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ORIGINAL ARTICLE / ÖZGÜN MAKALE

EVALUATION OF ROUNDUP® TOXICITY IN HUMAN LUNG CELLS

İNSAN AKCİĞER HÜCRELERİNDE ROUNDUP® TOKSİSİTESİNİN DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *In this study, toxic effects of Roundup, one of the most common glyphosate-based herbicides (GBHs), were assessed on human bronchial epithelial cells (BEAS-2B).*
Material and Method: *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and neutral red uptake assays were implemented for evaluation of cell viability at 24 and 48 h. Apoptosis detection was made by Muse analyzer while Hoechst staining was employed to detect apoptotic nuclear changes. In addition, dichlorofluorescein diacetate assay was used for the assessment of reactive oxygen species (ROS) formation.*
Result and Discussion: *Similar half maximal inhibitory concentrations were obtained from cytotoxicity assays. Results showed that significant reduction in the viability of BEAS-2B cells started to occur from 200 µM at 24 h and 50 µM at 48 h treatment times. Roundup treatments were found to cause apoptosis in a dose-dependent manner in both time points and apoptosis increased with increasing exposure time. Moreover, it was observed that cellular ROS formation was induced following Roundup exposure. These findings suggest that GBHs can stimulate ROS production, as well as apoptosis on healthy human lung cells which is important considering inhalation is one of the primary exposure routes to this group of chemicals.*

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ÖZ

Amaç: *Bu çalışmada, en yaygın olarak kullanılan glifosat bazlı herbisitlerden (GBH) biri olan Roundup'ın, insan bronşiyal epitel hücrelerine (BEAS-2B) toksik etkileri değerlendirilmiştir.*
Gereç ve Yöntem: *Hücre canlılığının 24 ve 48 saatte değerlendirilmesi amacıyla 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolyum bromür ve nötral red alım testleri uygulanmıştır. Apoptoz tespiti için Muse analiz cihazı; apoptotik nükleer değişikliklerin değerlendirilmesi için Hoechst boyama tekniği kullanılmıştır. Ayrıca reaktif oksijen türleri (ROT) oluşumunun değerlendirilmesi için diklorofloresin diasetat yöntemi uygulanmıştır.*
Sonuç ve Tartışma: *Her iki sitotoksisite testinden benzer yarı maksimal inhibisyon konsantrasyonları elde edilmiştir. 24 saatte 200 µM, 48 saatte ise 50 µM maruziyetten sonra BEAS-2B hücrelerinin canlılığında önemli ölçüde azalma olduğu tespit edilmiştir. Her iki zamanda da Roundup maruziyetinin doza bağlı olarak apoptoza neden olduğu ve apoptozun maruz kalma süresinin uzamasıyla arttığı bulunmuştur. Ayrıca, Roundup maruziyeti sonrasında hücresel ROT oluşumunun indüklendiği gözlenmiştir. Bu bulgular GBH'lerin sağlıklı insan akciğer hücrelerinde ROT üretimini ve apoptozu arttırdığını göstermektedir. GBH'lere başlıca maruziyet yollarından birinin inhalasyon olduğu göz önüne alındığında bu çalışmanın sonuçları önem arz etmektedir.*
Anahtar Kelimeler: *Akciğer hücresi, apoptoz, glifosat, reaktif oksijen türleri, toksisite*

INTRODUCTION

Glyphosate-based herbicides (GBHs), one of the most commonly used herbicides, exert their effects by interrupting shikimic acid pathway in plants [1,2]. Non-existence of this pathway in humans and animals was evaluated as a safety factor and resulted in more widespread consumption and environmental accumulation of these substances [3]. Increased usage of plants with glyphosate resistance further contributed to the environmental build-up of these compounds [4,5], which resulted in humans being exposed more to GBH residues [4,6]. In mammals, small amount of the absorbed glyphosate can be metabolized and excreted mostly as a parent compound [7]. Glyphosate (N-(phosphonomethyl) glycine), active component in GBHs, is a broad-spectrum, non-selective, post-emergent pesticide [8, 9]. Roundup is among the most well-known and widely used GBHs in the world [10].

Glyphosate-based herbicides were suggested as more toxic than single glyphosate because of their incorporation of adjuvants [11,12]. Several studies showed glyphosate and GBH exposure were associated with many adverse effects including cardiotoxicity, hepatotoxicity, nephrotoxicity neurotoxicity, immunotoxicity and reproductive toxicity [6-8,10,12,13]. Differences of opinion between authorities on carcinogenic properties of glyphosate led to uncertainties about the safety of this herbicide. International Agency for Research on Cancer (IARC) categorized glyphosate as probable carcinogen (Group 2A) [14] whereas United States Environmental Protection Agency [15] has not reported any relation between glyphosate exposure and human carcinogenicity. More recently, European Chemicals Agency (ECHA) declared current scientific evidence was not sufficient to classify glyphosate as neither carcinogenic, mutagenic or a reprotoxic chemical [16].

Inhalation is one of the primary human exposure routes for GBHs, especially in occupational context [17]. Previous epidemiological studies in farmers reported that glyphosate usage was associated with numerous diseases, including adverse effects on respiratory system. Glyphosate exposure was found to induce oxidative stress and affect lung function in farmers [18]. For this purpose, in the current study, human bronchial epithelial (BEAS-2B) cells were chosen as a model for investigation of Roundup toxicity on lung tissue.

Oxidative stress is among the proposed toxicity mechanisms for GBHs in humans [10]. Oxidative stress is considered to occur when antioxidant defenses in the biological systems were overwhelmed by reactive oxygen species (ROS) production [19]. GBHs might trigger generation of intracellular ROS, as well as cause apoptosis directly or as an indirect consequence of oxidative stress. Apoptosis is a programmed cell death which plays a critical role in regulating cell homeostasis [20]. Considering all these data, in this study, cytotoxicity of Roundup was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT), in addition to neutral red (NR) uptake assay for 24- and 48-hour periods in normal human bronchial epithelial cell line. Possible apoptosis-inducing and ROS producing effects of Roundup were also analyzed in this model system.

MATERIAL AND METHOD

Chemicals

Roundup Star was supplied from Monsanto (Antwerpen, Belgium). It contains 441 g/l (35.5%) of potassium salt of glyphosate and ethoxylated ether alkylamine (6%). RPMI, penicillin/streptomycin, Trypsin-EDTA and fetal bovine serum (FBS) were supplied by Gibco (Paisley, UK) while other chemicals by Sigma-Aldrich/Merck KGaA (Darmstadt, Germany).

Culture of Cells

Human bronchial epithelial (BEAS-2B) cells were bought from American Type Culture Collection. They were cultured in RPMI enriched by FBS, penicillin and streptomycin under 5% CO_2, 95% humidity and at 37°C.

Cytotoxicity Tests

Cytotoxicity was assessed with MTT and NR uptake assays. 96 well plates were used for seeding cells with 10^4 cells per well. Cells were exposed to 12.5-800 µM (2.1-135.2 µg/ml) Roundup for 24 and 48 hours. These selected concentrations are well below the agricultural usage level recommendations (2044-8200 µg/ml) [21,22]. MTT assay was performed according to the protocol of Mosmann [23]. For this assay, cells were incubated with 5 mg/ml MTT for 2 hours following Roundup treatment. Then, DMSO was used for dissolving formazan salts. In NR uptake assay, lysosomal integrity was evaluated based on protocol of Borenfreund and Puerner [24]. After Roundup exposure, cells were incubated with NR dye for 3 hours. Then, cells were fixed with formaldehyde (0.5%) in $CaCl_2$ solution and were exposed to acetic acid (1%) in ethyl alcohol solution. For both assays, absorbance of resulting colors at 540 nm was read using BioTek Synergy plate reader. Wells without cells were used as a blank. Viability of cells was measured using following equation:

$$Cell\ viability\ (\%) = \frac{\left(Abs.(treatment) - Abs.(blank)\right)}{\left(Abs.(control) - Abs.(blank)\right)} \times 100$$

Determination of Apoptosis

Apoptosis detection was conducted by Muse™ Annexin V and Dead Cell kit based on manufacturer instructions (Merck Millipore, USA). Cells at a density of 3.5×10^5 cells/25 cm^2 flasks were exposed to Roundup for 24 and 48 h. After exposure, cell suspension was obtained. Then, kit reagent was added to this suspension. Following the incubation of this mixture under dark conditions for 20 min, cells were assessed with Muse™ Cell Analyzer (Merck Millipore, USA).

Hoechst staining was employed to detect apoptotic nuclear changes. Cells with a density of 1.5×10^5 per well were seeded into 6-wells plates for Roundup treatment. After rinsing with PBS and fixing with paraformaldehyde (4%), cells were dyed with Hoechst 33258 (5 mg/ml) and washed with PBS. The cell morphology was evaluated under a fluorescence microscope (Eclipse Ti, Nikon). Chromatin condensation and nuclei fragmentation were assessed for apoptosis identification.

Determination of ROS

Intracellular ROS formation was assessed with dichlorofluorescein diacetate (DCFH-DA) assay based on method by Wang and Joseph [25] with some changes. Cells (2×10^4 cells per well) were treated with Roundup for 24 h on 96 well black plates. Following treatment and washing with HBSS, cells were incubated by 5 µM DCFH-DA. After incubation, cells were rewashed and then fluorescence was observed at 485 nm excitation and 530 nm emission wavelengths with BioTek Synergy plate reader.

Measurement of total protein content was made with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Wells without probe were used as a blank. Calculation was made according to following equation:

$$ROS\ Induction\ (fold) = \frac{\big(Fluor.(treatment) - Fluor.(blank)\big)/Protein\ conc.(treatment)}{\big(Fluor.(control) - Fluor.(blank)\big)/Protein\ conc.(control)}$$

Statistical Analysis

Statistical analysis was made by one-way ANOVA and Dunnett's Multiple Comparison tests using GraphPad Prism 9. *p* values less than 0.05 were deemed significant. Data were given as mean ± standard error (SEM) with n ≥ 3. Half maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism.

RESULT AND DISCUSSION

Induction of Cytotoxicity with Roundup

As shown in Figure 1, following exposure of cells to ≥200 µM concentrations of Roundup, cell viability decreased dose-dependently with MTT ($p < 0.001$), and NR assays at 24 h ($p < 0.01$ at 200 µM; $p < 0.001$ at 400 and 800 µM) as well as with NR assay at 48 h ($p < 0.001$). Following 48 h incubation, with MTT assay, Roundup caused dose-dependent decreases in cell viability ≥50 µM concentrations ($p < 0.05$ and $p < 0.01$, respectively, at 50 and 100 µM; $p < 0.001$ for higher concentrations). IC_{50} values were calculated as 281 µM (47.5 µg/ml) and 237 µM (40.1 µg/ml) for 24 and 48 h, respectively, for MTT, and 274 µM (46.3 µg/ml) and 213 µM (36 µg/ml), respectively, for NR uptake assay. Glyphosate-based herbicides had been shown to cause decreases in cell viability on many cell lines including liver, kidney, and nerve cells [11,26,27]. Although there are studies about the

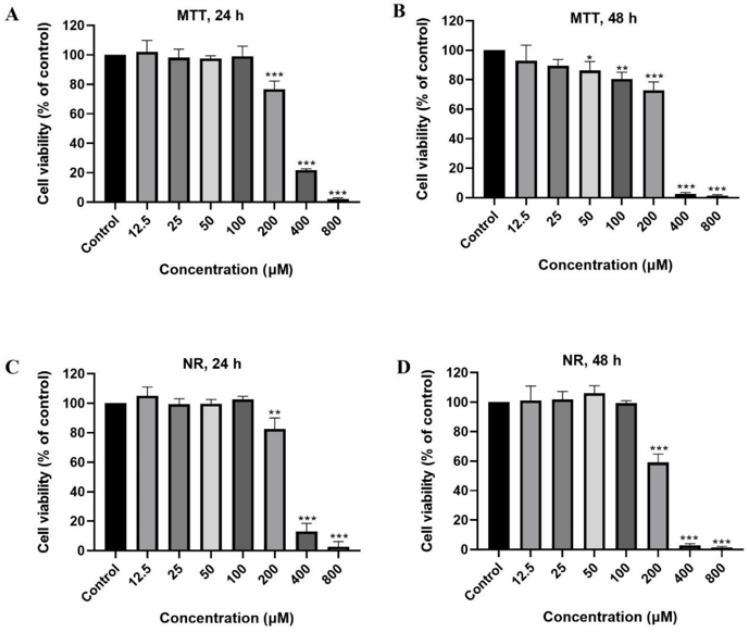

Figure 1. Viability of BEAS-2B cells after Roundup exposure. MTT, 24 h (A). MTT, 48 h (B). NR, 24 h (C). NR, 48 h (D). Data given as means ± SEM, n=3. *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* control

cytotoxicity of GBHs on different human lung cell lines, our data showed for the first-time cytotoxic effect of Roundup on healthy lung cells. In agreement with our results, in a study made with human alveolar carcinoma cells (A549) in which toxicity of different glyphosate formulations at 24 h were studied, IC_{50} values were calculated between 22.9 and 87.7 µg/ml [26]. In another study with the same cell lines, it has been shown that glyphosate significantly reduced cell viability at 1500 µM at 24 h [28]. As mentioned in many other studies, active ingredient glyphosate was considered less toxic than commercial glyphosate formulations with adjuvants [11,12]. Considering the two cytotoxicity test methods, MTT assay primarily detects mitochondrial damage whereas NR assay investigates lysosomal integrity [29]. Lopes et al. found MTT assay exhibited more sensitivity relative to NR assay when zebrafish hepatocytes were exposed to Roundup for 48 h [30]. Goulart et al., on the other hand, reported that lysosomes were more sensitive at low dose of Roundup (67.7 µg/ml) on the same line of cells [31]. In another study, cytotoxicity profiles of both assays were found similar after HepG2 cells were exposed to GBHs for 48 h which agreed with our observations [32].

Roundup Causes Apoptosis in Lung Cells

As seen in Figure 2, 150 µM ($p < 0.01$) and higher concentrations ($p < 0.001$) caused significant increase in total apoptosis at 24 h, while at 48 h, all doses led to significant cellular apoptosis compared to control ($p < 0.001$). In both time points, apoptotic cell ratio was augmented in dose dependent manner. Nuclear morphological changes including chromatin condensation and fragmentation in the apoptotic cells were also investigated using Hoechst 33258. The slightly colored, homogenous appearance of cells in control group changed to nuclei with bright stain, condensation and fragmentation after treatment with Roundup for 24 and 48 hours, as shown in Figure 3. Results of current study were in line with a

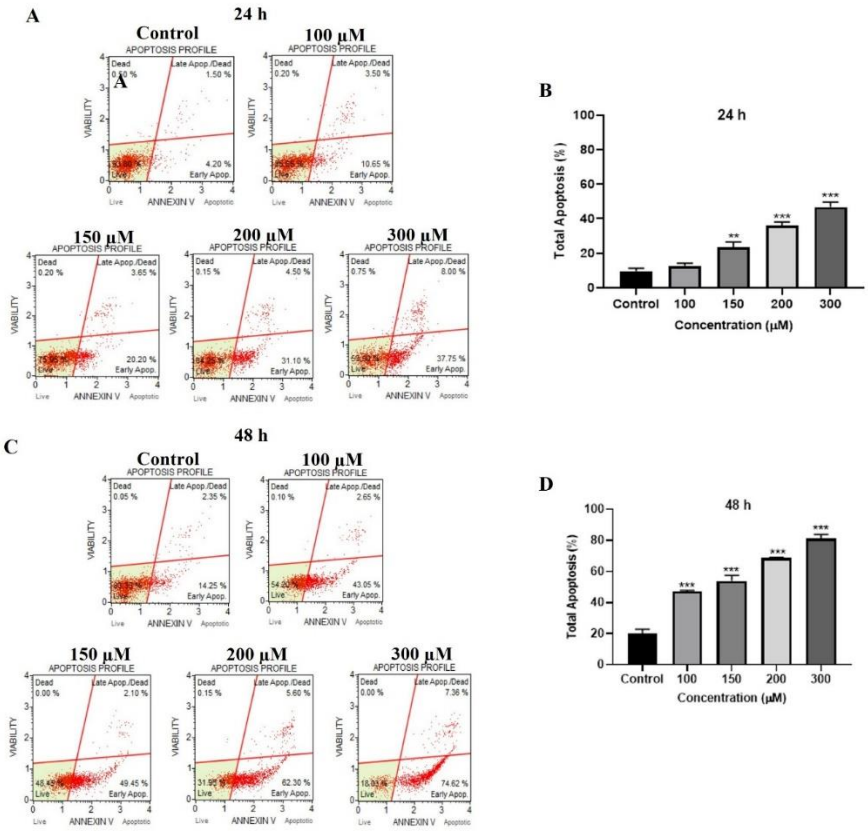

Figure 2. Apoptosis of BEAS-2B cells after Roundup exposure. Representative dot plots show the apoptosis profile at 24 h (A) and 48 h (C). Bar graph displays total apoptosis percentage at 24 h (B) and 48 h (D). Data shown in bar graph given as means ± SEM, n=3. **p < 0.01, ***p < 0.001 *vs.* control

study performed with A549 cells which showed that 50-125 µg/ml concentrations of Roundup led to apoptosis and morphological changes in nuclei [22]. Kwiatkowska et al. also demonstrated that glyphosate caused increases in the percentage of apoptosis and chromatin condensation in human peripheral blood mononuclear cells [33], which is also in agreement with our findings. In another study performed with 3T3-L1 fibroblasts, it has been shown that 1.05 mM GBHs for 24 h increased apoptotic cell rate [34].

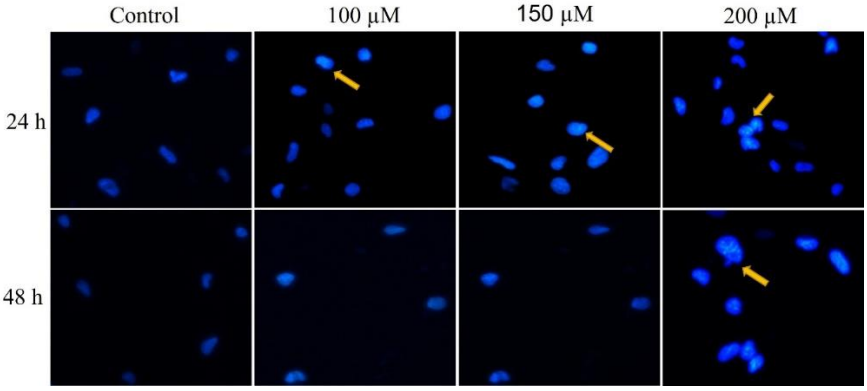

Figure 3. Assessment of nuclear morphological changes following Roundup exposure. Arrows show nuclear fragmentation (×20)

Roundup Induces ROS Formation

Reactive oxygen species have an important function in regulating apoptosis pathways [35]. Under normal physiological processes, a certain amount of ROS is produced in the body. Oxidative stress occurs when excessive ROS production overcomes antioxidant defense systems, causing lipid peroxidation, damage to cellular macromolecules and finally, dysfunction and death of cells [10]. In this study, ROS was found to be 1.24 and 1.47-fold ($p < 0.001$) higher compared to control group in 50 and 100 µM exposure groups, respectively, (Figure 4). However, ROS level declined 1.38-fold relative to control after 200 µM treatment ($p < 0.001$). In a previous study, it has been shown that 100 µg/ml Roundup treatment for 2 h increased ROS production in human alveolar carcinoma cells [36]. Bai et al. also found that 60 µg/ml Roundup exposure at 12 h caused ROS formation in porcine intestinal epithelial cells [37]. For comparison, in the current study, Roundup treatment led to increase in ROS levels at a lower dose, 8.45 µg/ml, although our exposure times were also longer relative to above-mentioned studies. In addition, the cell types used might be a factor in obtaining different results. In a study with pure glyphosate, however, ROS level was not changed by 250-750 µM concentrations at 24 h [38]. In our study, decline of ROS at higher dose (200 µM) might be related to excessive cytotoxicity at high doses.

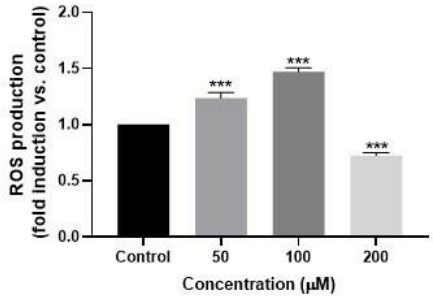

Figure 4. ROS formation after Roundup exposure at 24 h. Data given as means ± SEM, n=4. ***p < 0.001 *vs*. control

In conclusion, to the best of our knowledge, this is the first study which investigated toxic effects of a GBH on normal human lung cells. Roundup-induced mitochondrial and lysosomal cytotoxicity on BEAS-2B cells were shown following 24- and 48-hours incubation. In our model system, Roundup also caused cell death through apoptosis and led to ROS formation. Since inhalation is one of the primary exposure routes to this group of chemicals, the results of this study will contribute to the literature, in terms of the evaluation of glyphosate toxicity and its toxicity mechanisms especially in occupationally exposure groups. Furthermore, we believe that the results of current study are important due to the ongoing debate on the safety of GBHs. Although our results point that Roundup may induce ROS and apoptosis in BEAS-2B cells, other oxidative stress related parameters such as lipid peroxidation and antioxidant enzyme activities, as well as the pathways involved in apoptosis were not measured as a part of this work. We expect that investigations on these subjects will add more information to the toxic effect mechanisms of this group of substances and might set an example for future studies.

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AUTHOR CONTRIBUTIONS

Concept: B.Ü.E.; Design: B.Ü.E.; Control: B.Ü.E.; Sources: B.Ü.E., A.E; Materials: B.Ü.E., E.B., A.Ö.; Data Collection and/or Processing: B.Ü.E., E.B., A.Ö., Z.H.; Analysis and/or Interpretation: B.Ü.E., Z.H., A.G.; Literature Review: B.Ü.E.; Manuscript Writing: B.Ü.E., Z.H., A.E., A.G.; Critical Review: B.Ü.E., Z.H., A.E., A.G.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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