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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

LIDIANE PIGNATON AGOSTINI

ALTERAÇÃO DE EXPRESSÃO GÊNICA EM CÉLULAS MONONUCLEARES DO SANGUE PERIFÉRICO HUMANO SUBMETIDAS À EXPOSIÇÃO COM HERBICIDA À BASE DE GLIFOSATO

VITÓRIA

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia do Centro de Ciências da Saúde da Universidade Federal do Espírito Santo e à Rede Nordeste de Biotecnologia, como requisito parcial para a obtenção do título de Doutora em Biotecnologia.

Orientador: Prof. Dr. Iúri Drumond Louro

VITÓRIA

2018

![renorbio - rede nordeste de biotecnologia]

Programa de Pós-Graduação em Biotecnologia
Ponto Espírito Santo - Universidade Federal do Espírito Santo

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TÍTULO DO PROJETO: "ALTERAÇÃO DE EXPRESSÃO GÊNICA EM CÉLULAS MONONUCLEARES DO SANGUE PERIFÉRICO HUMANO SUBMETIDAS À EXPOSIÇÃO COM HERBICIDA À BASE DE GLIFOSATO"

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TIPO DE SESSÃO: Aberta

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ESTRUTURA DA TESE

Esta Tese está organizada em formato de artigo científico e de acordo com o exigido pelo Regimento desse Programa - com a comprovação de submissão de dois artigos em periódicos Qualis B1 ou superior.

As Listas de Figuras, Siglas, Abreviaturas e as Referências Bibliográficas contemplam as informações descritas na Introdução e Revisão de Literatura.

RESUMO

AGOSTINI, L. P. **Alteração de expressão gênica em células mononucleares do sangue periférico humano submetidas à exposição com herbicida à base de glifosato.** 2018. 121f. Tese (Doutorado em Biotecnologia) - Programa de Pós-Graduação em Biotecnologia, vinculado à Rede Nordeste de Biotecnologia (Renorbio), UFES, Espírito Santo. Brasil.

O Glifosato [N-(fosfonometil)glicina] é um herbicida pós-emergente, não seletivo e sistêmico. No processo de criação das formulações comerciais de herbicidas a base de glifosato (GBHs, do inglês *glyphosate-based herbicides*), como o Roundup®, são adicionados surfactantes com o intuito de aumentar a eficiência do composto base. A rota prioritária de degradação do glifosato por micro-organismos no solo resulta na formação do ácido aminometilfosfônico (AMPA). As respostas moleculares ao glifosato têm sido extensivamente estudadas em espécies de plantas e em alguns vertebrados. Em humanos, apesar dos estudos até agora realizados, não se conhece exatamente quais os riscos e mecanismos de atuação que explicariam a toxicidade ao glifosato relatada em alguns experimentos. Sendo assim, a hipótese dessa tese é de que a exposição rápida ao Roundup® e ao AMPA leva à alterações de expressão gênica em importantes processos celulares. Dessa forma, o objetivo desse trabalho é identificar genes diferencialmente expressos (DEGs, do inglês *differentially expressed genes*) em células mononucleares do sangue periférico (PBMCs, do inglês, *peripheral blood mononuclear cells*) humano submetidas à exposição rápida com herbicida à base de glifosato (Roundup®) e AMPA. O teste de MTT [3(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio brometo], realizado em triplicatas, foi utilizado para avaliar a viabilidade celular e para a escolha das condições de tratamento utilizadas na técnica de *microarray* (*GeneChip® Human Transcriptome Array 2.0*, Affymetrix). As condições analisadas foram controle (3 *chips*), AMPA (10 mM; 3 *chips*) e Roundup® (0,05%; 2 *chips*), expostos durante 3 horas. Utilizando um valor de $p < 0,05$ e *fold-change* de 1,5 foram identificados 5 DEGs no tratamento com o AMPA e 26 no tratamento com Roundup®. As análises de enriquecimento mostraram que os genes com expressão

alterada após exposição ao Roundup® estavam associados a 33 processos celulares, principalmente relacionados à regulação destes processos. A plataforma digital *Pathview* foi utilizada para identificar a atuação dos DEGs após exposição ao Roundup® em diferentes vias. Os genes *TNF, LTA, TAB2 e ATM* foram relacionados à via de sinalização NF-kappa β; *BCL2L11 e ATM* à via de sinalização FoxO; *SESN3 e ATM* à via de sinalização p53; e *TNF, BCL2L11 e ATM* à apoptose. Dessa forma, os resultados sugerem que o Roundup® altera o padrão de expressão gênica de diversos genes associados com o controle do ciclo celular, regulação de processos celulares e apoptose.

Palavras-chave: Expressão gênica, *microarray*, MTT, Roundup®, PBMC, humanos.

ABSTRACT

AGOSTINI, L.P. **Gene expression alteration in human peripheral blood mononuclear cells submitted to glyphosate-based herbicide exposure.** 2018. 121f. Thesis (Doctoral in Biotechnology) - Postgraduation Biotechnological Programme, UFES, Espírito Santo. Brazil.

Glyphosate is a post-emergent, non-selective and systemic herbicide. In the creating process of glyphosate-based herbicides (GBHs) such as Roundup®, surfactants are added to improve efficiency. The priority route of glyphosate's degradation in soil results in aminomethylphosphonic acid (AMPA). Molecular responses to glyphosate were analyzed in some species of plants and in some vertebrates. In humans, it is not known exactly what risks and mechanisms of action would explain glyphosate's toxicity reported in some experiments. The hypothesis is that fast exposure to Roundup® and AMPA leads to the differentiated expression of genes related to important cellular processes. Thus, the aim was identified these genes in human peripheral blood mononuclear cells when exposed to Roundup® and AMPA. The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test was performed in triplicates to evaluate cell viability and the choice of treatment conditions used in the microarray technique (GeneChip® Human Transcriptome Array 2.0, Affymetrix). Eight chips were used: 3 for controls, 3 for AMPA (10 mM) and 2 for Roundup® (0.05%). The exposure time was 3 hours. Using a $p < 0.05$ and a fold-change of ≥ 1.5 and ≤ -1.5, there were 26 differentially expressed genes (DEGs) identified after Roundup® exposure (3h; 0.05%) and 5 DEGs after AMPA treatment (3h; 10 mM). DEGs after Roundup® treatment showed association with 33 Gene Ontology (GO) cellular processes (enrichment analysis), mainly related to regulation. Pathview web was used to identify the effect off DEGs in different pathways. Only genes differentially expressed in Roundup® treatment were included in the pathways. *TNF, LTA, TAB2 and ATM* genes are related to NF-kappa B signaling pathway; *BCL2L11 and ATM* genes to FoxO signaling pathway; *SESN3 and ATM* genes to p53 signaling pathway; and *TNF, BCL2L11 and ATM* genes to apoptosis. Our results suggest that Roundup® change

expression pattern of a several genes associated with cell cycle control, cellular processes regulation and apoptosis.

Keywords: Gene expression, *microarray,* MTT, *Roundup*®, PBMC, humans.

LISTA DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

AChE	Acetilcolinesterase eritrocitária
AHS	Agricultural Health Study
AMPA	Ácido aminometilfosfônico
BChE	Butirilcolinesterase plasmática
CARC	Comitê de Revisão das Avaliações de Câncer
CAS#	Chemical Abstracts Service (número de registro de produtos químicos)
DNA	Ácido Desoxirribonucleico
EFSA	Autoridade Europeia para a Segurança dos Alimentos
ELISA	Ensaio de imunoabsorção enzimática
EPA	Agência de Proteção Ambiental
EPSP	5-enolpiruvilchiquimato-3-fosfato
EPSPS	Enzima 5-enolpiruvilchiquimato-3-fosfato sintase
et al.	E outros
FAO	Organização das Nações Unidas para Agricultura e Alimentação
FCMIA	Imunoensaio de microbola (microbead) covalente por fluorescência
GBHs	Herbicidas à base de glifosato
GC-MS	Cromatografia gasosa com espectrômetro de massa
GOX	Enzima glifosato oxidoredutase
h	Hora(s)
HaCaT	Linhagem de queratinócitos
HepG2	Linhagem de câncer de fígado
HILIC	Cromatografia líquida com interação hidrofílica

HPLC	Cromatografia líquida de alta performance
IARC	Agência Internacional de Pesquisas sobre o Câncer
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
IC	Intervalo de confiança
IUPAC	União Internacional de Química Pura e Aplicada
LC-MS/MS	Cromatografia líquida acoplada à espectrometria de massas sequencial
LD	Limite de detecção
LPS	Lipopolisacarídeo
M	Molar
mg/kg	Miligrama/kilo
mg/ml	Miligrama/mililitro
min	Minutos
mM	Milimolar
NNG	N-Nitrosoglifosato
OMS	Organização Mundial da Saúde
OR	Odds ratio, razão de chances ou de possibilidades
p	Probabilidade de significância
PBMCs	Células mononucleares do sangue periférico.
PEP	Fosfoenolpiruvato
Pi	Fósforo inorgânico
PMIDA	Ácido N-(fosfonometil) iminodiacético
POEA	Polietoxilenoamina
ppm	Partes por milhão
RNAm	RNA mensageiro

S3P	Chiquimato-3-fosfato
SCE	Troca de cromátides irmãs
SINAN	Sistema de Informação de Agravos de Notificação
SINITOX	Sistema Nacional de Informações Tóxico-Farmacológicas
TOXCEN	Centro de Atendimento Toxicológico do Espírito Santo
µg/L	Micrograma/litro
µg/ml	Micrograma/microlitro

SUMÁRIO

1 INTRODUÇÃO

A partir da década de 1940 começaram a operar as primeiras unidades produtivas de agrotóxicos no Brasil com a finalidade de aumentar a produtividade agrícola. Em meados da década de 1970, o parque industrial de agrotóxicos passou a operar, aumentando a comercialização de agrotóxicos de forma contínua desde então (IBAMA, 2010). No ano de 2014, os herbicidas responderam por 58% de todos os tipos de agrotóxicos comercializados (IBAMA, 2016). O principal ingrediente ativo comercializado foi o glifosato, com 194 mil toneladas vendidas (IBAMA, 2016).

O Glifosato (N-(fosfonometil)glicina) é um herbicida pós-emergente, não seletivo e sistêmico, que mata ou suprime plantas (IARC, 2015; MYERS et al., 2016). No processo de criação das formulações comerciais, são adicionados surfactantes para melhorar a eficiência (VANDEMBERG et al., 2017). Os surfactantes mais utilizados são a polietoxilenoamina (POEA) e os ácidos sulfúrico e fosfórico (SZÉKÁCS; DARVAS, 2012). Os produtos compostos pelo glifosato e pelo surfactante podem ser chamados de herbicidas à base de glifosato (GBHs, do inglês *glyphosate-based herbicides*). O Roundup® é o GBH mais comum (IARC, 2015).

O mecanismo de ação do glifosato baseia-se na inibição da enzima 5-enolpiruvilchiquimato-3-fosfato sintase (EPSPS), que atua na via do chiquimato, catalisando a conversão do chiquimato-3-fosfato (S3P) em 5-enolpiruvilchiquimato-3-fosfato (EPSP) (BOOCOCK; COGGINS,1983).

A rota prioritária de degradação do glifosato por micro-organismos no solo resulta na formação do ácido aminometilfosfônico (AMPA) (RIBEIRO et al., 2015). A meia-vida do glifosato em solo varia de 2 a 197 dias, e do AMPA de 76 a 240 dias (GIESY; DOBSON; SOLOMON, 2000). Nos mamíferos, o glifosato não é metabolizado eficientemente e é, principalmente, excretado inalterado na urina (MYERS et al., 2016).

A Agência Internacional de Pesquisas sobre o Câncer (IARC, do inglês *International*

Agency for Research on Cancer) classificou o glifosato como "provavelmente cancerígeno para os seres humanos (Grupo 2A)" em 2015. Entretanto, outras agências sugerem outras classificações. A Organização Mundial da Saúde (OMS) e a Organização das Nações Unidas para Agricultura e Alimentação (FAO, do inglês *Food and Agriculture Organization*) classificam o glifosato como "improvável que apresente risco carcinogênico para os seres humanos devido à exposição através da dieta" (FAO/ OMS, 2016). O Comitê de Revisão das Avaliações de Câncer (CARC, do inglês *Cancer* Assessment Review *Committee*) classificou o glifosato como "provavelmente não cancerígeno para humanos" (EPA, 2016).

Em 1974, quando a Monsanto iniciou a comercialização do Roundup®, defendia-se que o princípio ativo glifosato era seguro para o meio ambiente e para a saúde humana e animal (WILLIAMS et al., 2000). A partir da década de 1980, diversos estudos foram realizados buscando a confirmação das informações divulgadas através de experimentos de avaliação de toxicidade, genotoxicidade e carcinogenicidade (VIGFUSSOM; VYSE, 1980; BOLOGNESI et al., 1997; WILLIAMS et al., 2000; KOLLER et al., 2012; KWIATKOWSKA et al., 2017). A exposição *in vivo* ao glifosato também foi avaliada em diferentes organismos e através de estudos epidemiológicos em humanos (TARAZONA et al., 2017).

A toxicidade aguda do glifosato é classificada como baixa em ratos, sendo que o LD_{50} oral do glifosato puro é de 5.600 mg/kg (FAO/OMS, 2016). As intoxicações agudas após ingestão de GBHs são observadas em casos de suicídios ou acidentes. Os sintomas relacionados à intoxicação são diversos, variando de leves à mais graves, e algumas vezes levam o indivíduo à morte (POTREBIĆ et al., 2009; ZOUAOUI et al., 2013; KAMIJO; TAKAI; SAKAMOTO, 2015).

Os registros sobre os casos de intoxicação por agrotóxicos no Brasil são obtidos através de dois sistemas: o Sistema Nacional de Informações Tóxico-Farmacológicas (SINITOX) e o Sistema de Informação de Agravos de Notificação (SINAN). Segundo os dados disponíveis no SINITOX (2017), em 2014 foram registrados 5116 casos de intoxicação por agrotóxico. Infelizmente, esses dados não representam a realidade do país pois nem todos os estados repassam os dados para o sistema nacional

(SINITOX, 2017).

Em humanos, o diagnóstico de contaminação por glifosato é confirmado por testes como a cromatografia líquida acoplada à espectrometria de massas sequencial (LC-MS/MS), cromatografia gasosa com espectrômetro de massa (GC-MS), ELISA e nível da enzima colinesterase (YOSHIOKA et al., 2011; KRUGER et al., 2014; PEARSON; PATEL, 2016).

As respostas moleculares ao glifosato já foram analisadas para algumas espécies de plantas e para alguns vertebrados. Em humanos, são poucos os estudos realizados. Pesquisas que identifiquem as alterações a nível molecular são importantes para determinar em quais processos biológicos o glifosato atua alterando a expressão gênica.

A hipótese dessa tese é de que a exposição rápida ao Roundup® e ao AMPA leva à expressão diferenciada de genes relacionados a importantes processos celulares, o que explicaria a toxicidade ao glifosato relatada na literatura disponível.

Dessa forma, o objetivo desse trabalho é identificar genes diferencialmente expressos em células mononucleares do sangue periférico humano submetidas à exposição rápida com herbicida à base de glifosato (Roundup®) e AMPA.

2 REVISÃO BIBLIOGRÁFICA

2.1 Agrotóxicos

O modelo agrícola contemporâneo, que apresenta elevados índices de produtividade, tem como parte fundamental o uso de agrotóxicos (IBAMA, 2010). A Lei nº 7.802, de 11 de Julho de 1989, define agrotóxicos como:

> Os produtos e os agentes de processos físicos, químicos ou biológicos, destinados ao uso nos setores de produção, no armazenamento e beneficiamento de produtos agrícolas, nas pastagens, na proteção de florestas, nativas ou implantadas, e de outros ecossistemas, e também de ambientes urbanos, hídricos e industriais, cuja finalidade seja alterar a composição da flora ou da fauna, a fim de preservá-las da ação danosa de seres vivos considerados nocivos (BRASIL, 1989).

Os agrotóxicos (defensivos agrícolas, pesticidas ou produtos fitossanitários) apresentam em sua composição ingredientes ativos, que são substâncias químicas tóxicas que interferem na atividade biológica normal dos seres vivos alvos de controle. São os ingredientes ativos que conferem eficácia aos agrotóxicos e afins, por ação química, física ou biológica (BRASIL, 1989).

A partir da década de 1940 começaram a operar as primeiras unidades produtivas de agrotóxicos no Brasil. Em meados da década de 1970 o parque industrial de agrotóxicos foi instituído, aumentando a comercialização de agrotóxicos de forma contínua desde então (IBAMA, 2010).

A figura 1 apresenta o consumo crescente de agrotóxicos e afins entre os anos de 2000 a 2014.
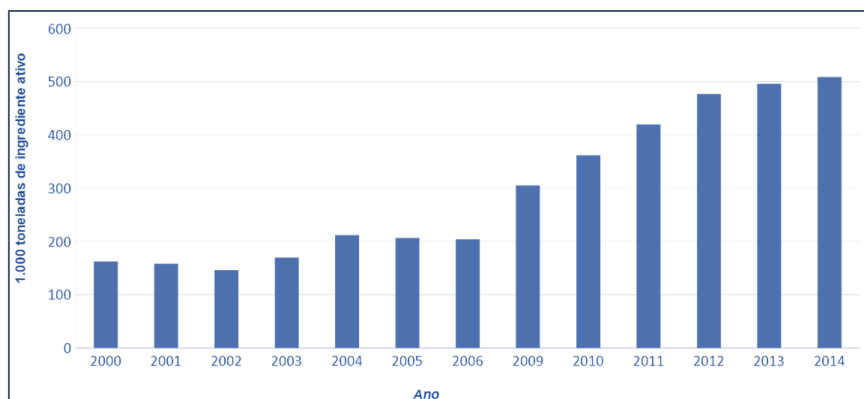
Figura 1. Consumo de agrotóxicos e afins (2000 – 2014). Fonte: Boletins anuais de produção, importação, exportação e vendas de agrotóxicos no Brasil (IBAMA, 2016).

A comercialização de agrotóxicos (Figura 2) ocorre de forma mais acentuada nos Estados de São Paulo e Goiás, seguidos por Espírito Santo, Minas Gerais, Mato Grosso e Santa Catarina (IBGE, 2015).
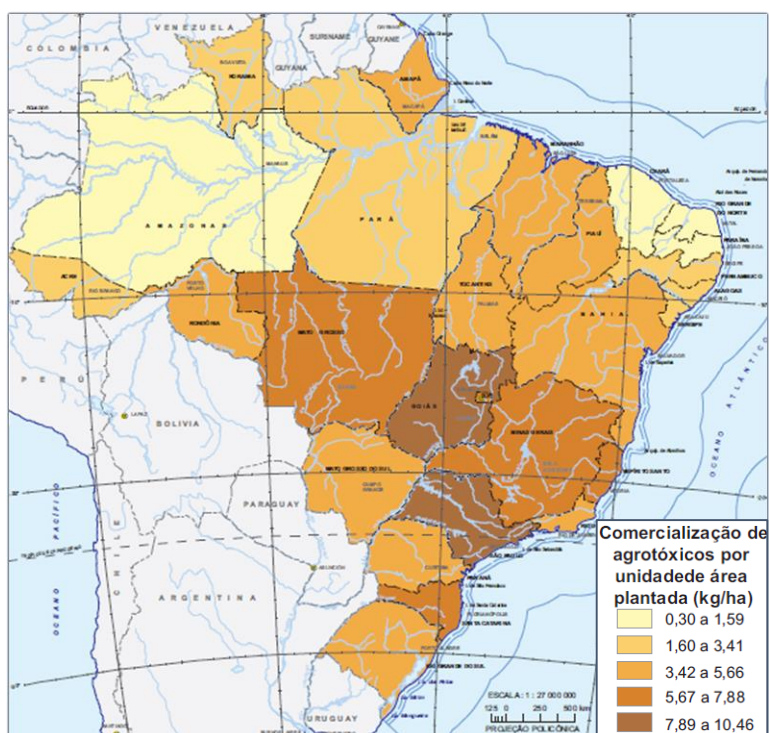
Figura 2. Comercialização de agrotóxicos, por área plantada (2012). Fonte: Indicadores de Desenvolvimento Sustentável – Brasil 2015 (IBGE, 2015).

Os agrotóxicos podem ser classificados de acordo com os mecanismos de ação contra os diferentes tipos de organismos: herbicidas (plantas), inseticidas (insetos), fungicidas (fungos), nematicidas (microorganismos de solo), moluscicidas (moluscos), dentre outros (IBAMA, 2010).

Os herbicidas são agentes químicos que evitam, reduzem ou eliminam plantas infestantes, chamadas comumente de ervas daninhas, que competem por água e nutrientes com a planta cultivada (IBAMA, 2010).

No ano de 2014, segundo o Boletim anual de produção, importação, exportação e vendas de agrotóxicos no Brasil (IBAMA, 2016), os herbicidas responderam por 58% de todos os tipos de agrotóxicos comercializados (294 mil toneladas).

O principal ingrediente ativo comercializado foi o glifosato, com 194 mil toneladas vendidas em 2014 (IBAMA, 2016).

No Espírito Santo, foram comercializadas 2,5 mil toneladas de herbicidas em 2014, sendo que o glifosato corresponde a 72% dessas vendas (IBAMA, 2016).

2.2 Glifosato

2.2.1 Histórico, características e comercialização

Os herbicidas à base de glifosato foram sintetizados pela primeira vez em 1950 como um potencial composto farmacêutico, mas suas atividades herbicidas não foram descobertas até serem ressintetizados e testados em 1970, sendo utilizados desde 1974 (SZÉKÁCS; DARVAS, 2012; WILLIANS et al., 2016). A patente original expirou em 2000 nos EUA, e em 1991 no restante do mundo (IARC, 2015).

O Glifosato, cujo nome IUPAC é N-(fosfonometil)glicina (CAS# 1071-83-6), é um herbicida pós-emergente, não seletivo e sistêmico, que mata ou suprime plantas, incluindo gramíneas, plantas perenes, videiras, arbustos e árvores. Quando aplicado a baixas doses, atua como regulador de crescimento e dessecante (IARC, 2015; FAO/WHO, 2016; MYERS et al., 2016).

Como apresentado na Figura 3, é uma molécula formada pelo aminoácido glicina e por uma fração fosfonometil, polar e solúvel em água (LI et al., 2013). A fórmula molecular do composto é $C_3H_8NO_5P$ e a massa molecular relativa é 169,07 g/mol (IARC, 2015).
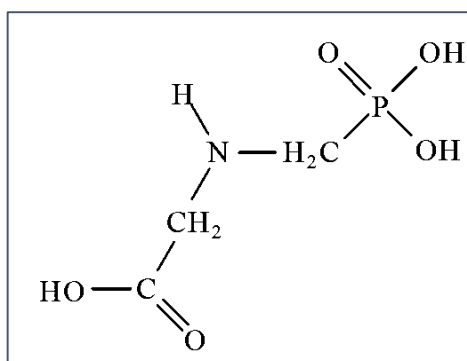
Figura 3. Estrutura molecular do Glifosato. Fonte: IARC, 2015.

O glifosato é um potente quelante que se liga a cátions divalentes (Ca, Mg, Mn e Fe) e forma complexos estáveis (CAKMAK et al., 2009). Ele se liga a macro e micronutrientes que são essenciais para vários processos nas plantas e para a resistência a patógenos (MERTENS et al., 2018). Ainda não está elucidada se a atividade quelante do glifosato contribui para os seus efeitos tóxicos em plantas e para os efeitos adversos no meio ambiente e para a saúde humana e de outros animais (MERTENS et al., 2018).

No processo de criação das formulações comerciais, o glifosato apresenta-se como um sal de isopropilamina, amônio ou sódio em concentrados solúveis em água (FAO,

2000). Nesse processo, são adicionados surfactantes (também chamados de adjuvantes) para melhorar a eficiência do agrotóxico. Essa melhora ocorre pois esses compostos permitem um transporte mais rápido do agrotóxico para a planta (através das folhas e meristemas), garantindo uma absorção mais eficaz do ingrediente ativo, protegendo-o da evaporação e retardando o processo de lixiviação (IPCS, 1996; SZÉKÁCS; DARVAS, 2012; VANDEMBERG et al., 2017).

Os surfactantes mais utilizados são a polietoxilenoamina (POEA) e os ácidos sulfúrico e fosfórico. Além disso, os GBHs podem conter outros ingredientes ativos, como ácido simasino, ácido 2,4-diclorofenoxiacético (2,4-D) ou ácido 4-cloro-2-metilfenoxiacético (IPCS, 1996). Geralmente, os GBHs causam mudanças mais fortes do que o próprio glifosato (MARTINEZ; REYES; REYES, 2007; MESNAGE; BERNAY; SERALINI, 2013; FOLMAR; SANDERS; JULIN, 1979).

O Roundup® é o GBH mais comum e é apresentado em muitas formulações (IARC, 2015). Outros nomes comerciais dos GBHs são: Abundit Extra, Credit, Xtreme, Glifonox, Glyphogan, Ground-Up, Rodeo, Touchdown, Tragli, Wipe Out e Yerbimat (FCI, 2015).

2.2.2 Mecanismo de ação

O mecanismo de ação do glifosato em plantas e algumas bactérias, baseia-se na inibição da enzima 5-enolpiruvilchiquimato-3-fosfato sintase (EPSPS), que atua na via do chiquimato (Figura 4). A enzima EPSPS, sintetizada no citoplasma, mas atuante no cloroplasto, catalisa a conversão do chiquimato-3-fosfato (S3P) em 5-enolpiruvilchiquimato-3-fosfato (EPSP), com liberação de fósforo inorgânico (Pi), utilizando fosfoenolpiruvato (PEP) como substrato (BOOCOCK; COGGINS,1983; CZELUSNIAK et al., 2012; VANDENBERG et al., 2017).
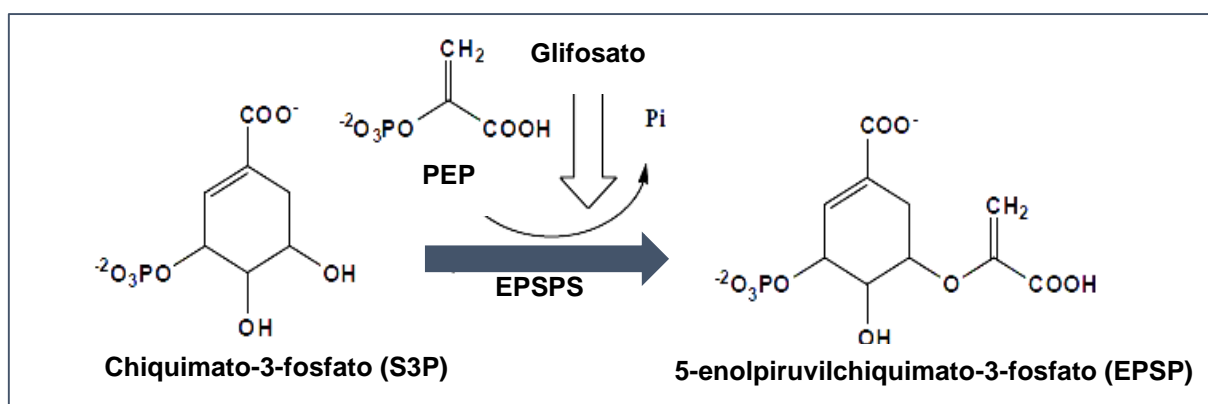
Figura 4. Inibição da enzima 5-enolpiruvilchiquimato-3-fosfato sintase (EPSPS) pelo glifosato em uma das etapas da via do chiquimato. Fonte: CZELUSNIAK *et al.*, 2012 (Modificada).

Os inibidores da enzima EPSPS competem com o fosfoenolpiruvato, provocando um aumento na concentração de chiquimato nas plantas e inibindo a produção de corismato, precursor dos aminoácidos aromáticos fenilalanina, tirosina e triptofano. Esses aminoácidos essenciais são utilizados na produção de compostos como a lignina, pigmentos, flavonoides, antocianinas, ácidos benzoicos e compostos aromáticos envolvidos em mecanismos de defesa da planta (BOOCOCK; COGGINS,1983).

Além disso, são utilizados na síntese de proteínas, vitaminas K e E, hormônios (auxina, etileno), antocianina e vários outros metabólitos secundários (MESNAGE et al., 2015; KWIATKOWSKA et al., 2016). Como consequência, esses eventos causados pela atuação do glifosato interrompem o crescimento de certas plantas (WILLIAMS et al., 2016).

A via do chiquimato ocorre em vários grupos de microorganismos, plantas e parasitas e está ausente em animais (RICHARD et al., 2005; THONGPRAKAISANG et al., 2013; VANDENBERG et al., 2017).

Culturas resistentes ao glifosato expressam a enzima EPSPS insensível ao herbicida (originada da *Agrobacterium spp.*), a enzima glifosato oxidoredutase (GOX) que o degrada, e/ou a enzima glifosato acetiltransferase que modifica o glifosato (SCHUTTE

et al., 2017).

2.2.3 Degradação

A degradação do glifosato por micro-organismos no solo pode seguir duas rotas metabólicas (Figura 5). A primeira consiste na transformação do glifosato em sarcosina por ação da bactéria *Agrobacterium radiobacter* ou da *Enterobacter aeroneges* (enzima C-P liase) (JUNIOR; SANTOS, 2002; POLLEGIONI; SCHONBRUNN; SIEHL, 2011). A segunda rota resulta na formação do ácido aminometilfosfônico (AMPA) como resultado da clivagem oxidativa do glifosato pela enzima GOX, sob a ação da bactéria *Anthrobacter atrocyaneus e Flavobacterium sp.* (RIBEIRO et al., 2015, POLLEGIONI; SCHONBRUNN; SIEHL, 2011).

A sarcosina é um metabólito de difícil detecção, já que em solo é rapidamente degradada pelos micro-organismos. O AMPA é considerado o metabólito principal por ser mais persistente, podendo se acumular no meio ambiente, e por ser a rota predominante nas bactérias presentes no solo (JACOB et al., 1988). A meia-vida do glifosato em solo varia de 2 a 197 dias, e do AMPA de 76 a 240 dias (GIESY; DOBSON; SOLOMON, 2000).

Nos mamíferos, o glifosato não é metabolizado eficientemente e é, principalmente, excretado inalterado na urina (MYERS et al., 2016). Em seres humanos, pequenas quantidades de AMPA foram encontradas no sangue após intoxicação por glifosato, degradado pela microbiota intestinal (MOTOJYUKU et al., 2008).
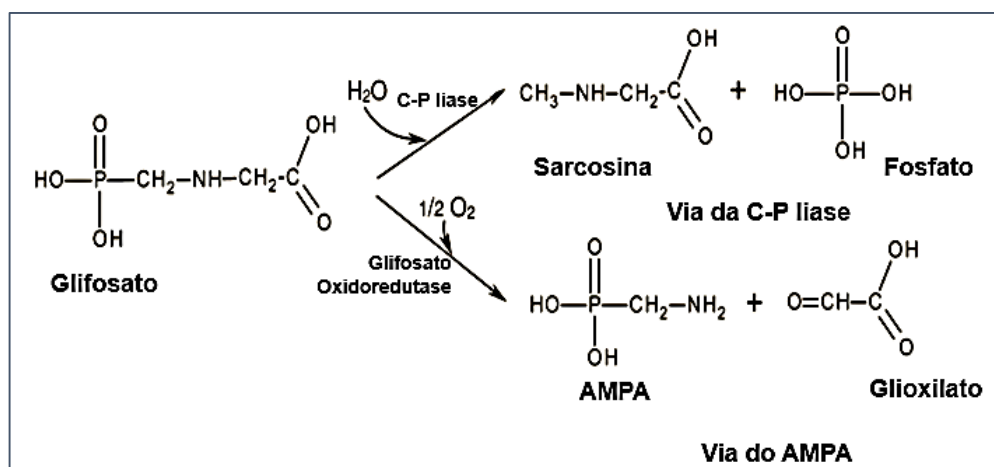
Figura 5. Mecanismos de degradação do glifosato. Acima: Via da C-P liase. Abaixo: Via do AMPA.
Fonte: POLLEGIONI; SCHONBRUNN; SIEHL, 2011 (Modificada).

2.2.4 Indicações de uso

Os GBHs têm usos agrícolas e não agrícolas. Na agricultura, o glifosato atua contra mais de 100 espécies de ervas daninhas (DILL et al., 2010). Sua aplicação pode ocorrer durante a pré-colheita, pós-plantio e em pré-emergência e pode ser tanto por pulverização área quanto por pulverização local (TOMLIN, 2000). Em doses menores atua como regulador de crescimento de plantas e dessecante (IARC, 2015).

Os usos não-agrícolas incluem: uso residencial (controle de ervas daninhas em jardins); aplicações industriais; controle de plantas em linhas de energia e em estradas; controle de espécies invasivas em sistemas aquáticos ou em zonas úmidas; no manejo florestal e no controle de plantações de cocaína e maconha (DILL et al., 2010; MANCE, 2012; LUBICK, 2009; SZÉKÁCS; DARVAS, 2012; WILLIAMS et al., 2016).

As mudanças na prática agrícola e no desenvolvimento de culturas geneticamente modificadas que são resistentes ao glifosato contribuíram para o aumento exponencial

do uso desse herbicida (MYERS et al., 2016).

2.2.5 Avaliações de risco

A IARC classificou o glifosato como "provavelmente cancerígeno para os seres humanos (Grupo 2A)" em 2015. Esta categoria é usada quando há evidências limitadas de carcinogenicidade em seres humanos e dados fortes sobre como o agente causa câncer em animais experimentais (IARC, 2015). De acordo com quatro painéis de especialistas que realizaram uma crítica detalhada da avaliação do IARC, as evidências não suportam esta conclusão, sendo consistente que "é improvável que o glifosato represente um risco carcinogênico para os seres humanos" (WILLIAMS et al., 2016).

A OMS e a FAO classificam o glifosato como "improvável que represente um risco carcinogênico para os seres humanos devido à exposição através da dieta (exposição a alimentos e água)" (FAO/OMS, 2016). De acordo com os estudos avaliados por essas organizações, a evidência indica que a administração de GBHs em doses tão altas quanto 2000 mg/kg, a via mais relevante para a exposição dietética humana, não foi associada a efeitos genotóxicos em uma esmagadora maioria de estudos em mamíferos (FAO/OMS, 2016).

O CARC classificou o glifosato como "provavelmente não cancerígeno para humanos" (EPA, 2016). Em novembro de 2015, a Autoridade Europeia para a Segurança dos Alimentos (EFSA, do inglês *European Food Safety Authority*) determinou que "era improvável que o glifosato representasse um risco carcinogênico para os seres humanos" (EFSA, 2015). A Agência de Proteção Ambiental (EPA, do inglês *Environmental Protection Agency*) apoia que o glifosato "provavelmente não é cancerígeno para os seres humanos" em doses relevantes (EPA, 2016).

2.2.6 Legislação brasileira sobre uso de agrotóxicos

A principal lei que regulamenta a produção, comercialização e uso de agrotóxicos é a "Lei dos Agrotóxicos e Afins" (Lei nº 7.802, de 11 de Julho de 1989) (BRASIL, 1989). São regidos por essa Lei:

> A pesquisa, a experimentação, a produção, a embalagem e rotulagem, o transporte, o armazenamento, a comercialização, a propaganda comercial, a utilização, a importação, a exportação, o destino final dos resíduos e embalagens, o registro, a classificação, o controle, a inspeção e a fiscalização de agrotóxicos, e seus componentes (BRASIL, 1989).

Essa lei é regulamentada pelo Decreto nº 4.074, de 04 de Janeiro de 2002 (BRASIL, 2002). Segundo esse decreto, as empresas são obrigadas a apresentar ao poder público relatórios de comercialização dos produtos agrotóxicos, com periodicidade semestral. O Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) é o órgão responsável pela divulgação desses relatórios (IBGE, 2015).

É responsabilidade do IBAMA realizar a Avaliação do Potencial de Periculosidade Ambiental de diversas substâncias químicas, incluindo os agrotóxicos. São avaliadas as propriedades físico-químicas e a toxicidade para os diversos organismos; o quanto o produto se acumula em tecidos vivos; se persiste por muito tempo no ambiente; e se consegue se deslocar (solo, ar ou água). São considerados ainda o risco de causar mutações, câncer, más-formações em fetos ou embriões, e se podem colocar em risco a reprodução de aves e de mamíferos (IBAMA, 2010).

Existem 4 classes de periculosidade: Classe I – produto altamente perigoso; Classe II – produto muito perigoso; Classe III – produto perigoso; e Classe IV – produto pouco perigoso (IBGE, 2015).

O Roundup Original® é classificado no grupo III, enquanto outros produtos da mesma

empresa (Roundup Ready®, Roundup Transorb®, Roundup Ultra®) se enquadram na classe II (IBAMA, 2010).

Atualmente, existe uma comissão especial da Câmara dos Deputados que analisa 18 projetos que alteram a Lei de Agrotóxicos. Os objetivos desses projetos são simplificar o processo para registro de pesticidas novos, facilitar o uso de genéricos, alterar o nome "agrotóxico" para "defensivo fitossanitário", criar novo órgão federal para administrar esse tema e diminuir o poder dos estados na fiscalização (BRASIL, 2017).

2.3 Efeitos biológicos do glifosato

Quando iniciou a comercialização do Roundup®, a Monsanto anunciou que o princípio ativo glifosato era seguro para o meio ambiente e para a saúde humana (incluindo o agricultor em contato direto) e animal, desde que utilizado de acordo com as recomendações técnicas e as indicações das bulas e rótulos dos produtos (WILLIAMS et al., 2000).

A partir da década de 1980, diversos estudos foram realizados buscando a confirmação das informações divulgadas pela Monsanto através de experimentos de avaliação de toxicidade, genotoxicidade e carcinogenicidade (VIGFUSSOM; VYSE, 1980; BOLOGNESI et al., 1997; WILLIAMS et al., 2000; KOLLER et al., 2012; KWIATKOWSKA et al., 2017).

Esses testes foram realizados com diversos tipos de vertebrados e avaliando as respostas *in vitro* ao glifosato e Roundup®, e também a exposição *in vivo* a esses compostos (TARAZONA et al., 2017).

No estudo de Romano et al. (2012), fêmeas de ratos foram tratadas com água contendo glifosato (50 mg/kg) entre o dia gestacional 18 e o quinto dia pós-natal, e os machos foram avaliados 60 dias após o nascimento. Os autores relataram que a

exposição materna ao glifosato afetou o desenvolvimento comportamental e reprodutivo em ratos machos, sendo este resultado relacionado à hipersecreção de andrógenos. Os resultados incluíram aumentos nas concentrações séricas de testosterona, estradiol e hormônio luteinizante; na produção de RNAm e proteína na hipófise; e na produção de esperma, além de puberdade precoce. Esses resultados foram contestados por DeSesso e Williams (2012) em relação à formulação do GBH, ausência de grupos de controle adicionais e uso de protocolo não padronizado para teste de preferência de parceiro sexual.

Resultados opostos aos de Romano et al. foram obtidos por Dallegrave et al. (2007). Neste estudo, os ratos Wistar foram expostos, via oral, a 50, 150 ou 450 mg/kg de glifosato durante a gravidez (21-23 dias) e lactação (21 dias). Os principais efeitos adversos foram observados na prole masculina: redução da contagem de espermatozoides e sua produção diária em adultos; elevação de espermatozoides anormais; e diminuição no nível sérico de testosterona na puberdade. Outro estudo do mesmo grupo avaliou os efeitos teratogênicos de Roundup® na prole de ratos Winstar. Os animais receberam por via oral 500, 750 ou 1000 mg/kg de glifosato do 6º ao 15º dia gestacional e a cesariana foi feita no 21º dia. A porcentagem de anormalidades esqueléticas (ausência de ossos ou partes de ossos, ossos dobrados, assimetria, fusões e fissuras) aumentou com o aumento da dose. Observou-se uma taxa de mortalidade de 50% em fêmeas gestantes tratadas com 1000 mg/kg de glifosato (DALLEGRAVE et al., 2003).

No estudo de Winnick e Dzialowski (2013), embriões de frango foram tratados com glifosato e Roundup® entre os dias 6 a 18. Os embriões expostos a 2% de glifosato e 1% de Roundup® apresentaram taxas aumentadas de mortalidade; diminuição do embrião, da massa cardíaca e hepática; e redução do tibiotarso e do comprimento do bico.

Lajmanovich, Sandoval e Peltzer (2003) observaram deformidades craniofaciais e bucais, anormalidades oculares e caudas curvadas em sapos expostos a glifosato e GBH. Tanto a mortalidade quanto os defeitos aumentaram com o tempo e a concentração de herbicidas. Jayawardena et al. (2010) relataram menor sobrevida,

metamorfose retardada e altas frequências (69%) de malformações como cifose, escoliose, úlceras da pele e edema também em sapos.

Em ostras, Akcha, Spagnol e Rouxel (2012) não encontraram efeitos sobre o desenvolvimento de embriões larvais ou espermatozoides dos animais mantidos em água do mar contendo glifosato e Roundup® durante 24 h em todas as concentrações testadas (0,5, 1,0, 1,5, 2,5, 5,0 µg/L). Também não foram detectados efeitos no desenvolvimento da larva embrionária de ostra em concentrações de glifosato, AMPA e Roundup® variando de 0,1 a 1000 µg/L, após exposição de 48 h (Mottier et al. 2013). Em relação à sobrevida, nenhuma larva foi observada acima da concentração de 10.000 µg/L de Roundup®. Apesar disso, não foram relatadas alterações na mortalidade independentemente das concentrações de glifosato e AMPA.

Peixes-zebra também foram utilizados para estudar os efeitos iniciais da exposição ao glifosato e Roundup Classic®, diluídos até a concentração de 50 µg/ml de glifosato (ROY; CARNEIRO; OCHS, 2016). Após 24h, foram encontradas anormalidades morfológicas (reduções encefálicas e oculares) e perda de ventrículos cerebrais delineados. Além de diminuições nos genes expressos nas regiões do olho, prosencéfalo e mesencéfalo.

O efeito de GBH sobre o sistema imunológico de jacarés-de-papo-amarelo foi avaliado por Siroski et al. (2016). Os répteis foram expostos durante dois meses a diferentes concentrações do herbicida que foram diminuindo progressivamente para simular a degradação do glifosato na água. No grupo 1, de 11 mg/L para 2,5 mg/L e no 2, de 21 mg/L para 5 mg/L. Além disso, foi injetada nos animais uma solução de lipopolisacarídeo (LPS) de *Escherichia coli* para desencadear uma resposta imune e avaliar os parâmetros associados a ela. Os resultados mostraram que os animais expostos ao GBH apresentaram menor atividade do sistema complemento do que os animais controle, mas não foram observadas diferenças nas células brancas do sangue.

2.4 Efeitos do glifosato em humanos

Como descrito anteriormente, muitas agências de avaliação de risco para a saúde humana classificam o glifosato como seguro para humanos. Essas avaliações são baseadas em, principalmente, estudos carcinogênicos com outros animais (MINK et al., 2012). Entretanto, nas últimas décadas, vários estudos foram publicados utilizando células humanas ou dados obtidos *in vivo*, que lançam dúvidas sobre a segurança dessas substâncias.

2.4.1 Experimentos *in vitro*

O teste *in vivo* dos efeitos do herbicida sobre o organismo humano é complexo. Não é possível realizar experimentos controlados com humanos utilizando agentes químicos que são tóxicos à saúde. Os modelos animais são utilizados, mas a resposta pode ser bastante diferente. Portanto, estudos *in vitro* com células ou linhagens celulares são essenciais para a compreensão dos efeitos dos herbicidas na saúde humana.

Não há consenso sobre a exposição de células humanas ao glifosato. As respostas variam de acordo com o tipo de célula, concentração, fórmula química, tempo de exposição e metodologia.

O primeiro estudo que avaliou os efeitos sobre o DNA causado por Roundup® em linfócitos humanos foi realizado por Vigfusson e Vyse, em 1980. Esses autores sugeriram que este produto químico é, no máximo, deficientemente mutagênico.

Alvarez-Moya et al. (2014) e Mladinic et al. (2009) também realizaram experimentos com linfócitos humanos. O primeiro estudo indica que o glifosato é genotóxico, dependendo do tempo e da concentração de uso. Os autores do segundo sugeriram

que menores concentrações de glifosato não apresentavam efeitos perigosos sobre o DNA.

Martinez et al. (2007) compararam a toxicidade do glifosato e Roundup® nas células mononucleares do sangue periférico humano (PBMCs) e identificaram que as formulações eram mais citotóxicas do que o componente ativo.

Em 2017, Kwiatkowska et al. observaram uma diminuição no nível global de metilação e aumento de danos ao DNA em PBMCs quando expostas ao glifosato.

A viabilidade celular das linhagens celulares prostáticas epiteliais normais imortalizadas utilizadas por Li et al. (2013) não diminuiu quando expostas ao glifosato.

Benachour e Seralini (2009) usaram linhagens de rim embrionário humano, além das células endoteliais da veia umbilical humana, para avaliar a toxicidade de quatro GBHs, com concentrações abaixo das recomendações agrícolas e correspondentes a baixos níveis de resíduos nos alimentos. Todas as formulações causaram morte celular total dentro de 24 h de incubação devido à inibição da atividade da succinato desidrogenase mitocondrial. Para os autores, os efeitos deletérios são proporcionais às concentrações de glifosato e dependem da natureza dos adjuvantes, o que mostra que os adjuvantes não são inertes.

Devido à facilidade de obtenção de linhagens celulares tumorais e à fácil manipulação dessas células, muitos estudos avaliam os efeitos do GBH nessas linhagens, conforme descrito abaixo. Além disso, fornecem informações sobre os possíveis efeitos do glifosato na progressão da doença.

Li et al. (2013) observaram que o glifosato e AMPA inibiram o crescimento celular em oito linhagens celulares: quatro de câncer de próstata, duas de câncer de ovário, uma de câncer cervical e uma de câncer de pulmão.

Efeito oposto foi observado por Kasuba et al. (2017), que observaram proliferação de células HepG2 quando expostas ao glifosato.

Richard et al. (2005) realizaram um estudo com Roundup® em uma linhagem celular

placentária humana derivada de coriocarcinoma e observaram que o GBH reduziu a viabilidade celular pelo menos duas vezes mais do que o glifosato.

Chaufan, Coalova e Molina (2014) examinaram os efeitos do glifosato, AMPA e GBH sobre o equilíbrio oxidativo e os *endpoints* celulares na linhagem HepG2. Observou-se que a exposição ao glifosato e ao AMPA não afetou a viabilidade celular, enquanto o GBH induziu uma rápida diminuição.

Desta forma, os dados atualmente disponíveis mostraram que os GBHs são mais tóxicos do que o próprio componente ativo, apoiando a ideia de que os aditivos nas formulações comerciais não são inertes e desempenham um papel na toxicidade do herbicida (CHAUFAN; COALOVA; MOLINA, 2014; COALOVA; MOLINA; CHAUFAN, 2014).

2.4.2 Exposição *in vivo*: estudos epidemiológicos

O aumento da exposição humana ao GBH é quase certo, considerando o aumento exponencial do uso desses produtos nas últimas décadas. O impacto do glifosato em doenças humanas foi avaliado por poucos estudos epidemiológicos. Esses trabalhos examinaram a associação entre exposições a GBHs e efeitos na saúde em humanos, incluindo câncer (VANDEMBERG et al., 2017).

O primeiro grande projeto que analisou a associação da exposição a pesticidas e o risco de câncer em cerca de 26.000 indivíduos (incluindo os trabalhadores diretamente expostos e suas famílias) foi o The Agricultural Health Study (AHS) (ALAVANJA et al., 1996).

Em 2003, em um dos estudos desse projeto, não foi encontrada nenhuma associação entre o uso de glifosato e o risco de câncer de próstata (ALAVANJA et al., 2003). De Ross et al. (2005) e Sorahan et al. (2015), utilizando os dados do AHS, também não

encontraram nenhuma associação entre ao uso de glifosato e o risco de diversos tipos de câncer.

Da mesma forma, outros estudos realizados com o AHS não encontraram associação entre o uso de glifosato e câncer colorretal, cólon, câncer retal, pâncreas e melanoma (LEE et al., 2007; ANDREOTTI et al., 2009; DENNIS et al., 2010).

A associação mais provável entre o uso de glifosato e o risco de câncer foi com Linfoma não-Hodgkin. Em diferentes populações, diversos estudos, como o de Cantor et al (1992), De Roos et al. (2003), McDuffie et al. (2008), Eriksson et al. (2008), sugeriram uma possível associação. Entretanto, nenhum deles conseguiu efetivamente provar.

Fatores que podem explicar essa ausência de associação entre câncer e glifosato é o baixo número de indivíduos utilizados nos estudos, bem como o pequeno número de indivíduos que utilizaram especificamente o glifosato. Muitos estudos são realizados através de questionários, e dessa forma, a quantificação e a identificação dos tipos específicos de herbicidas utilizados ficam comprometidas.

O glifosato, além da ingestão através do alimento e da exposição ocupacional, pode ser inalado do ar. Alguns estudos relacionaram o glifosato como fator de risco para o desenvolvimento de doenças respiratórias (CHANG; SIMCIK; CAPEL, 2011; WILLIAMS et al., 2016).

O glifosato foi avaliado como fator de risco para asma em mulheres com asma atópica (HOPPIN et al., 2008). Em agricultores de Iowa e Carolina do Norte, Slager et al. (2010) verificaram uma associação entre glifosato e rinite e episódios aumentados de rinite. Hoppin et al., em 2017, avaliou a associação do glifosato com sibilância alérgica e não alérgica em agricultores. Os resultados mostraram que o herbicida aumenta o risco para os dois grupos.

Já para Henneberger et al. (2014), existe uma associação inversa entre a exposição ao glifosato e o agravamento dos sintomas da asma, pois é possível que os pacientes com asma possam ser menos sensíveis ao herbicida ou que os indivíduos suscetíveis

evitem a exposição enquanto os outros permanecem expostos sem sintomas adicionais. Por outro lado, Faria et al. (2005) não detectou qualquer relação entre o glifosato e os sintomas de asma ou doença respiratória crônica em agricultores do Brasil.

Dentre os estudos epidemiológicos realizados, muitos avaliaram o risco de desenvolvimento de doenças em trabalhadores diretamente expostos ao glifosato. O objetivo do estudo de Jauhiainen et al. (1991) foi medir a exposição dos trabalhadores florestais ao herbicida glifosato durante o trabalho de limpeza feito com serras equipadas com pulverizadores de herbicidas. Nesse estudo, não foram identificados problemas de saúde relacionados ao glifosato.

Resultados opostos foram obtidos por Jayasumana et al. (2015), que observaram aumentos na frequência de doença renal crônica entre agricultores no Sri Lanka.

Diversos problemas de reprodução humana foram associados à utilização de glifosato. Curtis et al. (1999) identificaram uma diminuição da fertilidade (no mínimo 20%) de mulheres expostas à GBH e Sanin et al. (2009) observaram que mulheres expostas ao glifosato levaram mais tempo para conceber. Arbuckle, Lin e Mery (2001) relataram associação entre abortos espontâneos tardios (12-19 semanas) e exposição ao glifosato preconcepção.

Garry et al. (2002) examinaram a ocorrência de transtornos de nascimento e desenvolvimento em filhos de agricultores de Minnesota que aplicavam GBH. Os autores descobriram que a exposição ao glifosato/Roundup® aumenta o risco de déficit de atenção e transtornos de hiperatividade com déficit de atenção em crianças de 6 a 14 anos de idade. Curiosamente, ao investigar os níveis de exposição materna e fetal, Aris e Leblanc (2011) não detectaram o glifosato no sangue total materno nem no cordão umbilical.

Normalmente, o contato rápido da pele humana com GBH não causa grandes danos. No entanto, podem ocorrer lesões quando o contato é prolongado. Mariager et al. (2013) relataram queimaduras graves em uma mulher após prolongada exposição dérmica acidental (24 h) a GBH. A paciente desenvolveu inchaço local, bolhas e

feridas. Também apresentou deficiência neurológica na mão devido a uma condução nervosa reduzida. Para esses autores, o tempo de exposição prolongado poderia explicar o dano mais profundo do que o relatado anteriormente aos nervos e músculos.

2.4.3 Intoxicação aguda

A toxicidade aguda do glifosato é classificada como baixa em ratos, sendo que o LD_{50} oral do glifosato puro é de 5.600 mg/kg (FAO/OMS, 2016). Não existem valores de LD_{50} em humanos.

As intoxicações agudas após ingestão de GHBS são observadas em casos de suicídios ou de acidentes. Os sintomas relacionados à intoxicação são diversos, variando de leves à mais graves, e muitas vezes levam o indivíduo à morte. Muitos casos de intoxicação aguda são relatados na literatura.

Potrebić et al. (2009) descreveram um caso de envenenamento de uma mulher de 56 anos que ingeriu cerca de 500 mL de GBH. Os sintomas foram hipotensão, hipercalemia, insuficiência respiratória e renal. A paciente sobreviveu à fase aguda de envenenamento, mas desenvolveu um dano maciço no cérebro que levou ao coma e posteriormente à morte.

Ptok (2009) relatou um caso de uma professora de 26 anos que usou glifosato corretamente, mas sofreu de disfonia grave após algumas horas. Este problema foi causado por uma menor mobilidade da dobra vocal, sugerindo comprometimento de inervação. Os sintomas foram resolvidos espontaneamente 6 semanas depois e a mobilidade da dobra vocal retornou ao normal.

Malhotra et al. (2010) relataram o caso de um homem de 71 anos que tentou suicídio com GBH. Ao atendimento, o paciente estava em choque cardiogênico com acidose metabólica grave e apresentou falta de resposta clínica (> 7 dias). A tomografia

computadorizada cerebral foi normal. A leitura de eletroencefalograma no dia 8 pós-ingestão demonstrou dados consistentes com uma encefalopatia. Ele foi transferido da unidade de terapia intensiva no dia 10 e recebeu alta no dia 16, com recuperação clínica completa.

No estudo de Zouaoui et al, (2013) a finalidade foi associar as características clínicas de pacientes com intoxicação aguda com a concentração de glifosato no sangue e na urina para prever resultados clínicos. Os autores classificaram 13 casos de intoxicação por glifosato de acordo com a gravidade dos sintomas clínicos (sendo 3 laudos forenses). Entre os 10 pacientes, 5 tiveram intoxicação leve a moderada, 2 tiveram envenenamento grave e 3 morreram. Os sintomas mais comuns foram a ulceração orofaríngea (5/10), náuseas e vômitos (3/10), dificuldade respiratória (3/10), arritmia cardíaca (4/10), insuficiência renal (2/10) e consciência alterada (3/10). Outros sintomas foram choque cardiovascular, parada cardiorrespiratória, distúrbios hemodinâmicos, coagulação disseminada intravascular e, em casos fatais, falência múltipla de órgãos. Em casos de intoxicação leve a moderada e de casos fatais, as concentrações de glifosato no sangue apresentaram um valor médio de 61 mg/L (intervalo de 0,6-150 mg/L) e 4146 mg/L (intervalo 690-7480 mg/L), respectivamente.

As estratégias de tratamento para intoxicação por glifosato são principalmente de suporte, pois não há antídoto. A técnica de irrigação por sonda gástrica oral e a administração de carvão ativado são métodos que podem ser utilizados para descontaminação (LEE et al., 2008). Suporte ventilatório e inotrópico e hemodiafiltração venovenosa contínua também podem ser necessários de acordo o estado do paciente (MALHOTRA et al., 2010; ZOUAOUI et al., 2013).

2.4.4 Exposição e intoxicação por glifosato no Brasil

Os registros sobre os casos de intoxicação por agrotóxicos no Brasil são obtidos

através de dois sistemas: o Sistema Nacional de Informações Tóxico-Farmacológicas (SINITOX) e o Sistema de Informação de Agravos de Notificação (SINAN). O SINITOX tem como principal atribuição coordenar a coleta, a compilação, a análise e a divulgação dos casos de intoxicação e envenenamento notificados no país, e está vinculado à Fundação Oswaldo Cruz (SINITOX, 2017). O SINAN é alimentado, principalmente, pela notificação e investigação de casos de doenças e agravos que constam da lista nacional de doenças de notificação compulsória, sendo facultado aos estados e municípios incluírem nesse sistema outros problemas de saúde importantes em sua região (SINAN, 2017).

Segundo os dados disponíveis no SINITOX (2017) mais atualizados, em 2014 foram registrados no Brasil 2854 casos de intoxicação por uso agrícola de agrotóxico e 2262 por uso doméstico.

Infelizmente, esses dados não representam a realidade do país pois nem todos os centros estaduais de registro de intoxicação repassam os dados para o sistema nacional (SINITOX, 2017). Uma outra dificuldade é a falta de diagnóstico e a subnotificação, pois muitas pessoas não comparecem aos hospitais ou não identificam a causa da intoxicação (RIGOTTO; VASCONCELOS; ROCHA, 2014).

No Espírito Santo, o Centro de Atendimento Toxicológico do Espírito Santo (TOXCEN) é o responsável pelo registro dos casos de intoxicação por agrotóxicos. Em 2015, foram registrados 771 casos de exposição/intoxicação por uso agrícola de agrotóxico e 35 por uso residencial (TOXCEN, 2015).

Os dados apresentados pelo SINITOX e TOXCEN não especificam o tipo de agrotóxico ingerido. Dessa forma, não há um registro de intoxicação específico por GBH. Porém, considerando que o glifosato corresponde a uma grande porcentagem de todo o agrotóxico vendido, é provável que uma parcela considerável desses casos sejam por intoxicação por glifosato.

Em relação à intoxicação crônica, não existe um sistema de registro desses casos e toda a informação disponível sobre isso ocorre através de estudos epidemiológicos isolados.

2.4.5 Detecção de contaminação por glifosato

Existem vários métodos de detecção do glifosato e do AMPA no ar, água, solo, urina e soro sanguíneo (IARC, 2015). Em humanos, o diagnóstico de contaminação por glifosato é estabelecido pela ocorrência de quadro clínico compatível com a exposição e, nos casos de ingestão, confirmado por testes que detectam a presença do herbicida no corpo.

Yoshioka et al. (2011) desenvolveram um método para detectar glifosato no soro humano por cromatografia líquida acoplada à espectrometria de massas sequencial (LC-MS/MS) usando cromatografia líquida com interação hidrofílica (HILIC). O LD para o glifosato foi de 0,03 µg/mL. Mariager et al. (2013) também utilizaram essa técnica.

Acquavella et al. (2004) quantificaram o glifosato na urina usando cromatografia líquida de alta performance (HPLC) com reação pós-coluna e detecção de fluorescência. Antes do HPLC, o glifosato foi isolado e concentrado através de troca iônica. Por essa técnica, o LD foi de 1 µg/L. Curvin et al. (2007) também quantificaram o glifosato na urina, porém utilizaram um imunoensaio de microbola (microbead) covalente por fluorescência (FCMIA) e o LD foi 0,9 µg/L.

A cromatografia gasosa com espectrômetro de massa (GC-MS) e o ensaio de imunoabsorção enzimática (ELISA) para detecção de glifosato foram utilizados para medir a quantidade do herbicida em urina (KRUGER et al., 2014). A detecção por ELISA foi mais eficiente (mínimo de 0,1 µg/mL e máximo de 71,3 µg/mL) do que a quantificação por GC-MS (mínimo de 1,0 µg/mL e máximo de 40 µg/mL).

Um outro teste utilizado é a medida do nível da enzima colinesterase. Essa enzima atua na regulação dos impulsos nervosos por meio da degradação do neurotransmissor acetilcolina nas sinapses e nas junções neuromusculares (PEARSON; PATEL, 2016). Existem dois tipos dessa enzima: a acetilcolinesterase eritrocitária (AChe), que é encontrada nos eritrócitos, no pulmão e no tecido nervoso;

e a butirilcolinesterase plasmática (BChE), sintetizada no fígado e que circula pelo plasma sanguíneo (LOCKRIDGE et al., 2016).

Em casos de intoxicação por organofosforados, como o glifosato, a colinesterase é inibida e consequentemente ocorre um acúmulo de acetilcolina, resultando em depressão dos centros respiratórios e circulatórios na medula, fraqueza dos músculos respiratórios e edema pulmonar (LOCKRIDGE et al., 2016). Entretanto, níveis baixos dessa enzima também estão presentes em outras situações, como por exemplo, hepatites, estados de desnutrição, infecções agudas, anemias, infarto do miocárdio e dermatomiosite (FARIA; FASSA; FACCHINI, 2007).

Utilizando esse método, Oliveira-Silva et al. (2011) conseguiu detectar o glifosato. Outros estudos não foram bem sucedidos nessa detecção (ETGES et al., 2002; SALVI et al., 2003).

Além dessas variações na efetividade do teste, essa medida só é indicada para contatos recentes (até duas semanas). Um outro problema é determinar os limites aceitáveis da exposição ocupacional para populações expostas continuamente aos agrotóxicos (FARIA; FASSA; FACCHINI, 2007). Todos esses métodos de avaliação só detectam exposições mais imediatas ao glifosato e não permitem uma avaliação de exposição contínua e/ou crônica a esse herbicida.

Até o momento, não existem testes moleculares para detecção da contaminação por glifosato.

2.5 Expressão gênica alterada por glifosato

As respostas moleculares ao glifosato já foram analisadas para alguns organismos, como ostra-do-pacífico, peixe-chato europeu, mosquito *Aedes* e pulga-da-água (TANGUY *et al.*, 2005; MARCHAND *et al.*, 2006; RIAZ et al., 2009; LE *et al.*, 2010).

Em humanos, são poucos os estudos realizados.

Hokanson et al. (2007) avaliaram a expressão gênica em linhagens MCF-7 (adenocarcinoma de mama) expostas ao GBH e identificaram 680 genes desregulados. Desses, os três principais foram EGR1, HIF1 e CXCL12, que parecem impactar potencialmente as taxas de início de apoptose e alterar os níveis de vascularização associados à formação de tumor.

Thongprakaisang et al. (2013) identificou que o glifosato altera a expressão dos genes dos receptores de estrógeno em linhagens de câncer de mama. Esse resultado também foi obtido por Mesnage et al. (2017).

Alterações na expressão de proteínas atuantes na fase G1/S do ciclo celular e também de S100A6/S100A9 (proteínas reguladoras de Ca^{2+}), IP3R1 (receptor responsável por liberar Ca2+ do retículo endoplasmático) e SOD1 (superóxido dismutase) foram identificadas por George e Shukla (2013) em células HaCaT (queratinócitos). Essas alterações em conjunto induziram a proliferação das células e inibição da apoptose.

Estudos que utilizam técnicas que analisam a expressão gênica global das células expostas ao glifosato são raros. Também não foram identificados os padrões de expressão gênica em células humanas saudáveis quando expostas ao glifosato.

Dessa forma, pesquisas que identifiquem as alterações a nível molecular são importantes para determinar em quais processos biológicos o glifosato atuaria através de modificações no padrão de expressão gênica.

2.6 Hipótese

A hipótese testada neste estudo é de que a exposição rápida ao Roundup® e ao AMPA leva à expressão diferenciada de genes que governam processos celulares, podendo explicar a toxicidade relatada na literatura.

3 OBJETIVOS

3.1 Objetivo geral

Identificar genes diferencialmente expressos em células mononucleares do sangue periférico (PBMCs) humano submetidas à exposição rápida com herbicida à base de glifosato (Roundup®) e AMPA.

3.2 Objetivos específicos

- Avaliar viabilidade e morte das células submetidas aos tratamentos;
- Identificar alterações de expressão gênica nas células expostas ao Roundup® e AMPA;
- Identificar em quais processos biológicos os genes diferencialmente expressos estão atuando.

4 ARTIGOS CIENTÍFICOS DERIVADOS DA TESE

4.1 Manuscrito 1

O manuscrito intitulado "Glyphosate's effects in humans: an evaluation of in vitro and epidemiological studies" foi submetido para avaliação ao periódico Regulatory Toxicology and Pharmacology (Qualis Capes B1; Fator de Impacto: 2,221), de acordo com os critérios estipulados pelo Regimento do Programa de Pós-Graduação em Biotecnologia (Figura 6).

Figura 6. Comprovação de submissão do manuscrito 1 para a revista Regulatory Toxicology and Pharmacology.

Glyphosate's effects in humans: an evaluation of *in vitro* and epidemiological studies

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Abstract

Glyphosate (N-(phosphonomethyl)glycine) is a post-emergent, non-selective and systemic herbicide, whose commercial formulations are called glyphosate-based herbicides (GBHs). These herbicides can be found in soil, air, water and groundwater, as well as in food. Glyphosate is degraded by soil microbes to carbon dioxide and aminomethylphosphonic acid (AMPA). The aim of this review is describe *in vitro* and *in vivo* studies about human exposure to GBHs. *In vitro* studies with human cells are essential for understanding the effects of a chemical on human health, but there is no consensus. The responses change according to the cell type, chemical's concentration and formula, exposure time and methodology. Human exposure to GBHs can occur through food, soil, water and air by directly or indirectly contact at occupational exposure or even at household. The association between glyphosate and human diseases was evaluated by few epidemiological studies.

Key words: glyphosate-based herbicides, toxicity, risk evaluations, food.

Background

Glyphosate (N-(phosphonomethyl)glycine) [CAS# 1071-83-6] is a post-emergent, non-selective and systemic herbicide (IARC, 2015; FAO/WHO, 2016). This molecule consists of the amino acid glycine and a phosphonomethyl moiety (Li et al., 2013). Glyphosate has as trade names: Abundit Extra; Credit; Xtreme; Glifonox; Glyphogan; Ground-Up; Rodeo; Roundup®; Touchdown; Tragli; Wipe Out; Yerbimat and others (FCI, 2015). These products can be called GBHs. Roundup® is the most common GBH and is presented in many formulations. These pesticides can be found in soil, air, surface water and groundwater, as well as in food (IARC, 2015).

In the GBHs production, glyphosate is as an isopropylamine, ammonium or sodium salt in water soluble concentrates and water-soluble granules (FAO, 2000). The relevant impurities are formaldehyde (maximum, 1.3 g/kg), N-nitrosoglyphosate (maximum, 1 mg/kg), Nnitroso-N-phosphonomethylglycine

and N-(phosphonomethyl)iminodiacetic acid (PMIDA) (FAO, 2000; Kwiatkowska et al., 2016). Surfactants (also called adjuvants), most notably polyethyloxylated tallowamine (POEA) and sulfuric and phosphoric acids, may be added to the product to facilitate uptake by plants, being the type and concentration characteristic of each formulation (IPCS, 1996; Székács and Darvas, 2012).

Generally, GBHs cause stronger effects than glyphosate itself (Martinez et al., 2007; Mesnage et al., 2013; Folmar et al,, 1979). This may be due to significant toxicity of adjuvants present in herbicide preparations (Song et al., 2012). Martinez et al. (2007) showed that cytotoxicity caused by Roundup® were stronger than induced by glyphosate.

Glyphosate-based herbicides were first synthesized in 1950 as a potential pharmaceutical compound, but its herbicidal activity was not discovered until it was re-synthesized and tested in 1970, being in use since 1974 (Székács and Darvas, 2012; Willians et al., 2016).

Use of glyphosate-based herbicides

The GBHs have agricultural and non-agricultural uses. In agriculture, the glyphosate acts against more than 100 species of weeds and more than 60 of perennial weed plants (Dill et al., 2010). Its application can occur in pre-harvest, post-planting and in pre-emergence and can be both by spray and aquatic herbicide (Tomlin, 2000). In smaller doses, it acts as plant-growth regulator and desiccant (IARC, 2015; FAO/WHO, 2016). Non-agricultural applications include residential and industrial use; control of plants in power lines and on roads; in forest management and in the control of cocaine and marijuana plantations (Dill et al., 2010; Mance, 2012; Lubick, 2009; Székács and Darvas, 2012; Williams et al., 2016). Changes in farming practice and the development of genetically modified crops that are resistant to glyphosate have contributed to the increase in use (Myers et al., 2016).

Glyphosate's metabolization

Glyphosate competitively inhibits the activity of a key plant enzyme called synthase 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), present in shikamate pathway and which participates of chorismate's production, a precursor for aromatic amino acids using in the synthesis of a number of pigments as flavonoids and anthocyanins (Mesnage et al., 2015; Kwiatkowska et al., 2016). This pathway is present in various groups of microorganisms, plants and parasites and is absent in animals (Richard et al., 2005; Thongprakaisang et al., 2013). The inhibition of the synthesis of aromatic amino acids stops growth of certain plants (Williams et al., 2016).

Glyphosate is degraded by soil microbes to carbon dioxide and AMPA [CAS# 1066-51-9; Molecular Formula: CH_6NO_3P; Molecular Weight: 111.04 g/mol] as a result of oxidative cleavage by glyphosate oxidoreductase (GOX) (Ribeiro et al., 2015). AMPA can accumulate in the environment (Jacob et al., 1988). A minor pathway for the degradation of glyphosate in bacteria (*Pseudomonas* sp. Strain LBr) is via conversion to glycine (Jacob et al., 1988). In mammals, glyphosate is not metabolized efficiently and is mainly excreted unchanged into the urine (Myers et al., 2016). Is has suggest that metabolism in AMPA also occurs in humans because small amounts of this component have been found in the blood following glyphosate poisoning; however, it may be that this metabolization is carried out by gut microbial metabolism (Motojyuku et al., 2008). In agreement with this latter suggestion, a study in rats showed that after oral administration of glyphosate, a small amount of AMPA was detected in the colon 2 hours later, which was attributed to intestinal microbial metabolism (Brewster et al., 1991).

In 2015, the IARC has classified the glyphosate as "probably carcinogenic to humans (Group 2A)". According four Expert Panels witch conducted a detailed critique of the IARC evaluation, the evidences does not support this conclusion of the IARC, being consistent that glyphosate is "unlikely to pose a carcinogenic risk to humans" (Williams et al., 2016). The WHO and the Food FAO classifies the glyphosate as "unlikely to pose a carcinogenic risk to humans from exposure through the diet (food and

water exposures)" (FAO/WHO, 2016). In January 2018, IARC responded to criticisms of the Monographs and the glyphosate evaluation, and confirmed risk assessment (IARC, 2018).

Methodology

Articles were selected using PubMed database. There was no selection of publication time. Inclusion criteria were: utilization of human cells or epidemiological studies; exclusive use of glyphosate/GBH for *in vitro* studies or specific association with glyphosate's effects for epidemiological studies; exposure to glyphosate alone or GBH.

Effects of glyphosate and Roundup® *in vitro*

In vivo testing of the herbicide's effects on human organism is complicated. Animal models can be used, but the answer may be quite different. Therefore, *in vitro* studies with cells lines are essential for understanding the effects of these chemicals on human health.

Healthy human cells

There is no consensus on the exposure of human cells to glyphosate. The responses change according to the cell type, concentration, GBH's chemical formulation, exposure time and methodology.

The first study to evaluate the effects on DNA caused by Roundup® in human lymphocytes was conducted by Vigfusson and Vyse in 1980. They showed significant increases in the Sister-Chromatid Exchanges (SCE) index upon exposure to Roundup®, but only in high concentrations ($65X\ 10^{-5}$ M and $65X\ 10^{-4}$ M). These authors suggested that this chemical is at most weakly mutagenic based on the SCE test.

Alvarez-Moya et al. (2014) and Mladinic et al. (2009a and 2009b) also performed experiments with human lymphocytes. In the first study, the comet assay was used to examine the genotoxicity of isopropylamine glyphosate. The DNA damage increased with the glyphosate's concentration (0.0007, 0.007, 0.07 and 0.7 mM), usually proportionally, with an exposure time of 20h. For authors, this result indicates that glyphosate is genotoxic, depending on the time and concentration of use.

Mladinic et al. (2009a) evaluated the genotoxic and oxidative potential of glyphosate at concentrations likely to be encountered in residential (2.91 µg/ml) and occupational (3.50 µg/ml) exposure. The tests were done with and without metabolic activation. Ferric-reducing ability of plasma (FRAP), thiobarbituric acid reactive substances (TBARS) and the hOGG1 modified comet assay were used to measure glyphosate's oxidative potential and its impact on DNA. Genotoxicity was evaluated by alkaline comet and analysis of micronuclei and other nuclear instabilities applying centromere probes. Because of inefficient or incorrect DNA repair, chromosomal damage is expressed during the cell division and represents an index of accumulated genotoxic effects (Bolognesi et al., 2009). The alkaline comet assay showed significantly increased tail length (20.39 µm) and intensity (2.19%) for 580 µg/ml, and increased tail intensity (1.88%) at 92.8 µg/ml. With metabolic activation (S9), tail length was significantly increased for all concentrations tested: 3.5, 92.8 and 580 µg/ml. Using the hOGG1 comet assay, a significant increase in tail intensity was observed at 2.91 µg/ml with S9 and 580 µg/ml without S9. Without S9, the frequency of micronuclei, nuclear buds and nucleoplasmic bridges slightly increased at concentrations 3.5 µg/ml and higher. The presence of S9 significantly elevated the frequency of nuclear instabilities only for 580 µg/ml. FRAP values slightly increased only at 580 µg/ml regardless of metabolic activation, while TBARS values increased significantly. Since for any of the assays applied, no clear dose-dependent effect was observed, Mladinic et al. indicated that glyphosate in concentrations relevant to human exposure do not pose significant health risk.

In the third one (Mladinic et al., 2009b), glyphosate's concentrations and the use or not of metabolic activation were similar to the previous study and an evaluation of the possible clastogenic and aneugenic effects was performed. Frequency of the micronuclei, nuclear buds and nucleoplasmic bridges in

cultures treated with glyphosate slightly increased from 3.5 µg/ml onward. The presence of metabolic activation significantly elevated cytome assay parameters only at 580 µg/ml. No concentration-related increase of centromere (C+) and DAPI (4',6-diamidino-2-phenylindole) signals (DAPI+) was observed for glyphosate treatment. As the previous study, Mladinic et al. suggested that lower concentrations of glyphosate have no hazardous effects on DNA.

Martinez et al. (2007) compared the toxicity of glyphosate and Roundup® on peripheral blood mononuclear cells (PBMCs). Cells were exposed to different glyphosate's (1, 50, 100, 200, 300, 400, 500, 1000, 1500 and 2000 µg/ml) or Roundup®'s concentrations (1, 10, 20, 40, 60, 80 and 100 µg/ml) for 24, 48, 72 and 96 h. Both herbicides were toxic to PBMCs. The Roundup® cytotoxicity was greater than glyphosate, since the LC_{50} determined by the trypan blue exclusion method, at 24 h was the equivalent of 56.4 µg/ml for Roundup® and 1640 µg/ml (1.64 mg/ml) for glyphosate. According toauthors, this *in vitro* study confirmed the toxic effects on human cells by glyphosate and its commercial preparations. Furthermore, they reported that commercial formulations were more cytotoxic than the active component alone, supporting the concept that additives in commercial formulations play a role in the toxicity attributed to GBHs.

Kwiatkowska et al., in 2016, performed experiments with PBMCs. Was observed a decrease in PBMCs viability (2.7%) after 24 h of incubation with glyphosate at 10 mM. A decrease in adenosine-5'-triphosphate (ATP) level also was observed in cells treated with glyphosate from 5 mM after 24h of incubation. Measurement of cell morphology revealed changes in PBMCs granularity that occurred after 24 h of incubation with 0.5 mM of glyphosate. The conclusion was that glyphosate caused toxic effects on PBMCs only at very high concentrations. These concentrations correspond to those resulting from acute or subacute intoxication with GBH.

In 2017, Kwiatkowska et al. observed a decrease in global DNA methylation level in PBMCs at 0.25 mM of glyphosate for 24 h. Concentrations at 0.25 mM and 0.5 mM increased p53 promoter methylation, while it did not induce statistically significant changes in methylation of p16 promoter.

The study also observed that glyphosate induced DNA damage in PBMCs in the concentrations range from 0.5 to 10 mM. Moreover, we noticed that PBMCs significantly repaired glyphosate-induced DNA damage (mainly after 0 min post incubation), but they were unable to repair completely DNA strand-breaks after 120 min post incubation.

Kwiatkowska et al. (2014a) evaluated the effect of glyphosate on human erythrocytes. Authors noticed that even with long incubation time up to 24 h did not affect the level of hemolysis and were not observed morphological changes induced by glyphosate in these cells (0.01–5 mM). Therefore, they concluded that glyphosate do not have hemolytic properties.

Kwiatkowska et al. (2014b) also performed experiments with erythrocytes to evaluate acetylcholinesterase (AChE) activity. The results showed that glyphosate (0.25–5 mM),)caused statistically significant inhibition of AChE activity after 1 h and 4 h of incubation. Glyphosate, in the concentrations ranging from 0.5 mM to 5 mM for 1h treatment and from 0.25 mM to 5 mM after 4h incubation, respectively, caused decrease (about 20%) in this parameter. This change in AChE activity was observed only at high glyphosate's concentrations (0.25–5 mM), which is resulting only of an acute poisoning, according to Kwiatkowska et al.

Another study that analyzed erythrocytes (Pieniazek et al., 2004), showed that after 1 h of incubation the Roundup Ultra 360 SL® increased the level of methemoglobin and the products of lipid peroxidation at 500 ppm and hemolysis at 1500 ppm. Already, the glyphosate increased the level of methemoglobin at 1000 ppm and caused hemolysis only after a long time of exposure (24 h) to high doses (1500 ppm). Both Roundup Ultra 360 SL® and glyphosate did not cause statistically significant changes in the level of glutathione (GSH), but increased the activity of catalase.

Gehin et al. (2006), Heu et al. (2012) and George and Shukla (2013) investigated the effects of glyphosate in Human Skin Keratinocytes (HaCaT). Gehin et al. (2006) showed that glyphosate alone or included in Roundup 3 plus® (0-25 mM), induced significant changes in cellular antioxidant status as a glutathione depletion (dose dependent depletion, which was more sensitive with Roundup® than with

glyphosate), enzymatic (catalase, glutathione-peroxidase and superoxide dismutase) disorders, and increased lipid peroxidation.

The aim of Heu et al. (2012) was examine glyphosate's cytotoxic effects in intracellular mechanisms of apoptosis. They conducted different incubation periods. From 6 to 18 h of incubation periods, cytotoxic profiles were superimposed and presented an LC_{50} of approximately 30 mM. For shorter incubation periods (0.5 and 1 h), the LC_{50} increased to 53 mM. They observed an increase in the number of early apoptotic cells at a low cytotoxicity level (15%) and then, at 45% cytotoxicity, lot of cells reached not only early but also late apoptotic state and even a necrotic state. They also showed that the glyphosate-induced mitochondrial membrane potential disruption could be a cause of apoptosis in keratinocyte cultures.

George and Shukla (2013) aimed to clarify if the imbalance between $[Ca^{2+}]$ levels and oxidative stress is associated with glyphosate-induced proliferation in HaCaT cells. The $[Ca^{2+}]$ levels, reactive oxygen species (ROS) generation and expressions of G1/S cyclins, IP3R1, S100A6, S100A9 and SOD 1 and apoptosis-related proteins were investigated upon glyphosate exposure in HaCaT cells. Glyphosate (0.1 mM for 72h) significantly induced proliferation, decreases [Ca2+], and increased ROS generation. This pesticide also enhanced the expression of G1/S phase's proteins and the cell proliferation. Additionally, glyphosate also triggers S100A6/S100A9 and decreases IP3R1 and SOD 1 expressions. Notably, Ca2+ suppression also prevented apoptotic related events including Bax/Bcl-2 ratio and caspases activation. According to authors, this study highlights that glyphosate promotes proliferation in HaCaT cells probably by disrupting the balance between [Ca2+]i levels and oxidative stress which in turn facilitated the down regulation of mitochondrial apoptotic signaling pathways.

The immortalized human normal prostatic epithelial cell lines RWPE-1 and pRNS-1-1 were used by Li et al. (2013) to evaluate cell viability when exposed to glyphosate. Glyphosate's concentrations of 15, 25, and 50 mM did not significantly decrease the cell viability, when compared with the untreated control group.

To evaluated human cell toxicity for GBHs and glyphosate alone (concentration 0.1 to 10000 ppm), Mesnage et al. (2013) measured mitochondrial activities, membrane degradation and caspases 3/7 activities. The chemicals were cytotoxic, inducing similar dose-dependent patterns on the human embryonic kidney 293 cell line (HEK 293) after 24 h of incubation.

Benachour and Seralini (2009) also used HEK 293, besides Human Umbilical Vein Endothelial Cells (HUVEC), to evaluate the toxicity of four GBHs, with concentrations below agricultural recommendations (1-2%) and correspondents to low levels of residues in food. All formulations caused total cell death within 24 h of incubation, because of the inhibition of the mitochondrial succinate dehydrogenase activity and also caused necrosis, by release of cytosolic adenylate kinase measuring membrane damage. The GBHs also induced apoptosis via activation of caspases 3/7 enzymatic activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis) and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. Glyphosate caused only apoptosis. For the authors, the deleterious effects are proportional to glyphosate's concentrations and dependent on the nature of the adjuvants, which confirms that the adjuvants are not inert.

Tumor cells

Studies with healthy cells are important for assessing the glyphosate's effects on human health, as previously discussed. However, due to the facility of obtaining tumor cell lines and the ease of working with these cells, many studies evaluate GBHs's effects on tumor lines, as described below. These studies provide insights into the possible effects of glyphosate on disease progression.

Li et al. (2013) observed that glyphosate and AMPA inhibited cell growth in eight cancer cell lines, including four prostate cancer cell (C4-2B, LNCaP, DU-145, and PC-3), two ovarian cancer cell lines (SKOV-3 and OVCAR-3), one cervical cancer HeLa cell line and one lung cancer cell line (A549) at

concentrations up to 50 mM. Glyphosate at a concentration of 50 mM decreased 27%, 73.4%, 39.3%, 36.9%, 28%, 58.8%, 25% and 17% the cell viability in the LNCaP, CA-2B, DU-145, PC-3, SKOV-3, OVCAR-3, HeLa and A549 cell lines, respectively.

Some studies have demonstrated that glyphosate have a disrupting effect on estrogen receptor alpha (ERα) and beta (ERβ) transcriptional activities. In the study of Thongprakaisang et al. (2013), it was evaluated estrogenic and/or antiestrogenic effects of glyphosate compared with endogeneous estrogen (E2) in the estrogen dependent human breast cancer cells T47D, being observed that the herbicide caused the proliferative effects in T47D of approximately 15–30% in the absence of E2 condition. This study also demonstrated that glyphosate altered the levels of ERα and ERβ proteins: at 6 h of exposure, it increased the levels of both ERα and ERβ while at 24 h of exposure, only ERα showed a significant induction at 10^{-7}M glyphosate concentration.

Richard et al. (2005) realized a study with Roundup® in human placental cell line derived from choriocarcinoma (JEG3 cells) and observed that GBHs reduced JEG3 cell viability at least twice more efficiently than glyphosate. This effect increased with time and was obtained with Roundup®'s concentrations lower than that of the agricultural use. The toxicity increased with time (8-fold at 0.8% between 24 and 48 h), and the LD_{50} was approximately 1.8 times lower for Roundup® (0.7%) than for glyphosate.

In 2012, Koller et al. analysed the cytotoxic and genotoxic properties of glyphosate and Roundup® in the TR146 cell line, which is derived from a neck metastasis of buccal epithelial origin. In this study was observed that both products (20 min; 10–20 mg/L), induced strand breaks that led to formation of comets as well as nuclear anomalies that reflected DNA instability including chromosomal damage; presence of micronuclei markers; binucleated cells with micronuclei and increase of nuclear buds. The most sensitive endpoint was micronuclei induction. The treatment of the cells with highest dose of Roundup® (20 mg/L) caused a threefold increase over the background, and the corresponding concentration of glyphosate, a weaker effect was seen.

Besides these studies, several assays evaluated the impact of glyphosate in the HepG2 human hepatoma cell line, a pertinent model for xenobiotic actions. Gasnier et al. (2009), studying four different formulations of Roundup and pure glyphosate, verified that all glyphosate-based formulations, by contrast to glyphosate alone, induced a rapid decrease in cell viability according to the formulation and the test, within 24 h only. The R400 formulation caused around 50% of DNA strand breaks at 5ppm, a sub agricultural dilution. The caspases 3/7 were significantly activated with nontoxic doses of R450 (60 ppm) up to 156% in 24 h. Their levels were considerably enhanced to 765% within 48 h.

In a second study carried out by Gasnier et al. (2010) was observed that in the cell lines, mortality increased with time and glyphosate concentrations in GBHs (Express® 7.2 g/L; Bioforce® 360 g/L; GT® 400 g/L; GT+® 450 g/L), however the increase was not proportional to alone glyphosate concentration. In this way, glyphosate had no toxic action alone under the conditions used in studies. The direct glyphosate action was most probably amplified by vesicles formed by adjuvants or detergent-like substances that allowed cell penetration, stability, and probably changed its bioavailability and thus metabolism (Gasnier et al., 2010).

Kasuba et al. (2017) studied the toxic effects of glyphosate on HepG2 cells exposed for 4 and 24 h in low concentrations (0.5 µg/mL - acceptable daily intake; 2.91 µg/mL - residential exposure level and 3.5 µg/mL - occupational exposure level). The results obtained indicate that, at the tested concentrations, the herbicide stimulated cell proliferation at both exposure times. However, the effect was more pronounced after 4 h of exposure (8-9%). It seems that low concentrations effectively stimulated cell proliferation, especially in inherently unstable and proliferative tumor cells. In the comet assay analysis, it was consistently observed that HepG2 cells differed in the DNA contained in their nuclei, which also indicated an impact on cell proliferation. However, this study did not confirm DNA-damaging effects of glyphosate when tested as a pure active compound. The level of ROS did not change significantly after 4 and 24h treatments. Apoptosis and necrosis effect were not seen.

Chaufan et al. (2014) examined the effects on oxidative balance and cellular endpoints of glyphosate, AMPA and a GBH in HepG2 cell line. It was observed that glyphosate and AMPA exposure did not affect cell viability until 1000 mg/L, while GBH treatment induced a rapid decrease in cell viability depending on concentration and on duration of exposure. The ROS production was investigated and neither glyphosate nor AMPA treatment caused differences in ROS formation. In relation to cell death, it was observed that when cells were exposed to GBH, 23.5% of the nuclei showed a condensed and fragmented chromatin, while it was not modified by glyphosate alone or AMPA. In this way, this was verified that GBH induced dose-dependent cytotoxicity, while they were not observed toxic effects with glyphosate and AMPA at assayed concentrations. Therefore, this indicated that GBHs have adjuvants that, together with the active ingredient, caused toxic effects not observed with glyphosate itself.

In a study realized by Coalova et al. (2014), analyzing the influence of the spray adjuvant on the toxicity effects of a glyphosate formulation in HEp-2 cell line, was observed that all the agrochemicals assayed (glyphosate formulation, spray adjuvant and mixture) induced a decrease in cell viability depending on concentration and time exposure. Mixture (spray adjuvant with glyphosate formulation) resulted in significantly higher toxicity to cell cultures compared to each agrochemical alone suggesting additive effect. ROS production was investigated and was verified that glyphosate formulation and mixture treatment showed significantly higher levels of ROS (139% and 116% respectively). In relation to apoptotic cell death involving caspase 3/7 activation, was observed that these enzymes were significantly activated with LC_{20} concentration of all agrochemicals, what could be indicating that oxidative stress triggers caspase activation. Morphological changes occurred only with glyphosate formulation treatment. In this way, the currently available data showed that GBHs are more toxic than the active component itself, supporting the idea that additives in commercial formulations play a role in herbicide's toxicity (Chaufan et al., 2014; Coalova et al., 2014).

Effects of glyphosate and Roundup® *in vivo*

The human exposure to GHBs is increasing following the exponential rise of use of these products in the last decades. Both glyphosate and AMPA can be relatively persistent in the environment, which may result in a wide range of ecological risks (Bai and Ogbourne, 2016). Humans exposure to these chemicals can occur through food, soil, water, and air by directly or indirectly contact at occupational exposure or even at household (IARC, 2015).Glyphosate's impact on human diseases was evaluated by few human epidemiology studies. These works had examinated the association between exposures to GBHs and effects in human health, including cancer (Vandenberg et al., 2017).

Cancer

Alavanja et al. (1996) described the first big project, the Agricultural Health Study (AHS), to analyze the association of exposure to pesticides and the cancer risk. About 26.000 individuals, including the pesticides applicators and their families, composed the cohort. With this group, many studies were published.

In 2003, Alavanja et al. examined the association between pesticide use and risk of prostate cancer and found a correlation with the joint use of all pesticide analyzed (OR= 1.14, 95% IC: 1.05, 1.24). However, when analyzed only the use of glyphosate, was not found any association.

De Ross et al. (2005), using the AHS database, had studied all cancers described in that population and found no risk associated with glyphosate use, but the authors suggested an association (without significance) with multiple myeloma (RR=1.0; 95% IC: 0.9–1.2). Due this result, Sorahan et al. (2015) reanalyzed the same data with more complete information to prove the association. Again, was not found any significance (RR= 1.12; 95% IC: 0.50- 2.49).

Lee et al. (2007) studied the association of glyphosate's exposure with risk of colorectal cancer (OR= 1.2, 95% IC: 0.9-1.6.), colon cancer (OR= 1.0, 95% IC: 0.7-1.5) and rectal cancer (OR= 1.6, 95% IC= 0.9-2.9). Andreotti et al. (2009) studied cases of pancreas cancer (OR= 1.1; 95% IC: 0.6-1.7). Dennis et

al. (2010) studied melanoma (data not showed), but as well as all others studies done with AHS, did not found any association between the use of glyphosate and cancer. The groups suggested a possible correlation, but the fact was never proved.

Currently, the most plausible association of glyphosate and cancer was with Non Hodgkin Lymphoma. Many studies as Cantor et al. (1992) (OR= 1.1; 95% IC: 0.7-1.9), De Roos et al. (2003) (OR= 1.6; 95% IC: 0.9- 2.8), McDuffie et al. (2001) (OR= 1.20; 95% IC: 0.83–1.74), Eriksson et al. (2008) (OR= 1.51; 95% IC: 0.77–2.94) studied this cancer. All these groups evaluated different populations and in all cases, suggested a possible association, but none found a statistically significant association.

In 1998, Nordstrom et al. published a study realized at Sweden, where were analyzed 121 cases of hairy cell leukemia (HCL). An association was found between the exposure to any herbicides and the increased risk of HCL, but when the model was adjusted, the effect disappear (OR=1.8; 95% IC: 0.7-4.6). For glyphosate, was not found association (OR= 3.1; 95%IC: 0.8-12).

Lee et al. (2004) analyzed the effect of glyphosate on stomach and esophagus cancer. For both cases, was not found association (OR= 0.8.; 95% IC: 0.4-1.5 and OR= 0.7; 95% IC: 0.3-1.4, respectively).

It is very important to highlight that the number of subjects is usually very low when the count includes solely those who are exclusively exposed to glyphosate. Even with large sampling, it is complicate to estimate the exactly number of individuals who have used glyphosate alone to allow a perfect correlation between exposure and cancer development.

Respiratory diseases

Beyond the food and occupational exposure, glyphosate can be inhaled from the air. Even considering 100% absorption at maximum concentration, it's important to note that the exposure is about five times

smaller than the systemic acceptable daily intake proposed by European Food Safety Authority (EFSA) (Chang et al., 2011; Williams et al., 2016).

Hoppin et al. (2008) assessed glyphosate as risk factor for asthma in two groups: women with atopic asthma and those with nonatopic asthma. Glyphosate were significantly associated with atopic asthma (OR= 1.31; 95% CI= 1.02–1.67). Henneberger et al. (2014) found an inverse association between glyphosate exposition and asthma symptoms exacerbation. For authors it is possible that patients with asthma may be less sensitive to herbicide or that susceptible individuals to exacerbation avoid exposure while the others remain exposed without additional symptoms. Faria et al. (2005) didn't detect any relationship between glyphosate and asthma symptoms or chronic respiratory disease in farmers from Brazil.

In a posterior work, Hoppin et al. (2017) examined the association of glyphosate with allergic and non-allergic wheeze in male farmers. The results evidenced an exposure–response relationship in which the herbicide increases the risk to both allergic status (OR = 1.56; 95% CI = 1.19–2.03 for allergic and OR = 1.24; CI = 1.07–1.44 for non-allergic).

While studying the pesticide exposition as predictor of rhinitis in farmers from Iowa and North Carolina, Slager et al. (2010) verified an association between glyphosate and current rhinitis and increased rhinitis episodes (OR = 1.09; 95% CI = 1.05–1.13). Except Faria et al. (2005) all cited studies used data from AHS cohort study.

Others diseases or health problems

Jayasumana et al. (2015) observed increases in the frequency of chronic kidney disease among farmers in Sri Lanka. In the multivariable analysis the highest risk for this disease was observed among participants who used glyphosate (OR 5.12, 95% CI 2.33-11.26) as a pesticide.

A decrease of fertility (at minimum by 20%) was associated with women exposure to GBHs (Curtis et al., 1999). Differences in the time to get pregnant in populations exposed to GBHs from aerial spraying for the control of coca plants were noticed between Colombia's different regions. Reduced fecundability in some regions was not associated with glyphosate's spraying. However, women in Valle del Cauca, a sugar-cane region with a history of use of glyphosate and others chemicals for more than 30 years, took longer to conceive (OR =0.15; 95% CI: 0.12-0.18), (Sanin et al., 2009).

Bolognesi et al. (2009) also develop a study in these Colombia regions. A cytogenetic biomonitoring study was carried out in subjects from five regions, characterized by different exposure to glyphosate and other pesticides. Compared with Santa Marta (where organic coffee is grown without pesticides), the baseline frequency of binucleated cells with micronuclei (BNMN) was significantly greater in subjects from the other four regions, which means that these cells presented bigger defects in cytokinesis. The highest frequency of BNMN was in Boyacá (no aerial spraying of glyphosate) and in Valle del Cauca. There was no association between self-reported direct contact with sprays and frequency of BNMN. Four months after spraying, a statistically significant decrease in the mean frequency of BNMN, compared with the sampling of 5 days after spraying, was observed in Nariño, but not in Putumayo and Valle del Cauca. For these authors, these data suggest that genotoxic damage associated with glyphosate spraying as evidenced by micronuclei test is small and appears to be transient.

Arbuckle et al. (2001) reported an association between late spontaneous abortions (12–19 weeks) and preconception exposure to glyphosate (OR = 1.7; 95% CI 1.0–2.9). Interestingly, when investigating maternal and fetus exposure levels, Aris and Leblanc (2011) reported no detectable serum glyphosate in maternal whole blood nor umbilical cord.

There are rare studies that evaluated the association between neurological diseases and GBHs's use. Garry et al. (2002) examined the birth and developmental disorders occurrence in farm families. The

authors found that glyphosate/Roundup® exposition increases the risk of attention deficit and attention-deficit hyperactivity disorders in 6-14 years old children (OR = 3.6; 95% CI 1.35–9.65).

Usually, rapid human skin contact with GBHs does not cause large lesions. Nevertheless, lesions can occur when the contact is prolonged. Mariager et al. (2013) reported severe burns in a woman after prolonged accidental dermal exposure (24 h) to a GBH. The patient developed local swelling, bullae and exuding wounds. Neurological impairment followed affecting finger flexion and sensation with reduced nerve conduction. For these authors, long exposure time could explain the deeper symptoms than previously reported damage to nerves and muscles.

Acute Intoxication

Acute intoxications are observed in suicidal or accidental cases after ingesting GBHs. Symptoms, severe outcomes and fatalities related to intoxication are descript below.

Potrebić et al. (2009) described a case report poisoning of a 56-year old woman ingested about 500 mL of herbicide containing glyphosate isopropylamine salt. The most prominent manifestations of poisoning included hypotension, hyperkaliemia, respiratory and renal failure. The patient survived the acute phase of poisoning, but she developed massive white matter damage that led to vigil coma and lethal outcome. A similar outcome was presented by Chang et al. (2009), which reported the case of a 57-year-old woman who was admitted unconscious to the hospital after ingestion of GBHs (suicide attempt).

Ptok (2009) related a case of a 26-year-old teacher who used glyphosate correctly but suffered from severe dysphonia after some hours. This problem was caused by decreased vocal fold mobility, suggesting innervation impairment. Symptoms resolved spontaneously 6 weeks later and vocal fold mobility returned to normal.

Malhotra et al. (2010) reported a 71-year-old male who attempted suicide with a GBH. The patient suffered cardiogenic shock with severe metabolic acidosis and demonstrated prolonged clinical unresponsiveness (>7 days). Computed Tomography brain was normal. Electroencephalogram reading on day 8 demonstrated generalized slow wave activity and slow wave complexes consistent with an encephalopathy. He was transferred from the intensive care unit on day 10 and discharged home on day 16 with full clinical recovery.

The purpose of Zouaoui et al. (2013) was to describe the clinical feature and determinate the utility of the glyphosate's concentrations in blood and urine and the dose taken for predicting clinical outcomes. They classified 13 glyphosate poisoning cases by intoxication severity using clinical criteria (10 was clinical observations and 3 are forensic cases). Among the 10 patients, 5 had mild to moderate poisoning, 2 had severe poisoning and 3 died. Most common symptoms were oropharyngeal ulceration (5/10), nausea and vomiting (3/10), respiratory distress (3/10), cardiac arrhythmia (4/10), impaired renal function (2/10) and altered consciousness (3/10). Other symptoms were cardiovascular shock, cardiorespiratory arrest, hemodynamic disturbance, intravascular disseminated coagulation and multiple organ failure in fatalities. In mild–moderate intoxication and fatal cases, blood glyphosate concentrations had a mean value of 61 mg/L (range 0.6–150 mg/L) and 4146 mg/L (range 690–7480 mg/L), respectively.

The aim of Lee et al. (2008) study was to establish an early prognostic model of patients with GBH intoxication. Variables as age, sex, estimated amount of ingestion, symptoms/signs including first vital signs, chest x-ray, and biochemical studies (assays for serum urea nitrogen, creatinine, alanine aminotransferase, sodium, potassium) from 58 patients (19 men and 39 women) were analyzed. Seventeen patients died. Five variables (respiratory distress needing intubation, metabolic acidosis, tachycardia, elevated creatinine level and hyperkalemia) were found to be highly associated with poor outcome and mortality. The conclusion of the authors was that GBHs poisoning induced multiorgan toxicity and that pulmonary and renal toxicity seems to be responsible for its mortality. Useful

prognostic factors for predicting GBH's mortality was metabolic acidosis, abnormal chest x-ray, tachycardia and elevated creatinine level.

Many associations between glyphosate's concentrations found in human health remain indeterminate, without robust epidemiological and biomonitoring studies. These studies are essential to improve conclusions about the GBHs's safety.

Risk of glyphosate's presence in food

Humans are indirectly exposed to glyphosate through food, making worry the amount of glyphosate residues present in meat (and/or animals derived products), fruits and vegetables. There is also concern about the risks from this type of glyphosate exposure (Van Eenennaam and Young, 2017).

According to the Food and Agriculture Organization of the United Nations (FAO), glyphosate's residues amount in food must obey the dietary daily intake and the maximum residue limits (MRL), which range between 0.025 – 2 mg/kg among food types. Meat, bean and milk present a MRL of 0.05, 2 and 0.05 mg/kg, respectively. MRL is above 30 mg/kg for some cereals (including rice, wheat and oat) (FAO, 2016). Furthermore, Williams et al. (2000) suggested in their study a theoretical maximum value intake for glyphosate of 0.238 mg/kg body weight/day (adults) through the food .

The EFSA showed data glyphosate's residues concentration in different body parts of cattle and pig. No samples presented values above acceptable MRL (0.05 mg/kg) (EFSA, 2015). Granby et al. (2003) analyzed glyphosate's concentration in different cereals among 1998-2001. All samples (50 per year) showed glyphosate residual values lower than acceptable limits.

Reddy et al. (2008) analyzed glyphosate's concentrations in glyphosate resistant (GR) and non-GR corns. Results showed that non-GR corn presents a higher glyphosate concentration than GR-corn (0.871 and 0.308 mg/kg respectively), however, both values are under MRL (5 mg/kg). Arregui et al. (2003)

evaluated the presence of glyphosate residues in leaves, stems and grains of GR-soybean. Values ranged 0.1 – 4.4 mg/kg, bellow the MRL (5 mg/kg). However, some studies found that foods produced from genetically modified glyphosate resistant crops showed a higher residue concentrations compared with non-genetically modified crops (Bohn et al., 2014).

Chhapekar et al. (2014) showed that a transgenic variety of rice confers tolerance to glyphosate applications, and this kind of rice could tolerate 1% of Roundup® commercial, which is 5-fold more than that used to kill weeds under normal conditions. This data intensifies the concern with the indiscriminate use of glyphosate without the due concern of the residues accumulation in foods.

USDA GYPSA laboratory analyzed 300 soybeans samples and was detected 13 kinds of pesticides including glyphosate. Among these samples, 271 (90.3%) was positive for glyphosate's residues with levels ranging from 0.26 mg/kg – 18.5 mg/kg (USDA, 2013).

Kruger et al. (2014) tested urine samples of humans, dairy cows, hares and rabbits. Cows kept in genetically modified free area had significantly lower glyphosate concentrations in urine than conventional husbandry cows ($p<0.001$). Moreover, glyphosate concentration was significantly higher ($p<0.0002$) in urine of people consuming conventional food than in urine of people consuming predominantly organic food. In addition, chronically ill humans showed significantly higher glyphosate residues in urine than in the healthy population ($p=0.03$). Niemann et al. (2015) also evaluated glyphosate residues in human urine. Glyphosate was regularly found in urine at levels corresponding to a dietary daily intake of around 0.1-3.3 µg/kg body weight/day.

McGuire et al. (2016) evaluated the glyphosate concentration in maternal milk. None of samples was positive for glyphosate presence in lactating women. Thus, these authors suggest that dietary glyphosate exposure is not a health concern for breastfed infants.

On the other hand, McQueen et al. (2012) evaluated the presence of glyphosate's residues in foods consumed by 40 mothers. Although the presence of glyphosate in 75% of foods was confirmed, the total

concentration was 0.4% of the acceptable daily intake. MRL value is measure to analytical methods rather than on toxicology methods, thus, available MRLs may not necessarily suggest a safe level of a pesticide residue in food (Bai and Ogbourne, 2016).

Although international scientists and regulatory agencies still maintain the glyphosate as unlikely to be carcinogenic through food intake (JMPR, 2016), there are few studies about a low chronic exposure to glyphosate through food consumption. Thus cannot be discarded a potential risk to human health. The role of glyphosate exposition via food remains poorly explained, being necessary more studies that seek to achieve the potential of chronic glyphosate toxicity.

Conclusion

This review shows that many *in vitro* studies associate glyphosate with genotoxic and cytotoxic effects. On the other hand, many studies have not found such relationships, especially the epidemiological studies. Most agencies that assess the risk of exposure to glyphosate do not consider it to be carcinogen to humans. Despite this, there are no studies evaluating the chronic effects after decades of exposure. Thus, it is essential that robust epidemiological studies be conducted to confirm the assumed safety of glyphosate in relation to human health.

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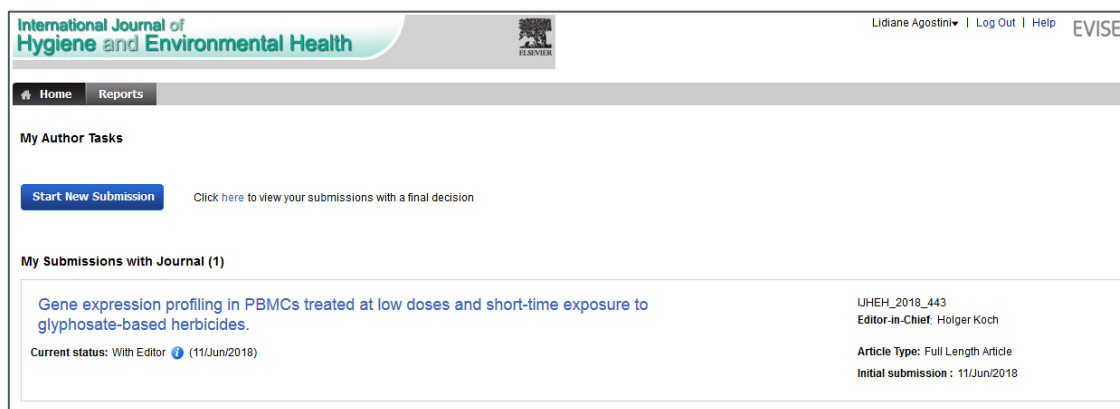
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4.2 Manuscrito 2

O manuscrito intitulado "Gene expression profiling in PBMCs treated at low doses and short-time exposure to glyphosate-based herbicides" foi submetido para avaliação ao periódico International Journal of Hygiene and Environmental Health (Qualis Capes A2; Fator de Impacto: 4,643), de acordo com os critérios estipulados pelo Regimento do Programa de Pós-Graduação em Biotecnologia (Figura 7).

Figura 7. Comprovação de submissão do manuscrito 2 para a revista International Journal of Hygiene and Environmental Health.

GENE EXPRESSION PROFILING IN PBMCS TREATED AT LOW DOSES AND SHORT-TIME EXPOSURE TO GLYPHOSATE-BASED HERBICIDES

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ABSTRACT

Glyphosate is a post-emergent, non-selective and systemic herbicide. Roundup® is the most common glyphosate-based herbicides (GBHs). The priority route of glyphosate's degradation in soil results in aminomethylphosphonic acid (AMPA). However, the effect of glyphosate on gene expression pattern in healthy cells remains unknown. Therefore, we aimed identify differentially expressed genes (DEGs) in human peripheral blood mononuclear cells (PBMCs) submitted to a short-time exposure with glyphosate-based herbicide (Roundup®) and AMPA, using microarray technique for gene expression profiling. There were identified 26 DEGs after Roundup® exposure (3h; 0.05%) and 5 DEGs after AMPA treatment (3h; 10 mM). The enrichment analysis of DEGS after Roundup® treatment showed association with 33 cellular processes. Pathview web was used to identify the effect of DEGs in different pathways. Only genes differentially expressed in Roundup® treatment were included in the pathways. *TNF, LTA, TAB2* and *ATM* genes are related to NF-kappa B signaling pathway; *SESN3* and *ATM* genes to p53 signaling pathway; and *TNF, BCL2L11* and *ATM* genes to apoptosis. Our results

suggest that Roundup® change the expression of genes associated with cell cycle control, regulation of cellular process and apoptosis.

Key words: GeneChip Human Transcriptome Arrays 2.0; cell cycle; herbicide; regulation of cell processes; peripheral blood mononuclear cells; GBH; AMPA.

INTRODUCTION

Glyphosate (N- (phosphonomethyl) glycine) is a broad spectrum, post-emergent, non-selective and systemic herbicide (IARC, 2015; MYERS et al., 2016). Commercial formulations add surfactants to improve efficiency (VANDENBERG et al., 2017). These products are known as glyphosate-based herbicides (GBHs), being the Roundup® the most common (IARC, 2015). The priority route of degradation of glyphosate by microorganisms in the soil results in the formation of aminomethylphosphonic acid (AMPA) (RIBEIRO et al., 2015). In mammals, glyphosate is not metabolized efficiently and is mainly excreted unchanged in the urine (MYERS et al., 2016).

Molecular responses to glyphosate have already been analyzed for some organisms, like pacific oyster, european flounder, mosquito, Daphnia magna (TANGUY et al., 2005; MARCHAND et al., 2006; RIAZ et al., 2009; LE et al., 2010). In humans, there are few studies.

Hokanson *et al*. (2007) evaluated gene expression in MCF-7 cell lines exposed to GBH and identified 680 deregulated genes. *HIF1*, *CXCL12* and *EGR1* were significantly dysregulated by glyphosate. According authors, altered EGR1 levels in response to glyphosate salts are less clear than for *HIF1* and *CXCL12*, but appear to potentially impact rates of apoptosis initiation and alter the levels of vascularization associated with tumor formation.

Thongprakaisang et al. (2013) identified that glyphosate alters the expression of estrogen receptor genes in breast cancer cell lines. Futhermore, for the authors the additive estrogenic effects of glyphosate may pose a risk of breast cancer because of their potential additive estrogenicity.

Mesnage et al. (2017) also evaluated the estrogenic potential of glyphosate in in breast cancer cell lines. Their results reveals that glyphosate activates ERalpha in breast cancer cells but only at relatively high concentrations, and that this activation is through a ligand-independent pathway.

George and Shukla (2013) identified that glyphosate changed expression pattern of proteins acting on the G1/S phase of cell cycle, also triggers S100A6/S100A9 expression and decreases IP3R1 and SOD 1 expressions in HaCaT cells. For authors, their study highlights that glyphosate promotes proliferation in HaCaT cells probably by disrupting the balance in between $[Ca2+]i$ levels and oxidative stress which in turn facilitated the downregulation of mitochondrial apoptotic signaling pathways.

Glyphosate' effects on gene expression pattern in healthy cells are poorly understood. Therefore, we propose identify differentially expressed genes in human peripheral blood mononuclear cells submitted to short exposure with glyphosate-based herbicide (Roundup®) and AMPA.

MATERIAL AND METHODS

Chemicals reagents

The commercial formulation of the herbicide glyphosate (N-phosphonomethyl-glycine) Roundup Original® [glyphosate 41% (360 g/L), POEA $\cong$ 15%, Monsanto] were considered in this study as 100% and, for the accomplishment of the experiments, it was diluted with water. Aminomethylphosphonic acid (AMPA) [99% (CAS # 1066-51-9, Sigma-Aldrich] was also diluted in water for the experiments. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [Sigma Aldrich] and dimethyl sulfoxide (DMSO) [Sigma Aldrich] were used in cell viability experiments.

Human peripheral blood mononuclear cells isolation and cell culture conditions

PBMCs were isolated from peripheral blood obtained from 3 healthy and non-smoking volunteers (2 female and 1 male; 28-33 years old), who do not have direct contact with herbicides. Blood were diluted with PBS (1:1). Cells were isolated by density gradient using Ficoll® Paque Plus (GE Healthcare). After centrifugation at 800G for 20 min, PBMCs were collected and PBS was added. The tube was centrifuged at 600G for 10 min and then the supernatant was discarded. This step was repeated. Pellet was resuspended and PBMCs were cultured in RPMI 1640 (Gibco-Invitrogen Life Technology) and supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, phytohemagglutinin (1ug/ml) and interleukin 2 (300U/mL). Cells were maintained at 37 °C with 95% air and 5% CO_2. PBMCs were cultured for 60 h before being used in the experiments.

Cell viability MTT Assay

MTT reagent assay were used to evaluated cell growth and viability. 3.5×10^4 cells were added in each well (96-well microplates), in triplicates. PBMCs were treated with different concentrations of Roundup® (0.01, 0.05, 0.1, 0.15; 0.2; e 0.3%) and AMPA (0.01, 0.05, 0.5, 1.0, 5.0 e 10 mM). After 3h, 15h, 24 and 48h of incubation, 0.5mg/mL of MTT was added into each well. DMSO was added after 3h to dissolve the precipitated dye and 48h later, color alterations were measured using FLUOstar Omega® (BGM LabTech) at 570 nm. Statistical analysis was performed using one - way variance analysis (ANOVA), followed by Bonferroni (post hoc) test to evaluate the differences between groups. Significance level considered was $p < 0.05$. Treatments were compared in relation to the control (CNT).

RNA Extraction

After treatment with Roundup® at 0.05% (1.1mM of glyphosate) and AMPA at 10mM for 3 hours, total RNA was extracted from PBMCs using RNeasy Mini Kit (Qiagen) according to manufacturer's protocols. RNA purity and quality was evaluated using a NanoDrop 2000 (ThermoFisher Scientific) and Agilent Bioanalyzer 2100 (Agilent Technologies), respectively. RNA integrity number (RIN) > 7 were considered for microarray analysis.

Microarray and data analysis

GeneChip Human Transcriptome Arrays 2.0 (HTA 2.0 - Affymetrix) is a highest resolution microarray for gene expression profiling of all transcript isoforms. Covered more than 245,000 coding transcripts, 40,000 non-coding transcripts and 339,000 probe sets covering exon-exon junctions (Friess et al. 2017). RNA was analyzed using HTA 2.0 according to the

manufacturer's instructions. Eight chips were used: 3 for controls, 3 for AMPA (10 mM) and 2 for Roundup® (0.05%).

The data were evaluated using the Partek Genomics Suite v 6.6 (Partek Inc., Louis, MO) and to assess the samples's distribution, Principal Component Analysis (PCA) was applied. Pre-processing of Affymetrix CEL-files was performed using the robust multi-chip analysis (RMA) algorithm, which performs background adjustment, quartile normalization and probe summarization. Differential expression analysis was realized using a two-way ANOVA. p-value <0.05 was considered as significant for the biological and molecular function analyses. Up and down-regulated genes were identified using a fold-change of 1.5.

An enrichment analysis (EA) was performed using MetaCore (GeneGo™, Thomson Reuters, NY), where genes with altered expression were mapped to Gene Ontology (GO). GO annotations were used as indicators of biological functions. Pathview (LUO et al. 2017) was used to construct the maps of different cellular pathways.

Validation of critical differential expressed gene

The quantitative real-time PCR (qPCR) was used to confirm the Microarray data. RNA from the samples was used for the reverse-transcription reaction according to SuperScript™ IV VILO™ Master Mix with ezDNase enzyme (Invitrogen). PCR reactions were carried out using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems®) on 7500 Fast Real-time PCR System (Applied Biosystems®). Primers of the selected genes are listed in Table 1. The relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method (LIVAK; SCHMITTGEN, 2001). GAPDH was used as a reference gene.

RESULTS

Cell viability

MTT analysis is shown in Figure 1. Survival rate was dose-dependent for both treatments (Figure 1). Cell death was less than 30% for all conditions. Roundup® at 0.01 and 0.05% showed similar viability after 15, 24 and 48 h. In general, cell viability reduced more with a bigger exposure times to Roundup®. Roundup® at 0.30% after 48 hours caused more cell death (~30%) than others concentrations and times. The small level of cell death were with 24h exposure to AMPA. AMPA at 10mM after 3 hours caused highest cell death.

Differential gene expression profile

There were 26 differentially expressed genes (DEGs) identified after Roundup® exposure. 21 genes were up regulated (13 protein coding, 5 RNA genes and 3 pseudogenes; Table 2). Top 10 up regulated DEGs were NFE2L3, TXK, MIR548L, RNU6-82P, MIR4439, OR2J2, TAB2, LRRC37A4P, LOC727896 and SESN3. Five genes were down regulated (Table 3): 4 protein coding (RHOU, TNF, LTA and FOSB) and 1 RNA gene (HIF1A-AS2).

After AMPA treatment, there were 5 DEGs (2 protein coding, 2 RNA genes and 1 pseudogene; Table 4). Combining both treatments, 2 DEGs were common (*HIF1A-AS2* and *LRRC37A4P*).

Roundup® exposure lead to a different gene expression patterns when compared to AMPA exposure and control (hierarchical distribution of the top 500 DEGs; Figure 2).

Enrichment and pathway analysis

DEGs after Roundup® treatment showed association with 33 Gene Ontology (GO) cellular processes. Table 5 present the top 10 GO cellular processes. DEGs are mainly related to the regulation of several processes: cell communication, signaling and signal transduction. Also, were related to positive regulation of cellular metabolic process, macromolecule metabolic process, cellular process, signal transduction and metabolic process. No significant results were obtained for AMPA treatment.

Pathview web (https://pathview.uncc.edu/analysis) was used to identify the effect of DEGs in different pathways (LUO et al., 2017). Only genes differentially expressed in Roundup® treatment were included in the pathways. *TNF, LTA, TAB2* and *ATM* genes are related to NF-kappa B signaling pathway (Figure 3). Nuclear factor-kappa B (NF-kappa B) is the generic name of a family of transcription factors that function as dimers and regulate genes involved in immunity, inflammation and cell survival (LIU et al, 2017; KEGG, 2018). This pathway is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products. Canonical pathway is induced by Tumor Necrosis Factor-alpha (TNF-alpha) and the non-canonical pathway is triggered by particular members of the TNFR superfamily, such as lymphotoxin-beta (; LIU et al, 2017; ZHANG et al., 2017).

BCL2L11 and *ATM* genes are related to FoxO signaling pathway (Figure 4). The forkhead box O (FOXO) family of transcription factors regulates the expression of genes in cellular physiological events including apoptosis, cell-cycle control, cellular differentiation, cell proliferation, glucose metabolism, DNA damage and repair oxidative stress resistance and longevity (FARHAN et al., 2017; KEGG, 2018).

SESN3 and *ATM* genes are related to p53 signaling pathway (Figures 5). The p53 signaling pathway is an important via that control cell cycle. p53 activation is induced by DNA damage,

oxidative stress and activated oncogenes. This protein is a transcriptional activator of p53-regulated genes related to cell cycle arrest, cellular senescence or apoptosis (MIRZAYANS et al., 2017; SLATTERY et al., 2018).

TNF, BCL2L11 and *ATM* genes are related to apoptosis (Figure 6). Apoptosis is a genetically programmed process for the elimination of damaged or redundant cells by activation of caspases. The 'extrinsic' pathway involves stimulation of members of the tumor necrosis factor (TNF) receptor subfamily by their specific ligands, such as TNF-alpha (ICHIM; TAIT, 2016; CAMPBELL; TAIT, 2018). TNF family of ligands activates anti-apoptotic or cell-survival signals as well as apoptotic signals (CORREIA et al., 2015; KEGG, 2018). The 'intrinsic' pathway is engaged by a wide array of stimuli that are sensed intracellularly, including cytokine deprivation, DNA damage, endoplasmic reticulum stress, metabolic stress, UV radiation and growth-factor deprivation (ICHIM; TAIT, 2016).

Quantitative real time PCR

Five DEGs were selected to be validated. *ATM, SESN3* and *BCL2L11* were chosen because were related to Pathview pathways. *NFE2L3* and *TXK* because they presented highest fold-change. All of the genes found to be similarly up- or down-regulated by both methods. Three genes (*ATM, BCL2L11* and *TXK*) confirmed microarray results (fold-change > 1.5) (Table 6).

DISCUSSION

Several studies showed that glyphosate is toxic to PBMCs, depending of the exposure time and concentration (MARTINEZ et al., 2007; ALVAREZ-MOYA et al., 2014; KWIATKOWSKA et al., 2016).

Martinez et al. (2007) compared the toxicity of glyphosate and Roundup® on peripheral blood mononuclear cells (PBMCs). Cells were exposed to different glyphosate's (1-2000 μg/ml) or Roundup®'s concentrations (1-100 μg/ml) for 24, 48, 72 and 96 h. Both herbicides were toxic to PBMCs. Alvarez-Moya et al. (2014) performed experiments with human lymphocytes. The comet assay was used to examine the genotoxicity of glyphosate. The DNA damage increased with the glyphosate's concentration (0.0007-0.7 mM), usually proportionally, with an exposure time of 20h. Ours MTT results showed similar results. Survival rate was dose-dependent for AMPA and Roundup® treatments; however, cell death was less than 30% for all conditions.

Kwiatkowska et al. (2016) showed a less decrease of cell viability. Authors observed a 2.7% decrease in PBMCs viability after 24h of incubation with glyphosate at 10 mM. The conclusion was that glyphosate caused toxic effects on PBMCs only at very high concentrations.

This is the first work that evaluated the effects of Roundup® and AMPA on global gene expression in PBMCs. Our results suggest that Roundup® change expression pattern of a several genes associated with cell cycle control, regulation of several cellular processes and apoptosis, which can explain glyphosate effects describes in literature.

ATM (ATM Serine/Threonine Kinase) belongs to the PI3/PI4-kinase family and is an important cell cycle checkpoint kinase. ATM plays a critical role in the response to DNA double strand breaks by phosphorylating a large number of downstream substrates that are involved in DNA repair, cell arrest, chromatin remodeling and apoptosis (DING et al., 2017). *ATM* were up

regulated after Roundup® exposure, which could indicate that these herbicide induced cellular stress.

BCL2L11 encodes a BH3-only protein named BIM. This protein, a key regulator of pro-apoptosis, is released from the cytoskeleton after activation by cytotoxic signaling, and is then translocated to the mitochondria, where it results in apoptosis (LEI; DAVIS, 2003). BIM has been shown to be critical for apoptosis in B and T lymphocytes, macrophages and granulocytes (STRASSER, 2005). Bim also leads to uncoupling of mitochondrial respiration and the subsequent increase in the cellular levels of reactive oxygen species (ROS) (SIONOV et al., 2015). Upregulation of BH3-only proteins can occur at transcriptional/post-translational levels in response to stress to trigger cell death (CAMPBELL; TAIT, 2018). Our results showed that BCL2L11 is up regulated after Roundup® treatment, which could leadto apoptosis as showed in MTT results.

Micro RNAs (miRNAs) are small non-coding RNAs (~ 20–24 nucleotides in length) that are involved in the post transcriptional control of gene expression (ABDI et al., 2017). Approximately, 60% or more of human protein coding genes may be subject to regulation by miRNAs (FRIEDMAN et al., 2009). Ji et al. (2018) showed that glyphosate (50 mg of glyphosate/kg/day) exposure during pregnancy and lactation altered microRNA expression in brain of mouse offspring, leading to neurological disorders.

The *MIR548L, MIR4439* and *MIR581* genes, that codifying miRNAs, were up regulated after exposure to Roundup®. Besides that, 9 of top 10 GO cellular processes altered after treatment with Roundup® are related to regulation of several cellular processes. Therefore, our results suggest that Roundup$^®$ affect strongly the regulation of several cellular process through up regulated miRNAs.

Long noncoding RNAs (lncRNAs) have regulatory roles in important biological processes, and many of them are deregulated in several human cancers. LncRNA hypoxia-inducible factor 1 alpha antisense RNA-2 (HIF1A-AS2) is a natural antisense transcript of hypoxia-inducible factor 1alpha (HIF-1α) (CHEN et al. 2015). HIF1A-AS2, via regulating the cancer-relevant HIF-1α pathway, plays a crucial role in cancer development (BERTOZZI et al., 2011).

Chen et al. (2016) suggested that silencing *HIF1A-AS2* could lead to cell proliferation inhibition, cell migration suppression, and apoptosis induction in bladder cancer cells. Our results showed that, after Roundup$^{®}$ and AMPA treatment, the HIF1A-AS2 gene was down regulated, which could be a sign for apoptosis.

CONCLUSION

Short exposure to Roundup® at low doses affect regulation of important cellular pathways (apoptosis, cell cycle control), leading cell to DNA repair or apoptosis. These alterations altered cellular functioning and could leading to development of health problems. Rapid exposure to AMPA does not significantly affect the pattern of gene expression of PBMCs.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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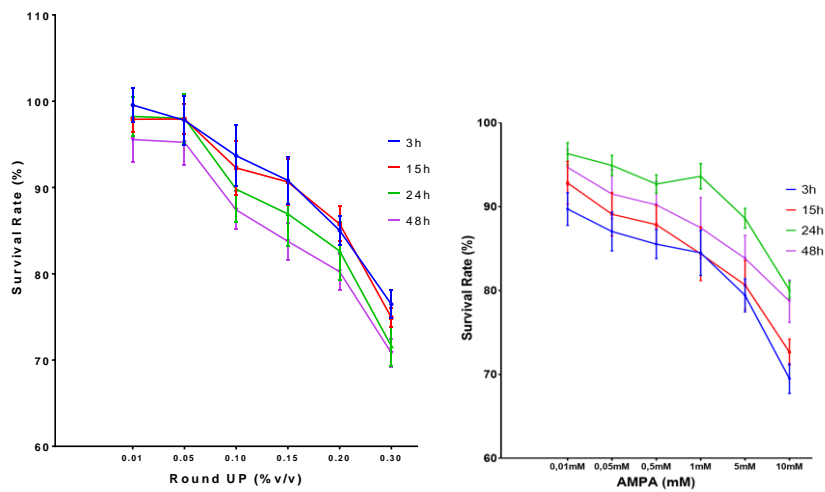
FIGURES AND TABLES

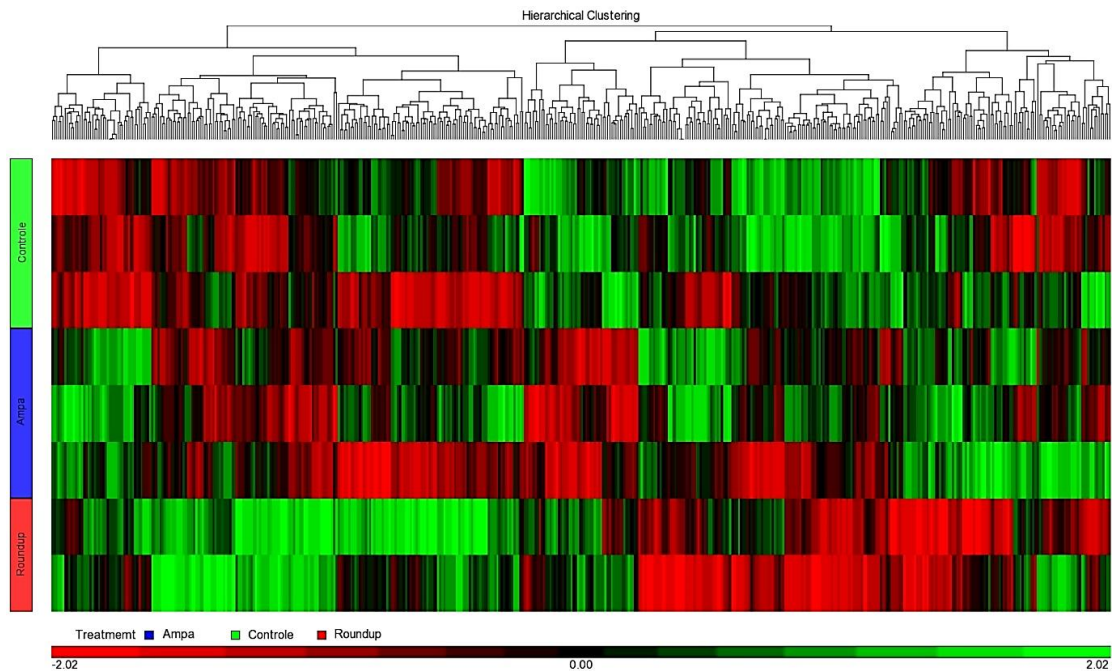Fig 1. Results of cell viability (MTT Assay) after treatments with Roundup® and AMPA.

Fig 2. The hierarchical distribution of the Top 500 DEGs.
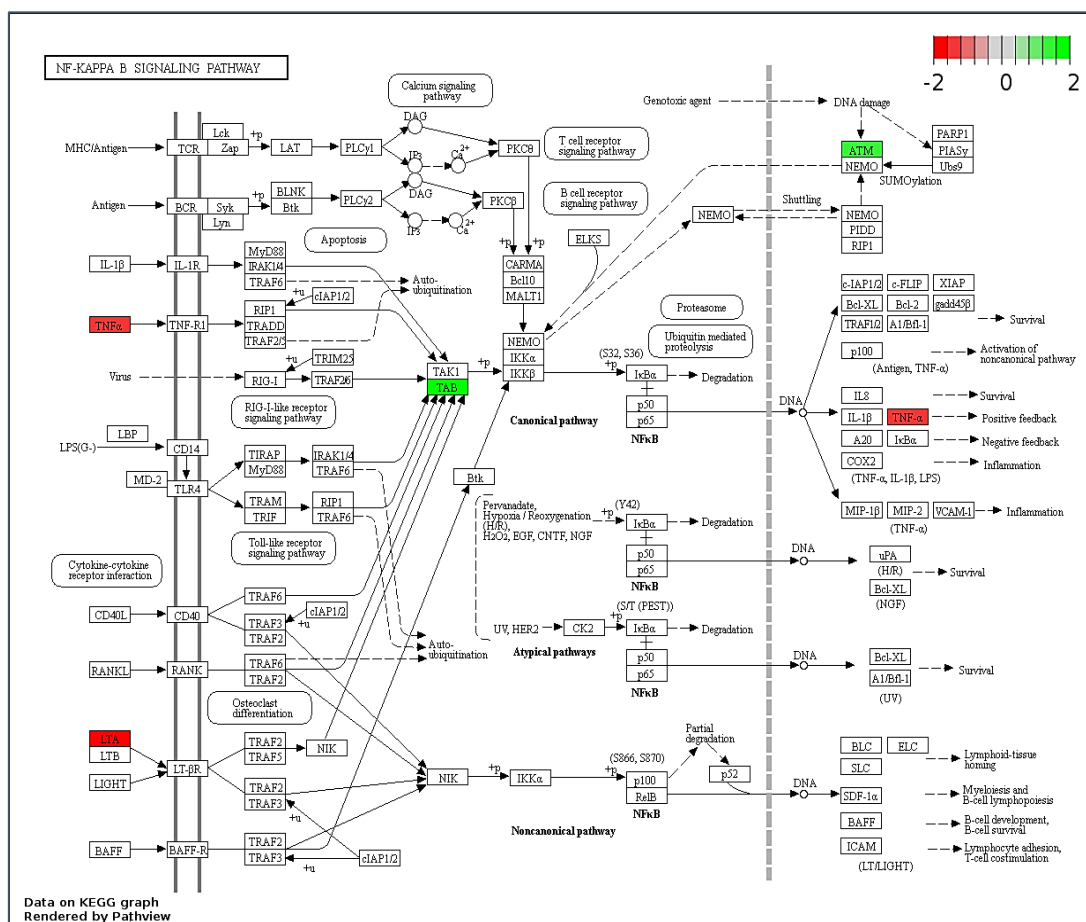
Fig 3. NF-kappa β signaling pathway. Associated genes (protein and fold-change): *TNF* (TNF-alfa, -1.54228); *TAB2* (TaB, 1.68298); *LTA* (LTA, -1.60701) and *ATM* (ATM, 1.57376).
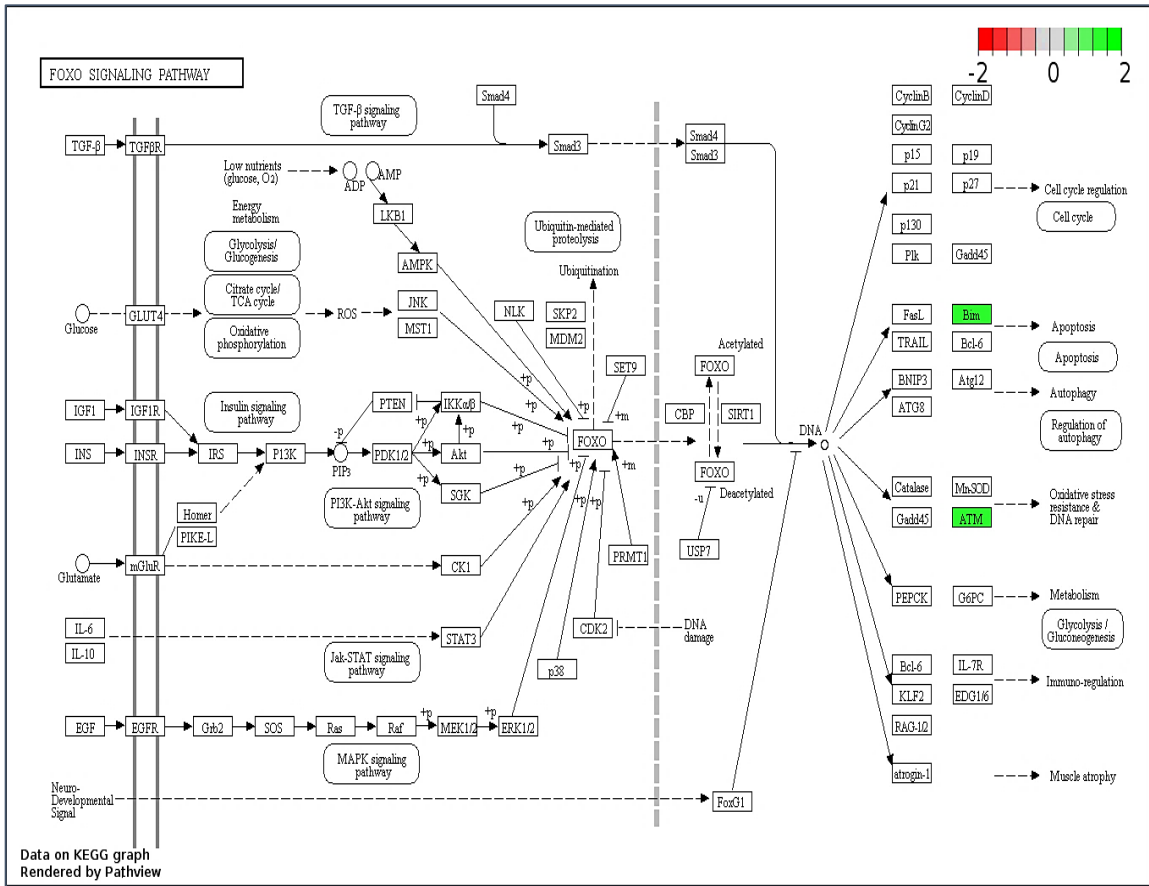
Fig 6. FoxO signaling pathway. Associated genes (protein and fold-change): *BCL2L11* (Bim, 1.57768) and *ATM* (ATM, 1.57376).
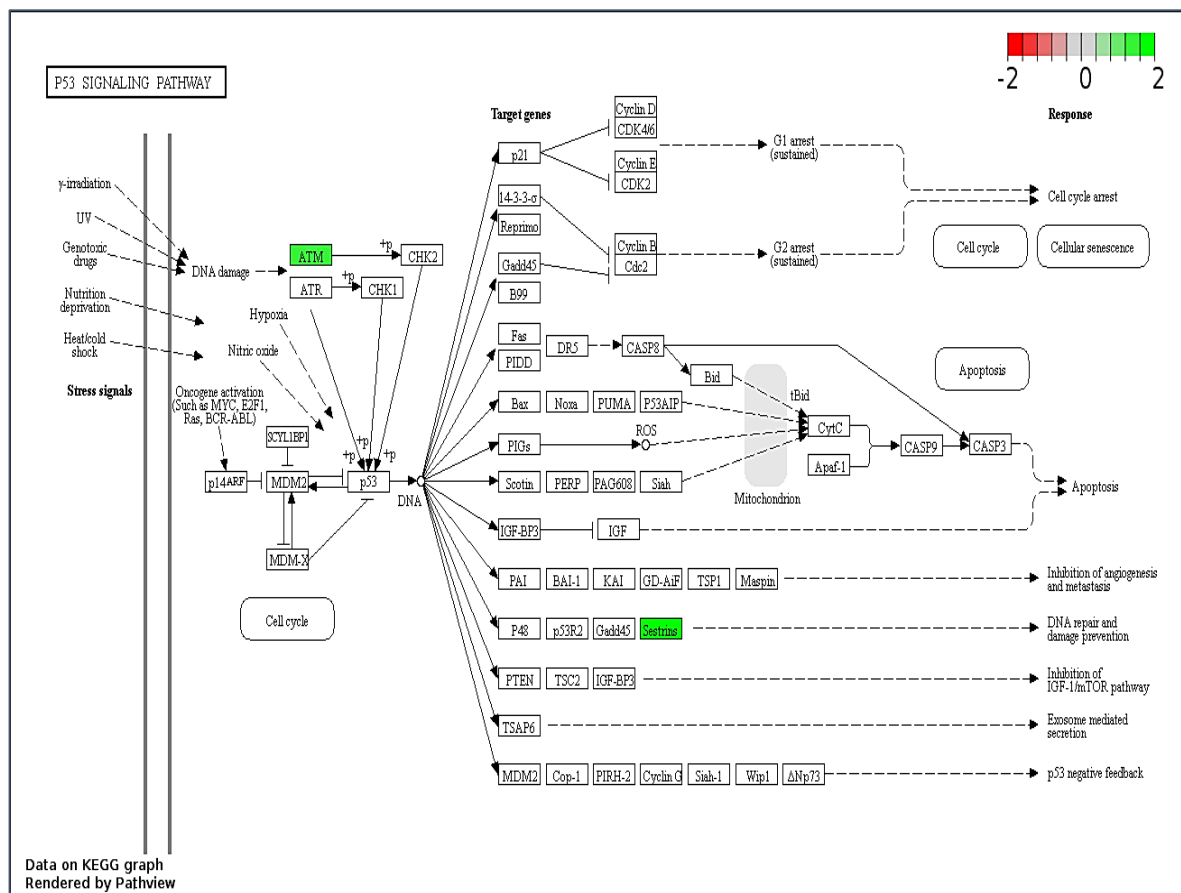
Fig 7. p53 signaling pathway. Associated genes (protein and fold-change): *SESN3* (Sestrin, 1.60232) and *AT*M (ATM, 1.57376).
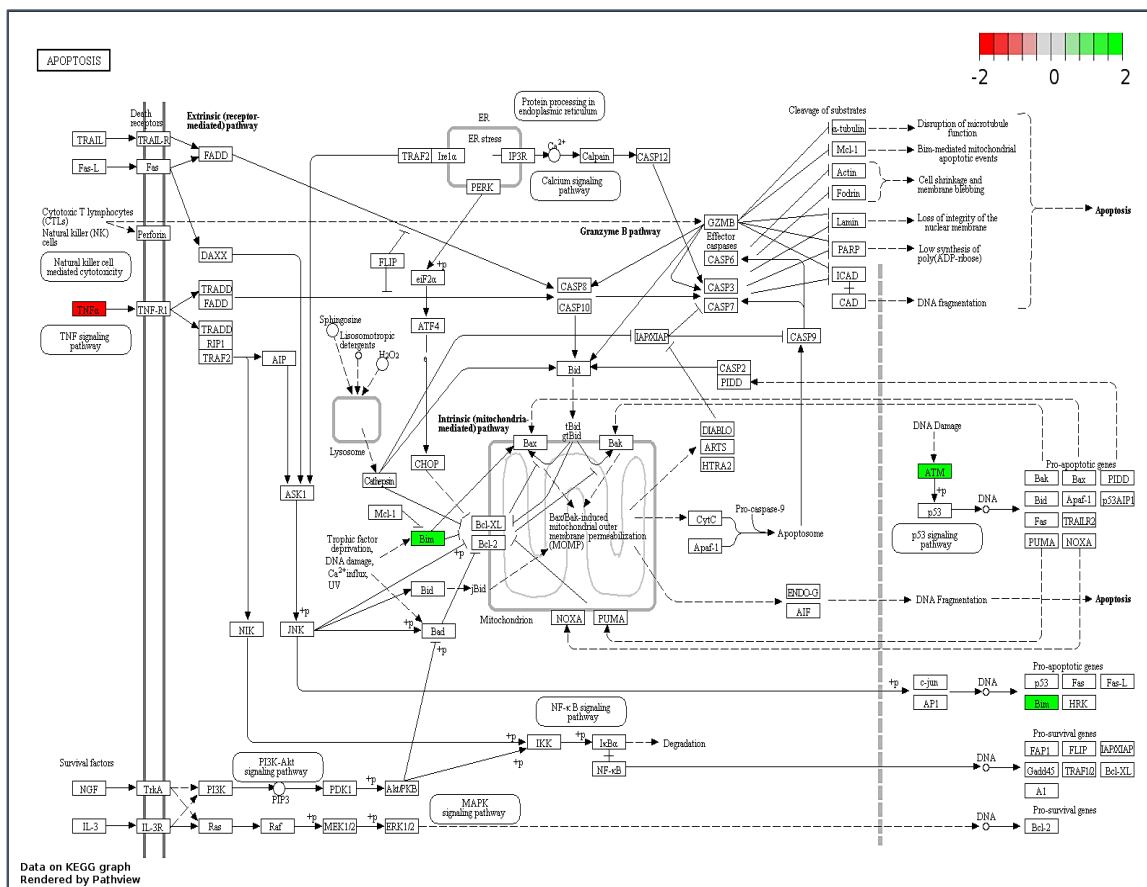
Fig 8. Apoptosis. Associated genes (protein and fold-change): *TNF* (TNF-alfa, -1.54228), *BCL2L11* (Bim, 1.57768) and *ATM* (ATM, 1.57376).

Table 1. Primers used in qPCR experiments.

Primer	Sequence (5' – 3')	Reference
gapdh-F	ACAACTTTGGTATCGTGGAAGG	Primer Bank
gapdh-R	GCCATCACGCCACAGTTTC	
atm-F	TTGATCTTGTGCCTTGGCTAC	Primer Bank
atm-R	TATGGTGTACGTTCCCCATGT	
sesn3-F	CTGGGAAAATCATGGGTTCTCC	Primer Bank
sesn3-R	GCATGGTTGTGTCAACATCCT	
nfe2l3-F	TTCAGCCAGGCTATAAGTCAGG	Primer Bank
nfe2l3-R	GCTCAGGATTGGTGGTATGAGA	
txk-F	ACGGAGGCTGCCATAAAACAT	Primer Bank
txk-R	GGATTGATTGAAAGGCGTGTCT	
bcl2l11-F	TGGATATTGTCAGGCCACTT	Primer Bank
bcl2l11-R	TTTCACTGCTCCCTAACCTG	

Table 2. Differentially expressed genes (p<0.05), up regulated after Roundup® treatment.

Gene Symbol	Name	Fold-change
NFE2L3	Nuclear Factor, Erythroid 2 Like 3 (Protein Coding)	1,91527
TXK	Tyrosine Kinase (Protein Coding)	1,83919
MIR548L	MicroRNA 548L (RNA Gene)	1,82434
RNU6-82P	RNA, U6 Small Nuclear 82 (Pseudogene)	1,81198
MIR4439	MicroRNA 4439 (RNA Gene)	1,79652
OR2J2	Olfactory Receptor Family 2 Subfamily J Member 2 (Protein Coding)	1,73639
TAB2	TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein 2 (Protein Coding)	1,68298
LRRC37A4P	Leucine Rich Repeat Containing 37 Member A4 (Pseudogene)	1,63838
LOC727896	Cysteine And Histidine Rich Domain Containing 1 (Pseudogene)	1,61015
SESN3	Sestrin 3 (Protein Coding)	1,60232
LOC100132686	Uncharacterized LOC100132686 (RNA Gene)	1,59648
BCL2L11	BCL2 Like 11 (Protein Coding)	1,57768
MIR581	MicroRNA 581 (RNA Gene)	1,57706
ATM	ATM Serine/Threonine Kinase (Protein Coding)	1,57376
OR5V1	Olfactory Receptor Family 5 Subfamily V Member 1 (Protein Coding)	1,5707
GPRIN3	GPRIN Family Member 3/ G protein-regulated inducer of neurite outgrowth 3 (Protein Coding)	1,561
LINC01004	Long Intergenic Non-Protein Coding RNA 1004 (RNA Gene)	1,55941
SMAD9	SMAD Family Member 9 (Protein Coding)	1,5569
ANKRD36C	Ankyrin Repeat Domain 36C (Protein Coding)	1,54102
YLPM1	YLP Motif Containing 1 (Protein Coding)	1,53064
MSMO1	Methylsterol Monooxygenase 1 (Protein Coding)	1,52546

Table 3. Differentially expressed genes (p<0.05), down regulated after Roundup® treatment.

Gene Symbol	Name	Fold-Change
RHOU	Ras Homolog Family Member U (Protein Coding)	-1,51073
TNF	Tumor Necrosis Factor (Protein Coding)	-1,54228
LTA	Lymphotoxin Alpha (Protein Coding)	-1,60701
HIF1A-AS2	HIF1A Antisense RNA 2 (RNA Gene)	-1,70298
FOSB	FosB Proto-Oncogene, AP-1 Transcription Factor Subunit (Protein Coding)	-1,73267

Table 4. Differentially expressed genes (p<0.05), up and down regulated after AMPA treatment.

Gene Symbol	Name	Fold-Change
SLC30A1	Solute Carrier Family 30 Member 1 (Protein Coding)	-1,93387
MIR29A	MicroRNA 29A (RNA Gene)	-1,75407
HIF1A-AS2	HIF1A Antisense RNA 2 (RNA Gene)	-1,5509
SLC39A10	Solute Carrier Family 39 Member 10 (Protein Coding)	1,52661
LRRC37A4P	Leucine Rich Repeat Containing 37 Member A4 (Pseudogene)	1,62566

Table 5: Top 10 GO cellular processes after treatment with Roundup®.

GO process	FDR
Regulation of cell communication	9.285E-03
Regulation of signaling	9.653E-03
Cellular response to stimulus	1.197E-02
Regulation of signal transduction	1.815E-02
Regulation of biological quality	1.828E-02
Positive regulation of cellular metabolic process	1.828E-02
Positive regulation of macromolecule metabolic process	1.828E-02
Positive regulation of cellular process	1.871E-02
Positive regulation of signal transduction	2.182E-02
Positive regulation of metabolic process	2.292E-02

Table 6. Comparison between fold-change results in Microarray and qPCR analysis.

Gene	Fold-change	
	Microarray	qPCR
TXK	1.83919	3.31214
ATM	1.57376	2.43361
BCL2L11	1.57768	2.01408
NFE2L3	1.91527	1.18129
SESN3	1.60232	1.07630

5 CONSIDERAÇÕES FINAIS

A literatura disponível sobre o glifosato é ampla, porém existem muitos pontos de divergência ou aspectos não abordados. Como os experimentos são realizados de forma independente, vários fatores como desenho experimental, qualidade dos reagentes, materiais disponíveis, modelo experimental e qualificação dos pesquisadores podem influenciar os resultados. Por outro lado, os efeitos da exposição crônica ao glifosato, principalmente em humanos, ainda não foram elucidados de forma satisfatória, o que pode ser explicado pela dificuldade na realização de estudos epidemiológicos e pela impossibilidade de expor pessoas saudáveis ao herbicida.

Baseada nas evidências atuais, muitas agências de avaliação de risco consideram o glifosato como provavelmente não carcinogênico, e não tóxico aos humanos, desde que utilizado de acordo com as instruções do fabricante. Entretanto, ainda não existe um consenso global sobre a classificação de risco.

Quanto às análises moleculares, foi observado que nas condições escolhidas, o AMPA alterou a expressão de 5 genes.

Em relação ao tratamento com Roundup®, foi possível perceber que uma curta exposição a baixas concentrações alterou o padrão de expressão gênica de 26 genes associados com controle do ciclo celular, regulação de processos celulares e apoptose, o que poderia explicar os efeitos biológicos do glifosato descritos na literatura.

Devido à escassez de estudos avaliando as alterações da expressão gênica em células humanas, esse trabalho foi pioneiro na tentativa de identificar os genes diferencialmente expressos quando PBMCs são submetidas ao Roundup®. Assim, as informações contidas nessa tese serão importantes para a realização de estudos adicionais que busquem elucidar o efeito do glifosato sobre a saúde humana.

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