). A Micronucleus is a small, chromatin-containing round-shaped body observed in the cytoplasm of cells that results from DNA damage or genomic instability such as chromosomal breakage and mitotic spindle damage (Terradas et al., 2016).

II. MATERIALS AND METHODS

A. Chemicals

All reagents, unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Giemsa stain, modified solution (CAS number 51811-82-6) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA).

B. Animals

The procedures and experimental design employed in the present study complied with the recommendations of the Collaborative Study Group for the MN Test (OECD, 2016). The protocols were approved by the Institutional Animal Care and Use Committee at Yarmouk University (IACUC/2021/4).

Males of Balb/c mice (6 weeks old, an average weight of 25 g) and Sprague-Dawley rats (55-60 days old, weighing 200-280 g) were obtained from the Animal House of the Department of Biological Sciences at Yarmouk University. All the animals had no signs of illness or visible deformities. Five animals were housed in separate plastic cages and maintained under standard conditions (Temperature: 23.0 ± 3.0 °C, relative humidity: 50% ± 10%, 12 h light/dark cycle, and air change: 10 times/h). Animals had free access to food and water. They were acclimated to the laboratory conditions for 1 week before starting the study.

C. Electromagnetic Field

Twenty-four mice were randomly distributed into six groups (4 animals per group). Every mouse in group 1 was individually placed in a 50 ml polypropylene tube in the complete absence of any unwanted source of EMR (Fig. 1). Animals in the second group were used sham-control (mouse in a tube, on the plastic tray, while mobile phone jammer switched off; no EMR to for possible stress factors). Animals in groups 3-6 were exposed to RFR generated by EMF transmitter at 1800 MHz and Specific Absorption Rate (SAR) value of 0.27 W/kg for 0.5, 1.0, 2.0 ad 4.0 h daily for 7 consecutive days. The tubes had circular drilled ventilation holes 3 mm in diameter at all sides to reduce the stress, allow air passage, and prevent overheating. Exposure sessions were accomplished at a fixed time every day.

![Fig. 1. Mobile phone jammer and the circular plastic tray show the experimental set up and the principle of exposure to mobile phone electromagnetic radiation at 1800 MHz, and SAR value of 0.27 W/kg. Each mouse was placed with the head oriented towards the center inside a separate perforated 50 ml polypropylene tube.](image1)

D. Plant Extracts

A. orientalis and P. majus (Fig. 2) were collected during the flowering season in March /2021 from Jerash and Ajloun-North of Jordan, respectively. They were identified by Jamil N El-Lahham (Professor of Flowering Plants Taxonomy, Department of Biological Sciences – Yarmouk University). Leaves of the two plants were washed under running water, then they were dried at room temperature and ground to powder to prepare extracts. A water extract was obtained by mixing a plant powder (1 g) with distilled water (10 ml) with continuous stirring for 15 min at 80 °C. After that, the mixture was soaked for 24 h at 25 °C, then centrifuged and filtered. The filtrate was stored at 4 °C until use.

![Fig. 2. (a) Ajuga orientalis and (b) Prasium majus.](image2)

E. Animal Dosing

Mice were divided to 6 groups (5 mice/group) for each of the extracts of both plants. Matching negative control (distilled water-treated animals) experiments were run
simultaneously. The other four groups were given one of the two extracts at three different doses: 370, 185, and 92.5 mg/kg b. w. The positive control was treated with Methyl Methane Sulfonate (MMS) 80 mg/kg and Mitomycin C (MMC) 14 mg/kg. All test groups were intraperitoneally (i. p) given 200 μl of an extract for 28 consecutive days.

F. Bentonite Nano-Clay Experiments

Twenty-five rats were divided into five groups (n=5/group). Bentonite Nanoclay powder (Chemical formula Al₂H₂O₃Si, 180.1 g/mol, sized less than 25 μm, Fig. 3) was diluted with normal saline (from local pharmaceutical companies in Jordan) to produce a suspension. This suspension was stirred and sonicated for 10 min and placed in 37 °C water bath. The suspension of the BNPs was freshly prepared and sonicated every day before injection. The suspension was i. p administered at the same time (10:00 am) daily for 28 consecutive days, with 0.25 ml suspension/animal containing 40, 80, 160, 320 mg Nanoclay/kg b. w. Equivalent volumes of normal saline were injected into the negatively controlled rats.

G. Blood Smears

After termination of the experiment, peripheral blood was collected in heparinized capillaries from the retro-orbital vein through a small puncture in the eye socket of the animal, smeared and stained as described previously (Khalil et al., 2017). Briefly, about 5μl of blood were immediately mixed with an equivalent volume of 3% Ethylene Diamine Tetraacetic Acid (EDTA) solution (1.5 mg per ml of blood) and smeared onto a clean prewashed glass slide. The blood films were air-dried, fixed in absolute methanol for 10 min, and then double-stained with Mayer’s hematoxylin (17 min) and 0.1 % Giemsa (15 min). Slides were rinsed thoroughly in tap water, then differentiated for 10 min in Sorensen’s buffer (pH 6.8) and allowed to air-dry. Observations were made by using a BioBlue microscope with an oil immersion lens. Under blind code, a total of 6000 cells in three slides (2000 cells/slide/animal) were examined to determine and percent MNE and percent echinocytes.

H. Statistical Analysis

The means, standard errors of the means (SEM), and percentages were calculated. Data were analyzed using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California USA). A Multiple t-test was used to compare MN and Burr cells for all data. Two-way ANOVA analysis was used for comparison between mean values (± SEM) of % MNE and % echinocytes, at different doses. Bonferroni’s multiple comparisons test was used to evaluate differences between the experimental and their corresponding controls. A difference of P<0.05 was considered significant.

III. RESULTS

Fig. 4 shows the presence of MN and the morphological changes of erythrocytes (echinocytes) following different exposure sessions.

Fig. 4. Photomicrographs of peripheral blood erythrocytes of animals treated with different agents. Thick arrows: Echinocyte; Tin arrow Micronuclei; Arrowhead Micronucleate echinocyte; Star: Discocyte with 2 micronuclei. Giemsa/ hematoxylin differential stain. Magnification: 1000X. Scale bar: 20 μm.

Fig. 5. Data are shown with the mean (± SEM) from a total of 6000 cells per treatment. All data are represented by averaging four treatments. DW: Distilled Water; EMR: Electromagnetic Radiation; MMC: Mitomycin C; MMS: Methyl Methane Sulfonate; MN: Micronucleate Polychromatic Erythrocytes; NS: Normal Saline. * Indicates significantly different from the negative control (student’s t-test; P < 0.05).

The results obtained from this study are shown in Fig. 5. Increases in the frequency of MN and the frequency of MNPCEs in animals receiving i. p injection with MMC or MMS were significantly (P<0.05) higher than that of the negative controls. Similar statistically significant (P<0.05) elevations in the percentage of induced echinocytes in the three experimental groups, relative to the matching negative controls, were recorded. The extent of these changes was extensive and time/dose dependent. Concerning RFR, significant reading was recorded only following 4h exposure period. The mean number of micronucleate echinocytes was followed in the case of bentonite-treated rats. The values were 10.55 ± 0.490, 9.70 ± 0.334, 5.80 ± 0.146, and 3.65 ± 0.128
for the doses 320 mg/kg, 160 mg/kg, 80 mg/kg and 40 mg/kg, respectively, versus normal saline (1.05 ± 0.146). Significance (p < 0.05) was calculated at the highest two concentrations.

The regression calibration curves for the correlation between percentages of echinocyte and MNE erythrocytes following different treatments are depicted in Fig. 6. Correlations between MN and echinocytosis induction showed a significantly positive relationship at high levels of exposure/treatment ($R^2 = 1.000$, 1.000, 0.999, and 0.563 for Ajuga extract, Prasium extract, bentonite, and for RFR, respectively). No significant relationships at shorter times or lower doses were observed.

Fig. 6. The correlation between erythrocytes echinocytosis and micronucleation under various experimental exposure conditions. MN-PCE: Micronucleated Polychromatic Erythrocytes.

IV. DISCUSSION

Many environmental pollutants have been associated with the mechanism of oxidative stress or reactive oxygen species (ROS) leading to high cytotoxicity and genotoxicity toxicity. Several studies have reported an increase in DNA damage/MN in various study systems exposed to RFR. This includes human peripheral blood lymphocytes (Siama et al., 2017), as well as rat bone marrow (Busljeta et al., 2004), and red blood cells (Kesari et al., 2011). Long-term constant or day-to-day repetitive total body exposure to RFR emitted from mobile phones resulted in disturbance of the cellular oxidant-antioxidant status at non-thermal power levels that led to an accumulation of oxidative DNA damage (Khalil et al., 2012; Stein et al., 2015; Yang et al., 2020; Alkis et al., 2021).

Electromagnetic and electrical radiation can generate a transient flow variations across cell membranes of various ionic species. This can displace electrolytes and ions in the body which disturb the body’s neurological and homeostasis maintaining systems (De Ninno2 and Pregolnato, 2017). An increase in intracellular Ca$^{2+}$ flux, almost immediately following EMF exposure. This produces a Ca$^{2+}$/calmodulin-dependent increase in nitric oxide. Nitric Oxide (NO) reacts with superoxide to form Peroxynitrite, a potent non-radical oxidant (Pacher et al., 2007), which can produce radical products, including hydroxyl radical and NO2 radical (Lymar et al., 2003). This proposal is strengthened by the findings that RFR emitted from mobile phones induces oxidative stress in human semen (De Iuliis et al., 2009). It is also supported by the fact that RFR-exposed individuals (Siama et al., 2017), and neuroblastoma cells (SH-SYSY) (Marjanovic Cermak et al., 2018) showed increased lipid peroxidation and alleviated glutathione contents, as well as catalase (CAT) and superoxide dismutase (SOD) activities. A similar effect has been observed in the rats exposed to RFR (Yang et al., 2020; Alkis et al., 2021).

As for BNPs, our results regarding the genotoxic effects agree with the previous studies (Fanizza et al., 2007; Bowman et al., 2011; Yuwen et al., 2013; Isoda et al., 2017; Paz et al., 2017; Wiemann et al., 2020). They are also in line with the epidemiological investigations (Demircigil, et al., 2010) that showed the potential of silica or quartz particles to induce significant genotoxic effects including DNA damage and MN formation. The samples employed in our study are known to exhibit positive surface potential and these properties may also contribute to the increased toxicity. We believe that bentonite positively charged Nanoparticles might attach to the cell surface disturb its function and diffuse into cytoplasm. Over time, they transform into biocompatible material that effectively binds with cellular DNA and causes DNA damage, as well.

Researchers (Karaman et al., 2007; Zhang et al., 2010; Yuwen et al., 2013; Maisanaba et al., 2015; Isoda et al., 2017) demonstrated that exposure to micro- or Nano-sized clays could induce significant ROS generation. Superoxide Dismutase (SOD) inhibition. BNPs also induced significant high levels of lipid peroxidation as measured by the Malondialdehyde (MDA) assay. More recently, bentonites were active in vitro and released Lactate Dehydrogenase (LDH), Glucuronidase, as well as H2O2 into the medium of the cultured rat alveolar macrophage cell line NR8383 and many other cells (Wiemann et al., 2020). Further, there is compelling evidence that the toxicity of silica material is linked with the steric organization of superficially located Silanol groups responsible for membranolytic activity, cell surface receptor interaction, and proteasome activation.

Furthermore, MDA can directly or indirectly affect functions of cellular and organ homeostasis as a result of oxidative stress in a variety of lipid systems including the cell membranes (Karaman et al., 2007). The free radical activity is associated with augmentation of the lipid peroxidation and deformation of the RBC membrane bilayer (Hashimoto et al., 1996). Thus, an alternative pathway for echinocytic transformation is the modification and destabilization of the RBC membrane and consequently the excessive increases of the intracellular Ca$^{2+}$ concentration as confirmed in previous studies (Hashimoto et al., 1996; Agroyannis et al., 2001; Ghazizadeh and Naziroğlu, 2014; Pall, 2016; Kaur et al., 2021). Calcium accumulation in RBCs has been associated with a wide range of toxic factors.

Based on the obtained results, it is tempting to speculate that bentonites adsorb large amounts of H2O2, leading to local ionic (Ca$^{2+}$ and/or Mg$^{2+}$) ions imbalances. Calcium is important for the normal function and survival of animal cells. It is capable of modulating many vital cellular functions, for example, membrane plasticity, cation exchange, and even cell death (Helperin et al., 1989). A rise in Ca$^{2+}$ influx has been demonstrated via the pore-forming toxins (Helperin et al., 1989). Two groups of researchers demonstrated the ability of Silicate Nano-clay (Lu et al.,

DOI: http://dx.doi.org/10.24018/ejbio.2022.3.3.363
provide data on the correlation between micronucleation and crenation of erythrocytes. The exact mechanism of action leading to MN formation and echinocytic transformation is not clear at present. It is questionable if the high intracellular Ca²⁺ and oxidative stress are only responsible for echinocytogenesis and cytogenotoxicity. Probably some other factors which evoke chromosome damage membrane changes may also participate. Overall, taken together, the two processes could be of diagnostic significance. Further research may prove helpful concerning the mechanism of action.

ACKNOWLEDGMENT

We appreciate the assistance of Professor Ahmad Alshamali, Department of Telecommunication Engineering, Yarmouk University, in the EMF exposure setup. Authors would like to thank Miss Noor Alyaqin Kanary and Miss Hanin Ghabash, from the Department of Biological Sciences, Yarmouk University, for their technical help in plant extraction.

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

REFERENCES


Ghazizadeh, V., and Nazroolu, M. (2014). Electromagnetic radiation (Wi-Fi) and epilepsy induce calcium entry and apoptosis through activation of TRPV1 channel in hippocampus and dorsal root ganglion of rats. Metabolic Brain Disease, 29, 787–799.


