

A person wearing a white protective suit and blue gloves is examining a plant stem in a field. The person is holding a long, green stem with a yellowish, bulbous part. The background is a blurred field of green plants.

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Full Length Research Paper

Spirulina protects against tacrolimus-induced hepatic and renal toxicity in rats: A biochemical and histological study

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Tacrolimus is a powerful immunosuppressive agent with hepatotoxic and nephrotoxic effects. It has a protective role against many toxicants. This study was conducted to evaluate the possible protective role of spirulina against tacrolimus induced hepatotoxicity and nephrotoxicity. Forty adult male albino rats divided into 4 groups. Group I, control group, Group II, spirulina group (received spirulina 500 mg/Kg body weight (bw)/day orally), Group III, tacrolimus group (received tacrolimus 12 mg/kg bw/day orally); and Group VI, prophylactic group (orally administered spirulina for 3 days before and 28 days concurrently with tacrolimus in the same previous doses). Tacrolimus induced adverse effects on both liver and kidney functions and structure that was manifested by elevated hepatic transaminases, total and direct bilirubin, albumin, blood urea nitrogen, serum creatinine and creatinine clearance. There was a significant decrease in serum total antioxidant capacity (TAC) and hepatic and renal total thiol molecules (TTM), with a significant increase in serum malondialdehyde in tacrolimus group. Histopathologically, tacrolimus induced swelling and granulation of hepatocytes, congestion of blood sinusoids and degeneration of bile ductules, glomerular hypertrophy and segmentation, swelling, degeneration and hyalinosis of renal tubules. Spirulina pre- and co-treatment significantly improved these deleterious effects. This was accompanied by partial restoration of the expression of PCNA near to the normal level observed in control rats. Moreover, spirulina treatment did not alter the trough blood tacrolimus levels or tacrolimus-induced immunosuppression. Further studies are warranted to evaluate whether transplant patients on tacrolimus treatment may benefit from the protective effects of spirulina.

Key words: Antioxidant, malondialdehyde (MDA), total antioxidant capacity (TAC), tacrolimus, total thiol molecules (TTM), proliferating cell nuclear antigen (PCNA), spirulina.

INTRODUCTION

Tacrolimus is an immunosuppressant macrolide produced by *Streptomyces tsukubaensis*. It is used to prevent rejection of transplanted organs by inhibiting

calcineurin enzyme that is crucial for the multiplication of T-lymphocytes which are vital to the immune process (Tanaka et al., 1987; Fruman et al., 1992). Protocols that

do not include calcineurin inhibitors often is associated with limited graft survival that makes tacrolimus considered as the backbone of most immunosuppressive regimens (Jantz et al., 2013). Despite its high clinical efficiency, tacrolimus has been well known for its adverse reactions. In particular, patients receiving tacrolimus chronically are at high risk to develop cholestasis and renal damage (Yadav et al., 2013; Banhara et al., 2015). Nephrotoxicity was reported in approximately 52% of kidney transplantation patients, 40% of liver transplantation patients receiving tacrolimus and in 59% of heart transplantation patients in US randomized trial (Boudjema et al., 2011). Moreover, tacrolimus toxicity clearly showed that induced lipid peroxidation can be partially reversed with antioxidants in children (Grunot et al., 2002). Histopathologic examination revealed that tacrolimus induces renal necrosis and apoptosis. It also increases reactive oxygen species production and decreases antioxidant status (Piao et al., 2014). Therefore, a big need arises to alleviate tacrolimus induced oxidative stress or to reduce its dose to a safer level. Conceivably, reducing tacrolimus dose can impair its therapeutic efficacy.

Spirulina is a great source of natural protein with all amino acids, phyto-nutrients, antioxidants, carbohydrate, mucopolysaccharides, vitamins and trace minerals. Many people use it as an effective natural appetite suppressant. It is known to have important beneficial effects on cellular metabolism and homeostasis (Abou Gabal et al., 2015). Spirulina was reported to have antioxidant, antimutagenic and antineoplastic effects (Premkumar et al., 2004; Khan et al., 2006; Abdel-Daim et al., 2016). The antioxidant and cytoprotective effects of spirulina can be attributed to its antioxidant active constituents including C-phyco-cyanins, β -carotene, vitamins, and minerals (Upasani and Balaraman, 2003; Abdel-Daim et al., 2013; Abdel-Daim, 2014; EL-Sabagh et al., 2014). Moreover, it was previously demonstrated that spirulina can protect against end organ toxicities induced by different chemotherapeutic agents as well as lead acetate-induced hepatotoxicity by ablating oxidative stress and lipid peroxidation (Khan et al., 2006; Hemalatha et al., 2012). Spirulina has also a cardioprotective effect against tilmicosin-induced cardiac toxicity in mice (Ibrahim and Abdel-Daim, 2015). Abdelkhalek et al. (2015) and Abdel-Daim et al. (2016) have reported the hepatorenal protective effects of spirulina platensis against deltamethrin-induced toxicity by minimizing lipid peroxidation and improving antioxidant capacity. Spirulina platensis also exerted antioxidant, anti-inflammatory and immunomodulatory effects in acetic acid-induced experimental ulcerative colitis (Abdel-

Daim et al., 2015). As far as immunosuppressive effects of tacrolimus are concerned; spirulina, was previously proved to have a remarkable immunosuppressive effect both *in-vivo* and *in-vitro*. Therefore, spirulina gains more and more attention from medical scientists as a natural treatment for allergic, autoimmune and transplant-related diseases (Hayashi et al., 1994; Kim et al., 1998; Ramirez et al., 2002; Rasool and Sabina, 2009; Kumar et al., 2010). Accordingly, this study aimed to investigate whether, and how, spirulina may alleviate tacrolimus induced hepatotoxicity and nephrotoxicity by assessment of liver and kidney function tests, oxidative stress markers as well as hepatic and renal histopathologic examination. Lastly, to verify any role for spirulina interaction with tacrolimus, we measured tacrolimus trough levels and lymphocytic proliferation assay in the presence and absence of spirulina.

MATERIALS AND METHODS

Spirulina tablets 500 mg were obtained from DXN Co., Malaysia. Tacrolimus 1 mg capsules were from Hikma Pharmaceutical Co., Jordan. Alanine amino transferase (ALT) and aspartate amino transferase (AST) kits were purchased from Diamond diagnostics (Cairo, Egypt). Alkaline phosphatase (ALP), total and direct bilirubin kits were from Biodiagnostic (Dokki, Giza, Egypt). Albumin kit was obtained from spectrum-diagnostics albumin-BCG kit (Egyptian Company for Biotechnology "S.A.E", Obour city Industrial area, Cairo, Egypt). Blood urea nitrogen (BUN) and creatinine colorimetric kits were purchased from Biomerieux (Lyon, France).

Experimental design

The present study was carried on 40 adult male albino rats, weighing about 180 to 200 g. Rats were caged under standardized environmental conditions. They were housed in a spate well ventilated cages, under standard conditions, with free access to standard diet and water *ad libitum*, throughout the whole period of the experiment (28 days). The experiment was performed in accordance with the guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, 1996). Rats were classified into four groups received the following for 4 weeks. Group I (control group) included 10 animals which did not received any medications. Group II (spirulina group) included 10 animals that were treated with spirulina dissolved in distilled water in a dose of 500 mg/kg body weight orally *via* orogastric tube (Khan et al., 2006; Abdel-Daim et al., 2013). Group III (tacrolimus group) included 10 animals. The animals received orally tacrolimus (6.7 mg/kg body weight) once daily by orogastric tube. Tacrolimus was dissolved in distilled water. This dose was equivalent to 1/20 of LD50; 134 mg/kg (NIIRDN, 1994; Lewis, 2004). Group IV (prophylactic group: Tacrolimus + Spirulina) included 10 animals that were treated with spirulina and tacrolimus. Spirulina was given in a dose of 500 mg/kg body weight orally 3 days before and 28 days concomitantly with tacrolimus according to Khan et al. (2006) and Abdel-Daim et

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al. (2013).

At the end of the experiment, the animals were weighed, then subjected to light ether anesthesia. Blood was collected through microcapillary tube from retro-orbital plexus and used for biochemical analysis. Rats were then sacrificed by decapitation. The obtained specimens from liver and kidney were divided into two parts. One part was frozen in liquid nitrogen (-170°C) and kept at -80°C for the determination of total thiol molecules (TTM). The other part was fixed immediately in 10% neutral buffered formalin and processed to get paraffin blocks for light microscopy examination. Five micrometers were stained with Haematoxylin and Eosin (H&E), and proliferating cell nuclear antigen (PCNA) immunostaining.

Biochemical study

Liver function tests

The activities of ALT and AST enzymes in serum were determined as described by Reitman and Frankel (1957). ALP activity was assayed according to the method of Belfield and Goldberg (1971). Total bilirubin and direct bilirubin were measured by the method of Walter and Gerade (1970). Serum albumin was determined colorimetrically according to the modified bromocresol green binding assay (BCG) (Tietz, 1990).

Kidney function tests

BUN and serum creatinine levels (mg/dl) have been measured according to the methods of Kaplan (1965) and Bjurosson (1979), respectively. Creatinine clearance (ml/min) as an index of glomerular filtration rate was calculated from serum creatinine and a 24 h urine sample creatinine levels using the formula: Creatinine clearance = (Urine creatinine (mg/dl)/Serum creatinine (mg/dl)) × (Urine volume (ml)/Urine collection time (h) × 60).

Oxidative stress markers

Serum total antioxidant capacity (mmol/l): The determination of the anti-oxidative capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide. The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide. The residual hydrogen peroxide is assayed colorimetrically by enzymatic reaction which involves the conversion of 3,5-dichloro-2-hydroxyl benzene sulphonate to a colored product (Koracevic et al., 2001).

Serum malondialdehyde (MDA, µmol/l): MDA was determined by measuring thiobarbituric reactive species using the method of Yagi (1998) in which the thiobarbituric acid-reactive substances react with thiobarbituric acid to produce a red colored complex with peak absorbance at 532 nm.

Total thiol molecules (TTM): TTM were measured in hepatic and renal tissues according to Sedlak and Lindsay's method (1968). Briefly, 0.2 ml Tris-HCl, 0.02 M EDTA buffer and 5,5'-Dithiobis-2-nitrobenzoic acid (in pure methanol) were added to test tubes containing tissue homogenate. The tubes were mixed and incubated for 15 min at room temperature, the samples were centrifuged at 3000 g for 10 min and ultimately the absorbance of the supernatant was measured at 412 nm. The TTM capacity was expressed as nmol per mg of protein in samples. Biodiagnostic kit (Dokki, Giza, Egypt) was used for the colorimetric determination of total protein in tissue homogenate.

Therapeutic drug monitoring: Tacrolimus trough levels (ng/ml)

were evaluated in blood at the end of the experiment 8 h after the last injection of tacrolimus by double antibody radioimmunoassay method (Winkler et al., 1995).

The lymphocyte proliferation assay (*in vitro*): The lymphocyte proliferation assay was done *in vitro* parallel to the experiment to investigate the influence of spirulina on the immunosuppressive effect of tacrolimus. It was done by isolation of peripheral blood lymphocytes by Histopaque density gradient centrifugation technique, the mononuclear cell layer was collected and washed three times with Hank's Balanced Salt Solution (300 ×g, 10 min) and resuspended in RPMI-1640 (Lonza, Germany). Isolated lymphocytes were incubated with tacrolimus at a concentration of 35 µg/L and combined tacrolimus and spirulina in a concentration of 35 and 250 µg/L, respectively for 2 h. The lymphocyte proliferation was measured by using XTT cell proliferation assay kit (ATCC) cat. no. 30-1011K according to the instruction manual and measuring the absorbance of the assay by ELISA BrdU (Colorimetric) kit (Roche Diagnostics, Penzberg, Germany).

Histological study

Specimens from the liver and kidney for light microscopy examination were fixed in 10% saline formalin and processed to prepare serial sections of 5-µm-thickness paraffin sections for (1) Haematoxylin and Eosin (H&E) stain (Wilson and Gamble, 2002), (2) immunohistochemically staining for localization of proliferating cell nuclear antigen (PCNA) reactivity (Ramos-Vara et al., 2008). PCNA was carried out by means of the avidin biotin-peroxidase complex method (Dako ARK™, Peroxidase, Code No. M0879, Dako, Glostrup, Denmark) following the manufacturer's instructions. Paraffin sections (5 µm) were dewaxed, hydrated and microwave-treated (0.01 M Trisodium citrate). Endogenous peroxidase was eliminated by incubation in 10% H₂O₂ in phosphate-buffered saline (PBS), pH=7 and 4. Sections were incubated with the specific primary antibody mouse monoclonal anti-PCNA antibody PC 10 (Dako, Santa Barbara, CA) at 1:20 dilution for 1 h. After 3 PBS washes, sections were incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulin. After repeated washes with PBS, slides were incubated with avidin and biotinylated horseradish peroxidase (1:200) for 30 min. Diaminobenzidine tetrahydrochloride (DAB) was used as chromogen substrate-chromogen that resulted in a brown-colored precipitate at the antigen site. After repeated PBS washes, slides were counterstained in diluted hematoxylin and rehydrated. Sections of human lymph node with germinal centers served as positive control slides. All steps of immunohistochemistry were performed at room temperature in a humidity chamber. Negative control slides were made using the same previous steps except the primary antibody was replaced by buffer.

E-Morphometric analysis

Using image analyzer at Faculty of Dentistry, Ain Shams University, the mean number of PCNA positive cells were measured. It was measured in randomly chosen five fields/section in five sections in all rats in each group at magnification of 400.

F-Statistical analysis

Data were represented as means ± standard deviation (SD). The differences were compared for statistical significance by analysis of variance (ANOVA) and student's t-test. Difference was considered significant at p < 0.05. The statistical analysis was performed using Epi-Info version 6.1 (Dean et al., 2000).

Table 1. Changes in the liver and kidney function tests in the studied groups.

Variable	Groups				ANOVA
	Control group (N= 10)	Spirulina group (N= 10)	Tacrolimus group (N= 10)	Tacrolimus + Spirulina group (N=10)	F-value
ALT (IU/l)	60.33 ± 6	58.19 ± 5.06 ^{NS}	128.28 ± 1.33 [#]	74.33 ± 1.76* ^{\$}	646.98
AST (IU/l)	119.17 ± 1.15	118.50 ± 1.22 ^{NS}	220.88 ± 1.35 [#]	130.85 ± 1.35* ^{\$}	15087.69
ALP (IU/l)	75.70 ± 1.67	74.51 ± 1.89 ^{NS}	175.18 ± 1.40 [#]	90.00 ± 0.52* ^{\$}	10759.67
Total bilirubin (mg/dl)	0.61 ± 0.03	0.59 ± 0.02 ^{NS}	1.63 ± 0.05 [#]	0.77 ± 0.01* ^{\$}	2495.73
Direct bilirubin (mg/dl)	0.31 ± 0.02	0.30 ± 0.01 ^{NS}	0.75 ± 0.04 [#]	0.35 ± 0.03* ^{\$}	622.56
Serum albumin (gm/dl)	5.6 ± 0.30	5.5 ± 0.20 ^{NS}	2.0 ± 0.04 [#]	5.2 ± 0.10* ^{\$}	840.63
Blood urea nitrogen (mg/dl)	41.40 ± 3.35	40.52 ± 2.55 ^{NS}	94.20 ± 5.89 [#]	58.40 ± 4.09* ^{\$}	364.47
Serum creatinine (mg/dl)	0.98 ± 0.09	0.99 ± 0.01 ^{NS}	1.80 ± 0.08 [#]	1.03 ± 0.03* ^{\$}	414.11
Creatinine clearance (ml/min)	42.1 ± 0.3	42.0 ± 0.5 ^{NS}	22.4 ± 0.1 [#]	37.8 ± 0.32* ^{\$}	2416.23

Data are expressed as mean ± standard deviation (SD). ^{NS}Non-Significantly different from control group. [#]Significantly different from the control group P < 0.001. *Significantly different from tacrolimus group P < 0.001. ^{\$}Significantly different from Spirulina group P < 0.001.

Table 2. Comparison of tacrolimus trough level between rats of tacrolimus group and protected group (spirulina + tacrolimus).

Variable	Groups		t-test	P-value
	Tacrolimus (N= 10)	Tacrolimus + Spirulina (N=10)	Student t-test value	
Tacrolimus trough level (ng/ml)	31 ± 4	28 ± 3 ^{NS}	1.9	> 0.05

Data is expressed as mean ± SD. ^{NS}Non-Significantly different from tacrolimus group.

RESULTS

Biochemical changes

General observation and body weight gain

During the whole period of the study, rats treated with tacrolimus showed decreased food intake as compared to other studied groups. There was a significant decrease (P < 0.05) in body weight gain (BWG%) for tacrolimus group as compared to the control group (8.7 g ± 2.1 vs. 36.5 g ± 3.5). On the other hand, there was a significant increase in BWG% in prophylactic group compared to the tacrolimus group (27.1 g ± 2.5 vs. 8.7 g ± 2.1, respectively).

Liver and kidney function tests

There was no statistical significant difference between control group and spirulina group regarding liver and kidney function tests as shown in Table 1. Rats treated by tacrolimus showed a significant increase in serum ALT, AST, ALP, total and direct bilirubin, as well as BUN, serum creatinine and a significant decrease in albumin and creatinine clearance compared to control rats. Pre- and co-treatment with Spirulina showed a significant improvement in these functional parameters in

comparison with tacrolimus-treated rats (Table 1).

Oxidative stress markers

There was a significant decrease in serum MDA and an increase in TAC as well as hepatic and renal TTM in spirulina-treated rats as compared with control group as shown in Table 2. Rats treated by tacrolimus showed a significant increase in serum MDA and a significant decrease in serum TAC as well as hepatic and renal TTM compared to control rats. Prophylactic group (spirulina + tacrolimus) showed a significant decrease in serum MDA and an increase in TAC as well as hepatic and renal TTM in comparison with tacrolimus-treated rats (Figure 1).

Therapeutic drug monitoring

As shown in Table 2, tacrolimus trough level did not differ in rats treated by tacrolimus either alone or in combination with spirulina.

Lymphocyte proliferation assay

There was a non-statistical significant difference in

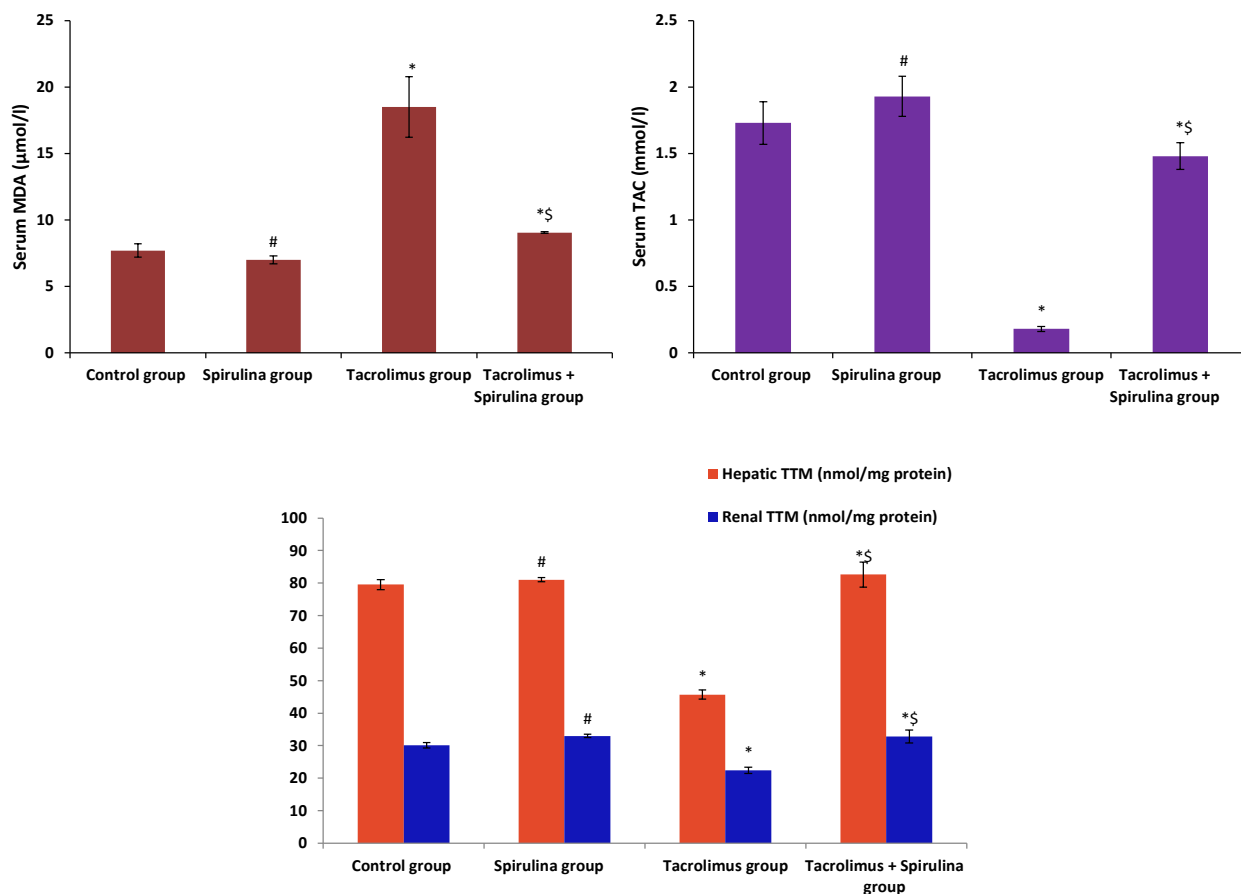


Figure 1. Changes of the oxidative stress markers in the studied groups. Data are expressed as mean \pm SD. #Significantly different from the control group $P < 0.05$. *Significantly different from tacrolimus group $P < 0.001$. $^{\circ}$ Significantly different from Spirulina group $P < 0.001$.

Table 3. Statistical comparison of lymphocyte proliferation assay between adult male albino rats of the treated group (tacrolimus) and the protected group (spirulina + tacrolimus).

Variable	Groups		t-test	P-value
	Tacrolimus	Tacrolimus + Spirulina	Student t-test value	
Lymphocyte proliferation assay	0.31 \pm 0.02	0.33 \pm 0.06 ^{NS}	1	> 0.05

Data is expressed as mean \pm SD. ^{NS}Non-Significantly different from tacrolimus group.

lymphocyte proliferation assay between tacrolimus group and protected group (spirulina + tacrolimus) (Table 3).

Histopathological changes

Histopathological changes in H&E stained sections

Groups I and II (Control and Spirulina groups): Light microscope examination of the liver of the control rats and spirulina treated rats showed hepatic lobules with

cords of hepatocytes with central vesicular nuclei radiating from the central vein and separated by blood sinusoids (Figure 2a). Examination of the renal cortex of the control and spirulina treated rats under light microscope showed normal renal corpuscles with glomeruli, Bowman's capsules lined by simple squamous epithelium. Proximal convoluted tubules (PCT) had eosinophilic cuboidal epithelium and narrow lumen, whereas distal convoluted tubules (DCT) had wide lumen (Figure 3a).

Group III (Tacrolimus group): Light microscope

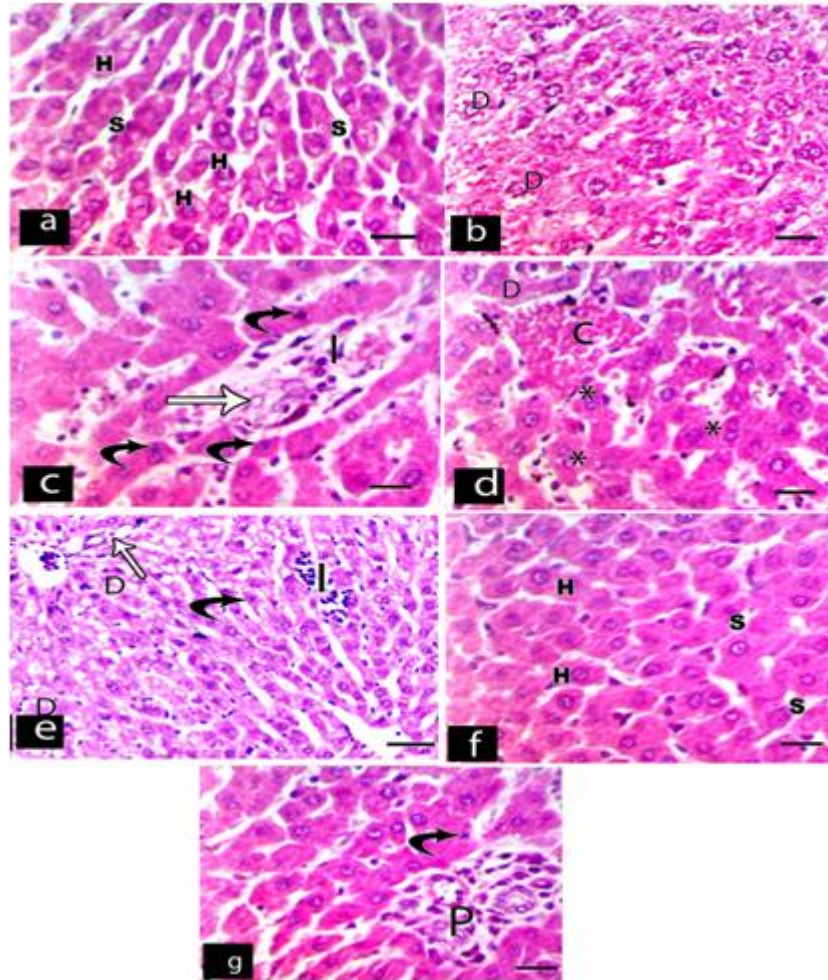


Figure 2. A photomicrograph of sections in the liver of adult male rats of different studied groups: a: group I (untreated control group); hepatocytes (H) well arranged in radiating cords, separated by blood sinusoids (S). b, c, d, e: group II (Tacrolimus group); degenerated, swollen hepatocytes (D) with granulated cytoplasm, multiple apoptotic figures (curved arrows), severe sinusoidal congestion (C) and inflammatory cellular infiltrates (I), and degenerated bile ductules (white arrow). f, g: group III (tacrolimus and Spirulina group); normal hepatocytes (H) well arranged in radiating cords, separated by blood sinusoids (S), and few apoptotic figures (curved arrows), and portal tracts showing bile ductules proliferation (P). H&E stained sections X 400 scale bar= 50 μ m.

examination of the liver revealed different changes in the hepatic lobule. Some hepatocytes showed swelling, degeneration and granulation of cytoplasm. Many degenerated bile ductules and sinusoidal congestion were also seen. There are also inflammatory cellular infiltrates and multiple apoptotic figures (Figure 2b to d). The renal cortex revealed different changes. Most of glomeruli are distorted. Some glomeruli are hypertrophied with enlarged malpighian corpuscles with congestion of glomerular capillaries. Others have widening of the capsular space or segmentation of the glomeruli. The glomeruli showed vacuolation. Proximal convoluted tubules lined with exfoliated degenerated cells and

presence of hyaline casts, some cells showing pyknotic nuclei were also observed. DCT showed vacuolation of cytoplasm and hyalinosis. There was also inflammatory cellular infiltrates (Figure 3b to d). There was also inflammatory cellular infiltrates. Peritubular hemorrhage, capillary and vascular congestion were also seen (Figure 2b to d).

Group IV (Tacrolimus + spirulina group): Light microscope examination of liver sections of group IV revealed that hepatocytes preserved normal appearance and normal liver architectural, some cells showed mild degeneration with few apoptotic figures (Figure 2f and g).

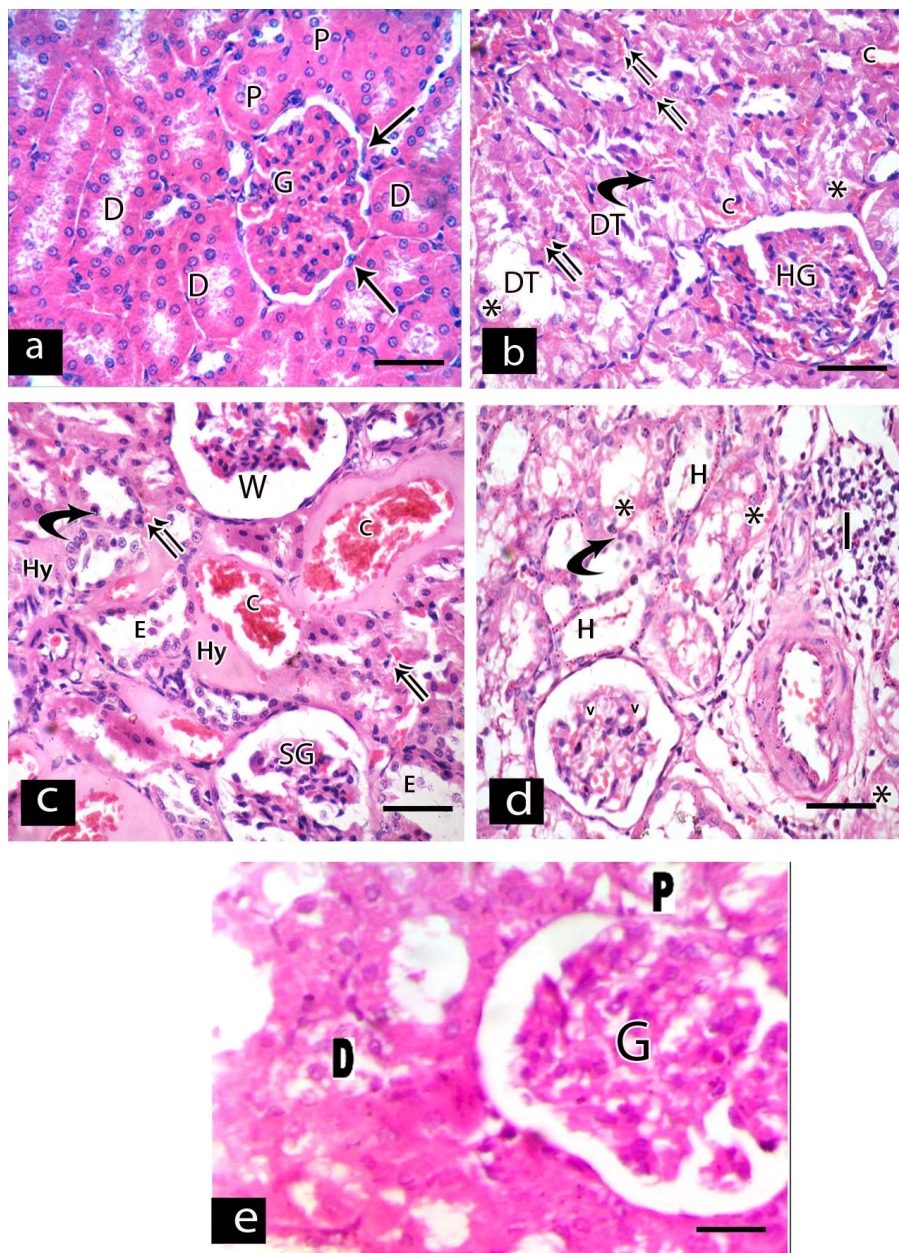


Figure 3. A photomicrograph of sections in the renal cortex of adult male rats of different studied groups. a: group I (untreated control group); showing normal glomeruli (G), with its capillary tufts surrounded by Bowman's capsule that are lined by simple squamous cells (arrows). Note the proximal convoluted tubules (P) with narrow lumen & intense acidophilic cells and distal convoluted tubules (D) with wide lumen & the pale acidophilic cells. b, c, d : group II (Tacrolimus group); showing that most of glomeruli are distorted . Some glomeruli are hypertrophied (HG) with enlarged malpighian corpuscles with congestion of glomerular capillaries. Others have widening of the capsular space (w) or segmentation of the glomeruli (SG). Glomerular cytoplasmic vacuolations (v) is seen. Most of renal tubules are distorted (DT). Exfoliated degenerated tubular cells (E), hyaline casts in the lumen, dark-stained pyknotic nuclei (curved arrow), numerous intracellular vacuoles in the tubular cells of proximal convoluted tubules & distal convoluted tubules (asterisk), and hyalinosis of the convoluted tubules (H) are observed. A focal areas of dense interstitial mononuclear cellular inflammatory cellular infiltrations (I), peritubular hemorrhage (double arrow) and peritubular capillary & vascular congestion (c) are seen. e: group III (Tacrolimus and Spirulina group); nearly normal glomeruli (G), mild degeneration in proximal convoluted tubules (P) and distal convoluted tubules (D). H&E stained sections X400; scale bar=50 μ m.

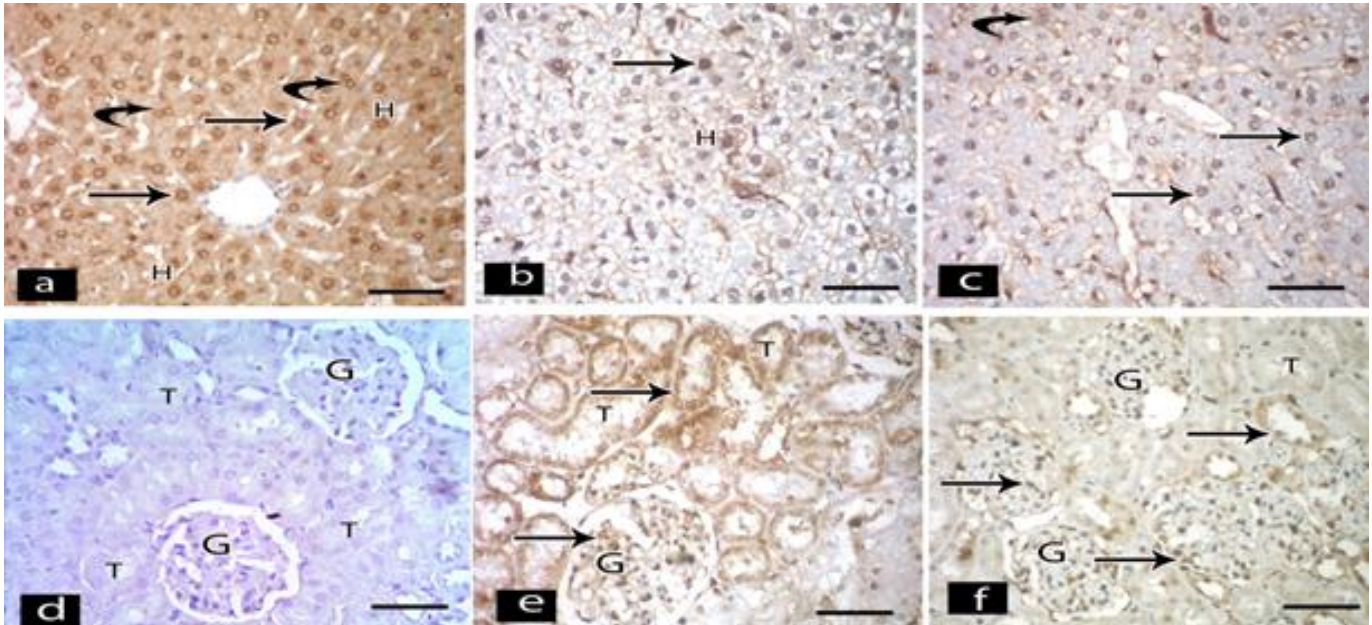


Figure 4. PCNA immunohistochemically stained sections in the liver (a-c) and in the renal cortex (d-f) of albino rats of different groups. (a & d) Control group; (b & e) tacrolimus group; (c & f) Tacrolimus + Spirulina group. PCNA stained sections in the liver (a-c); hepatocytes (H), multiple mitotic figures (curved arrows), positive nuclear immune reaction (arrows). a: showing strong expression of PCNA in most of nuclei & cytoplasm of hepatocytes (H) and mitotic figure was seen. b: showing disturbed hepatic structure, low expression of PCNA (arrows) in the irregular nuclei of hepatocytes (H). c: showing moderate expression of PCNA (arrows) in normal hepatocytes nuclei. Note multiple mitotic figures (curved arrows). PCNA stained sections in the renal cortex (d-f); Glomerulus (G), proximal & distal convoluted tubules (T), positive nuclear immune reaction for PCNA (arrows). d: showing negative immune reaction of PCNA in glomerular and in cells of the proximal & distal convoluted tubules in the control group e: showing strong positive nuclear & cytoplasmic reaction in many glomerular cells and tubular cells. f: showing positive nuclear reaction in few glomerular cells and few distal convoluted tubules cells. PCNA immunoperoxidase X 400, Scale bar: 50 µm.

Renal cortex of prophylactic group showed early mild hydropic degeneration and a few lesions (Figure 3e).

Histopathological changes of PCNA immunostained sections

The hepatic sections stained for proliferating cell nuclear antigen (PCNA) antibodies showed strong immune reaction in hepatocytes in the control and spirulina groups (Figure 4a). Tacrolimus group sections showed mild immune reaction in disrupted hepatocytes with irregular intended nuclei separated by irregular dilated hepatocytes (Figure 4b). Spirulina protected group revealed moderate nuclear reaction in most of hepatocytes with multiple mitotic figures (Figure 4c). The kidney sections stained for PCNA antibodies showed negative immune reaction in glomerular, PCT and DCT cells in the control and spirulina groups (Figure 4d). Tacrolimus group sections showed strong positive nuclear reaction in many glomerular cells and some tubular cells (Figure 4e). Spirulina protected group revealed nuclear reaction in few glomerular cells and positive immunoreaction in few PCT and DCT cells (Figure 4f).

Morphometric results

The mean number of PCNA immunostained cells/high power field (HPFs) showed a non-significant difference between control group and spirulina group in both liver and kidney specimens. Regarding the mean number of PCNA immunostained hepatocytes/HPFs in tacrolimus group compared with the control, there was a highly significant decrease, but tacrolimus plus spirulina group showed a highly significant increase compared with tacrolimus group that was non-significant compared with control (Table 4). However, there was a highly significant increase in the mean number of PCNA immunostained renal tubular cells/HPFs in tacrolimus group compared with the control, but tacrolimus + spirulina group showed a highly significant decrease compared with tacrolimus group and a non-significant increase compared with control (Table 4).

DISCUSSION

Tacrolimus is an immunosuppressive drug that binds to protein and inhibits the phosphatase activity of calcineurin in T lymphocytes to reduce the activity of the

Table 4. The mean number of PCNA positive cells/high power field (HPFs) in different groups.

Group	Control	Spirulina	Tacrolimus	Tacrolimus + Spirulina
Area percentage of PCNA stained hepatocytes/HPFs	10.09±1.03	10.28±1.00	2.04±0.47 [#]	6.10±0.03 ^{\$}
Area percentage of PCNA stained renal tubular cells/HPFs	1.4±0.13	1.73±0.14	19.28±1.27 [#]	4.52±0.66 ^{\$}

Data Values are expressed as mean± SD of n=6 animals. [#]Significantly different from the control group P < 0.001. ^{*}Significantly different from tacrolimus group P < 0.001. ^{\$}Significantly different from Spirulina group P < 0.001.

patient's immune system and so lower the risk of organ rejection (Naesens et al., 2009). It is a potent immunosuppressive agent that is used to treat solid organ transplant recipients, and it has played a large role in the improvement of graft survival rates. However, especially in high doses, it can induce renal toxicity and cholestatic hepatitis (Taniai et al., 2008). Therefore, the objective of the present work was to demonstrate the possible protective role of spirulina against the hepatic and renal damage induced by tacrolimus.

In the present study, tacrolimus treatment induced variable toxic effects, evidenced with a marked reduction in the BWG%; more than 75% decrease compared to control; there was also significant impairment in liver and kidney function tests. Tacrolimus administration induced significant elevations in AST, ALT, ALP, total and direct bilirubin which reach 1.5 or more times the upper limit of control group. These results were in agreement with studies of Taniai et al. (2008) who reported that tacrolimus produced increase in ALT, AST activities and total bilirubin level. Singh and Watt (2012) found also that many patients taking tacrolimus had a long term mild increase in liver enzymes. Elevated serum level of hepatic enzymes indicate liver damage, cellular leakage and loss of functional integrity of hepatocytes (Mishra et al., 2015). Supporting these notions, we found that tacrolimus induced histopathological changes including swelling of hepatocytes, granulation of cytoplasm, liver congestion, degenerated bile ductules, inflammatory cellular infiltrates and inflammatory cellular infiltrates. Similar findings were detected by Yadav et al. (2013) who found that tacrolimus induced hepatotoxicity in the form of cholestatic hepatitis and liver congestion.

Pre- and concomitant administration of spirulina with tacrolimus here significantly reversed tacrolimus induced changes in liver function tests. Thus, this reduction in the hepatic enzymes activities clearly pointed to the membrane stabilizing activity of spirulina. Reduction in the levels of AST, ALT, ALP and bilirubin towards the control values is an indicator of the protective effects of spirulina. The histological examination of the liver sections confirmed the aforementioned results where spirulina pre- and co-administration along with tacrolimus can restore the normal cellular architecture of the liver and reverse tacrolimus induced histopathological effects. In line with this, previous studies showed that spirulina returned the elevated serum levels of hepatic enzymes

near to normal levels in deltamethrin-intoxicated rats and other models of toxicity through its potent antioxidant and free radical-scavenging activities (Abdel-Daim et al., 2013; Abdel-Daim, 2014; Abdel-Daim et al., 2016).

Regarding tacrolimus induced nephrotoxicity in the current study, there were also significant elevations in BUN, serum creatinine and a significant reduction of creatinine clearance in tacrolimus treated group, in agreement with Abdel-Daim et al. (2013, 2016). Similar results were also reported by Di Benedetto et al. (2009) who found a significant increase of serum creatinine (>1.8 mg/dl) in patients developing renal dysfunction following liver transplantation due to calcineurin inhibitors. In concordance, the results obtained from the present study showed that microscopical examination of the kidney of adult albino rats treated with tacrolimus showed vacuolation of glomeruli and distal tubule. Banhara et al. (2015) reported that distal tubular dysfunction is prevalent among kidney transplant patients using tacrolimus. Moreover, Boudjema et al. (2011) suggested that tacrolimus induced nephrotoxicity is dose-dependent in transplant patients. Nephrotoxicity is a major clinical obstacle related to tacrolimus and is usually responsible for the discontinuation of treatment (Porayko et al., 1994). This is in agreement with Gaston (2006), where tacrolimus induced nephrotoxicity as manifested by severe interstitial fibrosis, peritubular calcification, and focal glomerulosclerosis; these changes may result in irreversible chronic renal failure in patients undergoing renal transplantation patients. Furthermore, other changes were observed in the kidney including swelling of proximal tubules, hyalanosis and presences of hyaline casts in proximal and distal tubules. These changes were similar to the results of Randhawa et al. (1997).

Although spirulina has demonstrated protection against multiple drug and toxin-induced systemic toxicity (Khan et al., 2006; Alam et al., 2013; Abdel-Daim et al., 2016; Bashandy et al., 2016) its protective effect on tacrolimus-induced toxic injury has never been investigated. This prompted us to evaluate whether and how, spirulina may ameliorate tacrolimus-induced hepato and nephrotoxicity. Accordingly, when rats administered spirulina concomitantly with tacrolimus, liver and kidney function tests returned near to control values, suggesting the cytoprotective ability of spirulina in liver and kidney cellular integrity, restoring their normal functions.

Spirulina was previously proven to have potent

antioxidant activities (Romay et al., 1998; Lissi et al., 2000; Chu et al., 2010). These activities were largely related to phycocyanin protein of spirulina. This protein contains a tetrapyrrole phycocyanobilin, which has been reported to have a significant antioxidant and radical scavenging properties, offering protection against oxidative stress (Bashandy et al., 2016). Similarly, in the current study, spirulina treatment had significantly improved the antioxidant parameters (serum TAC, hepatic and renal TTMs) compared to the control group. In confirm, a recent study has indicated that spirulina shows free radical scavenging and potent antioxidant activity during deltamethrin intoxication (Abdel-Daim et al., 2015; Abdelkhalek et al., 2015; Abdel-Daim et al., 2016). Furthermore, spirulina contains superoxide dismutase that exerts indirect action by retarding oxygen radical generating reactions rate (Belay, 2002; EL-Sabagh et al., 2014).

Supportive data were provided from the present histologic and immunohistochemistry studies, where spirulina co-administration ameliorated tacrolimus induced hepatocellular and renal cellular regeneration and proliferation in H&E stained section that were further supported by PCNA immunostaining. Spirulina protected group showed partial restoration of immunoreaction to PCNA in most of the hepatocytes and renal cells comparable to control rats. Ozaki et al. (2001) studied the role of spirulina in reducing nephrotoxicity, cellular hyperplasia and PCNA overexpression in peroxisome proliferators. Moreover, Makhoulf and Makhoulf (2012), tested the hepatoprotective effect of spirulina against ionizing radiation induced liver injury; they found spirulina could significantly increase hepatocytes DNA content and proliferation, the authors explained these effects by abundant content of spirulina of beta carotene and superoxide dismutase.

An additional objective in this study was to evaluate the possibilities of interaction between tacrolimus and spirulina that can reduce therapeutic efficacy of tacrolimus. Both the trough level of tacrolimus and lymphocyte proliferation assay did not change significantly in absence and presence of spirulina.

Conclusively, it was shown that orally administered spirulina may be associated with a decrease in tacrolimus induced hepatotoxicity and nephrotoxicity in adult male albino rats. Further studies are warranted to evaluate whether transplant patients on tacrolimus treatment may benefit from the protective effects of spirulina.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Hypothetical adjustment of the acceptable daily intake and correction of the underrated risk: A case study of glyphosate-based herbicides

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Following the introduction of genetically-engineered glyphosate-resistant (GEGR) crops, commercially known as Roundup Ready (RR), no pesticide's active principle has been used as much as glyphosate; yet its safety measures have been sternly disputed. After its classification by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans in 2015, scientists, activists, regulators and the general public revisited voluminous studies that outweighed the risk of this herbicide and raised ferocious concerns that warranted serious attention. Recently published studies on glyphosate established at least four toxicological principles. First, glyphosate exhibited severe mammalian toxicity at concentrations orders of magnitude lower than its regulatory-promulgated 'No Observed Adverse Effect Level' (NOAEL) or even its 'Chronic Reference Dose' (cRfD) and 'Acceptable Daily Intake' (ADI). Second, even though not transparently scrutinized or officially required for toxicological testing and risk assessment, glyphosate co-formulants and glyphosate-based herbicides (GBHs) are orders of magnitude more toxic than the principle active ingredient alone. Third, glyphosate and GBHs are cytotoxic and endocrine disruptors, and the latter explains why ultra low concentrations - yet environmentally relevant-cause severe chronic toxicity. Fourth, the endocrine disruption likely leads to epigenotoxicity that may be extended to offspring and unexposed descending generations. Taken all together, it can be fairly said that confidence in the regulatory-certified ADI values is highly eroded. To resolve the paradoxical discrepancy between regulatory safety measures and elicited toxicities at concentrations far below these measures, ADI was refined using two safety or adjustment factors. Together, these two factors scale down ADI by four orders of magnitude and bring it to an Adjusted ADI (AADI) value of 2.5 ng/kg bw/day. Contrary to regulatory ADI, the new AADI successfully explains many research findings which demonstrated severe mammalian toxicity at concentrations in the neighborhood of nanograms a.i./kg bw/day. This distills confidence in the new AADI value, as well as the magnitude of the proposed safety factors. Glyphosate uses as per human capita, in two countries representing the extremes of adopting RR crops (the USA) or not-adopting these crops (Egypt), were compared. The comparison confirms the association between growing RR crops and the escalated use of glyphosate, and shows that the American public is likely exposed to glyphosate residue at forty times higher levels than the Egyptian public.

Key words: Acceptable daily intake, adjuvant, chronic reference dose, co-formulants, food quality protection act, hazard, glyphosate, glyphosate-based herbicides, no observed adverse effect level, risk, roundup, roundup ready crops.

INTRODUCTION

Glyphosate is the active ingredient in Monsanto's first commercial herbicide (Roundup), and many other proprietary glyphosate-based brands (Monsanto, 2005). Worldwide, glyphosate is considered to be the most used herbicide in agriculture, horticulture, viticulture, forestry, parks, industrial and public sites, aquatic environments, gardens, sports fields, school grounds, etc. A US-patent also covers the use of glyphosate for antibiotic treatment of animal and human pathogenic infections (Organic NZ Magazine, 2015). The unprecedented use of GBHs provides uncountable exposure pathways, and increasingly raises concerns over their possible adverse outcomes in human-health and the environment. Regardless of the IARC classification of glyphosate as probably carcinogenic to humans (Guyton et al., 2015), and of the serious scientific and public concerns over its safety, pesticide industry and regulatory authorities complacently claim that when GBHs are used as recommended, the public is exposed to only 'safe' levels that pose no serious toxicological risks to humans (FAO/WHO, 2016).

To interpret the level of risk of any pesticide, its actual exposure is compared to a reference safety threshold, e.g., ADI; calculated for experimental animals and extrapolated to humans. ADI is the amount of a substance, expressed on a body-mass basis, daily ingested in food or drinking water over lifetime without imposing any appreciable risk to human health (The Detox Project, 2016; WHO, 1987). The calculation to set the ADI is based on one hundredth (1/100) the dose considered to be non-toxic in animal feeding trials; toxicologically known as NOAEL (Faustman and Omenn, 2001).

NOAEL-generating experimental studies are usually run by pesticide companies according to protocols set in consultation with the Organization for Economic Cooperation and Development (OECD), an agency mainly dedicated to facilitating international trade, not to shielding public health. Besides, since the data are generated by, or provided through, pesticide companies, conflict of interest may not be preventable or avoidable.

Glyphosate, which was ironically considered to be as safe as caffeine and table salt (Charry, 1997; Preston, 2014; The Credible Hulk, 2015) for four decades, was recently classified by IARC/WHO and added to the A2-carcinogenic category (Guyton et al., 2015). This paradigm shift in glyphosate toxicology is due to many reasons including: (i) the escalated use of GBHs and obviously the subsequent high residues and elevated human and environmental exposures (Benbrook,

2016; Myers et al, 2016), especially after the first adoption of RR crops in 1996 (Monsanto, 2015), that is, the post- era of RR biotechnology; (ii) a growing body of solid evidence indicating that experimental animals and humans face serious risks as a result of their exposure to concentrations far below the regulatory-claimed-to-be safety thresholds (Jayasumana et al., 2015; Mesnage et al., 2015); (iii) safety thresholds or limits are set for the active ingredient 'alone' which is generally less toxic than the formulation blends actually polluting the environment and affecting human life. The third reason (iii) implies two things that are strongly supported by research findings: (a) the safety thresholds are erroneously overestimated; (b) the mammalian toxicity of glyphosate is bestowed by co-formulants. The ultimate result is that what is assessed to be safe in laboratory testing is not actually safe under field conditions. Therefore, one cannot use regulatory-adopted safety measures as a reference for the interpretation of risk under real-life situations of human and environmental exposures.

It is generally accepted that pesticide formulations are up to three orders of magnitude acutely or chronically more toxic than their active principles (Mesnage et al., 2014; Defarge et al., 2016) due to the toxic and/or synergistic effect of co-formulant(s). The co-formulant effect factor can be further complicated by the diversity of used glyphosate-based generic brands. For example, over 750 formulations are registered for glyphosate use in the USA alone (Henderson et al., 2010), and more than 500 adjuvant/co-formulant substitutes are commonly used in glyphosate end-use products (The Greens-EFA-EU, 2016). Unfortunately, most of these co-formulants earn commercial confidentiality rights and are not totally scrutinized or accessible to scientists or even regulatory agents, let alone the lack of studies regarding their hazard to human health and the environment. It is surprising that regulatory authorities are sometimes misled or deceived by pesticide industry and accept the notion of co-formulants as toxicologically-inert materials that pose no toxicological risk to human health and the environment. This notion is not only inaccurate; it is also misleading and extremely dangerous if we consider that levels of GBHs for which the active principle is claimed to be safe are not actually safe over the long term or for recently-discovered toxicological endpoints, e.g., endocrine-mediated epigenetic toxicity review by Ibrahim (2016).

For example, disturbances of functional genes were observed in kidney and liver of rats treated with glyphosate at as low as 4.0 ng/kg bw/day (Mesnage et

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al., 2015). This dosage level is five orders of magnitude lower than the regulatory-held safe exposures or ADI levels (0.30 to 1.75 mg/kg bw/day) for this herbicide (Center for Food Safety, 2015). The fact that regulatory ADI values fail to explain recent findings which demonstrated serious animal-health outcomes at ultra-low concentrations, far below the ADI levels (Defarge et al., 2016) indicates that these levels lack the criteria and qualification of being used as a reference safety threshold. More importantly is that the public health cannot afford the adoption of what is claimed and clamored by 'professional' pesticide regulatory authorities or agencies to be an acceptable exposure level when in fact five orders of magnitude lower concentrations can induce serious human-health defects (Bonn, 2005). Let alone, the spread of epidemiological incidences of chronic diseases thought to be causally related to GBHs (Jayasumana et al., 2015). Gasnier et al. (2009) found that GBHs presented DNA damages and carcinogenic-mutagenic-reprotoxic (CMR) effects on human cells and *in vivo*. Exposure to low doses of GBHs may result in reproductive and hormonal problems, miscarriages, low birth weights, pre-term deliveries, and birth defects. It is strange that the safety of public health can sometimes be in the hands of individuals rather than professional pesticide regulatory authorities, e.g., US-EPA and EU-EFSA. This statement applies perfectly to Glyphosate; as for the time these regulatory authorities maintain glyphosate re-registration for weed control, the newly elected president of Sri Lanka, Maithripala Sirisena, announced in one of his first decisions that the country's importation of glyphosate was to be banned immediately and that the release of any stocks already present in the country was to be halted as well (Heyes, 2015). Due to all the discrepancies between the regulatory-certified safety measures (e.g., ADI values) and reputable scientific research findings, as well as the epidemiological incidences that greatly contradict and challenge these measures, it was the intent of the author to reexamine these measures and find ways to adjust them within the scope of published research, reports and observations.

MATERIALS AND METHODS

This manuscript shed some lights on some serious problems inherent in the traditional approach of establishing the acceptable exposure thresholds of pesticides (NOAEL, cRfD, ADI, etc.). For a multitude of reasons, glyphosate has been used as a case study to support the rationale, theme and conclusions of the present study. In order to work this case and achieve the objectives of this manuscript, the author has taken permission from Dr. Charles M. Benbrook to use some data from his landmark article that has been recently published in Environmental Sciences Europe on the trends of glyphosate use in the USA and worldwide (Benbrook, 2016). For the sake of comparisons, data for the volume of glyphosate used in Egypt during 2014 was obtained from the database of the Agricultural Pesticide Committee (APC), Ministry of Agriculture and Land Reclamation, Egypt. The methods used in this manuscript

were mainly based on the philosophy and calculations of risk assessment and management that were remarkably compiled by Purdue Pesticide Programs, Purdue University Cooperative Extension Service (Whitford et al., 2016). In particular, the relationship between the acceptable exposure thresholds and the risk posed by actual exposure under real-life situations was worth considering. Besides, the author has attempted to establish a new equation to calculate the daily human exposure from the volume of the national and global use of glyphosate.

RESULTS AND DISCUSSION

The main objective of this manuscript is to create a quasi-mechanistic model to possibly adjust the pesticide safety measures (NOAEL, ADI, cRfD, etc.) that are routinely calculated from the empirical risk assessment model. The empirical model uses data collected from experimental studies that, unfortunately, use low-resolution tools and endpoints to calculate these measures. According to the empirical model, risk assessment of any pesticide to human health and the environment relies on two principal factors: (1) its innate or potential hazard of the active ingredient; and (2) its actual level of exposure to humans and the environment. The first factor is more or less based on fixed and experimentally-defined toxicological safety measures (e.g., NOAEL or ADI), while the second one depends on actual human and environmental exposure stemming from how much pesticide is being applied in a region on a given crop, collectively across all crops, and in other places. If perfectly determined, the potential hazard is static for each toxicological endpoint, while the experienced exposure is momentarily dynamic. In line with these two factors, the results and discussion section is divided into two subsections (I & II). The first subsection contains a literature-based justification approach for the importance of refining ADI values measured for the active ingredient 'alone' using glyphosate as an exemplary model. This subsection is supported by two novel figures that clearly show how erroneously overestimated ADI value leads to enormously underrated risk, especially in the era of RR biotechnology. The second subsection is dedicated to comparing some data for glyphosate use in the USA and Egypt, as representatives of countries adopting or not adopting RR crops, respectively. This comparison allows the author to see how much of the escalated use of glyphosate can be attributed to growing RR crops, and how this escalated use can seriously threaten the safety reputation this herbicide with reference to an adjusted or miniaturized ADI value. In this subsection, the global use of glyphosate is also included.

The underlying principles of adjusting glyphosate ADI values

There are several reasons that led the author to question and challenge the reliability and validity of the currently-

known and regulatory-certified ADI values of glyphosate and its formulations (GBHs). The same and other reasons have encouraged the author to seek ways to refine the currently-accepted but evidently-overestimated ADI values. The six reasons that create the underlying principles of this manuscript are as discussed in the following:

First, ADI values have been determined by testing the active principle *'alone'* on laboratory animals; yet the regulatory authorities enforce these values on all used GBHs; barely known for the identity and toxicity of their individual components. That is in spite of the fact that people and the environment are genuinely exposed to formulations, not just their isolated active ingredient. Several Studies confirmed that glyphosate formulations administered to rats and pigs at levels - deemed safe for glyphosate active ingredient alone - were extremely harmful to treated animals (Adam et al., 1997; Antoniu et al., 2012; Benedetti et al., 2004; Lee et al., 2009; Romano et al., 2010).

Second, ADI values are based on studies conducted on adult animals mostly failed to test or observe the effects of exposure during vulnerable windows of development, e.g. foetal development and unexposed descending generations. The issue of trans generational or epigenetic inheritance of adverse human-health and environmental effects of endocrine disrupting pesticides was strongly emphasized when the well-known fungicide vinclozolin was given at a single time to mice with testis in a critical period of development. As discovered by Anway et al. (2005), vinclozolin produced an adverse effect on the developing testis that was passed on to the following three generations of mice. The epigenetic inheritance was also found with other pesticides and pesticide mixtures. For example, Manikkam et al. (2012) showed clearly that the epigenotoxic effects of an insecticidal mixture (permethrin + DEET) lasted for three successive generations. A subtle endocrine disruption during early life can modify the morphologies and functions of many organs and eventually cause reprotoxicity and cancer (Vandenberg et al., 2012).

Third, regulatory-accepted risk assessment protocols are based on the 15th century old adage of Philippus von Hohenheim (globally known as Paracelsus, the father/founder of toxicology) who stated that : *"the dose makes the poison"* and implied that the higher the dose, the greater the degree of toxicity (The Detox Project, 2016; Wikipedia, 2016). Although it fully applies to acute toxicity and related endpoints, this adage does not apply to some chronic toxicity, especially what is related to endocrine-disruption, wherein the dose-response relationship is not always monotonic and safe levels cannot simply be extrapolated from high doses (Heindel et al., 2013; Lagarde et al., 2015; Vandenberg et al., 2012; Zoeller and Vandenberg, 2015). Ultra-low concentrations of some endocrine-disrupting pesticides are more toxic than NOAELs which are commonly

expected or extrapolated from higher concentrations. Besides, NOAEL itself may still cause serious response or damage on the same or different endpoints, if the dose matches the vulnerability window(s) and/or exhibits a biphasic or concaved relationship with its response. In the light of the endocrine-disrupting potential of glyphosate (Babalola, 2016), the author prefers to rephrase the well-known Paracelsus toxicology norm to make it applicable to any pesticide chemicals regardless of the shape of its dose-response curve (monotonic or non-monotonic). The rephrased toxicological principle states that *"the dose unfolds the actual risk of its potential or tacit hazardousness."* The dose required for some toxicological outcomes or endpoints does not have to be only in the range of high doses.

Fourth, the potential endocrine-disruption by glyphosate and its commercial formulations (Séralini et al., 2014; Séralini, 2015; Thongprakaisang et al., 2013) indicates that the standard long-term animal studies and traditional endpoints required by regulatory authorities and executed by pesticide companies are inadequate to accurately determine valid and reliable ADI values. In a comprehensive review including 314 references, Fuhrman et al. (2015) compiled and discussed the uncertainties and unknown that regulators may face when considering the risk assessment of endocrine disruptors and indicated clearly that there is no definitive risk assessment tool for these chemicals; a situation that will enforce regulators to accept data from loosely designed testing protocols and poorly defined, even distant or irrelevant, endpoints.

Fifth, several studies demonstrated additive or synergistic effects of different types of endocrine disruption, e.g., estrogenic, antiandrogenic, or thyroid-disrupting agents, when used in mixture at concentrations far below their NOAELs. A dramatic enhancement of endocrine effects not predicted from tests on individual compounds (Rajapakse et al., 2002; Silva et al., 2002, 2011) has been observed for some estrogenic chemicals. When three estrogenic test systems were used (Seeger et al., 2016), similar outcomes on mixtures of endocrine-disrupting pesticides were confirmed. The additive/synergistic behavior of endocrine disruptors is likely to be the case with glyphosate and additives in glyphosate-based formulations.

Sixth, commercially used formulations of glyphosate contain additives (adjuvants or co-formulants), which are either toxic in their own right and/or increase the toxicity of glyphosate (Mesnage et al., 2013; Séralini, 2015).

The six abovementioned reasons, along with their solid research evidence supporting them, challenge the validity and reliability of regulatory-enforced ADI values. These values seem to be highly overestimated and the risk of exposure assessed with reference to them is significantly underestimated. This has been simply and conceptually illustrated in Figure 1. Like many toxicologists from around the world, the author believes that the EPA's

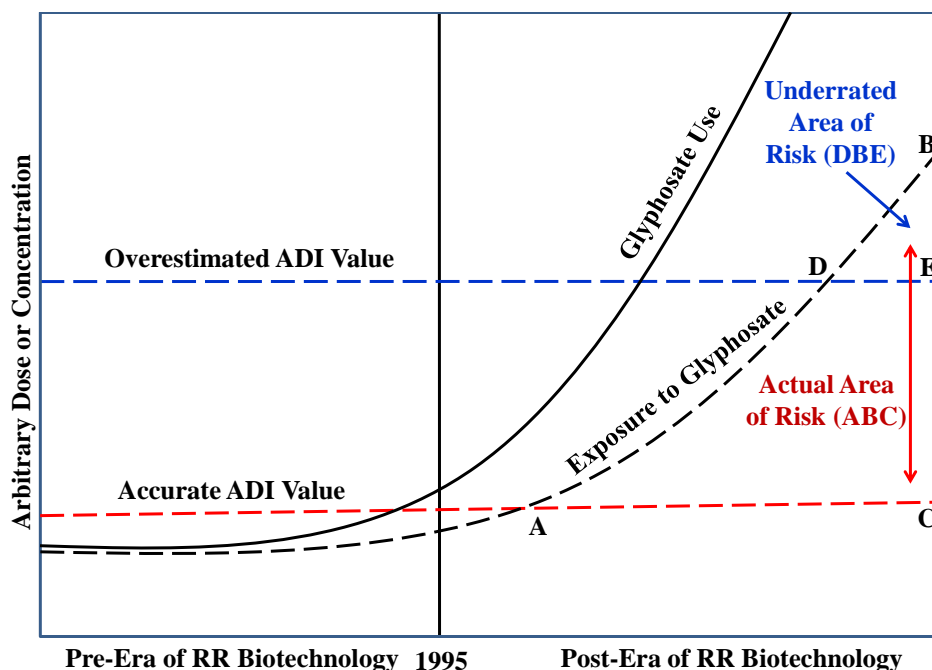


Figure 1. Scenarios of glyphosate use in the pre- and post-era of RR biotechnology. Note that the overestimated ADI value makes the risk of exposure underrated (DBE). With an accurate ADI value, the actual risk is precisely determined (ABC). Using an overestimated ADI value induces false safety or uncountable risk ($ADEC = ABC - DBE$).

cRfD or the European ADI values for glyphosate are overly estimated. The range of these values (0.30 to 1.75 mg/kg bw/day) is considered to be too high to mark any acceptable or conservative human-exposure threshold. Based on these values, the safety margin or ceiling of this herbicide is likely wider or higher than the actual case scenarios especially in the light of the highly vulnerable endocrine system and its mediated epigenetic effects or outcomes (Defarge et al., 2016; Ibrahim, 2016). The endpoints of these outcomes are likely: (a) inflicted by ultra-low doses; and (b) appeared in maternally exposed offspring or unexposed descending generations (Ibrahim, 2016). To simply explain the danger of relying on overestimated ADI value while assessing the risk of actual pesticide exposure, Figure 1 was generated. Although highly simplified, this figure superbly illustrates the risk situation of glyphosate exposure in the pre- and post-era of RR Biotechnology. It also illustrates the author's renovated toxicological principle which states: "Once the ADI value is erroneously overestimated for any pesticide, the risk from exposure to this pesticide will always be enormously underestimated."

It also shows that there is a huge area of actual risk (the ABC area) when exposures are compared to an accurately-determined safety measures (accurate ADI value). To the contrary, this risk is underrated and shrunk to the DBE area when exposures are compared to overestimated regulatory safety measures or ADI values. Therefore, it is highly critical that the current ADI values

of glyphosate are reassessed and refined, while taking endocrine disruption and the likely heritable epigenetic havoc into consideration. Since this has not been experimentally done yet, the author will provide some hypothetical adjustment of the acceptable exposure threshold of GBHs, specifically the ADI. It is within our understanding that the relationship between the exposure level to any pesticide and its used quantity is not perfectly straight - but certainly correlated. It is also understood that the interface of pesticide use, human and environmental exposure and observation, biologically-responsive system(s) and adverse outcomes is very complex. Obviously, the nature and severity of these outcomes vary depending on the overall health of the exposed organism, its physiological and psychological state, the level, timing and duration of exposures, the tissues exposed, their vulnerability, the consequent human health outcomes, to count just a few. In particular, the timing of pesticide exposure that temporally and spatially matches the sensitivity window is a key determinant, especially with endocrine-disruption and epigenetically-mediated outcomes (Ibrahim, 2016).

ADI-adjusting factors

Two safety factors were introduced to adjust or scale down glyphosate ADI values. The first factor (10x) is to compensate for the unlikely certainty of no harm in the

light of elevated environmental and human exposure and the repeated epidemiological incidences of glyphosate-related health effects. The second factor (1000x) is to compensate for the bestowed toxicity of glyphosate in the presence of co-formulants. The introduction of the co-formulant safety factor is extremely important due to the fact that even though ADI is determined for glyphosate alone, people are exposed to the whole formulation simply because glyphosate can never be used alone and by itself for weed control.

FQPA factor

According to researchers, cell damage and/or cell death, especially, embryonic, placental and umbilical cord cells, can occur at residue concentrations commonly found on Roundup-treated crops, yards, lawns, parks and gardens for weed control (Scientific America, 2009). It is important to note that the US Food Quality Protection Act (FQPA) requires the Environmental Protection Agency (EPA) to assure that a pesticide can be used if only its residues demonstrate "A Reasonable Certainty of No Harm." This assurance requires the EPA to introduce a tenfold (10x) safety factor when setting and reassessing tolerances unless adequate data are available to support a different factor (EPA, 1996; McDonald Jr., 2000). This factor is also used to compensate for dietary exposures and higher risk of glyphosate or any pesticide to extra-sensitive groups in the population, e.g., pregnant women, infants, children, and elderly people living in or nearby heavily exposed areas. Considering the uncertain safety of safety measures set for GBHs, and of the continual and high exposure of pesticide applicators, farm workers and bystanders in residential areas close to RR fields, one can introduce, for partial adjustment of glyphosate ADI, a safety factor of 10X, similar to that of the 1996 mandate of US-FQPA Act.

Adjuvant factor

Based on a diversity of recent studies, a second safety factor of 1000X was introduced in the present study to further adjust the thought- and also found-to-be overestimated ADI values. This factor possibly compensates for the bestowed toxicity of glyphosate induced by adjuvants or co-formulants which are mistakenly believed to be inert additives. It has been recently mentioned (Mercola.com, 2016) that certain GBH adjuvants cause human cell toxicity, adding to the hazards inherent in the active principle (glyphosate). In a study of the effects of glyphosate and its adjuvants on hepatic (HepG2), embryonic (HEK293) and placental (JEG3) cell lines, Mesnage et al (2013) found that the toxicity of commercial formulations was due to adjuvants rather than the active ingredient itself, and the toxicity

was in fact proportional to the concentration of these adjuvants. Mesnage et al. (2014) found out that this has also been the case with other herbicides, as well as some insecticides and fungicides. The formulations in almost all the tested pesticides were up to 1000 times more toxic than their active ingredients to human cells *in vitro*. Polyethoxylated tallow amine (POEA), a major adjuvant/surfactant in Roundup formulations, has been shown to be 1,200 and 2,000 times more cytotoxic than glyphosate (Defarge et al., 2016). The bestowed toxicity of the formulated vs. active principle of glyphosate is emphasized not only for human-health outcomes but also for environmental disruption (Martini et al., 2016; Székács et al., 2014). For example, glyphosate at 50 ppb was shown to have significant negative impacts on the aquatic invertebrate, *Daphnia magna* (Cuhra et al., 2013; Myers et al., 2016). This concentration is orders of magnitude lower than the range of the Maximum Contaminant Level or eco-toxicological threshold (700-27000 ppb) assigned by regulatory authorities in the USA and Canada (Canadian Council of Ministers of the Environment, 2012). Based on the aforementioned studies, the author chose to use a safety factor of 1000X to compensate for the bestowed toxicity of glyphosate induced by co-formulants.

Adjusted ADI (AADI) value

A group of scientists has compiled evidence supporting a miniaturized ADI value of 0.025 mg/kg bw/day (Antoniou et al., 2012). Although this value is 12 to 70 times lower than the EU and EPA reference values, it is still four orders or magnitude higher than what was found to inflict gene disturbance or epigenetic disorder in rats (Mesnage et al., 2015). Therefore, Antoniou's ADI value requires further refinement. When this value was taken as a baseline for adjustment, and divided by the combined safety factors of 10⁴X, as proposed in the present study, an Adjusted ADI (AADI) value of only 0.0000025 mg/kg bw/day or 2.50 ng/kg bw/day was obtained for glyphosate in the context of its formulated blends. A recent finding by Mesnage et al. (2015) clearly showed that genes in kidney and liver of rats treated with glyphosate at 4.0 ng/kg bw/day were functionally disturbed. The fact that this dose is only 1.6 times that of the AADI value from the present study indicates that this value is reasonably calculated and conservatively adjusted and refined. After rationally adjusting the ADI value based on this manuscript's quasi-mechanistic model, the danger of relying on an overestimated glyphosate ADI value as a yardstick for risk assessment of GBHs deserves further emphasis. Figure 2 compares the calculated risk of exposure to GBHs when an overestimated and adjusted ADI values of glyphosate are taken into consideration.

By looking at Figure 2, one can easily extract two intimately related points: (1) the higher the magnitude of

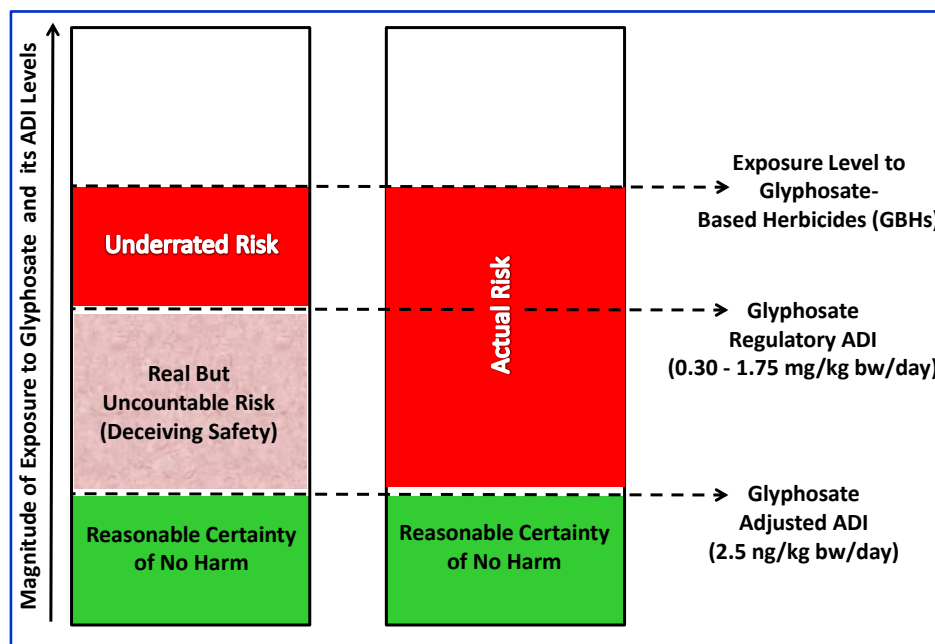


Figure 2. An illustration shows how overestimated level of acceptable exposure to glyphosate (ADI) leads to underestimated or underrated risk of actual exposure to glyphosate-based formulations (GBHs); thereby any uncountable risk becomes a deceiving safety. It also indicates the importance of adjusting and refining the regulatory-certified, yet overestimated, glyphosate ADI values. As seen in the right side of the figure, the adjusted ADI value corrects for the underrated risk.

overestimation, the bigger the chance of missing the assessment of a significant part of the actual risk; (2) the bigger the difference between the inaccurate and accurate ADI values, the bigger the area of deceiving safety. Obviously a result like this one erodes confidence in regulatory-promulgated ADI values, at least in the case of GBHs. With this conception in mind, it appears that levels of GBHs, for which the active principle is claimed to be safe, may in fact pose serious risk to humans over the long term. It is, therefore, believed that people are misled by the current safety measures (ADI values) of pesticides' active ingredients when these measures are applied to interpret and assess the risk of end-use products or formulations. Even if the safety thresholds or measures adopted by regulatory authorities for glyphosate were accurate, the overuse of this herbicide in the past two decades and after the introduction of RR crops may have driven its exposure levels far above these measures, thereby the certainty of no harm is becoming foggy or uncertain.

RR crops: Glyphosate overuse and risk concerns

Successes in developing RR crops allow farmers to overuse glyphosate either forcibly, voluntarily or even irresponsibly. Statistics have shown that no pesticide in

the history of plant protection has been used as widely as glyphosate (Benbrook, 2016; Van Hoesen, 2016), especially after the introduction of RR crops to agriculture. It is no wonder why voluminous research studies indicate that glyphosate is predominantly found in the air, the water, the soil, the food, the feed and the human body (Myers et al., 2016 for citations), sometimes at levels far-exceeding the regulatory-allowed thresholds. To make the theme of this study clear and intact, the 2014 consumption of glyphosate has been compared in the USA, wherein RR crops are heavily cultivated; in Egypt, wherein these genetically-engineered crops have never been introduced to the Egyptian agriculture; and worldwide, wherein these crops are adopted in some countries and are not adopted in others. Table 1 shows glyphosate use in these three comparisons, along with the corresponding populations to calculate this use as per human capita. The arbitrary human exposure (the last column in Table 1) was calculated according to the following equation:

$$\text{Human Exposure (ng glyphosate a.i./kg bw/ day)} = \frac{[(\text{mg a.i. used as per capita} \times 10^6)]}{(365 \times 70 \times 10^6)} \quad (1)$$

Wherein 365 is the number of days in the year; 70 is an assumed average weight of working adults (kg/adult) who are either fractionally at risk or directly exposed to

Table 1. Glyphosate use in Egypt, the USA, and worldwide during 2014, and the corresponding populations that may hypothetically be exposed to this herbicide.

Country	Total glyphosate Use in 1000 kg Active Ingredient (a.i.)	2014 Population in Millions*	Glyphosate Use in mg a.i. as per Human Capita	Arbitrary of Human Exposure in ng/kg bw/ day (Equation 1)
Egypt	831.38	86.9 ^a	9567	00.37
USA	125384.00	318.9 ^b	393177	15.39
World	825804.00	7174.6 ^c	115101	04.50

*The population sizes were obtained from Index Mundi (2015a, b, and c).

glyphosate; the 10^6 in the numerator is for the conversion of mg to ng; and the 10^6 in the denominator is a hypothetically suggested fraction of glyphosate that may find its way to human body or a hypothetical fraction of population that may receive an exposure above the average population in a normal distribution. Even though the exposure levels were mostly arbitrary, comparing the data of Egypt and the USA to examine the effect of RR adoption on glyphosate use and human exposure still holds. In this regard, just by looking at the amount of glyphosate used as per capita (Table 1), one can easily find that this amount in the USA is 40.8 times that of Egypt's amount and 3.4 times that of the global amount. In short, the comparison implies that: (1) the overuse of glyphosate, especially in the USA, is concomitant with heavily growing RR crops; (2) it is legitimately accepted to raise concerns over glyphosate overuse; (3) reassessment of the actual risk of glyphosate in areas heavily growing RR crops is highly justifiable and irresponsibly overdue; (4) countries not growing RR crops and do not experience the spread of resistant weed biotypes, like Egypt, may still use glyphosate with some 'severe' label restrictions as previously suggested by Ibrahim (2015). Comparing the arbitrary exposure levels in Table 1 with the AADI value (2.50 ng a.i./kg bw/day) shows that the US person in the highest sector of glyphosate exposure receives daily concentration 6.2 times higher than the AADI value (15.39/2.50).

This indicates that this sector of the population is at actual glyphosate risk, and may explain the recently documented correlation between the application of GBHs in the USA and the spread of several human diseases. In their study, Swanson et al. (2014) found positive and highly significant correlation between annual glyphosate use and the spread of hypertension, stroke, diabetes prevalence, diabetes incidence, obesity, lipoprotein metabolism disorder, Alzheimer's, senile dementia, Parkinson's, multiple sclerosis, autism, inflammatory bowel disease, intestinal infections, end stage renal disease, acute kidney failure, cancers of the thyroid, the liver, the bladder, the pancreas, the kidney and myeloid leukemia. On the extreme end of the comparison, the exposure of the Egyptian person in the highest glyphosate exposure sector is only 0.148 times that of the AADI value (0.37/2.50). Contrary to the US, the Egyptian

person in this sector is 6.8 times further down the acceptable daily threshold or AADI value. The average person in the highest exposure sector in the world is exposed to almost twice ($4.50/2.50 = 1.8X$) as much as the reference AADI dose. The world exposure is lower than that of the USA due to the fact that some countries in the world are still growing traditional crops, that is, not genetically-engineered for glyphosate-resistance. The total amount of glyphosate used is not expected to be evenly distributed among: days of the year; cropland areas; or population. To the contrary, people exposed to glyphosate either occupationally (farm-workers, that is, applicators and pickers), or by virtue of their rural residence in areas heavily cultivated with RR crops, are expected to incur relatively higher exposure levels than the average arbitrary values calculated in Table 1. Besides, the risk to pregnant woman, infants, children, and elderly people may be actually higher.

Conclusion

This manuscript indicates conclusively the erroneous and misleading model of assessing the risk of pesticides based on the hazard of their active principles or active ingredients and on the preassumption of a monotonic dose-response relationship for such hazard. This error erodes our confidence in regulatory-authorized safety measures, especially as we know almost nothing about the identity, toxicity, and joint action of co-formulants within themselves and/or with the active principles. Glyphosate is used in this manuscript as an exemplary model to prove the wrong regulatory policy of using the ADI calculated for the active ingredient to evaluate the risk and establish the precautionary principles for the end-use formulations which contain many structurally-unknown and toxicologically-untested adjuvants along with the active ingredient. Many studies indicate conclusively that GBHs are more toxic than the active ingredient (glyphosate) alone. Therefore, the NOAEL, cRfD and ADI, originally assessed for glyphosate alone are overestimated and cannot be applied to interpret the risk of exposure to glyphosate-containing formulations or GBHs under real-life situations. When ADI is erroneously overestimated for any pesticide the risk of exposure to

this pesticide will be enormously underrated; thus drives pesticide-related activities or agricultural practices to areas of false or deceiving safety. It is worth-reemphasizing here that there is no toxicological basis whatsoever to accept the practice of applying NOAEL, cRfD or ADI measured for the active ingredient of any pesticide alone to assess the risk of exposure to its end-use formulations and mixtures. Besides, registration eligibility decisions for mixtures should never be blindly relied on the risk of individual pesticides. Last, but certainly far from least, Pesticide Regulatory Authorities should revisit all their promulgated safety measures and challenge their validity and reliability.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations: **a.i**, Active Ingredient; **ADI**, Acceptable Daily Intake; **AADI**, Adjusted Acceptable Daily Intake; **Bw**, Body Weight; **cRfD**, Chronic Reference Dose; **FQPA**, Food Quality Protection Act; **EPA**, Environmental Protection Agency; **GBHs**, Glyphosate-Based Herbicides; **GEGR**, Genetically-Engineered-Glyphosate-Resistant [crop]; **IARC**, International Agency for Research on Cancer; **Kg**, Kilogram; **LOAEL**, Lowest Observed Adverse Effect Level; **NOAEL**, No Observed Adverse Effect Level; **RR**, Roundup Ready; **WHO**, World Health Organization.

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Full Length Research Paper

Fast food premium toys as a significant source of lead and chromium to the environment

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Electronic fast food premiums were investigated for their ability to leach toxic metals into the environment. Low levels of the metals barium, cadmium, chromium, and mercury were detected. Significant quantities of lead were found, up to 177 mg/L, over thirty five times the permissible levels.

Key words: Waste, management, toxic, metal, leachate, lead, toys.

INTRODUCTION

The Federal Trade Commission (FTC) describes a premium as any non-food item distributed in connection with a company's food products. The history dates back to 1977 when Dick Brams invented the 'Happy Meal'. Developed over two years and debuting in 1979 the first Happy Meal was a circus-wagon-themed box with the standard hamburger or cheeseburger option, as well as French fries, cookies, a soft drink and a toy (Webley, 2010). The first toys were simple, a stencil, eraser, bracelet, puzzle, or spinning top. In 1987 the first licensed Disney toy appeared, since that time hundreds of licensing agreements have spawned a seemingly endless array of collectible toys. Other Fast Food Premiums (FFP) include coupons, game cards, beverage containers and special offers. FFP are now in use by most fast food restaurants around the world. Examples of other fast food franchises in the US include Burger King, Carl's Junior, Wendy's, Subway, and Hardee's. The suitability of using FFP to market food to children has long been an issue (Otten, 2014).

According to Time Magazine 1.2 billion Happy Meals

were sold worldwide in 2012, that equates to slightly more than 3.2 million Happy Meals sold per day. In the United States, there are about 220 million Happy Meals sold each year, which is about 602,000 Happy Meals per day. Data concerning FFP in general is considered a trade secret and for that reason is difficult to monitor and use for any quantitative analysis. In 2011, Happy Meal and other child meal sales were down by 6%, from 1.3 billion to 1.2 billion orders (Tuttle, 2012).

FFP toys are around three inches in height or length and may be static, dynamic (push action), wind-up, or electronic (Figure 1). Released in batches of around 6 to 8 variants per batch, between just one or all FFP toys may have some variety of electronic components or metal spring. Electronic components may be lights, sound, or both; and may or may not include a Printed Wire Board (PWB). FFP toys featuring lights or sound may use a rudimentary spring and lever system to activate them.

A PWB generally consists of an epoxy glass board used as a surface to attach components and

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Figure 1. Examples of fast food premium toys.

interconnections to form a functioning electronic circuit. Tin/lead solder (63% tin and 67% lead) is the prevalent solder alloy used in PWBs (Nordic Council of Ministers, 1995). Lead is a well-known environmental contaminant with recommended environmental levels established by various governmental bodies throughout the world. These boards are small, typically between 1 and 2 cm², and have attached cell batteries, running either a wired speaker, LED, or sometimes both. Previous work in this area has shown much larger PBWs with between 0.0 and 72.5 mg/L leached from them under the EPA Toxicity Characteristic Leaching Procedure (TCLP). Subsequently, PWBs regularly exceed the lead toxicity characteristic (TC) limit of 5 mg/L in TCLP leachates. Alternative forms of solder are accessible containing mixtures of tin/copper and tin/silver/copper (Townsend et al., 2008).

The earliest consideration of leaching of harmful pollutants from children's toys (Milana et al., 1993) found 'limited danger', the toys were of the period (1990s) and none of the toys investigated were electronic. Conversely, the only widely reported incidence of toxic metals found in FFP toys was the 2010 case of cadmium found in 13.4 million painted Shrek glasses sold by McDonalds (Neuman, 2010). These levels were deemed high enough to illicit concern, but the exact amounts went unreported in the press. Lead and cadmium have long been used as stabilizers in plastic toys (Kumar and Pastore, 2007). More recently toxic metals have been found in a variety of children's toys and jewelry bought on the North American (Guney and Zagury, 2013) and Turkish market (Charehsaz et al., 2014). Preliminary household and commercial items identified an electronic (TCLP) work done by this laboratory on a variety of FFP toy as a significant source of lead (10,000 times the earlier reports), along with trace amounts of other metals,

amount of lead leached from the toys featured in the most notably chromium. Further investigation of FFP toys manufactured during the years 1997 to the present was undertaken in order to ascertain their potential for leaching toxic metals, those results are presented here. Whilst the concentrations measured in this study should not be considered indicative of what will be observed in landfill leachate, the results do provide an insight into potential problems with these toys being discarded into the household waste stream. It is hoped that this data can also be helpful for performing life-cycle assessment (LCA) of FFP.

Leaching procedures

In the United States, the Resource Conservation and Recovery Act (RCRA) of 1976 led to the establishment of federal standards for the disposal of solid waste and hazardous waste. A waste is considered hazardous when it exhibits one or more of the following characteristics: Ignitable, corrosive, reactive, toxic, or listed in the code of federal regulations. RCRA requires that industrial wastes and other wastes must be characterized for toxicity following testing protocols published by the Environmental Protection Agency (EPA). Tostar et al. (2013) describes several variations of leaching tests for inorganic solids, including dilute nitric acid, dilute sodium hydrogen tartrate, and dilute citric acid. Since all of the food premiums were purchased in the United States. We chose to follow the U.S. EPA Test Methods for Evaluating Solid Waste (SW - 846) (U.S. Environmental Protection Agency, 1996). TCLP (method 1311) is designed to simulate material sitting in a landfill for a number of years (with an assumption of the acidic conditions found in most landfills), and then "determine the mobility of both

Table 1. Maximum concentration of D-List contaminants for toxicity characteristic identification.

EPA Haz. waste code (metals)	Contaminant	Regulated level (ppm)
D004	Arsenic	5.0
D005	Barium	100.0
D006	Cadmium	1.0
D007	Chromium	5.0
D008	Lead	5.0
D009	Mercury	0.2
D010	Selenium	1.0
D011	Silver	5.0

Table 2. List of TCLP ICP-OES standards.

Standard No.	Name	Composition	Elements Present	Concentration mg/L)
1	Zero DI	DI water	Ag, As, Cr, Pb/Ba/Cd, Se	0
2	Low Hg	18 mL H ₂ O + 2 mL TCLP Std#2	Hg	10
3	Med Hg	10 mL H ₂ O + 10 mL TCLP Std#2	Hg	50
4	High Hg	20 mL TCLP Std#2	Hg	100
5	Low TCLP Std #1	18 mL H ₂ O + 2 mL TCLP Std#1	Ag, As, Cr, Pb/Ba/Cd, Se	2.50/50/0.5
6	Med TCLP Std #1	10 mL H ₂ O + 10 mL TCLP Std#1	Ag, As, Cr, Pb/Ba/Cd, Se	12.5/250/2.5
7	High TCLP Std #1 (straight)	20 mL TCLP Std#1	Ag, As, Cr, Pb/Ba/Cd, Se	25/500/5
8	High Pb	20 mL of QC Std# 21	Pb	100

organic and inorganic analytes present in liquid, solid, and multiphase wastes" from the leachate that the material would produce. TCLP is the only leaching procedure specified by regulation for characterizing the hazardous waste toxicity characteristic (40 CFR 261.24: waste codes D004 - D043) under RCRA regulations. Method 1312, the synthetic precipitation leaching procedure (SPLP), is designed to simulate material left *in situ* (in or on top of the ground surface) exposed to rainfall (with an assumption that the rainfall is slightly acidic) and then "determine the mobility of both organic and inorganic analytes present in liquids, soils, and wastes" from the leachate the material would produce. SPLP is used to determine the leaching potential of soils, waste, and wastewater caused primarily by rainfall (precipitation).

Table 1 lists the regulatory thresholds one would utilize to compare against TCLP analytical results. If a solid waste fails the test for one or more of these compounds, the waste is considered to be a characteristic hazardous waste.

METHODS

EPA SW-846 Method 1311 'Toxicity Characteristic Leaching Procedure' was the chosen leaching method. Since the procedure calls for the analysis of low-level metal concentration, the utmost

caution was taken in order to avoid contamination. All plastic containers and funnels were acid-washed using 5 M trace metal grade Nitric Acid (Fisher Scientific), and triple rinsed with Type II deionized water.

The FFP toys were disassembled and all metallic and electronic components were separated from their plastic shells. These consisted of a mixture of screws, PWB, wires, speakers, LED, and cell batteries. All parts to be analyzed from each premium were weighed in order to assess the amount of TCLP extraction fluid to be added. TCLP solution was made in the appropriate quantity for each batch of 5-6 FFP toys plus TCLP blank sample by adding 5.7 mL laboratory grade glacial acetic acid (Fisher Scientific) to 1 L of deionized water obtaining a solution that was 2.88 ± 0.05 pH.

Sample containers (Nalgene bottles 250 mL, 8 oz) were then filled with the TCLP solution using a ratio of 20:1 volume of TCLP solution to toy FFP component mass. Each batch of samples was then placed on a rotator for a period of eighteen hours. At the end of the eighteen-hour rotation period the samples were removed from the rotator, filtered through a plastic funnel with No. 40 Whatman filter paper into a clean container and placed in the refrigerator. Samples were analyzed within 12 h of processing in order to avoid loss of analyte due to precipitation.

TCLP extracts from each toy FFP sample were measured using a Thermo iCAP 6300 ICP - OES equipped with autosampler and iTEVA software. Standards (SPEX CertiPrep) used for the analysis were Assurance TCLP Standard 1 (Ag, As, Ba, Cd, Cr, Pb, Se) and Assurance TCLP Standard 2 (Hg), and Assurance QC Standard 21. (Pb). Standard dilutions can be shown in Table 2. Standard calibration curves for each element were plotted with this method. Analyte concentrations were calculated by subtracting the TCLP blank concentration from the reported ICP-OES values. The Limit of Detection (LoD) was 0.002 mg/L for Ba, 0.04 mg/L for Cd, 0.02

Table 3. The analysis of FFP toys for metals.

Fast Food Premium (FFP) Toy	Barium (mg/L)	Cadmium (mg/L)	Chromium (mg/L)	Lead (mg/L)
Anastasia 'Bartok', McD 1997	0.03 ± 0.00	ND	0.15 ± 0.00	1.9 ± 0.00
Sega Game, McD 2003	0.01 ± 0.00	ND	0.33 ± 0.01	22.50 ± 0.04
Tony Stewart Racing Game, McD 2004	0.01 ± 0.00	ND	0.22 ± 0.00	10.3 ± 0.01
ESPN Football Game, McD 2004	0.04 ± 0.00	ND	0.24 ± 0.01	11.37 ± 0.02
NFL Football Game, BK 2005	0.05 ± 0.00	ND	0.70 ± 0.00	81.92 ± 0.14
Happy Feet 'Penguin', BK 2006 †	ND	ND	1.56 ± 0.01	ND
The Bee Movie 'Barney', McD 2007	0.01 ± 0.00	ND	0.37 ± 0.00	51.4 ± 0.08
The Simpsons 'Barney', BK 2007	0.28 ± 0.00	ND	0.27 ± 0.00	5.10 ± 0.01
American Idol 'Country Clay', McD 2008	0.28 ± 0.01	ND	0.23 ± 0.00	63.08 ± 0.38
Madagascar 'Lulu', McD 2008	0.08 ± 0.00	ND	0.40 ± 0.01	6.9 ± 0.10
Madagascar 'Alex', McD 2008	0.16 ± 0.00	ND	0.51 ± 0.01	9.17 ± 0.04
Madagascar 'Gloria', McD 2008	0.15 ± 0.01	ND	0.26 ± 0.02	14.06 ± 0.05
Madagascar 'Julian', McD 2008	0.17 ± 0.00	ND	0.57 ± 0.00	176.80 ± 0.30
Pacman 'Ghost Chomping Pac', BK 2013	0.64 ± 0.00	0.10 ± 0.00	0.25 ± 0.00	54.57 ± 0.08
Star Trek 'Klingon Battle Cruiser', BK 2009	0.10 ± 0.00	ND	0.30 ± 0.01	25.6 ± 0.11
Star Trek 'Communicator', BK 2009	ND	ND	0.46 ± 0.00	3.13 ± 0.03
Star Trek 'Tricorder', BK 2009	0.73 ± 0.00	0.90 ± 0.02	ND	62.72 ± 0.39
AATCTS ' 2009	3.86 ± 0.00	ND	0.68 ± 0.00	66.65 ± 0.12
AATCTS ' 2009	0.51 ± 0.00	ND	0.67 ± 0.01	7.00 ± 0.02
Avatar 'Neytiri', McD 2009	0.02 ± 0.00	ND	1.76 ± 0.05	53.24 ± 0.24
Avatar 'Dire Horse', McD 2009	ND	ND	0.93 ± 0.00	132.5 ± 0.90
Avatar 'Turok', McD 2009	0.09 ± 0.00	ND	0.45 ± 0.01	1.79 ± 0.01
G.I.Joe 'Walkie Talkie', McD 2009	ND	ND	0.49 ± 0.00	3.78 ± 0.00
Superhero Squad 'Ironman', BK 2009 †	ND	ND	1.97 ± 0.01	0.03 ± 0.03
Fantastic Four 'Thing', McD 2010	3.32 ± 0.00	ND	0.66 ± 0.00	86.43 ± 0.13
HTTYD 'Monstrous Nightmare', McD 2010†	ND	ND	2.0 ± 0.03	ND
HTTYD 'Gronckle', McD 2010	0.01 ± 0.00	ND	3.28 ± 0.00	ND
Shrek Forever After 'Donkey', McD 2010	0.38 ± 0.00	ND	0.49 ± 0.00	59.29 ± 0.23
Rio 'Rafael', McD 2011	0.08 ± 0.00	ND	0.92 ± 0.01	ND
Rio 'Blu', McD 2011	1.08 ± 0.01	ND	0.37 ± 0.00	28.29 ± 0.08
Minions, McD 2013 †	0.21 ± 0.00	ND	0.75 ± 0.01	11.87 ± 0.02
Amazing Spiderman 2 'Electro', McD 2014 †	0.01 ± 0.00	ND	1.20 ± 0.00	ND
The Peanuts Movie 'Lucy', McD 2015 †	0.58 ± 0.01	ND	0.90 ± 0.00	ND
Non-electronic wind-up toys				
Mattel 'Drex', BK 2010 †	ND	ND	1.27 ± 0.02	ND
HTTYD 'Toothless', McD 2010 †	0.01 ± 0.00	ND	2.77 ± 0.02	ND

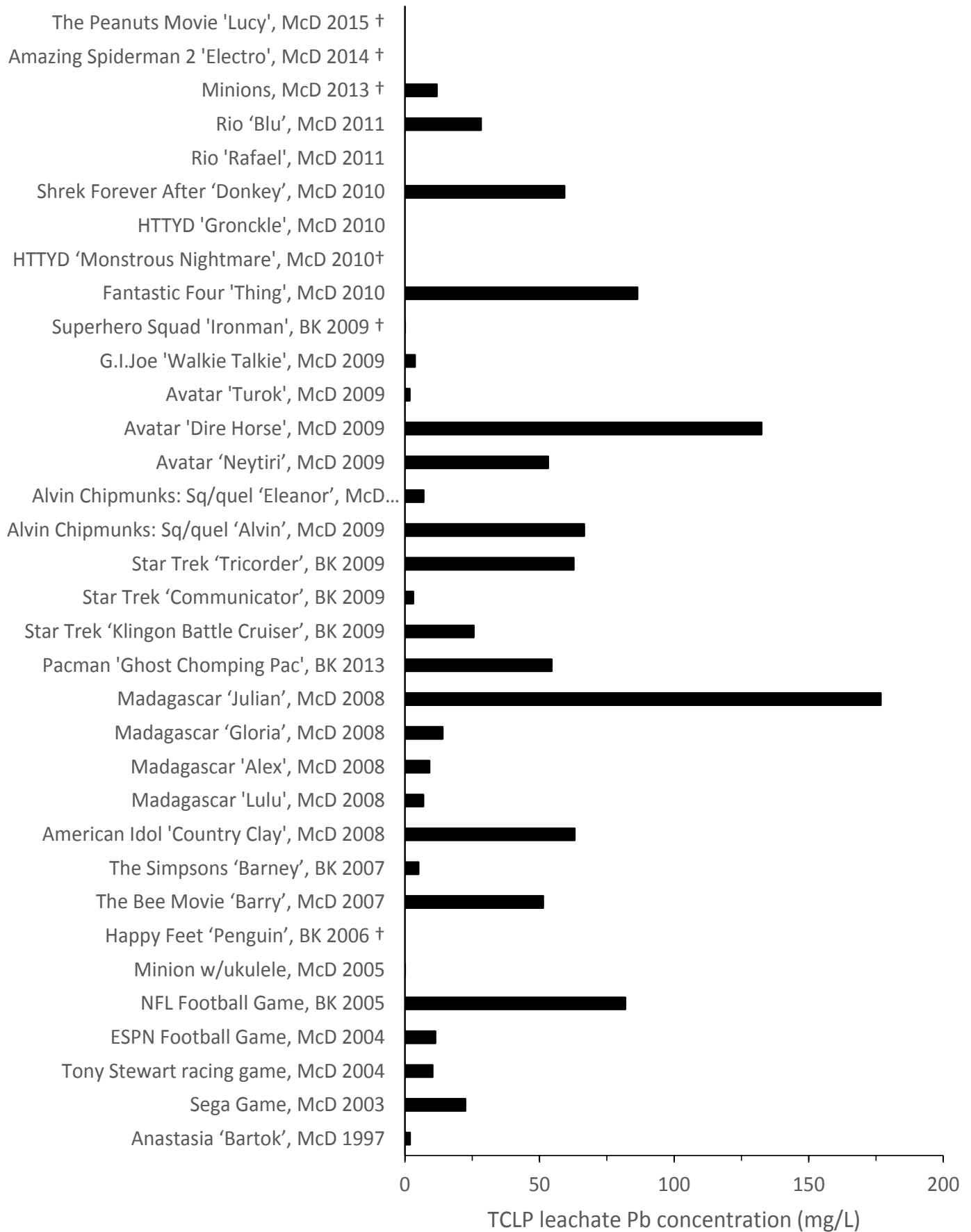
No amounts of silver, arsenic, and selenium were detected. Samples containing measured Hg are not shown as there were issues with contamination of the TCLP solution. BK - Burger King, McD - McDonalds, AATCTS - Avin and the Chipmunks: The Squeakquel, HTTYD - How to Train Your Dragon.

mg/L for Cr, 0.56 mg/L for Hg, and 0.32 mg/L for Pb. The Limit of the Blank (LoB) was 0.001 mg/L for Ba, 0.004 mg/L for Cd, 0.01 mg/L for Cr, 0.53 mg/L for Hg, and 0.3 mg/L for Pb. The response was linear up to 100 mg/L (R^2 analyzed by ICP - OES directly after each treatment was finished). Dilutions were made where necessary. Standard correlation R^2 values of > 0.9 were deemed acceptable. Results were classed as not detected (ND) below the instruments limit of detection.

RESULTS AND DISCUSSION

The results of the TCLP tests of 35 FFP toys are shown in Table 3 and Figure 2. There were 33 FFP toys containing electronics with 6 FFP toys that contained

electronics with no PWB, their LED lights being operated via springs and levers. No silver, arsenic, or selenium was detected in any of the FFP. Small amounts of mercury were found in some of the samples however since it was below the LoD those results are not reported. The TCLP results found that the two most commonly detected metals were lead and chromium, with trace amounts of barium, and cadmium. Lead was found to leach from PWB at concentrations greater than the RCRA TC limit (5.0 mg/L) for 22 out of the 27 tested FFP toys that contained PWB. The Pb concentration ranged from 0 to 176.8 mg/L with an average of 31.9 mg/L. The largest Pb concentrations were found in the extraction fluid from



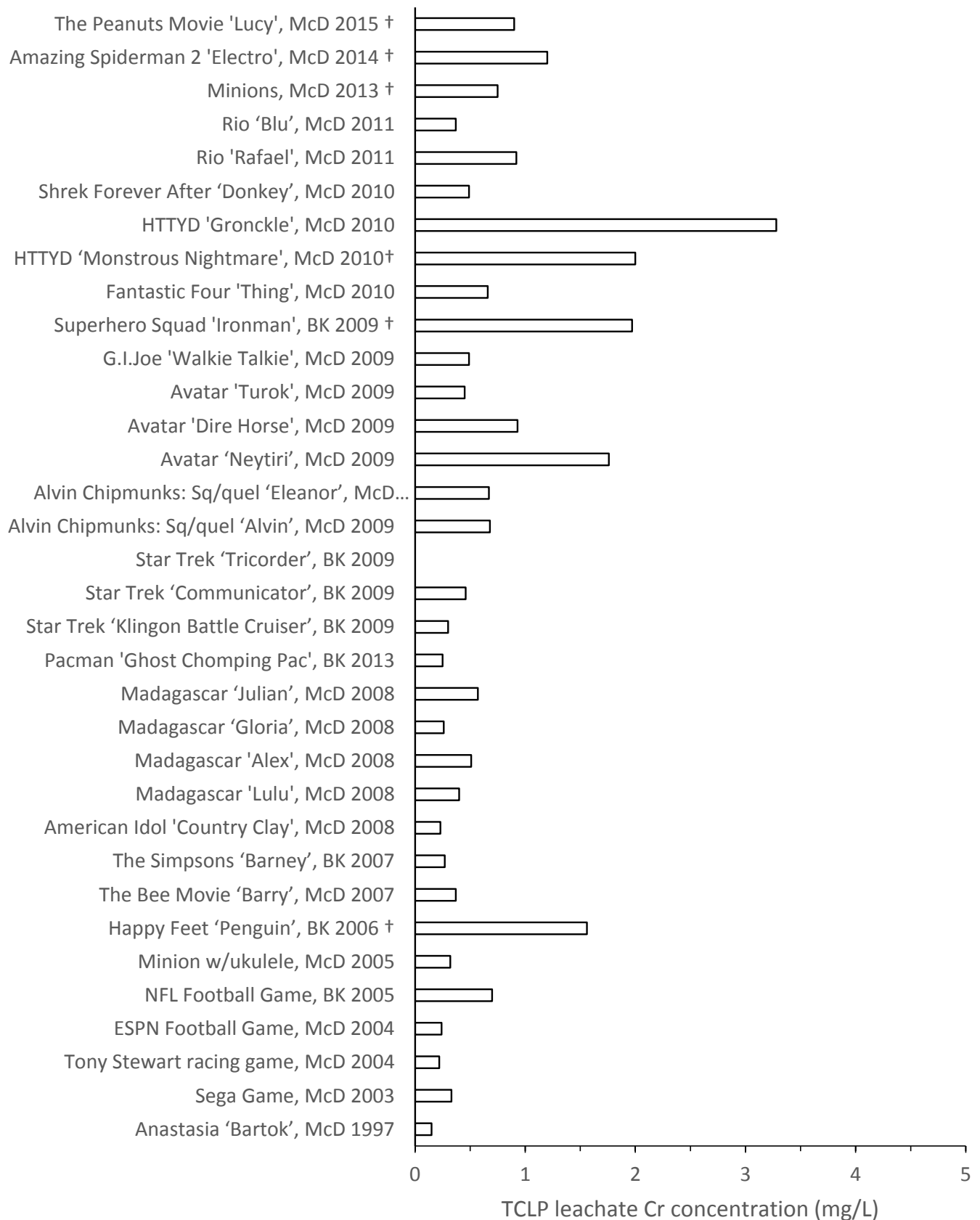


Figure 2. D-List contaminant metals investigated in this study. †: No circuit board.

Table 4. Standard reduction potentials.

Metal	E° (V)
$\text{Ba}^{2+} + 2\text{e}^- \rightleftharpoons \text{Ba}$	-2.912
$\text{Cr}^{2+} + 2\text{e}^- \rightleftharpoons \text{Cr}$	-0.913
$\text{Cr}^{3+} + 3\text{e}^- \rightleftharpoons \text{Cr}$	-0.714
$\text{As} + 3\text{H}^+ + 3\text{e}^- \rightleftharpoons \text{AsH}_3$	-0.608
$\text{Cd}^{2+} + 2\text{e}^- \rightleftharpoons \text{Cd}$	-0.4030
$\text{Pb}^{2+} + 2\text{e}^- \rightleftharpoons \text{Pb}$	-0.1262
$\text{Se} + 2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2\text{Se}$	-0.082
$\text{Hg}_2^{2+} + 2\text{e}^- \rightleftharpoons 2\text{Hg}$	+0.7973
$\text{Ag}^+ + \text{e}^- \rightleftharpoons \text{Ag}$	+0.7996
$\text{Hg}^{2+} + 2\text{e}^- \rightleftharpoons \text{Hg}$	+0.851

electronic toys containing PWB, whilst wind-up toys showed typically similar amounts of Cr but no Pb. The Cr concentration ranged from 0 to 3.7 mg/L with an well below the TC limit (5.0 mg/L). The remaining TC metals defined by RCRA silver, arsenic, mercury, and selenium were not detected in any of the leaching tests. This is likely a consequence of the limit of detection of our instrument and redox chemistry.

As shown in Table 3, no silver was detected in any of the samples. It has been demonstrated elsewhere (Townsend et al., 2008) that the presence of other metals in a sample can influence the relative leachability of a given metal, specifically those with high reduction potentials (Table 4). Although we did not quantify the levels of Fe in the leachate, we suspect that the inclusion of any screws, springs, or assorted metal parts in the leachate study may have prevented any significant amount of silver from being oxidized and detected in the leachate solution. Parts containing iron or having a chrome plating were found in many of the FFP toy samples. It was observed that after conducting the leaching procedure many of the metal components were noticeably oxidized as evidenced by a blackened or discolored surface.

Conclusions

We have shown that FFP toys are a previously unreported and significant source of TC metals lead and chromium to the environment and should be treated as E-Waste, to be disposed of in the appropriate way along with similar electric and electronic devices. In order to attract customers, the complexity of FFP has increased to a point where it is now common for them to include a PWB, LED's or even a rudimentary LCD display. Our results show 10,000 times the amount of lead than reported in the original 'Little Toys' work of Milana et al. (1993), a change resulting of the progression to simple toys to more sophisticated electronic devices. All TCLP lead results for the toys containing PWBs were higher

than the permissible EPA level of 5.0 mg/L, up to 35 times the permissible level. Barium, cadmium, chromium, and mercury were also detected at ppb levels. Chromium results were all less than the permissible 5.0 mg/L, but still as high as 3.3 mg/L.

The US EPA's design for the environmental program has worked with stakeholders to investigate and promote lead-free solders. The European Union Directive 2001/95/EC, Restriction of Hazardous Substances (RoHS) restricts the use of hazardous materials found in electrical and electronic products including the metals cadmium, chromium, lead, and mercury from 2006 onwards. Given that many of these FFP toys were manufactured after the RoHS initiative, the solder used in the PWB was expected to be lead free. The results may indicate that this was not the case. 78% (21 of 27) of the electronic FFP toys with PWB showed levels of lead in the leachate samples higher than the regulated levels. In comparison, only one of the six FFP toys without a PWB showed a level of lead higher than the regulated levels. Since lead was not detected in most of the samples without a PWB but was detected in samples containing a PWB, it is expected that the source of the lead is the solder used in the interconnects between components. This solder does not meet RoHS requirements. The source of the chromium is likely from the chrome plating of screws, springs, and wires. As demonstrated by the results of FFP toys that lack a PWB, but instead utilize levers or springs, these have a much lower average concentration of lead but higher average concentration of chromium.

While a majority of the FFP toy samples contained a battery, none of those samples showed elevated levels of mercury. This result is expected for two reasons. Firstly, both Europe and the United States effectively outlawed the used of mercury button cells between 1991 and 1992 it is expected that the battery chemistry would be free of mercury. Secondly, since the battery cells in the FFP toy samples were not mechanically broken open, it is likely that they did not contribute significantly to the leaching results. While the identity of the batteries were not confirmed, it is assumed that they were zinc-air, alkaline, or silver cells with the former being the least likely due to its discharge characteristics. However, over time the batteries contained in FFP toys will corrode to a point where the container will fail and the cathode and anode materials as well as the electrolyte will begin to leach into the environment. Future studies will include a separate analysis of the battery types and leaching properties.

Early and ongoing work on consecutive TCLP leaching tests of FFP toys has shown that there is potential for further leaching at levels exceeding the first leach. Since no account of the total metal content of FFP toys exists it is difficult to assess their potential for contamination. Our calculations suggest that the mass of lead leached from fast food premium toys in a standard 18 h TCLP test ranged between 0 and 55 mg. It is therefore important to

report that these FFP toys continue to contain levels of TC metals that exceed the permissible levels and present an ongoing threat to our environment.

Conflicts of Interests

The authors have not declared any conflict of interests.

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