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In vitro study of glyphosate effects on thyroid cells☆

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ABSTRACT

Glyphosate is a pesticide, which contaminates the environment and exposes workers and general population to its residues present in foods and waters. In soil, Glyphosate is degraded in metabolites, amino-methyl-phosphonic acid (AMPA) being the main one. Glyphosate is considered a potential cancerogenic and endocrine-disruptor agent, however its adverse effects on the thyroid were evaluated only in animal models and *in vitro* data are still lacking. Aim of this study was to investigate whether exposure to Glyphosate could exert adverse effects on thyroid cells *in vitro*.

Two models (adherent-2D and spheroid-3D) derived from the same cell strain Fisher-rat-thyroid-cell line-5 (FRTL-5) were employed. After exposure to Glyphosate at increasing concentrations (0.0, 0.1–0.25- 0.5-1.0-2.0–10.0 mM) we evaluated cell viability by WST-1 (adherent and spheroids), results being confirmed by propidium-iodide staining (only for spheroids). Proliferation of adherent cells was assessed by crystal violet and trypan-blue assays, the increasing volume of spheroids was taken as a measure of proliferation. We also evaluated the ability of cells to form spheroids after Glyphosate exposure. We assessed changes of reactive-oxygen-species (ROS) by the cell-permeant H2DCFDA. Glyphosate-induced changes of mRNAs encoding for thyroid-related genes (TSHR, TPO, TG, NIS, TTF-1 and PAX8) were evaluated by RT-PCR.

Glyphosate reduced cell viability and proliferation in both models, even if at different concentrations. Glyphosate at the highest concentration reduced the ability of FRTL-5 to form spheroids. An increased ROS production was found in both models after exposure to Glyphosate. Finally, Glyphosate increased the mRNA levels of some thyroid related genes (TSHR, TPO, TG and TTF-1) in both models, while it increased the mRNAs of PAX8 and NIS only in the adherent model.

The present study supports an adverse effect of Glyphosate on cultured thyroid cells. Glyphosate reduced cell viability and proliferation and increased ROS production in thyroid cells.

1. Introduction

Glyphosate is a broad-spectrum herbicide employed in agricultural and aquatic environments, as well as in weeding carried out in gardens and lawns almost all over the world (Meftaul et al., 2020). Glyphosate displays its herbicide action interfering with the shikimic acid pathway (Amrhein et al., 1980; Schönbrunn et al., 2001) by binding and inhibiting the enolpyruvylshikimate-3-phosphate synthase (EPSP), an enzyme exclusively found in plants. Glyphosate, however, also impacts non-target living organisms in which EPSP is not present (Strilbyska et al., 2022; Wang et al., 2022). This effect raised concerns because of potential hazards for other organisms. Exposure to Glyphosate in humans may occur due to occupational handling and/or through contaminated food or water (Gillezeau et al., 2019). Indeed, this herbicide was detected in 60–80% of the general population, being recovered in human urine, blood, and maternal milk, (Acquavella et al., 2004; Fernandes et al., 2019; Gillezeau et al., 2019; Peillex and Pelletier, 2020; Solomon, 2016; Soukup et al., 2020). The half-life of Glyphosate in soil was estimated to range from 2 to 197 days, but it may also persist for several months (Giesy et al., 2000; Laitinen et al., 2009), largely

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depending on weather conditions, such as the amount of rain and temperature. The compound is degraded to several metabolites, among which aminomethylphosphonic acid (AMPA), the main metabolite of Glyphosate, usually co-occurs with it (Al-Rajab and Schiavon, 2010; von Mérey et al., 2016; Wang et al., 2022)). Similarly, AMPA is detected in human urine (Buekers et al., 2022; Connolly et al., 2020; Soukup et al., 2020), serum (Yoshioka et al., 2011) and hair (Alvarez et al., 2022). Toxicokinetic studies of Glyphosate in rats showed an oral bioavailability around 23.21%, and after the ingestion of a single large dose of glyphosate (100 mg/kg bw), small amounts of AMPA were detected in plasma (6.49% of the parent plasma concentration)(Anadón et al., 2009; Bernal et al., 2010) with a recovered maximum plasma concentrations of 4.62 and 0.416 μg/ml of glyphosate and AMPA respectively (Anadón et al., 2009). Pharmacokinetics studies in laboratory animals suggest that a bioaccumulation of Glyphosate does occur in kidney and liver (Myers et al., 2016). In humans, the toxicokinetic of Glyphosate was poorly explored, but small amounts of AMPA (15.1 μg/ml) were detected in the serum after deliberate Glyphosate poisoning (Motojyuku et al., 2008). The excretion of both Glyphosate was assessed by few studies reporting excretion half-life of 9 h (Zoller et al., 2020), 5.5–10 h (Connolly et al., 2020); 6–9 h or 18–33 h (Faniband et al., 2021); being the excretion fraction of Glyphosate (as unchanged molecule) in human urine as low as 1%(Connolly et al., 2020; Faniband et al., 2021; Zoller et al., 2020). Recent data suggest that in humans the metabolism of Glyphosate to AMPA is negligible, and the small amount of AMPA detected in urine is likely to derive from direct ingestion of AMPA via its residues in food (Huch et al., 2022; Kohsuwan et al., 2022; Zoller et al., 2020). Increased risk of cancer, endocrine-disrupting effects, as well as several other adverse effects for human health were potentially attributed to Glyphosate (Romano et al., 2021; Tarazona et al., 2017) and to its metabolite AMPA(Eaton et al., 2022). However, regulatory agencies considered the current evidence insufficient to consider Glyphosate as an endocrine-disrupting chemical or a carcinogenic agent (A, 2019; EFSA, 2019). For this reason, Glyphosate is at present classified as "probably carcinogenic to humans" (Group 2A) by the International Agency for Research on Cancer (IARC/WHO) (IARC, 2015; Tarazona et al., 2017). On May 2022, the Risk Assessment Committee of the European Chemical Agency (ECHA) stated that the available scientific evidence did not meet the criteria to classify Glyphosate for specific target organ toxicity, or as a carcinogenic, mutagenic or reprotoxic substance (ECHA, 2022).

Several studies recognized that pesticides are potential thyroid-axis disrupting compounds (Kongtip et al., 2019; Shrestha et al., 2018). However, the current knowledge on the effects of Glyphosate on thyroid function is poor, being the results obtained in animal models inconsistent and epidemiological data still limited. Indeed, previous studies on amphibians showed no toxicity of Glyphosate for the hypothalamus-pituitary-thyroid (HPT) axis (Howe et al., 2004), a decrease in TRHβ expression (Lanctôt et al., 2014) and increase of deiodinases (Navarro-Martín et al., 2014). On the other hand, studies in rats and mice suggested an interference of Glyphosate with HPT axis, however showing different results including unmodified (Phusate, 2021; T, 2021; Phusate, 2021; Vaishali, 2021), higher (Hamdaoui et al., 2020); (Manservisi et al., 2019), or decreased (de Souza et al., 2017; Zhang et al., 2021) TSH circulating levels after exposure to Glyphosate. In humans, a prospective cohort study concluded that the risk of developing hypothyroidism was greater in subjects self-reporting the use of Glyphosate (Shrestha et al., 2018). This assumption was not shared by other investigators who analyzed data obtained in the same cohort study (Goldner et al., 2013). Even less explored are the effects of Glyphosate at the cellular level. In 2015, the EFSA declared that there is no straightforward evidence sustaining cytotoxicity and genotoxicity of Glyphosate, thus *in vitro* studies on different type of cells are required to better clarify the issue ((EFSA), 2015). Since then, a few studies showed that Glyphosate can be cytotoxic in several types of cells, and other *in vitro* adverse effects were described (Kwiatkowska et al., 2016; Kwiatkowska et al., 2017; Martínez et al., 2007; Nagy et al., 2019; Santovito et al., 2018). However, no information is available on thyroid cells. Based on the results obtained in animal models, which suggested a possible perturbation of the thyroid axis by Glyphosate, our hypothesis is that Glyphosate could *in vitro* disrupt thyroid cells similarly to what reported for other cell types. Moreover, we also hypothesized that the exposure to Glyphosate could modify the expression of some genes, which are critical for thyroid development and function.

Thus, aim of the present study was to investigate the potential adverse effects of Glyphosate on thyroid cells being cultured both in two-dimensional (2D, i.e. adherent) and three-dimensional (3D, i.e. floating) conditions. After exposure to Glyphosate, we evaluated cell viability, proliferation, production of reactive oxygen species (ROS) and modulation of the mRNAs encoding for thyroid-related genes. This novel information could be the starting point for subsequent mechanistic studies.

2. Materials and methods

2.1. Cultures of thyroid cells

The Fisher-Rat-Thyroid cell Line-5 (FRTL-5) (ATCC CRL 8305, F1 subclone) was used for the present study. This is a well characterized thyroid cell line that expresses most of the tissue-specific functions of thyroid follicular cells. Among these functions FRTL-5 cells synthetize and secrete thyroglobulin, actively transport iodine and display numerous cell functions, which, as it occurs for *in vivo* thyroid cells, are dependent on TSH stimulation (Meli et al., 1999). Cells were grown for one week in a medium (Coon's Modified Ham's F-12 Medium (Sigma Chemical Co.) enriched with a hormone mixture containing TSH, somatostatin, insulin, transferrin, hydrocortisone and glycyl-histidyl-lysine (Sigma Chemical Co.) (Thomasz et al., 2015). This medium was also supplemented with adult calf serum a 5% concentration and Gentamicin (BioWhittaker, Inc., Walkersville, MD).

2.2. Viability assay using tetrazolium dye WST-1

Thyroid cells were seeded at 2×10^3 cell/well concentration in flat-bottom 96-well plates. After adherence, we treated FRTL-5 cells with Glyphosate at increasing concentrations 0; 0,1; 0,25; 0,5; 1; 2; 10 mM, which derived from previous studies performed on other cell types. (Kwiatkowska et al., 2016; Kwiatkowska et al., 2017; Martínez et al., 2020; Nagy et al., 2019). Moreover, we took into account that acceptable daily intake (ADI) of 0.5 mg/kg was considered by EFSA corresponding to 0.500 μg/mL (3 mM) (EFSA, 2015; Santovito et al., 2018). Thus, we designed the Glyphosate exposure range according to both previous experience in other types of cells and the maximal acceptable daily intake. Also considered were the IDs 50 of Glyphosate for hygromycin-B resistant plants (1.70 ± 0.03 mM) and for control plants (0.45 ± 0.02 mM) (Peñaloza-Vázquez et al., 1995).

Glyphosate was purchased from Sigma Aldrich (CAS number 1071-83-6; molecular weight 169.07 mol/L; molecular formula: (HO)2P(O) CH2NHCH2CO2H) and dissolved in water. Fig. 1 shows the chemical structure of the compound. We assessed FRTL5 cells viability after exposure to Glyphosate for 24, 48 and 72 h. Water-soluble tetrazolium salt (WST-1) was used to evaluate cell viability as previously reported (Coperchini et al., 2020). The colorimetric agent WST-1 reacts with mitochondrial dehydrogenases, resulting in the production of formazan by viable cells only. We measured the amount of formazan produced by

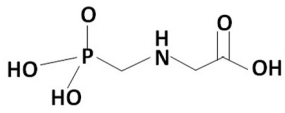

Fig. 1. Chemical structure of Glyphosate.

absorbance, using a multimode plate reader (Victor NIVO Multimode Plate Reader, PerkinElmer) at 450 nm. All experiments were performed in triplicates.

2.3. Evaluation of cell proliferation

We seeded FRTL5 cells at a density of 3000 cells per well in 96 flat-well plates. After adhesion, we exposed FRTL-5 cells to increasing concentrations of Glyphosate (0.0, 0.1, 0.25, 0.5, 1.0, 2.0, 10.0 mM) for 24, 48 and 72 h. At the end of the incubation, we fixed FRTL-5 cells with cold methanol (100% concentration for 20 min) and then stained them with crystal-violet dye (0.5%) for 5 min. Afterwards, we washed wells with deionized water and incubated the stained cells with Sodium dodecyl sulfate (SDS (1% concentration for 2 h). SDS released crystal violet from cells, which was then measured at an optical density (OD) of 570 nm (Crowley et al., 2016). All experiments were performed in triplicates.

2.4. Trypan blue assay for cell proliferation

Trypan blue exclusion test (TB) was used to confirm results of crystal violet assay. After treatments, FRTL-5 cells were detached with trypsin, centrifuged and, 10 μl of the TB dye was mixed with 10 μl of cell suspension. An inverted microscope Olympus BX51 (Olympus, Deutschland GmbH, Hamburg) was used to evaluate differences in cell proliferation among treated cells counted by.Burker chamber. The proliferation was expressed as a percentage of untreated cells that were arbitrarily considered as 100% (da Silva et al., 2020). All experiments were performed in triplicates.

2.5. Formation of spheroids

Spheroids were generated based on the method of Kelm et al. with some modifications (Kelm et al., 2003). Agarose was dissolved in PBS at a 1% concentration, heated in a microwave, and then 200 μl of the solution were dispensed in each well of a 96- round-bottom-well plate. After a few seconds we removed the agarose solution and plates were left to dry for at least 1 h. Cultures of 3D spheroids were established starting from a dispersed cell suspension, which was obtained by trypsinizing monolayer cultures of FRTL-5 cells. Then, 200 μL of the cell suspension (5000 cells/well) were dispensed to each agarose-coated well. Cells were monitored daily and after four days the formation of spheroids was confirmed by microscopy. A digital camera-equipped inverted microscope (Olympus IX83, Olympus Corporation, Tokyo, Japan, plus digital camera Nikon Instruments) was used to capture phase-contrast pictures and to evaluate the morphology of spheroids.

2.6. Spheroids viability assessment

The viability of spheroids was assessed by WST-1 after exposure to increasing concentrations of Glyphosate (0; 0.1; 0.25; 0.5; 1.0; 2.0; 10.0 mM) for 72 h. In this cell viability test, 100 μl of cell culture medium were carefully removed from each well (without touching spheroids) and substituted for by 100 μl of culture medium containing Glyphosate at the desired concentration. After 72 h of incubation, 20 μl of the WST-1 reagent were added to each well and a microplate reader at the OD of 450 nm was used to measure the amount of formazan produced (Victor nivo PerkinElmer). All experiments were performed in triplicates.

2.7. Propidium Iodide (PI) staining for the detection of cell death

After exposure to Glyphosate, FRTL-5 spheroids were stained with PI-Hoechst to detect died cells. After Glyphosate exposure at the desired concentrations, DMSO (10%) was used as positive control. A mix of Hoechst 33,258 + 20 μg/ml PI (Thermofisher) was used to stain spheroids at room temperature for 10 min in the dark. Spheroids' pictures were obtained by a florescent microscope. (Olympus IX83).

2.8. Evaluation of the spheroid volume

Spheroids were exposed to Glyphosate (0; 1.0; 2.0; 10.0 mM) for 3 different incubation periods (24, 48 and 72 h). Pictures to analyze the spheroid size were obtained using an Olympus IX83 microscope equipped with a digital camera. We measured the area (A) and the perimeter (P) of spheroids using the CellSens software. For calculating the shape factor describing sphericity of aggregates, measured parameters were exported to MS EXCEL. The following formula: $V = [(a^2)x\ (b)]/2$, (a = minor diameter; b = major diameter) was used to assess the spheroid volume (V). Spheroid volume was then expressed as a ratio of control being considered as 1 (no Glyphosate in the culture medium) (Lazzari et al., 2018). All experiments were performed in triplicates.

2.9. Effect of Glyphosate on the formation-time of spheroids

To assess whether cell exposure to Glyphosate might affect the formation of spheroids, FRTL-5 cells were seeded in 96 agarose-coated and incubated with increasing concentrations of Glyphosate. After 4 days of culture, we observed cells at the microscope and the sphericity index (SI) was calculated according to the formula $SI = \frac{\pi\sqrt{4A/\pi}}{P}$. Spheroids with an SI $\geq$ 0.90 were considered spherical (Zanoni et al., 2016). All experiments were performed in triplicates.

2.10. Detection of reactive oxygen species (ROS) production

To assess the production of reactive oxygen species (ROS) by FRTL-5, we used the cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) (Sigma Aldrich). At the end of the incubation period H2DCFDA was added for 45 min. Positive control cultures were treated with H_2O_2. Cells were then washed with PBS and fluorescence was measured by a microplate reader (492-495exc/517–527 em) (Victor nivo PerkinElmer). ROS production was calculated as a percentage of the mean Fluorescence Intensity (FI) compared with that of untreated cells, which were arbitrarily taken as 100%. All experiments were performed in triplicates.

2.11. Real time PCR

2D and 3D FRTL-5 cells were incubated with Glyphosate at the non-cytotoxic concentration of 0.5 mM for 24 h. The RNA was extracted from both adherent and spheroid FRTL-5 cells by using a commercial kit for RNA extraction (Norgen Biotek, Canada). cDNA was then reverse transcribed by a SensiFast c-DNA synthesis kit (Bioline, London, UK). The sensi-Fast SYBR Green Hi-ROX kit (Bioline, London, UK) was employed to perform RT-PCRs that were run on a StepOne Plus Applied Biosystems real-time PCR system (Thermofisher). Pre-designed primers targeting rat Thyroglobulin (Tg: ENSRNOG00000006104.7, F: acgatgggcttatcaacagg; R: atatggcagcagcaaggatg), thyroid-stimulating-hormone receptor (TSH-R: ENSRNOT00000005671.4, F: aggacatggtgtgtaccccc; R: aatctgcaaaggc-caggttg), thyroid transcription factor gene Pax8 (Pax8: ENSR-NOT00000031058.6, F: ggccaccaaatctctgagcc; R: tgggaatcgatgctcagtcg), TTF-1 (TTF-1: ENSRNOT00000011453.5, F: ggacgtgagcaagaacatgg; R: gggtgtcaggtgaatcatgc) NIS (NIS: ENSRNOG00000018822, F: tgcaccttgta-cactaccgt; R: ccgaggatcagggtcaaagt), TPO (TPO: ENSRNOG00000004646, F: ccacaattgccaacctgtca; R: tgggctgactgaaaccatct) and the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH: ENSR-NOT00000050443.4, F: aacgacccctcattgacct; R: ccccatttgatgttagcggg) were obtained from Biomers.net GMBH (Soflinger, Germany). Primers were chosen based on previously published studies (Coperchini et al., 2020). GAPDH was taken as the reference gene. We chose GAPDH because it is one of the most used reference genes. Moreover, in previous studies on FRTL-5 cells we found that GAPDH provides reproducible results after

toxic treatment (Coperchini et al., 2020). Data were normalized for GAPDH and analyzed using the ΔΔCt method. All experiments were performed in triplicates.

2.12. Statistical analysis

We used the SPSS (SPSS, Inc., Evanston, IL) software to perform statistical analysis. To test the potential dose-dependent effect of Glyphosate on FRTL-5 cells within a specific time point, results of viability and proliferation assays were entered into separate analyses of variance (one-way ANOVA) for the comparison of Mean group values. *Post hoc* analysis was then performed (*p* value was assigned according to the Dunnett's correction for multiple comparisons). ANOVA for repeated measures was employed among the three time points on which cells were exposed to increasing concentrations of Glyphosate. If the Glyphosate effect differed significantly between the various time points, *post hoc* comparisons were performed by Bonferroni's correction. For genes analysis, Student *t*-test for paired and unpaired data was used. Values are reported as median and 25th and 75th percentiles. Statistically significance was given considering a p value < 0.05. Figures were drawn as box plots according to Weissgerber et al. (2015).

3. Results

3.1. Effect of Glyphosate exposure on the viability of FRTL-5 cells (2-D model)

Adherent FRTL-5 cells were exposed to increasing concentrations of Glyphosate for 24, 48 and 72 h. The viability of FRTL5 was significantly reduced by Glyphosate already after 24 h (Fig. 2, T1) (ANOVA; $F = 25.328$; $p < 0.0001$, *Post Hoc* analysis by Dunnett $p < 0.05$ for 2 mM and 10.0 mM Glyphosate *vs.* 0.0 mM). A significant reduction of the cell viability was also observed starting from 2 mM Glyphosate both at 48 (ANOVA: $F = 12.196$; $p < 0.0001$; *Post Hoc* analysis by Dunnett $p < 0.05$ for Glyphosate 2.0 and 10.0 mM vs. 0.0 mM), and 72 h (ANOVA: $F = 13.961$; $p < 0.0001$; Post Hoc analysis by Dunnett $p < 0.05$ for Glyphosate 2.0 and 10.0 mM vs. 0.0 mM) (Fig. 2, T2 and T3, respectively). To assess whether the magnitude of the effect of Glyphosate was dependent upon the exposure time, results of the cell viability assay were compared by ANOVA for repeated measures for all the concentrations of Glyphosate at 24, 48 and 72 h. No significant effect of the time*-CONCENTRATIONS was found on cell viability after exposure to Glyphosate (ANOVA for repeated measures: Greenhouse-geisser $F = 1.093$; $p = 0.373$). Since the WST-1 assay was employed for assessing cell viability, this result also implies that Glyphosate induces a significant decrease in mitochondrial activity in FRTL-5 cells, as compared to untreated controls.

3.2. Effect of Glyphosate exposure on the proliferation of adherent FRTL-5 cells (2-D model)

After exposure to increasing concentrations of Glyphosate, cell proliferation was assessed after 24, 48 and 72 h by crystal violet staining. While after 24 h no change in the FRTL-5 cell proliferation rate was found (ANOVA; $F = 1.659$ $p = 0.147$) (Fig. 3, Panel A, T1), a reduction in cell proliferation was observed in cells exposed to the highest concentration of Glyphosate 10.0 mM after both 48 h (ANOVA $F = 7.417$ $p < 0.05$; Post Hoc analysis by Dunnett $p < 0.05$ for 10.0 mM Glyphosate vs. 0.0 mM) and 72 h (ANOVA: $F = 19.520$ $p < 0.05$; *Post Hoc* analysis by Dunnett $p < 0.05$ Glyphosate 10.0 mM vs. 0.0 mM) (Fig. 3, Panel A, T2 and T3, respectively). As performed for the cell viability assay, ANOVA for repeated measures was employed to assess a possible effect of time on the proliferation rate after treatment with Glyphosate. Thus, results of proliferation assay were compared for all the concentrations of Glyphosate at 24, 48 and 72 h. Significant differences were observed among the three times of exposure to Glyphosate in terms of reduction of cell proliferation. A significant interaction of time*CONCENTRATIONS was observed (ANOVA for repeated measures, Greenhouse-Geisser: $F = 3.974$, $p < 0.001$; Post Hoc by Bonferroni $p < 0.05$, for 72 and 48 h *vs.* 24 h) showing a greater increase of reduction in cell proliferation at 72 h of exposure.

The Trypan blue assay confirmed the results obtained with crystal violet by showing no reduction in cell proliferation after 24 h (ANOVA; $F = 0.409$; $p = 0.865$) (Fig. 3, Panel B, T1) and a significant reduction of FRTL-5 cells proliferation at the highest concentration of Glyphosate (10 mM) after both 48 (ANOVA: $F = 11.318$, $p < 0.05$; *Post Hoc* analysis by Dunnett $p < 0.05$ for 10.0 mM Glyphosate vs. 0.0 mM); and 72 h only (ANOVA: $F = 10.699$; $p < 0,05$ *Post Hoc* analysis by Dunnett $p < 0.05$ for

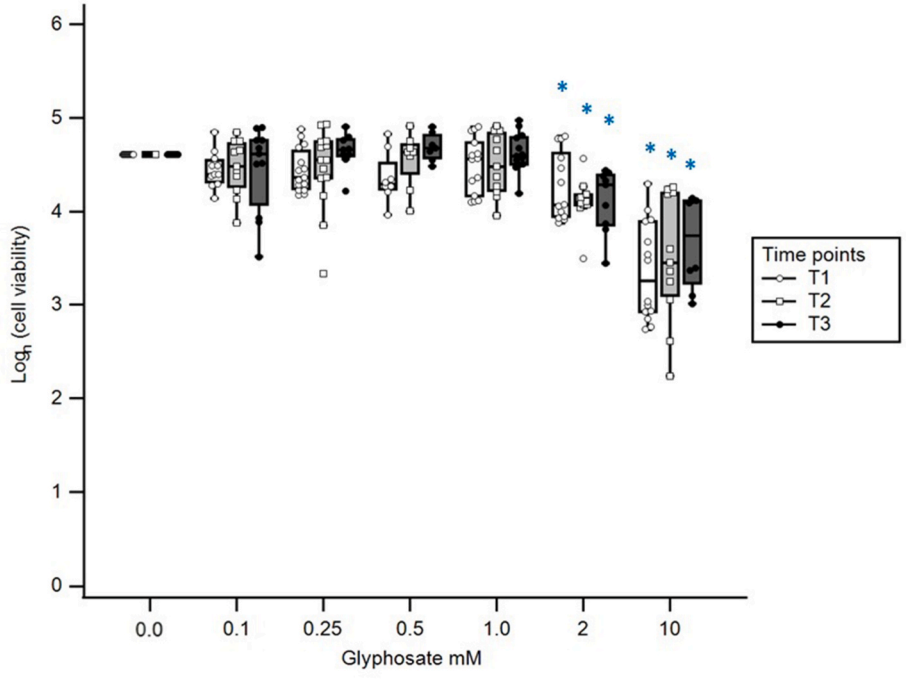

Fig. 2. Effect of Glyphosate on viability of FRTL-5 (Adherent bi-dimensional model).Reduction of FRTL-5 adherent cells viability after exposure to increasing concentrations of Glyphosate for 24, 48 and 72 h. T1: reduction of cell viability after 24 h starting from Glyphosate 2.0 mM (ANOVA; $F = 25.328$; $p < 0.0001$, Post Hoc analysis by Dunnett $p < 0.05$ for Glyphosate 2.0 and 10.0 mM vs. 0.0 mM). T2: reduction of cell viability after 48 h starting from Glyphosate 2.0 mM (ANOVA: $F = 12.196$; $p < 0.0001$; Post Hoc analysis by Dunnett $p < 0.05$ Glyphosate 2.0 and 10.0 mM vs. 0.0 mM). T3: reduction of cell viability after 72 h starting from Glyphosate 2.0 mM ANOVA: $F = 13.961$; $p < 0.0001$; Post Hoc analysis by Dunnett $p < 0.05$ Glyphosate 2.0 and 10.0 mM vs. 0.0 mM). No significant differences were observed between T1, T2 and T3 (ANOVA for repeated measures: Greenhouse-geisser $F = 1.093$; $p = 0.373$).Data are expressed as natural logarithm (Log_n) of ODs of treated cells calculated on the OD of untreated samples (Glyphosate 0.0 mM) considered as 100%. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. Superimposed dots indicate the univariate scatterplot of data * Post Hoc analysis by Dunnett $p < 0,05$.

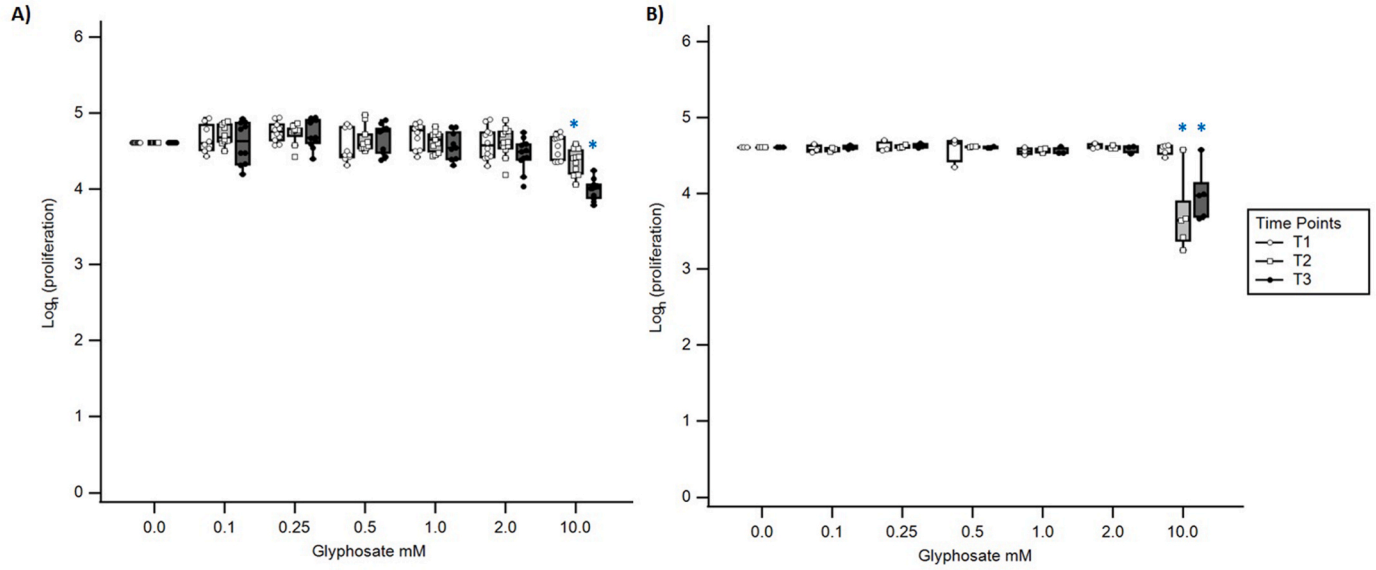

Fig. 3. Effects of Glyphosate on the proliferation of FRTL-5(Adherent bi-dimensional model).Panel A) Results of crystal violet assay. T1: no changes in the FRTL-5 proliferation rate was observed after 24 h (ANOVA: $F = 1.659$; $p = 0.147$). T2: after 48 h a reduction in cell proliferation was observed in cells exposed to Glyphosate 10.0 mM (ANOVA: $F = 7.417$; $p < 0.05$; Post Hoc analysis by Dunnett $p < 0.05$ Glyphosate 10.0 mM vs. 0.0 mM). T3: the proliferation rate was reduced also after 72 h of exposure to Glyphosate (ANOVA: $F = 19.520$; $p < 0,05$; Post Hoc analysis by Dunnett $p < 0.05$ Glyphosate 10.0 mM vs. 0.0 mM). The comparison of the effect among T1 and T2 and T3 showed significant differences in terms of reduction of cell proliferation observing a significant interaction of time*CONCENTRATIONS (ANOVA for repeated measures, Greenhouse-geisser: $F = 3.974$, $p < 0.001$; Post Hoc by Bonferroni $p < 0.05$ 72 h vs. 24 and 48 h). Panel B) Results of Trypan blue assay. T1) after 24 h no reduction in cell proliferation was observed (ANOVA: $F = 0.409$; $p = 0.865$). T2: after 48 h a significant reduction of FRTL-5 cells proliferation was showed for Glyphosate 10.0 mM (ANOVA: $F = 11.318$; $p < 0.05$; Post Hoc analysis by Dunnett $p < 0.05$ Glyphosate 10.0 mM vs. 0.0 mM). T3) the proliferation rate was reduced after 72 h by Glyphosate 10.0 mM (ANOVA: $F = 10.699$; $p < 0.05$ Post Hoc analysis by Dunnett $p < 0.05$ Glyphosate 10.0 mM vs. 0.0 mM). The comparison of the effect among T1, T2 and T3 showed a significant interaction of time*CONCENTRATIONS (ANOVA for repeated measures: Greenhouse-geisser $F = 3.260$; $p < 0.05$).Data are expressed as natural logarithm (Log$_n$) of ODs of treated cells calculated on the OD of untreated samples (Glyphosate 0.0 mM) considered as 100%. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. Superimposed dots indicate the univariate scatterplot of data * Post Hoc analysis by Dunnett: $p < 0,05$ vs. Glyphosate 0.0 mM.

10.0 mM Glyphosate vs. 0.0 mM) (Fig. 3, Panel B, T2 and T3 respectively). ANOVA for repeated measures confirmed results of the crystal violet assay showing a significant interaction of time*CONCENTRATIONS (ANOVA for repeated measures: Greenhouse-geisser $F = 4.524$ $p < 0.05$; Post Hoc by Bonferroni $p < 0.05$, for 72 and 48 h vs. 24).

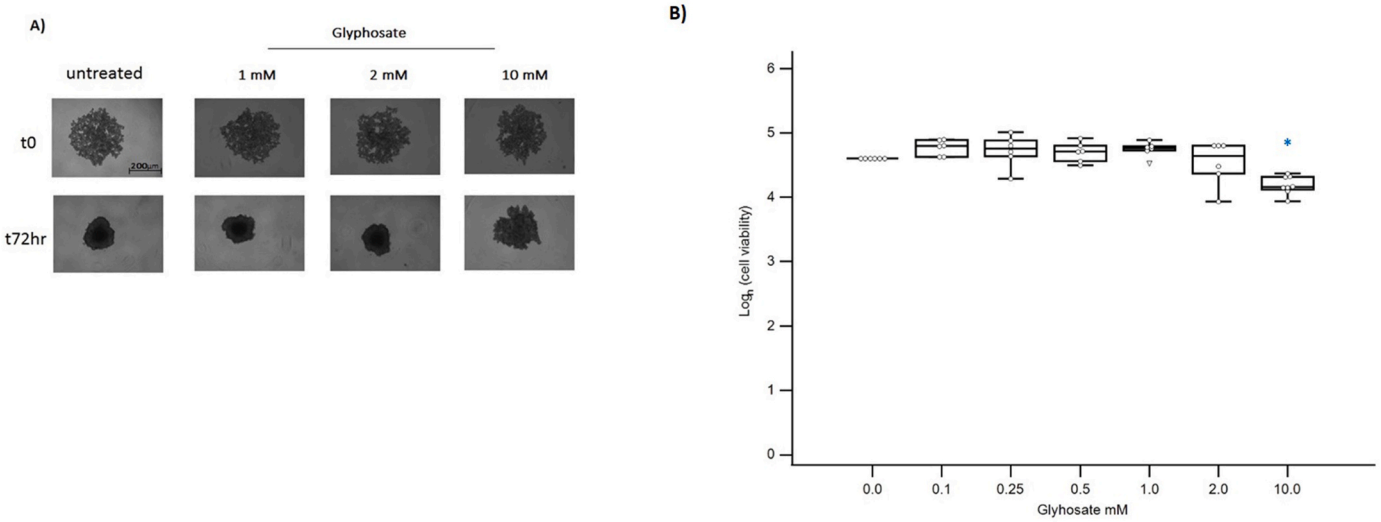

Fig. 4. Effect of Glyphosate on the ability of FRTL-5 to form spheroids.FRTL-5 cells were seeded in 96-well coated with agarose and left for 24 h in incubator at 37° and 5% of CO2 and were treated with Glyphosate at increasing concentrations (0.0; 0.1; 0.25; 0.5; 1.0; 2.0; 10.0 mM) for four days. Panel A) Phase contrast images: cells exposed to Glyphosate 10.0 mM did not make spheroids (Magnification 4X); Panel B) WST-1 assay for cell viability showed that Glyphosate 10.0 mM induced cell death in those samples that did not make spheroids (ANOVA: $F = 8.506$; $p < 0.001$; Post Hoc analysis by Dunnett $p < 0.05$ Glyphosate 10.0 mM vs. 0.0 mM).Data are expressed as natural logarithm (Log$_n$) of ODs of treated cells calculated on the OD of untreated samples (Glyphosate 0.0 mM) considered as 100%. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. Superimposed dots indicate the univariate scatterplot of data * Post Hoc analysis by Dunnett: $p < 0,05$ vs. Glyphosate 0.0 mM.

3.3. Effect of Glyphosate exposure on the ability of FRTL-5 cells to form spheroids (3-D model)

These set of experiments were performed treating FRTL-5 cells with increasing concentrations of Glyphosate for four days (i. e., the time required for spheroid formation). As shown in the representative pictures of Fig. 4 (Panel A), only the highest concentration of Glyphosate was able to inhibit spheroid formation by FRTL-5 cells. The sphericity index (SI) was calculated and was ≥0.9 for 0.0 Glyphosate vs. 2.0 mM and 0.6 for 0.0 Glyphosate vs. 10.0 mM, confirming a reduction of spheroid formation by of FRTL-5 cells. The detection of a "non-perfect sphericity", which is considered as the diameter of a circle having the same area as the spheroid section being imaged (Zanoni et al., 2016), could suggest a direct interference of Glyphosate in the spheroid formation process. To assess if the reduction of FRTL-5 cells spheroid formation was dependent upon the decrease of cells viability, the WST-1 assay was performed. Results showed that cell viability after exposure to Glyphosate 10 mM was reduced (ANOVA: $F = 8.506$; $p < 0{,}001$; Post Hoc analysis by Dunnett $p < 0.05$ for 10.0 mM Glyphosate vs. 0.0 mM). This finding suggests that the imperfect formation of spheroids by FRTL-5 cells was, at least in part, due to cell death (Fig. 4, Panel B).

3.4. Effect of Glyphosate exposure on the viability of FRTL-5 cells (3-D model)

Glyphosate reduced the viability of FRTL-5 cells spheroids only at the highest concentrations of 10 mM at 24 h (ANOVA; $F = 48.595$, $p < 0{,}001$; *Post Hoc* analysis by Dunnett $p < 0.05$ for 10.0 mM Glyphosate vs. 0.0 mM); 48 h (ANOVA: $F = 10.230$ $p < 0{,}001$; *Post Hoc* analysis by Dunnett $p < 0.05$ for 10.0 mM Glyphosate vs. 0.0 mM)) and 72 h (ANOVA: $F = 7.872$ $p < 0{,}001$; *Post Hoc* analysis by Dunnett $p < 0.05$ Glyphosate 10.0 mM vs. 0.0 mM) (Fig. 5, upper panel, T1, T2, T3, respectively). As previously showed in adherent cells, the potential time-dependency of the effect of Glyphosate was evaluated by ANOVA for repeated measures, by comparing results of the viability assay among the three time points (24, 48 and 72 h). No significant effect of the time was found (ANOVA for repeated measures, Greenhouse-geisser: $F = 1.937$, $p = 0.051$). To confirm these results spheroids were stained with PI and Hoechst. PI positive cells were observed only after exposure to Glyphosate at 10 mM or DMSO 10%, the latter used as positive control (Fig. 5, lower panel).

3.5. Effect of Glyphosate exposure on the volume of FRTL-5 cells spheroids (3-D model)

To assess whether Glyphosate exposure could modify the proliferation of FRTL-5 cells being aggregated in spheroids, changes in spheroid volume were assessed. As shown in Fig. 6, treatment with Glyphosate was not followed by changes in the volume of FRTL-5 spheroids after 0 (ANOVA: F = ANOVA: F = 2103; p = 0,153), 24 (ANOVA: F = 2661; p = 0,096 NS) (Fig. 6 T0, T1) and 48 h (ANOVA: $F = 1711$; $p = 0.218$). After 72 h of exposure to Glyphosate we observed a significant reduction

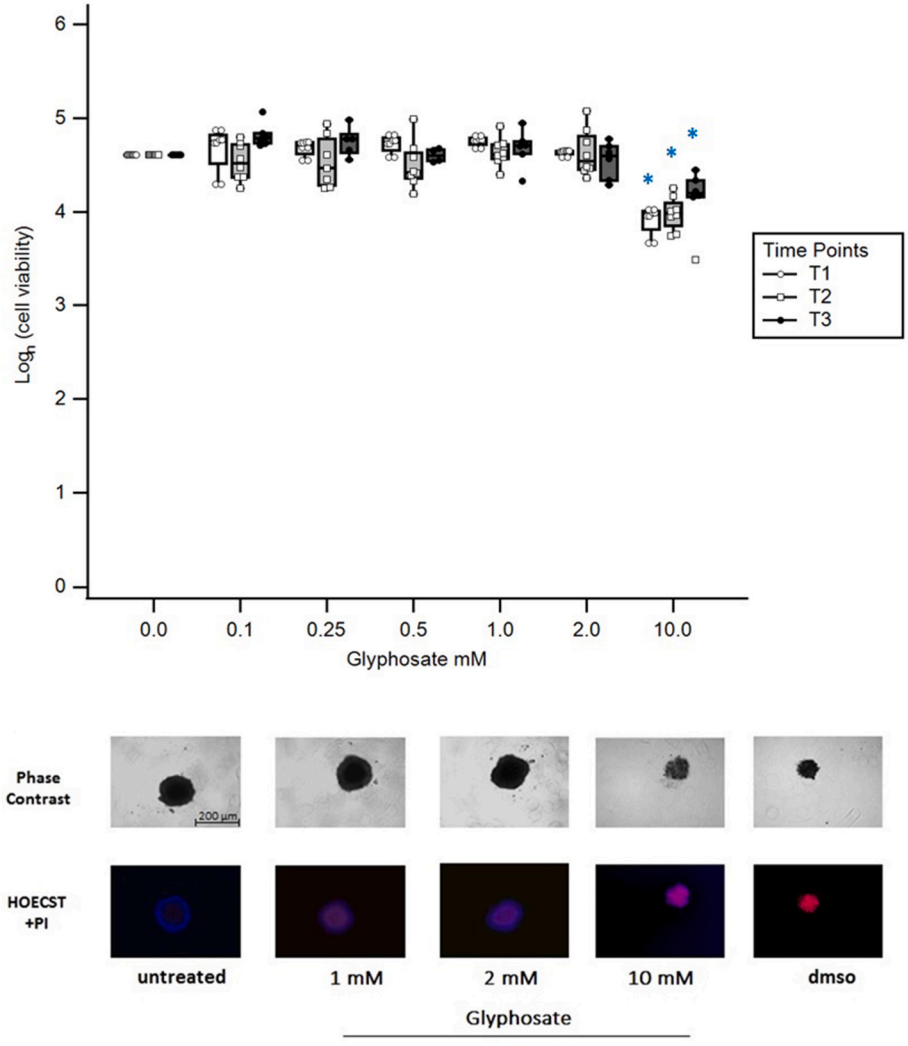

Fig. 5. FRTL-5 spheroid cells (tri-dimensional model) viability after Glyphosate exposure.Upper panel: spheroids were exposed to Glyphosate at increasing concentrations (0.0; 0.1; 0.25; 0.5; 1.0; 2.0; 10.0 mM) for 24, 48 and 72 h. Box plots derive from the results of WST-1 assay for cell viability. T1) Glyphosate significantly reduced the viability of spheroids after 24 h of exposure only at 10.0 mM (ANOV: $F = 48.595$; $p < 0.001$); T2) Glyphosate significantly reduced the viability of FRTL-5 cells after 48 h of exposure only at 10.0 mM (ANOVA: $F = 10.230$; $p < 0.001$), T3) Glyphosate significantly reduced the viability of FRTL-5 cells after 72 h of exposure only at 10.0 mM (ANOVA: $F = 7.872$; $p < 0.001$). No significant effect of the time was registered (ANOVA for repeated measures, Greenhouse-geisser: $F = 1.937$; $p = 0.051$).Lower panel: images of spheroids (magnification 4X) simultaneously stained with Hoecst 33,258+ Propidium Iodide (PI) after exposure to Glyphosate showed that at 10.0 mM spheroids were positive to PI + similar to the positive control (DMSO).Data are expressed as natural logarithm (Log_n) of ODs of treated cells calculated on the OD of untreated samples (Glyphosate 0.0 mM) considered as 100%. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. Superimposed dots indicate the univariate scatterplot of data.* Post Hoc analysis by Dunnett: $p < 0{,}05$ vs. Glyphosate 0.0 mM.

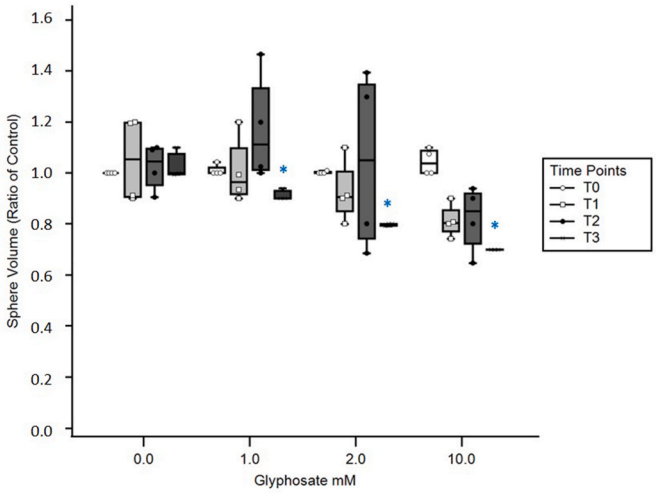

Fig. 6. Effect of Glyphosate exposure on spheroids volume.Spheroids were exposed to Glyphosate at increasing concentrations (0.0; 1.0; 2.0; 10.0 mM) for 0, 24, 48 and 72 h (T0, T1, T2, T3 respectively).Box plots show spheroids volume (median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers) after Glyphosate exposure at given concentrations and time points. At T0 Glyphosate did not change spheroids volume at any concentration tested (ANOVA: F = 2.103; p = 0.153); at T1 the exposure to Glyphosate did not change spheroids volume at any concentration tested (ANOVA: F = 2.661; p = 0.096) at T2 the exposure to Glyphosate did not change spheroids volume (ANOVA: F = 1.711; p = 0.218); at T3 the exposure to Glyphosate significantly reduced spheroids volume starting from 1.0 mM concentration (ANOVA: F = 60.064; p < 0.001). ANOVA for repeated measures did not show any significant interaction of time*CONCENTRATION (F = 1.172, p = 0.377). Superimposed dots indicate the univariate scatterplot of data * Post Hoc analysis by Dunnett: p < 0,05 vs. Glyphosate 0.0 mM.

in the volume of spheroids starting from 1.0 mM concentration (ANOVA: F = 60,064; p < 0.001; *Post Hoc* analysis by Dunnet p < 0.05 for Glyphosate 1.0, 2.0, 10.0 mM vs. 0.0 mM) (Fig. 6, T2 and T3). The potential effect of time on the reduction of spheroid volume exerted by Glyphosate was evaluated by analyzing data by ANOVA for repeated measures and by comparing results among the four (0 = T0, 24 = T1, 48 = T2, 72 = T3) time points. Results showed a trend of reduction that increased at T3 (73 h), however with a non-significant interaction of time*CONCENTRATIONS (ANOVA for repeated measures: Greenhouse-Geisser (F = 1.172, p = 0.377).

3.6. Effect of Glyphosate exposure on the production of reactive oxygen species (ROS) by adherent FRTL-5 cells (2-D model) and by spheroids (3-D model)

FRTL-5 were exposed to increasing concentrations of Glyphosate for 24 h. As shown in Fig. 7 (Panel A), the exposure to Glyphosate increased in a significant manner ROS production in the 2-D model starting from a 0.25 mM concentration (ANOVA: F = 15.085 p < 0.05; *Post Hoc* analysis by Dunnett p < 0.05 for all concentrations of Glyphosate vs. 0 mM). Exposure to Glyphosate produced a significant increase in the concentration of ROS also in the 3-D model starting from 2 mM concentration, (ANOVA: F = 6.604 p < 0,05; *Post Hoc* analysis by Dunnett p < 0.05 for 2.0 and 10.0 mM Glyphosate vs. 0.0 mM) (Fig. 7, Panel B)

3.7. Effect of Glyphosate exposure on the mRNA of thyroid-related genes in adherent FRTL-5 cells (2-D model) and in spheroids (3-D model)

The expression levels of two critical thyroid transcription factor genes (TTF-1 and Pax8) and of a panel of genes involved in thyroid differentiation and function (i. e. TSHR, TPO, TG and NIS) were evaluated in both the 2-D and the 3-D model of FRTL-5 cells. Cells were exposed to Glyphosate at a concentration of 0.5 mM (chosen according to previous studies and proven not to exert a cytotoxic effect).

When FRTL-5 were exposed to Glyphosate, differences were observed between adherent cells and spheroids. Indeed, the mRNA levels of all investigated genes increased in adherent cells after exposure to Glyphosate (Fig. 8, Panel A). At variance, in the spheroid model, PAX8 and NIS mRNAs did not show any change, whereas Glyphosate produced a significantly increased expression of the mRNAs for all the other genes (TSH-R; TTF-1; TPO; Tg) (Fig. 8, Panel B).

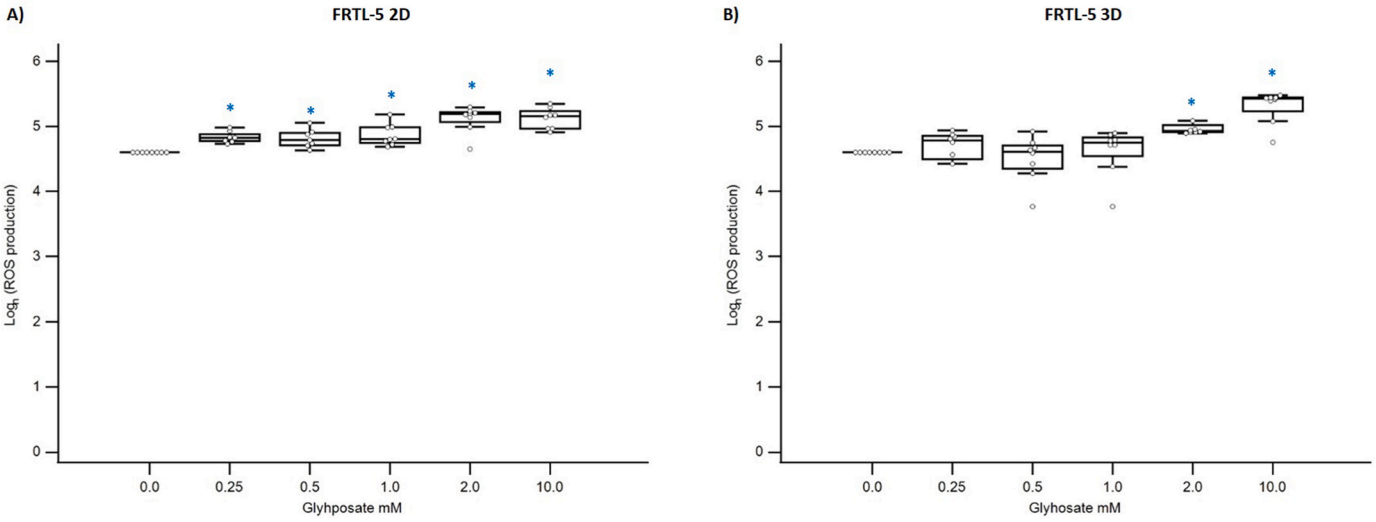

Fig. 7. Effects of Glyphosate on ROS production by FRTL-5 adherent cells and spheroids.Box plots show results of ROS detection by H2DCFDA. Panel A) FRTL-5 adherent cells (2D-model) were exposed to Glyphosate at increasing concentrations (0.0; 0.1; 0.25; 0.5; 1.0; 2.0; 10.0 mM) for 24 h. Glyphosate exposure induced an increase in ROS production starting from 0.1 mM concentration (ANOVA: F = 15.085; p < 0.05). Panel B) FRTL-5 spheroids (3D-model) were exposed to Glyphosate at increasing concentrations (0.0; 0.1; 0.25; 0.5; 1.0; 2.0; 10.0 mM) for 24 h. Glyphosate exposure induced an increase in ROS production starting from 2.0 mM concentration (ANOVA: F = 6.604; p < 0.05).Data are expressed as natural logarithm (Log$_n$) of ODs of treated cells calculated on the OD of untreated samples (Glyphosate 0.0 mM) considered as 100%. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. Superimposed dots indicate the univariate scatterplot of data. * Post Hoc analysis by Dunnett: p < 0,05 vs. Glyphosate 0.0 mM.

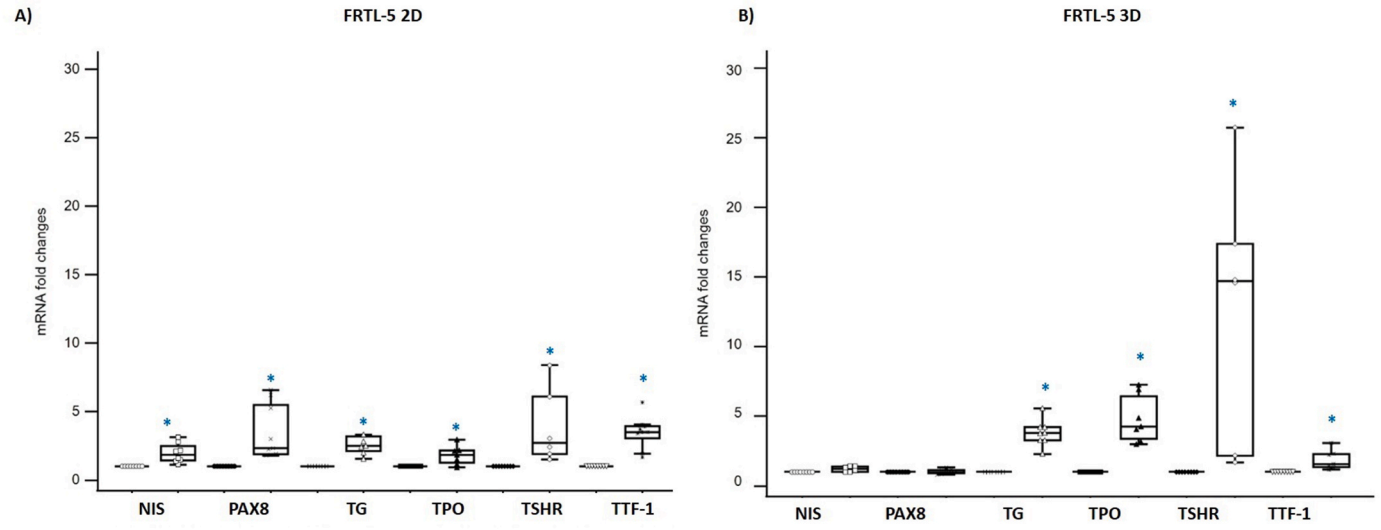

Fig. 8. Effect of Glyphosate exposure on mRNA levels of thyroid-related genes Adherent and spheroid FRTL-5 cells were exposed to Glyphosate 0.5 mM for 24 h. Panel A) Thyroid related genes mRNA levels after exposure to Glyphosate in adherent model: mRNA levels of all genes increased in adherent cells after exposure to Glyphosate (*Student T-test $p < 0.05$, treated vs untreated). Panel B) Thyroid related genes mRNA levels after exposure to Glyphosate in spheroid model: TSH-R, TTF-1, TPO, Tg, mRNA showed a significant increase after exposure to Glyphosate (*Student T-test $p < 0.05$ treated vs untreated). PAX8 and NIS mRNA did not show any changes.Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. Superimposed dots indicate the univariate scatterplot of data.

4. Discussion

The present *in vitro* study evaluated the potential adverse effects of Glyphosate exposure on a differentiated rat thyroid cell line (FRTL-5 cells) using a 2-D (adherent) and a 3-D (spheroid) model. Both in the 2-D and in the 3-D model, *in vitro* exposure to Glyphosate produced a significant: i) reduction in cell viability and mitochondrial respiration (as demonstrated by results of the WST-1 assay); ii) reduction of cell proliferation and; iii) increase of ROS production. Taken together, the above results would indicate a potential adverse effect of Glyphosate on FRTL-5 thyroid cells.

Our results are in line with some previous *in vitro* studies in other types of cells. Glyphosate exposure was shown to produce: i) adverse effects including citotoxicity in peripheral blood mononuclear cells (PBMC) (Martínez et al., 2007); ii) a reduction of the antioxidant defense ability of skin cells (Gehin et al., 2006); iii) death of keratinocytes, likely due to an increased oxydative stress (Heu et al., 2012); iv) a decreased viability of Hep-2 cells, prostatic cells and pluripotent cells (Mesnage et al., 2013); (Benachour and Séralini, 2009); (Coalova et al., 2014); (Martinez and Al-Ahmad, 2019); v) cytotoxic effects, increased ROS production and apoptosis in the neuroblastoma cell line SH-SY5Y (Martínez et al., 2020). At difference, no adverse effects of *in vitro* exposure of Glyphosate were observed in lymphocytes and ZFL cells (da Silva et al., 2020); (Mladinic et al., 2009). Thus, our findings suggest that thyroid cells should be included among those cells which are sensitive to the adverse effects of Glyphosate.

In the present study, the effects of Glyphosate were investigated on both adherent (2-D) and spheroid aggregated (thus floating) (3-D) culture systems, which are relevant experimental models for studying proliferation and differentiation of thyroid cells (Toda et al., 2011). While cultured adherent cells represent the best model for cytotoxicity tests, the 3-D model more closely resembles the native tissue. The use of spheroid-aggregated thyroid cells does increase the physiology of the culture system, thus allowing to reach more firm results. In the viability assay, only small differences emerged between the 2-D and 3-D model, being the concentration at which the effect started lower in the 2-D (2.0 mM) compared with 3-D model (10.0 mM). No impact of the incubation time was found in the two models. The 3-D model also allowed showing that exposure to Glyphosate reduced the ability to form spheroids. Different results were observed in the proliferation assay, as, in the 2-D model, Glyphosate produced an inhibition of cell growth at the maximal concentration (10.0 mM), which increased over time. On the other hand, any concentration of Glyphosate reduced the volume of spheroids, but only at the longest incubation time. The production of ROS increased in both models after exposure to Glyphosate, but it started at the lowest concentration used in the 2-D model. The increase of ROS detected is not to be underestimated also in view of the fact that Glyphosate was demonstrated to display a toxicity related to oxidative stress in several organs (Wang et al., 2022). It is important to observe that the effect of Glyphosate on cell viability was not dose-dependent. This is in line with previous findings in other cell types, in which both the cytotoxic and anti-proliferative effect were not dose-dependent (De Almeida et al., 2018; Kwiatkowska et al., 2016; Townsend et al., 2017). However, the fact that cytotoxicity was observed using two different assay techniques (WST-1 and Trypan blue) and both in the 2-D and the 3-D models, strongly support the concept that Glyphosate does exert a cytotoxic effect on thyroid cells, although it appears as a non-monotonic one.

Even more interesting is the observation that Glyphosate up-regulates the mRNA expression of several thyroid related genes: TTF-1, TSH-R, TPO and TG in the 3 D model, and in addition PAX8 and NIS in the 2-D model. These data are in contrast with those of Zhang et al., who reported a reduction in the expression of a few thyroid-related genes (NIS, TPO, TG, TSHR) in rats after exposure to Glyphosate(Zhang et al., 2021). The different results observed in adherent and spheroids cell model and the discrepancies with *in vivo* findings would suggest that the regulation of thyroid-related genes expression by Glyphosate is a complex issue which remains, at least in part, unsolved and should be more deeply investigated. However, the observed *in vitro* modulation of this panel of genes by Glyphosate should not be underestimated, mainly because PAX8 and TTF-1 encode for transcription factors, which are pivotal in the process of thyroid organogenesis (Fagman et al., 2011). Thyroid primordial cells derive from the embryonic foregut, and at this stage of differentiation they express the transcription factor TTF-1, which marks the differentiation towards the final thyroid fate of primordial endodermal cells (Lazzaro et al., 1991). When committed thyroid cells organize themselves into the characteristic thyroid follicles to start the biosynthesis of thyroid hormones, the transcription factor PAX8 is implicated in the control of this process. The

pivotal role of Pax8 in the early stages of thyroid development has been clearly documented by the phenotype of Pax8 knock-out mice (Macchia et al., 1998; Mansouri et al., 1998). Thus, thyroid development follows a well-defined sequence of transcription factor expression, which might be disrupted by a timely-improper activation of transcription factor genes by Glyphosate. Moving to the other genes, their up-regulation could strongly influence thyroid function, being the TSH-R responsible for growth and function of already differentiated thyroid cells and NIS responsible for the intracellular uptake of iodine (De Felice and Di Lauro, 2004, 2011; Zoeller et al., 2002). TG is the precursor protein for the final synthesis of triiodothyronine and thyroxine (T3 and T4) and is also the storage protein for iodine and synthetized thyroid hormones. TPO is a membrane-bound enzyme, which plays a critical role in the biosynthesis of thyroid hormones. Again, thyroid hormone biosynthesis involves a complex sequence of events, which requires a timely intervention of the involved proteins. Thus, any modifications in the expression of the respective encoding genes may potentially disrupt thyroid embryogenesis and function, mainly when exposure to Glyphosate occurs during fetal life.

The translational relevance of our findings regards the potential development of thyroid disease after Glyphosate exposure. Epidemiological studies in humans support the hypothesis that pesticides, including Glyphosate, may cause hypothyroidism in exposed farmworkers((EFSA), 2015). Indeed, some studies reported a relationship between self-reported exposure to Glyphosate and several patterns of thyroid dysfunction, which included higher TSH and lower T4 in one study (Piccoli et al., 2016), increased total T4 in another study (Kongtip et al., 2019) and lower TSH and higher TT3 and FT4 as compared with controls in a third study (Bernieri et al., 2019). A recent case-control study performed in California also suggested that exposure to Glyphosate could be linked to an increased risk of thyroid cancer, although the cancerogenic action could result from exposure to a mixture of chemicals (cocktail effect) (Omidakhsh et al., 2022). Different results were reported in a recent study, which did not observe any association between Glyphosate exposure and thyroid cancer incidence (Lerro et al., 2021). Based on our study, and on current *in vivo* evidence, the possible detrimental effect of Glyphosate on the thyroid could include: i) morphologic alterations of thyroid follicles hence producing hypothyroidism (Hamdaoui et al., 2020); ii) altered expression of thyroid related genes possibly producing a cancerogenic effect (Zhang et al., 2021); iii) altered expression of genes encoding for transcription factors (TTF-1 and PAX8), which suggests a disrupting effect on fetal thyroid embryogenesis when exposure occurs during the thyroid organogenesis window; iv) alterations in the oxidative phosphorylation process, that is closely linked to the development of autoimmunity and cancer (Romano et al., 2021); v) alterations of microbiome status and metabolism (micro-organisms express the EPSPS enzyme that is inhibited by glyphosate) (Leemans et al., 2019). Indeed, recent studies suggest that the gastrointestinal tract plays an important role in the control of thyroid function, since it regulates iodide absorption, the conversion of T4 to T3, and does modulate the immune system(Virili et al., 2018).

Some limitations of the present study should be acknowledged: i) the design does not allow drawing mechanistic conclusion, which appear mandatory to further elucidated the mechanisms responsible for the adverse effects of Glyphosate; ii) based on current knowledge, it remains hard to ascribe the effects of Glyphosate exposure to a specific Adverse Outcome Pathways (AOP), which is currently regarded as crucial step for transition towards mechanistic toxicology (Groh et al., 2015); iii) moreover, it could be that, at least some of the effects of Glyphosate might be mediated by its metabolite (AMPA), which was not evaluated in the present study. Indeed, high urinary levels of both Glyphosate and AMPA were associated with oxidative stress and other adverse effects (Buekers et al., 2022; Eaton et al., 2022). Nevertheless, the present study constitutes a first step for the assessing the adverse effect of Glyphosate on thyroid cells, and solves the current lack of *in vitro* data on thyroid cells.

5. Conclusions

In conclusion, the results of the present study further support a potential adverse effect of Glyphosate exposure on thyroid cells, which appears in line with previous evidence obtained in animal models. *In vitro* models are a useful tool for studying the cellular response to a given compound in a system where the experimental conditions are closely controlled. The effect of Glyphosate observed in the present study should not be underestimated, indeed, due to the widespread and constant increase of Glyphosate use in the world, it is extremely important to assess the effect of this compound that is currently detected not only in the serum of exposed workers, but also in the general population. Moreover, the modulation of thyroid related genes would suggest a potential thyroid-disrupting effect, mainly during fetal life. The lack of data on the *in vitro* thyroid effects of Glyphosate prompted the present study, aimed at providing a first step to further characterize the possible disrupting effects of Glyphosate on the thyroid system. The next step will be to characterize the mechanisms involved in the observed *in vitro* effects, as well as the potential role of Glyphosate metabolites (mainly of AMPA) on the thyroid. Taken together, data from our and other studies suggest that exposure to Glyphosate might produce adverse effects on the thyroid, which should not be regarded as negligible. Harmful effects might also derive from the prolonged exposure to even low Glyphosate concentrations and should be investigated by future studies.

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Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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