

Lung Inflammation from Single and Repetitive Exposure to Glyphosate

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Abstract

Background: Glyphosate is an active ingredient in herbicides used in agriculture worldwide. Exposure to glyphosate has been associated with respiratory dysfunctions in agricultural workers. However, the ability of glyphosate to induce inflammation in the lung is not well studied. Therefore, we evaluated lung inflammatory response to glyphosate at agricultural relevant dose for single and repetitive exposures.

Methods: Male C57BL/6 mice were intranasally exposed to glyphosate (1 µg/40 µl) for 1-day or once daily for 5-days, and 10-days. After the exposure periods, mice were euthanized to collect the bronchoalveolar lavage (BAL) fluid and lung tissue.

Results: Repetitive exposure to glyphosate for 5-days and 10-days showed an increase of neutrophils in BAL fluid and eosinophil peroxidase levels in lungs, a marker for eosinophils. Leukocyte infiltration in lungs was further confirmed through lung histology. Th2 cytokines including IL-5 and IL-13 were increased in BAL fluid after 10-days of glyphosate exposure whereas IL-4 was not increased. Lung sections from all glyphosate groups showed higher expression for ICAM-1, VCAM-1, and vWF adhesion molecules. TLR-4 and TLR-2 expression was increased in lungs after repetitive exposure to glyphosate.

Conclusions: We conclude that repetitive exposure to glyphosate induces migration of neutrophils and eosinophils and release of Th2 cytokines. This study, for the first time, provides evidence for the role of ICAM-1, VCAM-1 and vWF in lungs of glyphosate-treated animals.

Background

Glyphosate [N-(phosphonomethyl) glycine] is the most common active ingredient in herbicides. Glyphosate-based herbicides are extensively used in agriculture worldwide (1). Glyphosate has been detected in urine samples of agricultural workers and their family members, indicating exposure to glyphosate (2–4).

Glyphosate exposure has been associated with increased risk of rhinitis and allergic and non-allergic wheeze among pesticide applicators (5–9). Moreover, glyphosate exposure is linked with exacerbation of existing asthma in workers (10). Glyphosate has been detected at a level of 17.33 µg in air from the farms sprayed with glyphosate (11). Although there is an evidence of glyphosate exposure as a risk factor for human respiratory problems, the ability of glyphosate to induce inflammation in lungs is not well studied.

Recently, Kumar and colleagues challenged female C57BL/6 mice intranasally with different doses of glyphosate (100 ng, 1 µg or 100 µg) for 7-days and did not observe dose dependent effect of glyphosate on markers of airway inflammation and lung pathology (11). Glyphosate exposure for 7-days induced an increase of neutrophils in bronchoalveolar lavage (BAL) fluid, eosinophils in lungs, and IL-5 (BAL fluid), IL-13, IL-10, IL-33 and TSLP cytokines in blood samples of challenged mice (11). The leukocyte recruitment is facilitated through an increase in expression of endothelial adhesion molecules due to release of

cytokines. The pulmonary endothelial cells show constitutive expression of various adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and von Willebrand factor (vWF) (12). However, there is no data on the expression of adhesion molecules in the lungs of glyphosate-exposed animals.

The present study was designed to characterize the lung inflammation induced with exposure to agricultural relevant dose of glyphosate (1 µg) for different periods (1-day, 5-days, and 10-days) by using mice model. Our data shows that repetitive exposure to glyphosate increased the inflammatory markers and expression of adhesion molecules in lungs.

Materials And Methods

Mice exposures

The experimental protocols were approved by the Animal Ethics Research Board of the University of Saskatchewan (Protocol # 20160106). Male C57BL/6 mice (Charles River Laboratories, Montreal, QC Canada), 6–8 weeks old, were maintained at the Laboratory Animal Services Unit of the University of Saskatchewan. Mice were fed *ad libitum* and were acclimatized for one week after arrival.

Mice were divided into glyphosate and control treatment groups (n = 5 per group). The 1 µg dose of glyphosate treatment was selected based on glyphosate levels found in the agricultural environment and has been utilized in other studies (11). The stock solution of glyphosate (0.8 M; analytical grade PESTANAL standard, Sigma, St. Louis, MO USA) was prepared in Hank's Balanced Salt Solution (HBSS). It was vortexed for 10 minutes and syringe filtered (0.22 µm; Fisher Scientific). Mice received 40 µL of either glyphosate (1 µg/40 µl) or saline intranasally for 1-day or daily for 5-days or 10-days. Mice were lightly anesthetized using isoflurane before treatments. There were no differences in the weight of mice in the control and the glyphosate-treated groups. After 4-hours of last treatment, mice were euthanized by CO₂ inhalation, and BAL fluid and lung samples were collected.

Bronchoalveolar lavage collection and processing

BAL fluid was collected by washing the airways three times with 0.5 ml ice-cold HBSS. The collected BAL fluid was centrifuged at 1000 *g* for 10 minutes at 4° C, and supernatants were stored at -80° C for cytokine analysis. Cells from BAL fluid was resuspended in HBSS and kept on ice until used for leukocyte counts.

Total and differential leukocyte count

The total and differential leukocyte counts in BAL fluid were performed using a hemocytometer and cytospin stained with Protocol Hema 3 kit (ThermoFisher Scientific, Waltham, MA USA), respectively.

Cytokine levels

Multiple cytokines in BAL fluid were measured using a Custom Mouse Procartaplex Multiplex Immunoassay (ThermoFisher Scientific, Waltham, MA USA), according to manufacturer's instructions for magnetic bead-based ELISA. Plates were read using a Bioplex 200 system (Bio-Rad, Mississauga, ON Canada) and Bioplex Manager Software (Bio-Rad, Mississauga, ON Canada).

Lung tissue collection and processing

Following BAL fluid collection, the right lung was tied off at the primary bronchus, and the left lung was fixed in-situ through intratracheal instillation of 200 µl of 4% paraformaldehyde (PFA). The right lung was removed and snap-frozen in liquid nitrogen and stored at -80°C for eosinophil peroxidase (EPO) and RNA analysis. The fixative-instilled left lung was further submerged in 4% PFA for 16 hours at 4°C . Lung tissue was then washed through ascending grades of alcohol before embedding in paraffin. Lung sections of 5 µm thickness were cut from paraffin-embedded tissues. Hematoxylin & eosin staining, and immunohistochemistry were performed on these lung sections.

Eosinophil peroxidase quantification

Lung tissues were homogenized using 2 mm Zirconia beads (BioSpec, Bartlesville, OK USA) in tubes containing RIPA lysis buffer supplemented with 1X Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA USA) in a Mini- Beadbeater-24 homogenizer (BioSpec, Bartlesville, OK USA) for two, 1-minute rounds. The tubes were cooled on ice in between rounds of homogenization. The total protein concentration of lung homogenates was determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. Eosinophil peroxidase (EPO) was quantified using a Mouse Eosinophil Peroxidase DuoSet ELISA (LifeSpan Biosciences, Seattle, WA USA). Plates were read using a BioTek Synergy HT plate reader (BioTek, Winooski, VT USA) at 450 nm.

Histology and scoring for inflammation

Lung sections from all the mice were stained with hematoxylin & eosin stain. Two stained lung sections from each mouse were reviewed and scored for lung inflammation. Each of the sections on the slide was reviewed at different magnifications (x20, x40, x100). Each section's scoring was performed by multiple reviewers blinded to the exposure groups, and scores were averaged. Cellular infiltration in alveolar, perivascular, and peribronchiolar compartments of lungs were scored. Each parameter was given a score based on the intensity and was statistically analyzed (0: absent, 1: mild, 2: moderate, 3: severe).

Immunohistochemistry and analysis

Lung sections from all mice were stained with antibodies against ICAM-1, VCAM-1, and vWF markers. Briefly, lung sections were immersed in a series of xylene baths for deparaffinization and different alcohol grades for rehydration. Endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide in methanol for 20 minutes. Antigen unmasking and blocking were done for 30 minutes with 2 mg/ml pepsin and 1% bovine serum albumin, respectively. The lung sections were incubated overnight at 4⁰ C with the following primary antibodies: ICAM-1 (dilution 1: 100; rabbit monoclonal anti-mouse ICAM-1, ab79707, Abcam Inc., ON Canada), VCAM-1 (dilution 1:100; rabbit monoclonal anti-mouse VCAM-1, ab134047, Abcam Inc., ON Canada), and vWF (dilution 1:200; rabbit monoclonal anti-mouse vWF, ThermoFisher Scientific, Waltham, MA USA). Following overnight incubation, the secondary goat anti-rabbit antibody (dilution 1:200; ThermoFisher Scientific, Waltham, MA USA) was added onto tissue sections. Slides were incubated for 1 hour at room temperature in a humidified chamber. The color was developed using a peroxidase kit (Vector laboratories, Burlington ON, Canada) according to manufacturer's instructions and counterstained with methyl green (Vector laboratories, Burlington ON, Canada). In the end, slides were dehydrated through a series of ethanol concentrations and were fixed with xylene before mounting. Controls with the omission of the primary antibody or secondary antibody were run at the same time.

The expression of ICAM-1, VCAM-1, and vWF was reviewed in five random fields of the lung sections from each mouse (N = 3). Expression of ICAM-1 was scored in the vasculature, and bronchial epithelium. Each parameter staining was given a score based on staining intensity by a reviewer blinded to exposure groups (0: no or occasional staining, 1: weak staining, 2: moderate staining, 3: intense staining).

RNA isolation and real-time PCR

Lung homogenates were prepared using 2 mm Zirconia beads (BioSpec, Bartlesville, OK USA) in tubes containing RLT lysis buffer (Qiagen, Chatsworth, CA USA) in a Mini-Beadbeater-24 homogenizer (BioSpec, Bartlesville, OK USA). RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA USA) according to manufacturer's instructions. Purified mRNA was quantified using a Take3 plate and BioTek Synergy HT plate reader (BioTek, Winooski, VT USA). cDNA was generated using iScript Reverse Transcription Supermix (BioRad, Hercules, CA USA) with 0.5 µg mRNA. PCR was conducted in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA USA) using the following protocol: 25⁰ C for 5 minutes, 46⁰ C for 20 minutes, and 95⁰ C for 1 minute.

Real-time PCR was performed using probes for mouse ICAM-1 (Mm00516023_m1), TLR-4 (Mm00445273_m1), TLR-2 (Mm00442346_m1), Hsp72, (Mm01159846_s1) and A20 (Mm00437121_m1) (Life Technologies, Grand Island, NY USA). Each reaction was carried out in duplicate using ribosomal RNA (Life Technologies, Grand Island, NY USA) as an endogenous control. PCR was conducted in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA USA). PCR reactions were carried

out as follows: 50° C for 2 minutes, 95° C for 10 minutes followed by 40 cycles at 95° C for 15 seconds and 60° C for 1 minute. Relative quantification was estimated from each target gene's cycle threshold obtained from real-time PCR data followed by analysis with the $\Delta\Delta C_t$ method.

Cell culture experiment and treatments

A549 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA USA). A549 cells were grown in Dulbecco's Modified Eagle Media (DMEM, ThermoFisher Scientific, Waltham, MA USA) supplemented with 10% fetal bovine serum containing penicillin and streptomycin (ThermoFisher Scientific, Waltham, MA USA) at 37° C and 5% CO₂ until confluence was reached. Cells were dissociated using Tryp- LE Select (ThermoFisher Scientific, Waltham, MA USA) and counted using a hemocytometer. For treatments, A549 cells were seeded at a density of 0.5×10^6 per well in 96 well plates and allowed to attach overnight. Cells were washed with 1X PBS (ThermoFisher Scientific, Waltham, MA USA) before treatments. Glyphosate treatment solutions were prepared in DMEM serum-free media using the same stock solution used for mice experiments. Treatment with dimethyl sulfoxide (DMSO, 0.5%) in serum-free DMEM media was used as a positive control. Untreated cells in serum-free DMEM were used as a negative control. A549 cells were treated for 24 hours at 37° C and 5% CO₂ with different glyphosate concentrations or positive control.

Cytotoxicity

The viability of treated A549 cells was tested using a Cell Proliferation Kit 1 (MTT based, Roche, Catalog #11465007001) according to manufacturer's instructions. Briefly, cells were incubated with 10 μ l of MTT labeling reagent for 4 hours at 37° C and 5% CO₂. Next, 100 μ l of solubilization buffer was added to each well and incubated for 24 hours in the incubator. Plates were read using a BioTek Synergy HT plate reader (BioTek, Winooski, VT USA) at 450 nm.

Data analysis and statistics

Data was analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Error bars represent mean \pm SD. For values outside the assay limit of detection, either the LLOD/2 (lowest limit of detection) or a minimum value below the lowest attained value was designated. Statistical significance was determined using one-way ANOVA with a follow-up Tukey test for multiple comparisons. If the assumption of equal variance was not met, the data was either log-transformed, followed by one-way ANOVA and multiple comparison tests, or a non-parametric Kruskal-Wallis test was conducted. A p-value < 0.05 was considered significant for differences between groups. For graphing of data, "a" indicates a significant difference compared with the control group; "1" indicates a significant difference compared with the 1-day exposure group; "2" indicates a significant difference compared with the 5-days exposure group.

Results

Leukocyte counts in bronchoalveolar lavage fluid

Glyphosate exposure for 5-days and 10-days significantly increased the total leukocyte counts in BAL fluid as compared to control exposures (Fig. 1A). Total leukocytes count in 10-days glyphosate exposure group was significantly higher than 1-day glyphosate treatment group.

Neutrophils were significantly higher after glyphosate exposure for 5-days and 10-days as compared to same days of control exposure (Fig. 1C, D). Macrophage and lymphocyte count in any of the glyphosate treatment groups were not significantly different than control exposures (Fig. 1B-D).

Cytokine levels in bronchoalveolar lavage fluid

Glyphosate exposure for 10-days resulted in a significant increase in IL-13 (Fig. 2I) and IL-5 (Fig. 2J) compared to the control group. There were no significant differences in TNF- α (Fig. 2A), IL-6 (Fig. 2B), KC (Fig. 2C), MCP-1 (Fig. 2D), MIP-2 (Fig. 2E), IL-1 β (Fig. 2F), IL-10 (Fig. 2G), IL-4 (Fig. 2H) or IL-33 (Fig. 2K) levels between 1-day, 5-days, or 10-days glyphosate exposures and their respective control exposures.

Eosinophil peroxidase in lungs

Eosinophil peroxidase (EPO) is released by activated eosinophils and acts as a marker for the presence of eosinophils. Glyphosate exposure significantly increased the EPO levels in the lungs after 1-day, 5-days, and 10-days exposures as compared to the respective control exposure (Fig. 3).

Lung histology

Lungs of control mice showed normal architecture without any leukocyte infiltration in 1-day (Fig. 4A), 5-days (Fig. 4B), and 10-days (Fig. 4C) exposure groups. Lungs of glyphosate treated mice in the 1-day exposure group showed occasional sloughing and increased thickness of bronchial epithelium, and slight leukocyte infiltration (Fig. 4D, G). Glyphosate exposure in the 5-days (Fig. 4E, H) and 10-days (Fig. 4F, I) exposure groups showed a greater leukocyte infiltration in the alveolar, perivascular, and peribronchiolar regions of the lungs. Moreover, both group's lung sections, 5-days, and 10-days glyphosate exposure groups showed cellular binding to the vascular endothelium, occasional sloughing, and increased thickness of the bronchial epithelium.

Semi-quantification data showed that glyphosate exposure in the 1-day group had significant leukocyte infiltration in the perivascular region as compared to 1-day control exposure (Fig. 4J). Glyphosate exposure for both the 5-days and 10-days significantly increased the leukocyte infiltration in the

perivascular, peribronchiolar (Fig. 4K), and alveolar (Fig. 4L) regions as compared to the respective control exposure.

ICAM-1, VCAM-1, and vWF staining

Lungs of control mice in 1-day, 5-days, and 10-days exposure groups showed positive endothelial staining in large blood vessels for ICAM-1 (Fig. 5A-C), VCAM-1 (Fig. 6A-C), and vWF (Fig. 7A-C) adhesion molecules. Staining for all these adhesion molecules was minimal in the bronchial epithelium and alveolar septa regions of control mice lungs.

Glyphosate exposure for 1-day (Fig. 5D), 5-days (Fig. 5E), and 10-days (Fig. 5F) increased staining for ICAM-1 in alveolar septa and endothelium in large blood vessels. ICAM-1 staining was also induced in bronchial epithelium of lungs from all periods of glyphosate groups. Semi-quantification data showed that ICAM-1 blood vessel staining was significantly increased after glyphosate exposure for 1-day, 5-days, and 10-days compared to the respective control exposures (Fig. 5G). ICAM-1 bronchial epithelium staining significantly increased in the glyphosate 5-days and 10-days exposure groups compared to control groups (Fig. 5H).

Similarly, the staining for VCAM-1 (Fig. 6D-F) and vWF (Fig. 7D-I) was also increased in bronchial epithelium, alveolar septa, and endothelium of large blood vessels in lung sections of glyphosate groups for 1-day, 5-days, and 10-days.

ICAM-1, TLR-4, TLR-2, Hsp72 and A20 mRNA

Glyphosate exposure for 5-days significantly increased the ICAM-1 mRNA as compared to 5-days control and 1-day glyphosate exposure (Fig. 8A). ICAM-1 expression in the 10-days glyphosate exposure group showed a significant reduction compared to the 5-days glyphosate exposure group.

Glyphosate exposures for 5-days and 10-days showed a significant increase in TLR-4 as compared to the control exposures (Fig. 8B). TLR-4 mRNA in the 5-days glyphosate group was significantly higher compared to the 1-day glyphosate exposure group.

TLR-2 mRNA expression in the glyphosate group was significantly higher after 5-days as compared to both the 5-days control and 1-day glyphosate exposure groups (Fig. 8C).

Hsp72 mRNA expression was significantly higher after glyphosate exposure for 5-days than 5-days control and 1-day glyphosate exposures (Fig. 8D). Hsp72 was significantly lower in the glyphosate 10-days exposure group as compared to the 5-days glyphosate exposure group.

Expression of A20 was not significantly different between 1-day, 5-days, or 10-days glyphosate exposures compared to control exposure (Fig. 8E).

Cytotoxicity of A549 cells

Glyphosate treatment at different concentrations showed dose-dependent toxicity on A549 cells (Fig. 9). The reduction in viable cell percentage was observed with the increase in glyphosate treatment concentration (1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M, 100 μ M, 1 mM, and 10 mM). Glyphosate treatment at 1 mM and 10 mM showed a significant cell death compared to the untreated cells. The dotted line in the graph represents LD₅₀ after glyphosate treatment.

Discussion

We report the first study where mice were treated for single and multiple days with glyphosate. The data reported in this paper show that multiple exposure to glyphosate induced migration of neutrophils and eosinophils and release of IL-5 and IL-13 cytokines. Staining for ICAM-1, VCAM-1, and vWF was increased in the alveolar septa and endothelium of large blood vessels in all glyphosate treated lungs, suggesting their role in leukocyte migration after glyphosate exposure. These data add to the evidence on the ability of glyphosate to induce lung inflammation and therefore it may play a role in human respiratory problems.

Lung inflammation is characterized by recruitment of inflammatory cells such as neutrophils and eosinophils in response to inflammatory agent. Glyphosate herbicides are used in agriculture worldwide and glyphosate exposure has been associated with respiratory dysfunction in agricultural workers (1, 7). Glyphosate's ability to induce inflammation in the lungs is not well understood. Therefore, we used mice to evaluate lung inflammation after single (1-day) and repetitive exposure (5-days and 10-days) to glyphosate at agricultural relevant dose. Our results show that repetitive exposure to glyphosate compared to single exposure induced greater cellular infiltration in lungs. The induction of inflammation was further underscored by neutrophils in BAL fluid and levels of eosinophil marker in lungs after repetitive exposure to glyphosate. The data from an earlier study (11) showed similar findings after 7-days of glyphosate exposure. While Kumar and colleagues examined lung effects at only one time-point (7 days) (11) we did so at 1-day, 5-days, and 10-days of glyphosate exposure. The lung inflammation caused by chronic exposure to glyphosate may be associated with lung function impairments observed in pesticide applicators as high prevalence of chronic respiratory symptoms and decline in lung function were reported (13–15).

The recruitment of inflammatory cells is a multi-step process and is facilitated through the release of cytokines and upregulation of endothelial adhesion molecules. Repetitive exposure to glyphosate for 10-days significantly increased Th2 cytokines IL-5 and IL-13 whereas there was no increase in IL-4. These Th2 cytokine results are similar to the findings of others reported after 7-days of glyphosate exposure (11). IL-13 deficient mice exposed to glyphosate for 7-days and 21 days (3 times exposure for 3 weeks) showed less cellular infiltration and diminished production of IL-5 in lungs (11). These results suggests that IL-13 signaling in lungs may be critical in observed inflammation after glyphosate exposure. Both IL-5 and IL-13 have important roles in lung inflammation associated with asthma or COPD (16–18). IL-5 is

involved in the recruitment of eosinophils in the asthmatic lung; Interestingly we did notice an increase in eosinophils in the lung tissues from glyphosate-treated mice.

Kumar and colleagues showed an increase of IL-33, and IL-10 after 7-days of glyphosate exposure (11). Our same dose of glyphosate and longer exposure periods showed no differences in these cytokines. However, glyphosate-treated lungs showed damage to lung epithelium which is one of the potential sources of these cytokines (19, 20). These differences in cytokine results could be possible that we missed the time point for their increase in 5-days gap between our exposure periods. Secondly, the two studies used different gender of mice for glyphosate exposure. There is a known difference in inflammatory response between the different genders (21, 22). Gender response may be an important factor for differences in cytokine responses after glyphosate exposure which needs further studies.

The activation of pulmonary endothelium by cytokines leads to the expression of adhesion molecules which are important for the tissue recruitment of inflammatory cells such as neutrophils and eosinophils (12). There is no data on expression of adhesion molecules in lungs of animals treated with glyphosate. Our study provides first immunohistochemical data showing increased pulmonary expression of ICAM-1, VCAM-1 and vWF proteins in glyphosate treated mice. It is well known that ICAM-1 and VCAM-1 engage selectins to slow-down rolling neutrophils and vWF secreted from Weibel-Palade bodies in endothelial cells facilitates recruitment of platelets (23, 24) and is a marker of inflammation (25–27). The higher levels of cytokines such as IL-13 and IL-5 likely caused the observed increase in the expression of adhesion molecules in lungs of animals treated with glyphosate. Nevertheless, the increased endothelial expression of ICAM-1, VCAM-1 and vWF provides molecular evidence of vascular inflammation in the lungs of glyphosate exposed mice.

For agricultural crop production workers, glyphosate is often co-exposed with other well-known lung inflammation stimulants, most notably endotoxin (LPS) as a component of organic dust. Grain dust inhalation has been shown to induce neutrophilic inflammation in exposed individuals as well as in animal models, and endotoxin (LPS) in the grain dust is often associated with respiratory dysfunction among grain farmers (28–30). Because bacterial molecules activate cells upon binding with TLRs (31), we examined the expression of TLR4 and TLR2 in lungs from mice in our experiments and found an increase in their expression following multiple exposure to glyphosate. While it is not known whether glyphosate directly activates TLR-mediated cell activation, the increased expression of TLRs will enhance sensitivity of the lung to bacterial challenges. Activation of the TLR receptors is critical for inflammatory signaling resulting in the release of cytokines and upregulation of endothelial adhesion molecules, which are necessary for leukocyte migration (32). These results suggest the need to study glyphosate exposure by using TLR receptor knock-out mice which would provide a better understanding on mechanistic role of TLRs in glyphosate induced lung inflammation.

Finally, our in vitro data show cytotoxicity of glyphosate (1 nM to 10 mM) on A549 cells. The dose-dependent effect of glyphosate on A549 cellular toxicity suggests that lower doses of glyphosate are less likely to have a toxic effect on lung epithelial cells. Therefore, these cellular results support our lung

inflammatory findings of mice experiment after exposure to a lower dose of glyphosate. Recent data show that isopropylamine salt of glyphosate did not contribute to apoptotic and genotoxic effects on A549 cells (33). Therefore, our results need to be interpreted with caution as they are based on pure glyphosate. Further studies are needed to examine not only the mechanism of glyphosate-induced cytotoxicity but also the cytotoxicity in vivo. For example, we would like to examine apoptosis and cell death in the lungs of glyphosate-treated animals.

Conclusions

Repetitive exposure to glyphosate induced a migration of neutrophils and eosinophils and release of IL-5 and IL-13. It was concomitantly associated with increased pulmonary expression of ICAM, VCAM-1, and vWF adhesion molecules and TLR-4 and TLR-2 receptors. Exposure to glyphosate could be a risk factor for respiratory dysfunctions observed among agricultural workers. Future studies are needed to investigate the chronic effects of glyphosate on markers of airway inflammation and lung pathology.

Abbreviations

BAL: Bronchoalveolar lavage; GLY: Glyphosate; ICAM-1: Intercellular adhesion molecule-1; IL: Interleukin; TLR: Toll-like receptor; TLR-2: Toll-like receptor 2; TLR-4: Toll-like receptor 4; VCAM-1: Vascular adhesion molecule-1; vWF: Von-Willebrand factor

Declarations

Ethics approval and consent to participate

The study was approved by the Animal Ethics Research Board of the University of Saskatchewan (Protocol# 20160106), and animal research was conducted according to Canadian Council on Animal Care guidelines.

Consent for Publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

UP conceptualized and designed the study, conducted the experiments, analyzed the samples, interpreted the data, and prepared and revised the manuscript. SK contributed to the study concept, design, data management and interpretation, reviewed and revised the manuscript. DS contributed to study methodology, laboratory analysis, reviewed, and revised the manuscript. BT contributed to the study methodology, reviewed and revised the manuscript. RSS, GA, BS contributed to the study methodology, results interpretation, reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Figures

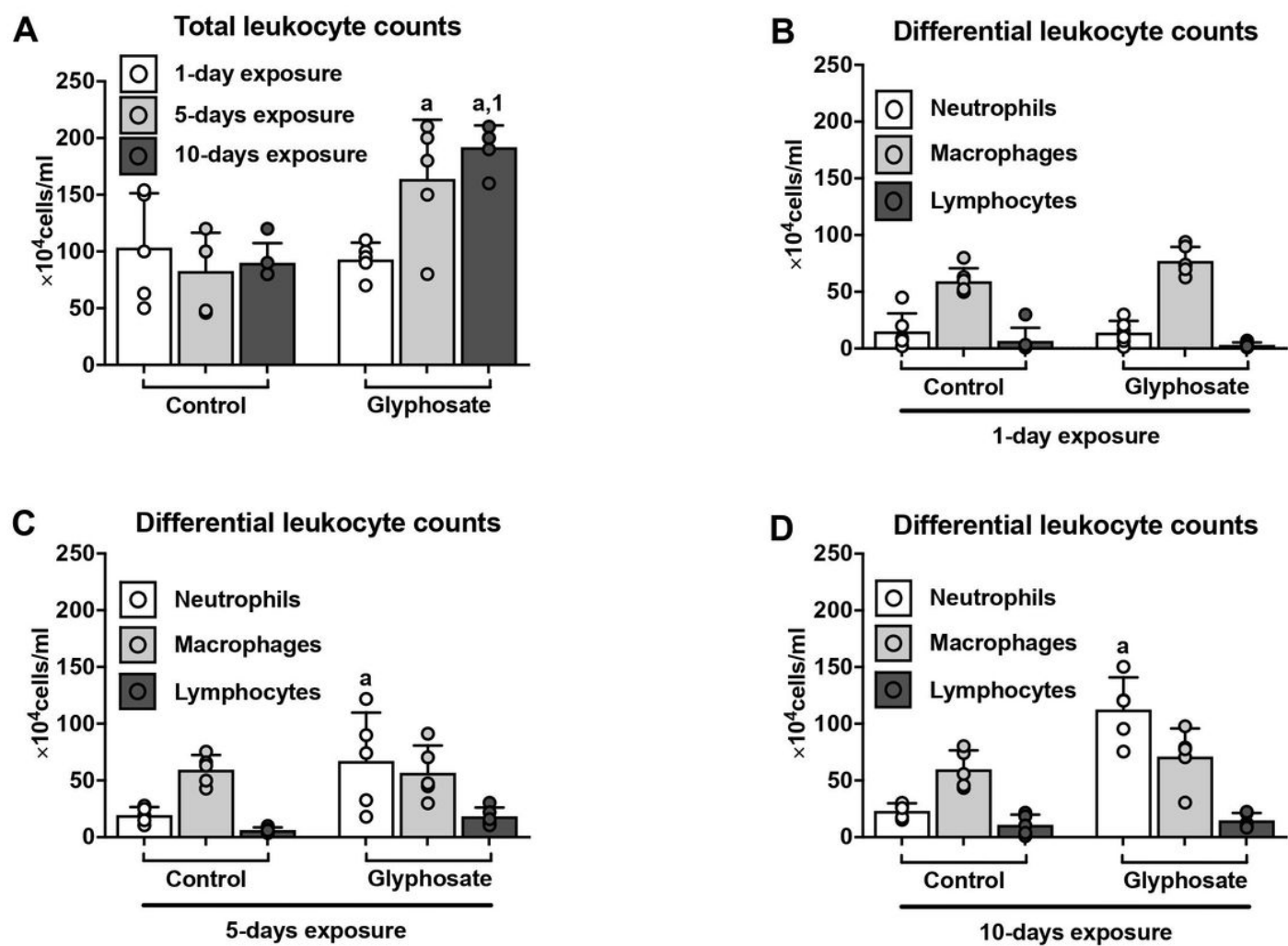


Figure 1

Total leukocyte count (A) and differential leukocyte count (B-D) in BAL fluid of mice after exposure to glyphosate or control for 1-day, 5-days and 10-days. Total leukocyte counts measured using hemocytometer and differential leukocyte counts were measured on cytopins stained with Protocol

Hema 3 kit. Data presented as mean \pm SD (N = 5 mice per group). “a” indicates significant difference ($p < 0.05$) compared with control group.

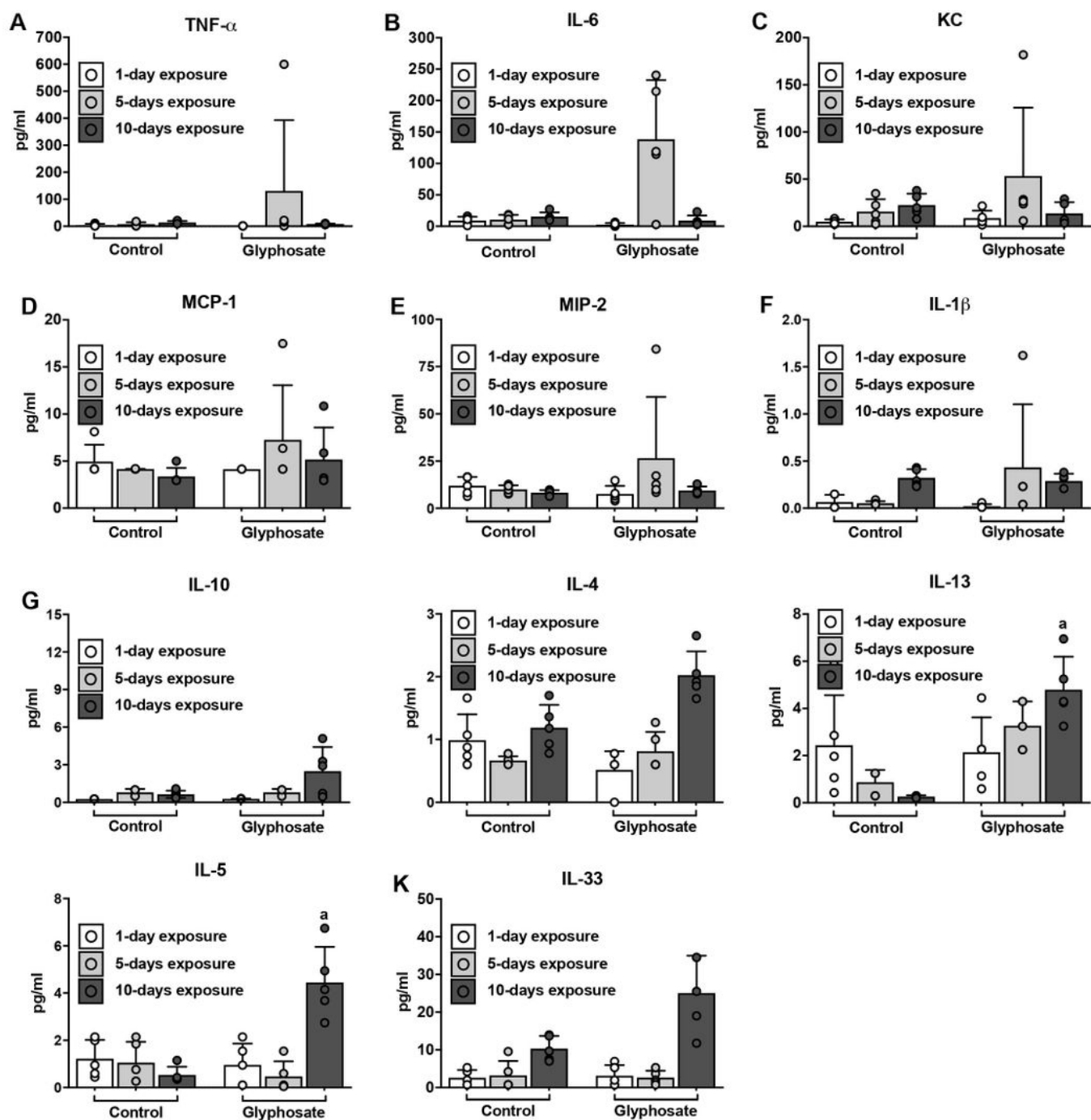


Figure 2

Cytokine levels in supernatant of BAL fluid of mice after exposure to glyphosate or control for 1-day, 5-days, and 10-days (A-K). Cytokine levels were assessed using a Custom Mouse Procartaplex Multiplex

Immunoassay. Data presented as mean \pm SD (N = 5 mice per group). "a" indicates significant difference ($p < 0.05$) compared with control group.

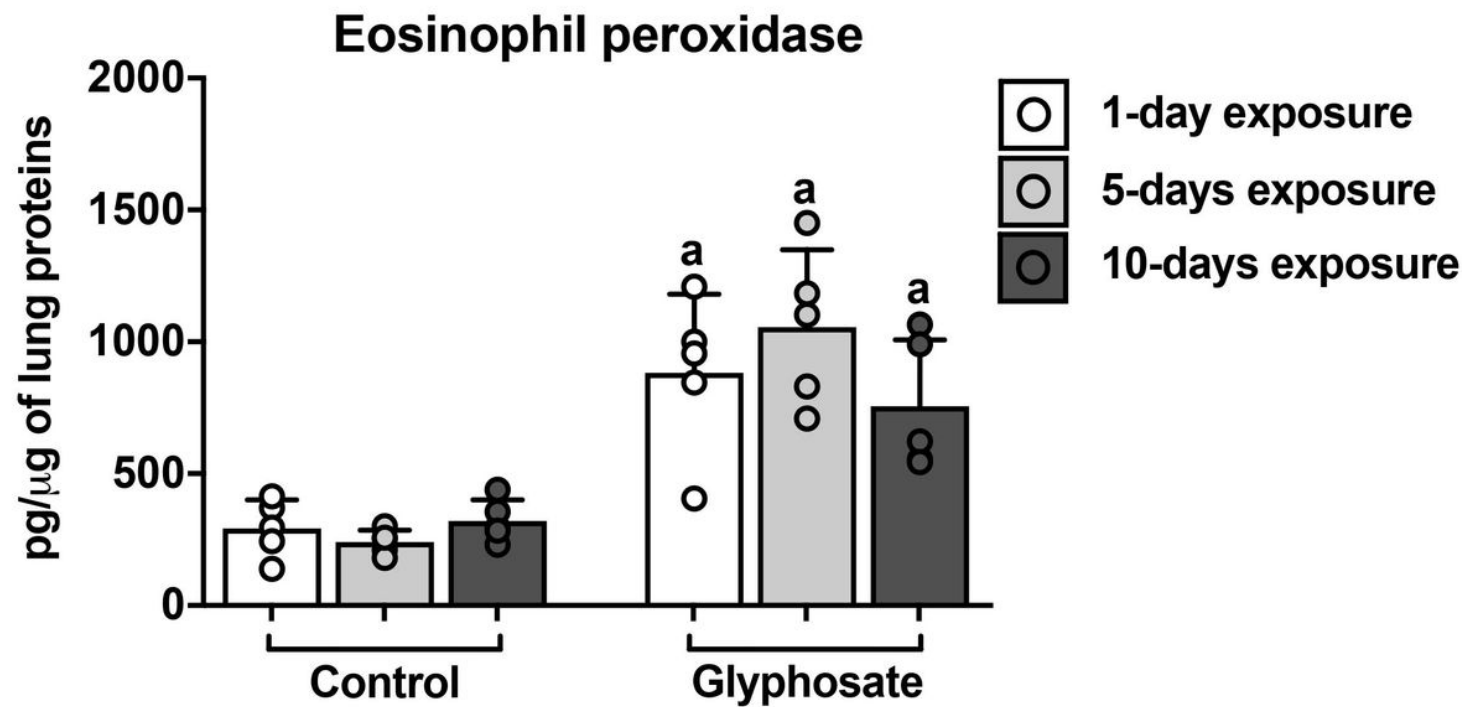


Figure 3

EPO levels in mice lungs after exposure to glyphosate or control for 1-day, 5-days, and 10-days. Lung EPO levels were measured using Mouse Eosinophil Peroxidase ELISA. Data presented as mean \pm SD (N = 5 mice per group). "a" indicates significant difference ($p < 0.05$) compared with control group.

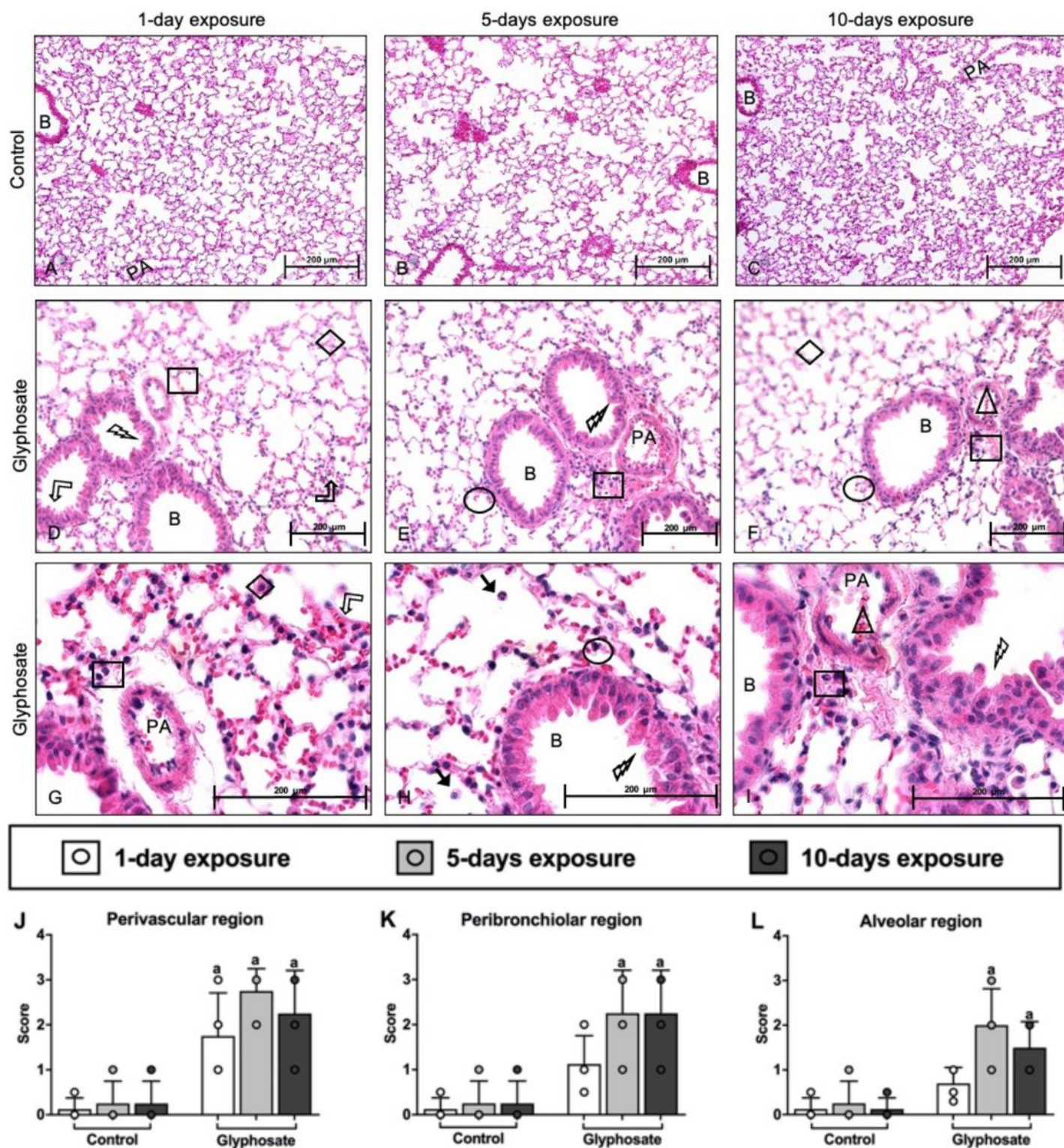


Figure 4

Hematoxylin and eosin stained lung sections of mice after 1-day, 5-days and 10-days of exposure to control (A-C) or glyphosate (D-I) and scoring of lung sections for inflammation (J-L). Group representative images showing perivascular infiltration (square), peribronchiolar infiltration (circle), alveolar infiltration (diamond), perivascular space increase (double arrow), blood vessel congestion (triangle), bronchial epithelium thickness increase (bent up arrow) and sloughing of bronchial epithelial surface (lightning

bolt). Data presented as mean \pm SD (J-L, N = 5 mice per group; 5 fields per section). “a” indicates significant difference ($p < 0.05$) compared with control group. Magnification: $\times 200$ (A-C); $\times 400$ (D-F); $\times 1000$ (G-I). Scale bar: 200 μ m (A-I). PA: Pulmonary artery; B: Bronchus.

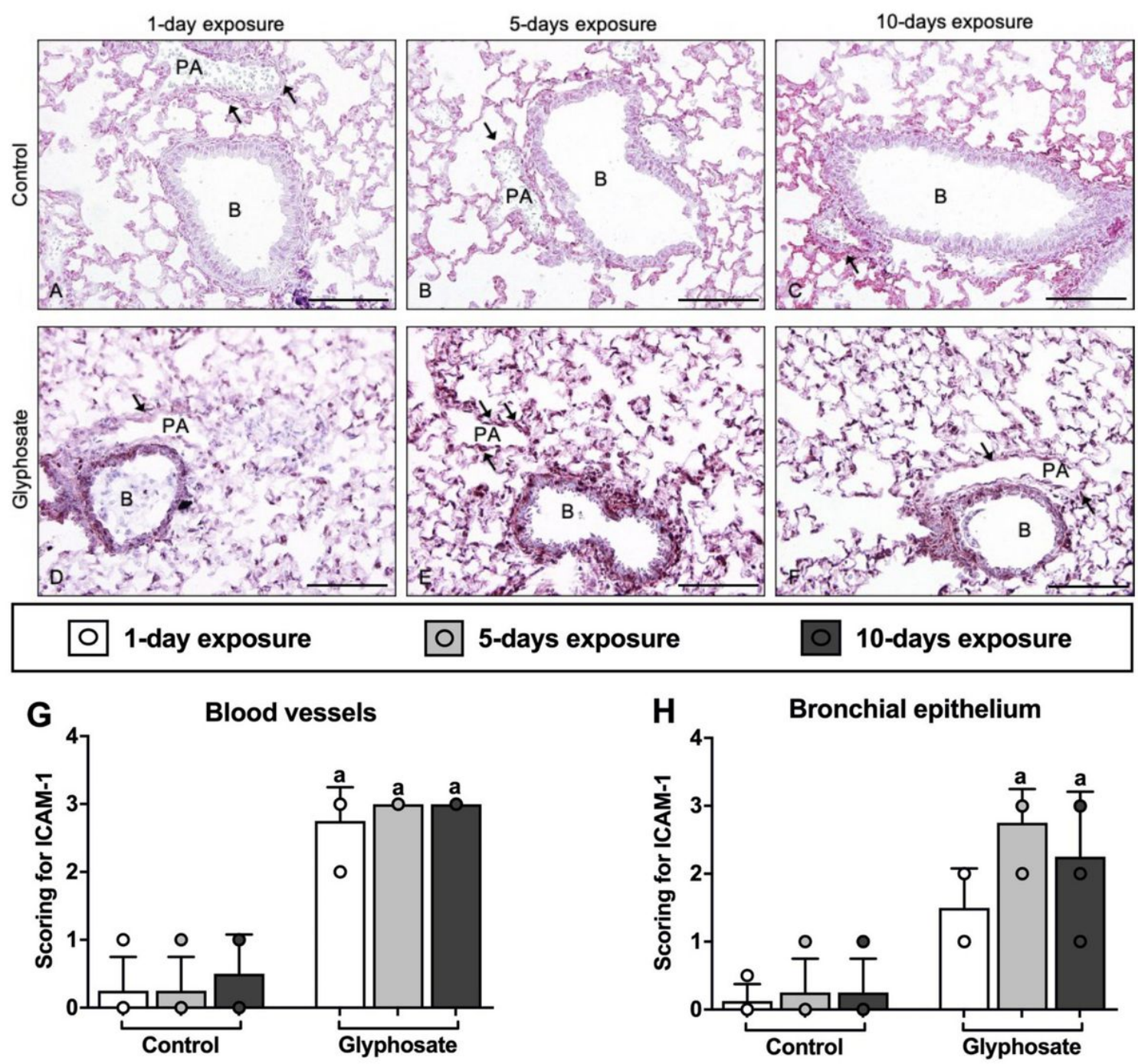


Figure 5

Immunostaining for ICAM-1 in lungs of mice after 1-day, 5-days and 10-days exposure to control (A-C) or glyphosate (D-F) and ICAM-1 scoring (G-H). Group representative images showing ICAM-1 positive staining (arrows). Data presented as mean \pm SD (N = 3 mice per group; 5 random fields per section). “a” indicates significant difference ($p < 0.05$) compared with control group. Magnification: $\times 400$ (A-F). Scale bar: 50 μ m (A-F). PA: Pulmonary artery; B: Bronchus.

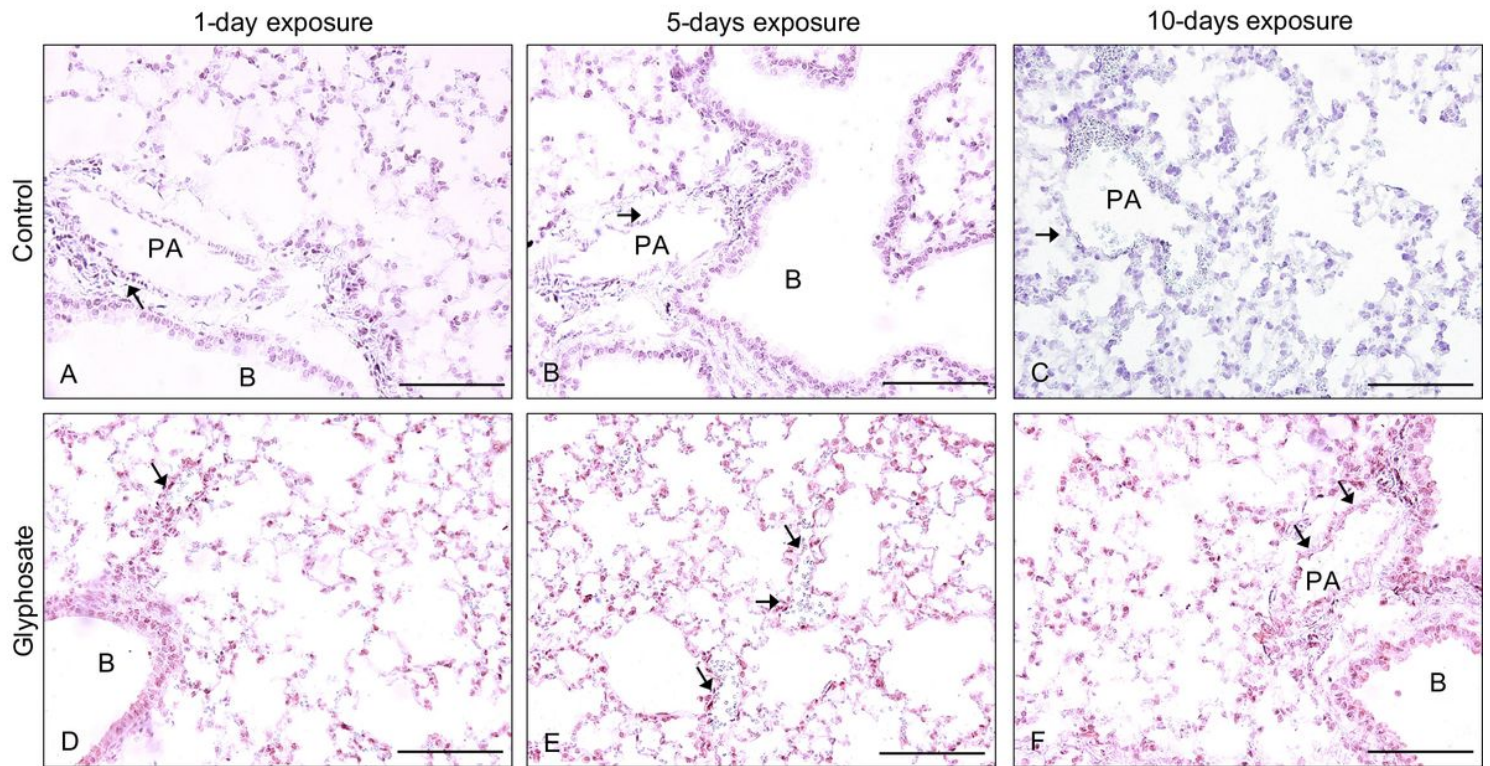


Figure 6

Expression of VCAM-1 in mice lungs after exposure for 1-day, 5-days, and 10-days to control (A-C) or glyphosate (D-F). Group representative images showing VCAM-1 positive staining (arrows; N = 3 mice per group; 5 random fields per section). Magnification: $\times 400$ (A-F). Scale bar: 50 μm (A-F). PA: Pulmonary artery; B: Bronchus.

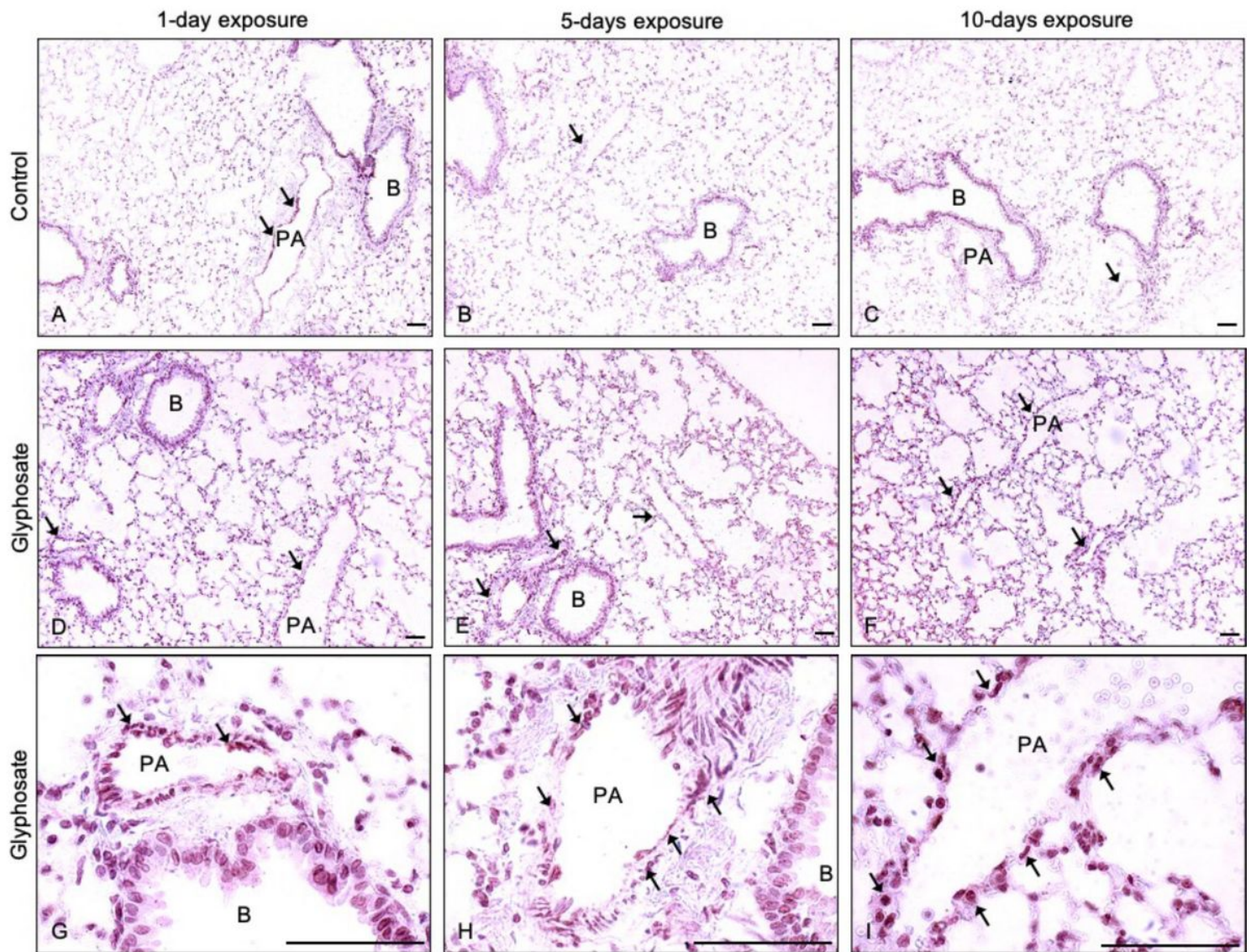


Figure 7

Immunohistochemistry staining for vWF in lungs of mice exposed for 1-day, 5-days, and 10-days to control (A-C) or glyphosate (D-I). Group representative images showing vWF positive staining (arrows; N = 3 mice per group; 5 random fields per section). Magnification: ×400 (A-F). Scale bar: 50 μm (A-F). PA: Pulmonary artery; B: Bronchus.

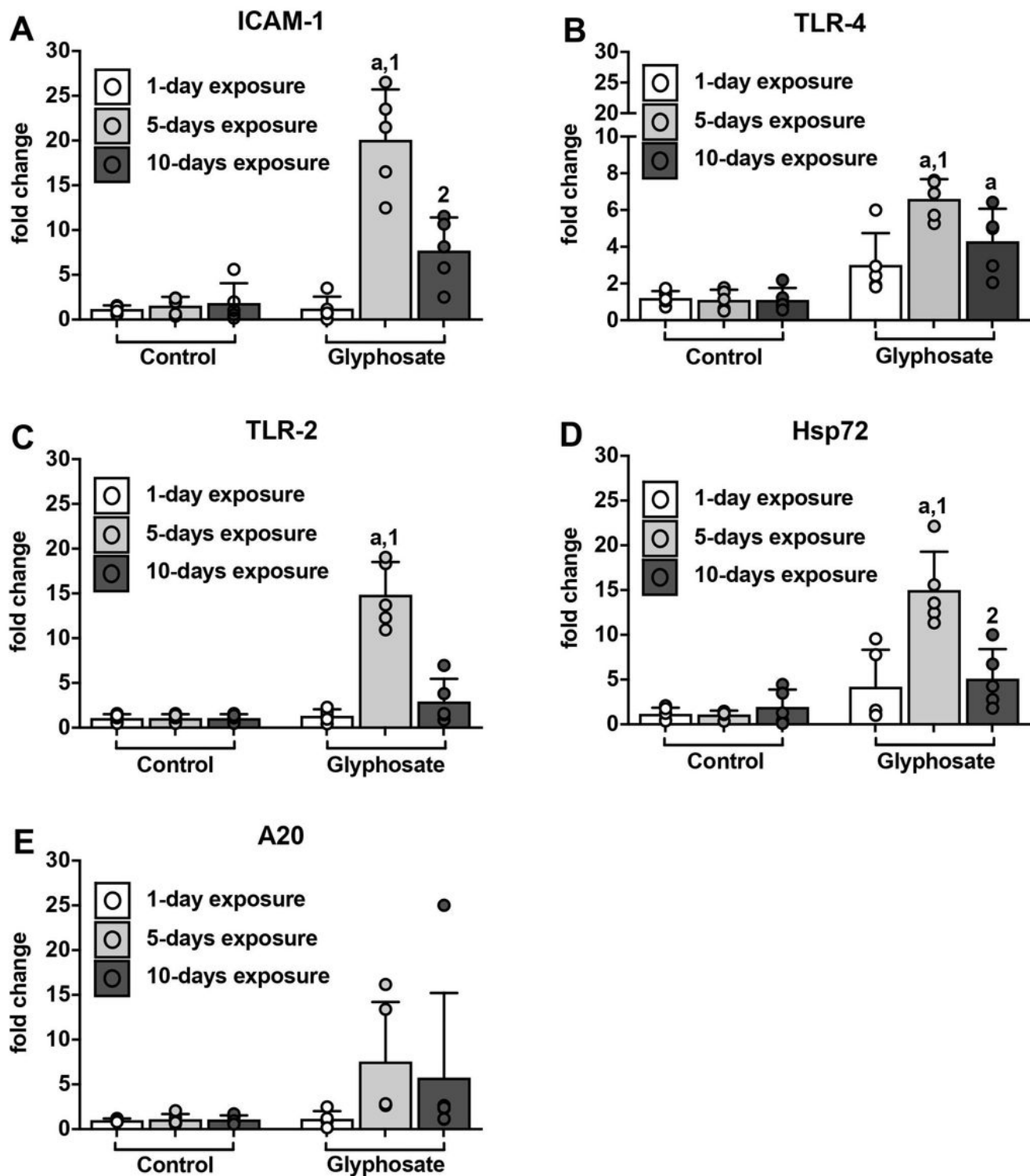


Figure 8

Fold change of ICAM-1 (A), TLR-4 (B), TLR-2 (C), Hsp72 (D), and A20 (E) in lungs from control or glyphosate exposure for 1-day, 5-days, and 10-days. Lung mRNA changes were measured using probes with real-time PCR. Data presented as mean \pm SD (N = 5 mice per group). "a" indicates significant difference ($p < 0.05$) compared with control group; "1" indicates significant difference compared with 1-day exposure group; "2" indicates significant difference compared with 5-days exposure group.

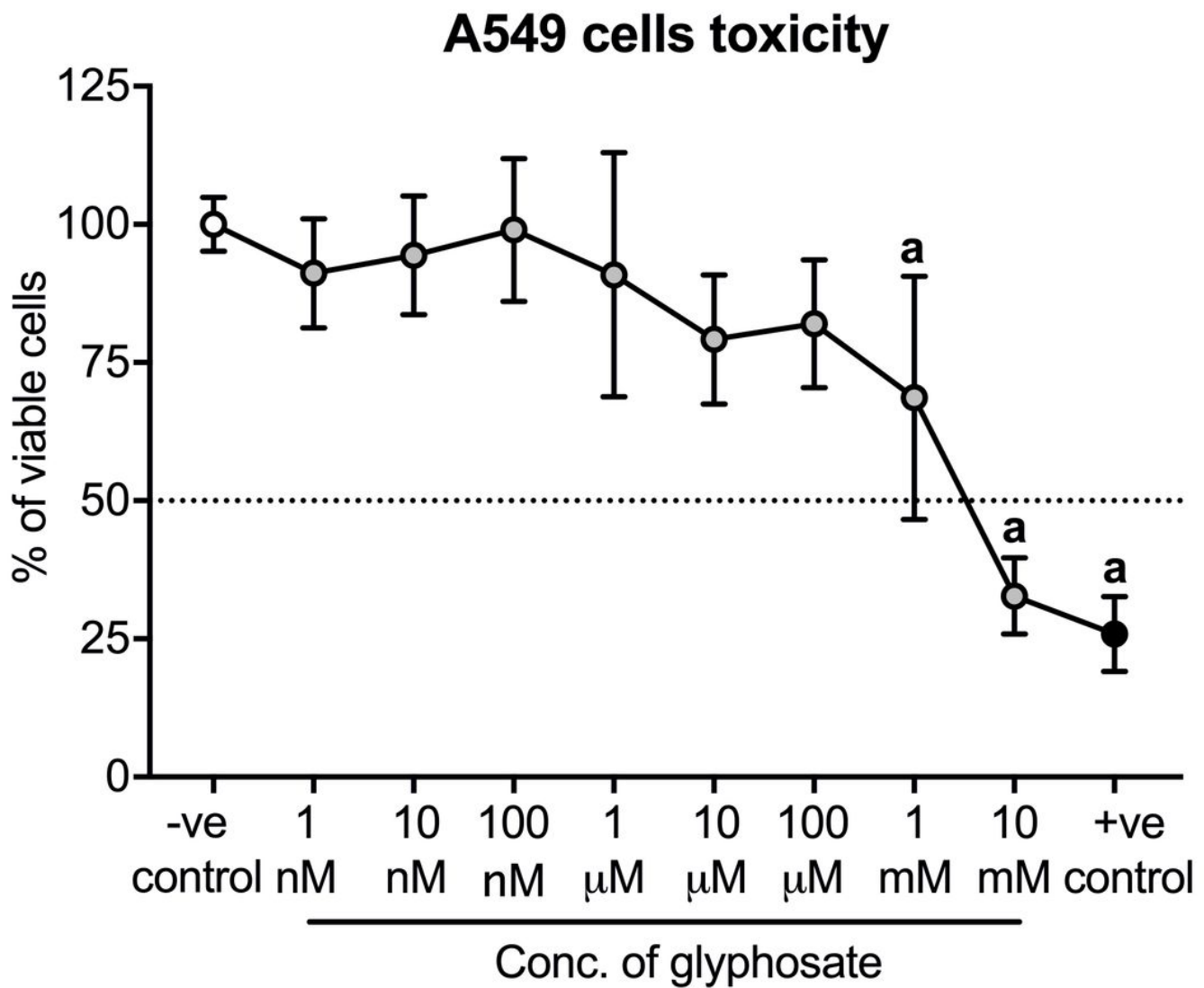


Figure 9

A549 cells viability after treatment with different concentrations of glyphosate. DMSO treated cells act as a positive control. The cell viability was measured using Cell Proliferation Kit 1. A dotted line shows the LD50. Data presented as mean \pm SD of three independent experiments with each treatment performed in duplicate. "a" indicates significant difference ($p < 0.05$) compared with negative control group.