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Developmental neurotoxic effects of Malathion on 3D neurosphere system

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ABSTRACT

Developmental neurotoxicity (DNT) refers to the toxic effects induced by various chemicals on brain during the early childhood period. As human brains are vulnerable during this period, various chemicals would have significant effects on brains during early childhood. Some toxicants have been confirmed to induce developmental toxic effects on CNS; however, most of agents cannot be identified with certainty. This is because available animal models do not cover the whole spectrum of CNS developmental periods. A novel alternative method that can overcome most of the limitations of the conventional techniques is the use of 3D neurosphere system. This in-vitro system can recapitulate many of the changes during the period of brain development making it an ideal model for predicting developmental neurotoxic effects. In the present study we verified the possible DNT of Malathion, which is one of organophosphate pesticides with suggested possible neurotoxic effects on nursing children. Three doses of Malathion (0.25 μM, 1 μM and 10 μM) were used in cultured neurospheres for a period of 14 days. Malathion was found to affect proliferation, differentiation and viability of neurospheres, these effects were positively correlated to doses and time progress. This study confirms the DNT effects of Malathion on 3D neurosphere model. Further epidemiological studies will be needed to link these results to human exposure and effects data.

1. Introduction

Pesticides are important chemicals used widely in agriculture, and their use has improved the yield of plants for food leading to reduced food costs. However, the problem with the wide use of pesticides is that they generally persist in the environment. This in turn causes severe environmental pollution and potential acute and chronic cases of human poisoning (Dallegrave et al., 2007; Yen et al., 2000). Organophosphorus pesticides (OPs) are currently the most commonly utilized pesticides worldwide. They combine almost 40 different chemical members registered by the US-EPA (EPA, 2002). About 70% of all insecticides used in USA are OPs, constituting about 73 million pounds in 2001 (Kiely, 2004). Besides, OP toxicity is considered a massive concern in developed as well as low-income countries (Rohlman et al., 2011). The cholinesterase enzymes inhibiting effects of OPs represent the main mechanism underlying their acute toxicity. However, other possible toxic effects have been linked to chronic exposure to OPs (e.g. delayed neurotoxicity, developmental neurotoxicity and other organs' toxicity) (Abou-Donia, 2003). The OP Malathion (O, O-dimethyl dithiophosphate of diethyl mercaptosuccinate) is one of the OPs with variable toxic effects (RED, 2006). Malathion has several applications worldwide to control major arthropods in public health programs e.g. for animal ectoparasites, human head and body lice, also it is used as a household insecticide and to protect grain in storage (Maroni et al., 2000). Malathion bioactive metabolite, malaoxon, is known to induce excitotoxicity (Hazarika et al., 2003). It is noteworthy that, many researches proved neurotoxic effects of malathion both in humans and experimental animals (Vidair, 2004; Abdel-Rahman et al., 2004). One of the possible risks of Malathion is its effect during early childhood. Recent studies showed that exposure of postnatal pups to Malathion via lactation inhibits the activity of brain, plasma and erythrocyte cholinesterase in the pups (Selmi et al., 2012). Additionally, similarities exist between Malathion and lead (pb) (which is a known neurotoxicant that affects early brain development) regarding their increasing BBB permeability by similar mechanisms (Balbuena et al., 2011). Likewise, recent findings suggest that Malathion exposure during lactation induced biochemical cerebral alterations and oxidative

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stress in rat pups. Further, clinical manifestations (e.g. decrease in motor coordination, vestibular function and muscular strength/coordination) in rat pups (Acker et al., 2011) and behavioral and morphological deformities in fresh water fish (Patil and David, 2010) have been observed as a result of Malathion exposure in postnatal animals and by analogy human neonates. These may be correlated to the lower levels of the enzymes involved in organophosphate deactivation in this critical developmental age. As described previously, OP (including Malathion) exert their toxic effects by inhibiting acetylcholinesterase enzyme (AChE) through phosphorylation of the serine residue at its active site. The main function of AChE is to hydrolyze the neurotransmitter acetylcholine (ACh) to choline and acetate during neurotransmission at cholinergic synapses (Wang et al., 2009). The developing brain is extremely vulnerable to neuroactive chemicals that elicit or block neurotransmitter responses during periods of rapid brain growth. Hence, AChE, through affecting ACh, will affect brain development (Yanai, 1984). Since the tragedy of Minamata-Japan, the term of developmental neurotoxicity (DNT) has been coined to verify chemical-induced toxic effects of substances on growing brains of children (Lein et al., 2007). This has been recognized as a serious threat to human health (Needleman et al., 2002). Current DNT testing guidelines (Organization for Economic Cooperation and Development, 2007) propose investigations in rodents, mainly rats. Such testing strategy, however, implies the use of 140 dams and 1000 pups, which is extremely time- and cost-intensive (Lein et al., 2005). Relying mainly on the existing in-vivo testing strategies for DNT of the many toxic substances would demand huge and unacceptable costs in terms of animals and person-years (Lein et al., 2007). Therefore, according to the "3R principle" (reduction, replacement, and refinement) of Russel and Burch (1959), alternative testing strategies are needed to create affordable, sensitive, and mechanism-based methods suitable for high- or medium-throughput screening (Collins et al., 2008).

The search for better alternatives for animal models advocated some potential in vitro models for toxicity testing that mimic most of the basal processes of brain development in the culture dish. The most promising models for DNT testing are based on three-dimensional animal or human cell culture systems: the embryonic stem cell test (EST) (Genschow, 2002), the whole embryo culture (WEC) and neural progenitor cells (NPCs), grown as neurospheres (Zhang et al., 2001). The three-dimensional neurosphere system mirrors the basic processes of brain development, namely proliferation, migration, differentiation and apoptosis. So far, however, research with neurospheres has largely focused on their application for neuroregeneration in disease states of the central nervous system (Iwanami et al., 2005). Nevertheless, a few studies have also utilized neurospheres for toxicity studies in vitro by analyzing a variety of endpoints such as viability, proliferation, migration, differentiation, neurite outgrowth and apoptosis (Meacham et al., 2005). These provide support for their use in hazard identification screens for chemicals that may cause developmental neurotoxicity. Since effect of Malathion on growing brain is one of the areas of concern in neurotoxicology, in the present study, we investigated the impact of Malathion on neurosphere endpoints (e.g. proliferation, differentiation and neurite outgrowth) trying to extrapolate the resulting data to developmental toxic effects leading to gross morphological brain changes. In addition, the role of two suggested mechanisms; oxidative stress and acetyl cholinesterase (AchE) inhibition, for this neurotoxic effect of Malathion on cultured neurospheres was investigated (Selmi et al., 2012).

2. Materials and methods

All chemicals were of molecular biology grade and were obtained from Sigma-Aldrich (USA) unless otherwise stated.

2.1. Cell culture

Rat neural progenitor cells were isolated from rat embryos (E14) extracted from placental tissue. The cortices were aseptically dissected out from the brains of the fetuses and the tissues were triturated by repeated passage through a fire-polished constricted Pasteur pipette. The dispersed tissues were allowed to settle for 3 min. The supernatant was, then, transferred to a fresh tube and centrifuged at 1000 g for 5 min. The pellet was placed in Hank's balanced salt solution cultured as free-floating neurospheres in proliferation medium [Dulbecco's modified Eagle medium and Hams F12, (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany)], 20 ng/ml epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), 20 ng/ml recombinant human fibroblast growth factor (rhFGF; R&D Systems, Wiesbaden-Nordenstadt, Germany), and penicillin and streptomycin (1:100 vol/vol; Invitrogen) at 37 °C with 7.5% CO as previously described (Balbuena et al., 2012). Differentiation was initiated by growth factor withdrawal in differentiation medium [Dulbecco's modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen)] and plating onto a poly-D-lysine/laminin matrix.

2.2. Chemical exposure

For proliferation analysis, neurospheres were treated for 2 weeks with Malathion (El Nasr for Chemical supplies, Egypt) (0.25, 1, or 10 μM) in proliferation medium. The doses were concluded following preliminary study using at least five concentrations in serial two-fold dilutions around the EC 50-Death (half-maximal effective concentration inducing neuronal cell death) for Malathion. This is to reach the optimum dose that can affect functions of neurospheres without affecting viability of the cells.

2.3. Proliferation analyses

For proliferation analysis, spheres were cultured in proliferation medium. After 0, 3, 7 and 14 days, sphere size was determined by software analyses (Cell Profiler, version 2.1; Broad Institute, freely downloaded from http://www.cellprofiler.org). Diameter of each neurosphere was measured and exported to excel file further to statistical analysis.

2.4. Differentiation analyses

For analysis of differentiation, following initiation of differentiation as described previously, images of plated neurospheres were evaluated regarding distinct neuronal morphology with fasciculation of neurites that radiate from the central aggregation of neuronal perikarya.

2.5. Reduced glutathione (GSH) determination

Total GSH was determined using glutathione reductase and NADPH coupled reaction with 5,5′-dithiobis (2-nitrobenzoic acid) as described previously (Valentovic et al., 2004); values were expressed as nmol/g tissue. Glutathione disulfide (GSSG) was measured following 2-vinylpyridine derivatization and expressed as nmol/g tissue. The results were expressed as percentage of control values.

2.6. Lipid peroxidation

Lipid peroxidation was measured as described previously (Valentovic et al., 2004). The amount of malondialdehyde (MDA) was calculated based on a standard curve (range 1–40 nmol) using MDA (Aldrich, St. Louis, MO) and expressed as μmol MDA/mg protein. The results were expressed as percentage of control values.

2.7. Superoxide dismutase (SOD)

SOD activity was measured using the BIOXYTECH SOD-525 kit (Oxis International), using the method described previously (Takahashi, 2000). The results were expressed as percentage of control values.

2.8. Cholinesterase enzyme assay

Cholinesterase activity was determined following the method of Ellman et al. (1961). The assay was carried out in the following steps: For each sample, two tubes containing 4 ml substrate solution [20 mN Tris buffer at pH 8.2 with 20 mM magnesium chloride ($MgCl_2$) and 100 mM NaCl, 0.001 M Dithio-2-nitrobenzoic acid (DTNB) as reagent], and 0.001 M acetylcholine iodide, as substrate, were pre-incubated at 37 °C in a water bath for 10 min. Into each tube, 20 μl of cell culture was added. The sample was vortexed and transferred into a cuvette for absorption rating. Absorption reading was taken at one-minute time intervals for 3 min using a spectrophotometer (Shimadzu UV-3000; Shimadzu Corporation; Kyoto, Japan) at 412 nm. A substrate free blank was run in parallel with each sample tested.

3. Statistical analyses

The experiments were made in triplicate. All data were given as the mean of the three samples ± standard deviation (SD). Two groups of data were analyzed by Student's t-test. Three groups of data were analyzed by analysis of variance (ANOVA) with a Tukey post hoc test. For all tests, $p < 0.05$ was deemed significant while $p < 0.001$ was considered highly significant.

4. Results

4.1. Proliferation analysis

As shown in Table 1; control group of neurospheres showed normal pattern of proliferation, where applying size analysis for the cell cultured revealed progressive increase from 176 ± 56.3 μm in day 0 till reaching 800.6 ± 117 μm. Malathion exposed cell cultures, revealed arrest in proliferation which was positively correlated to the increase in Malathion concentrations. Cells exposed to Malathion (0.25 μM) showed arrest of neurosphere diameter in days 7 and 14, so that their sizes were highly significantly lower when compared to control group. Proliferation of cells exposed to Malathion (1 μM) was significantly arrested as indicated by significant decrease in size as compared to controls on days 3 and 7, while on day 14 the neurospheres lost their shape. The same pattern of proliferation arrest was noticed for Malathion at 10 μM but not 0.25 μM.

4.2. Differentiation analysis

As shown in Fig. 1; following initiation of differentiation, neurospheres in the control group showed distinct neuronal morphology with fasciculation of neuritis. However, Malathion treated neurospheres failed to show this neurogenic differentiation.

4.3. Oxidative stress assays

As shown in Table 2; parameters measure oxidative stress revealed no difference compared to control. MDA, GSH and SOD were all comparable to the levels of control group in the Malathion exposed groups.

Table 1
Proliferation analysis of different groups.

Days	Control	Malathion 0.25 μM	Malathion 1 μM	Malathion 10 μM
0	176 + 56.3 μm	165 + 77.4 μm	175 + 64.2 μm	155 + 56.8 μm
3	302.4 + 42.8 μm	287 + 84 μm*	331.2 + 71 μm	214.2 + 27.8 μm
7	633 + 12.3 μm	413 + 21.3 μm**	408.1 + 24 μm**	309.8 + 4.8 μm**
14	800.6 + 117 μm	411.3 + 16 μm**	N/A	N/A

* Statistically significant ($p < 0.05$) compared to control.
** Highly significant ($p < 0.001$) compared to control.

4.4. AchE assay

As shown in Table 3; AchE activity declined in a highly significant pattern compared to control in all Malathion treated groups. This decline in esterase activity was dose dependent.

5. Discussion

Developmental neurotoxicity (DNT) comprises a broad category of neurological disorders that may be of unknown etiology. Examples include learning defects, psychological disorders (e.g. schizophrenia and autism) and morphological damage (e.g. decrease in brain size) (Hass, 2006). The potential contribution of environmental chemical exposure is a major concern; hence, it is of paramount importance to accurately predict the neurotoxic effects of these chemicals using more advanced high throughput systems alternatives to animal studies (Rice and Barone, 2006). In the present study, we investigated the DNT of Malathion with different concentrations on 3D neurosphere system. Neurospheres are thought to trace the predicted effects on different stages of brain development. This advantage of neurospheres in-vitro system over traditional animal models, improves our ability to verify the putative adverse effects of Malathion on children brain development in case of exposure in early life. The current study used low doses (0.25 μM, 1 μM and 10 μM) of Malathion to imitate the regular life exposure, especially through lactation. This is in contrast to most preceding studies where much higher doses were used (1–20 mM) (Balbuena et al., 2011, 2012; Galántai et al., 2011; Moore et al., 2010). Although previous studies showed neurotoxic effects of Malathion on animal models and cell cultures, results could not be extrapolated to normal exposure due to larger doses used in these studies (Selmi et al., 2012; Vidair, 2004; da Silva et al., 2006; Ostrea et al., 2012). Since most of exposure studies revealed very low concentrations of Malathion in the environment compared to acute exposure doses (Sankararamakrishnan et al., 2005; Agarwal et al., 2015), it is more appropriate to study lower dose than those used in previous researches. In the current study, a preliminary dose response study was performed to choose the doses that are capable of affecting different neurosphere functions with minimal impact on cell viability. This approach is more suitable for developmental studies, since the impact of toxic agents in such scenario is usually due to chronic exposure to very low doses. This is contradictory to using large doses that may lead to acute effects and usually affects cell viability. Moors et al. (2009) have shown that: a) proliferation, migration, differentiation, and apoptosis of human neurospheres can be quantified; b) in vivo effects of the developmental neurotoxicant could be imitated in vitro using Mercury as a prototype of developmental neurotoxicant; and c) the methods applied are suitable for medium-throughput screening. Thus, the three-dimensional neurosphere system offers a useful method for DNT hazard identification. However, neurophere utility as a screening system for developmental neurotoxicity is limited to basic processes of brain development including; brain growth and proliferation, migration, differentiation and apoptosis. This is due to the fact that they do not simulate complex higher brain structure development. Furthermore, neurosphere system cannot perform drug metabolism, compared to in vivo studies. This advocates the use of complimentary strategies e.g. incubation with S9 mixes or hepatocyte co-culture to include "maternal metabolism" in neurosphere system (Moors et al., 2009).

The effects of Malathion were tested regarding proliferation, differentiation capacity and viability of neurospheres. Furthermore, the mechanisms of such neurotoxic effects (e.g. AchE inhibiting and prooxidant effects) of Malathion were verified in cell culture. The effects of Malathion on proliferation were evident as noticed through the arrest of normal increase in neurosphere diameter with progress of time in a proliferation culture medium. Meanwhile, differentiation of neurospheres to neuritis was inhibited by addition of Malathion to the differentiation medium. Also, Malathion decreased the viability of

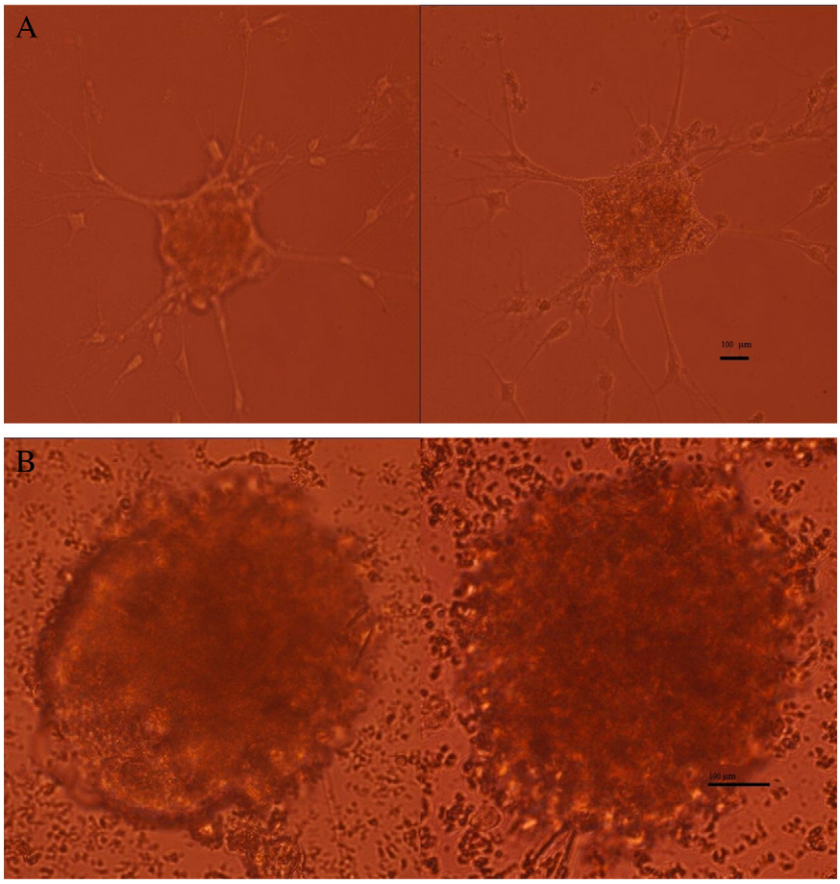

Fig. 1. Image of plated neurospheres after 1 week. Panel A (control × 40) shows distinct neuronal morphology with fasciculation of neurites that radiate from the central aggregation of neuronal perikarya. In contrast, panel B (Malathion 1 and 10 μM × 20) shows the absence of such neurological differentiation with neurospheres of the same appearance as in day 0. Scale bar: 100 μm.

neurospheres significantly in a dose and time related pattern. These effects suggest strong developmental neurotoxic effects of Malathion during the critical period of brain development (Moors et al., 2009; Schreiber et al., 2010). The multiple effects induced by Malathion on neurosphere system underscore the more global effects of Malathion compared to other tested neurotoxic agents evaluated in other studies (Moors et al., 2009; Schreiber et al., 2010), hence, its more powerful sequelae for children exposure. Two potential mechanisms have been suggested for these DNT effects of Malathion viz.; oxidative stress (Fortunato et al., 2006; Delgado et al., 2006) and inhibition of AchE enzyme (Selmi et al., 2012). The present study shows that assays of oxidative stress had no difference in comparison to control. This finding can be attributed to the fact that the current study used smaller doses compared to previous similar works by Delgado et al. (2006) and Moore et al. (2010) which used higher concentrations of Malathion (1–24 mM). The use of these smaller doses may be helpful to elucidate the toxic effects and mechanisms in real life exposure. Moreover, previous studies comparing between human neural progenitor cells (hNPCs) and the human neuroblastoma cell line SH-SY5Y showed that hNPCs were less sensitive towards oxidative stress (Moors et al., 2009), which is in concert with the knowledge that primary cells are less sensitive to various stressors than cancer cells (Hileman et al., 2004; DiMasi, 2001). This was confirmed shortly after by the work of Schreiber et al. (2010) who attributed the better defense mechanisms of neurospheres than do postmitotic neural cells to the high GSH content of associated astrocytes (Madhavan et al., 2006). These previous studies suggest that oxidative stress' role may be minor in Malathion induced DNT due to the less vulnerability of growing brains than in adults when used with regular doses. In contrast, AchE level was significantly decreased in Malathion treated groups. This finding supports the role of esterase lowering effects of Malathion as a potential mechanism of its developmental neurotoxic effects. The lower levels of AchE in growing brains would make them more vulnerable to any further decline in activity as in the case of Malathion exposure (Selmi et al., 2012; Acker et al., 2011; Vidair, 2004; da Silva et al., 2006). So, based on our findings, low dose exposure to Malathion causes its neurotoxic effects on growing brain neurons through inhibiting the protective AchE activity. AChE is a critical player in the regulation of several

Table 2
Oxidative stress assays.

	MDA	Reduced GSH	SOD
0.25 μM Malathion — 1 week	99 + 4%	92 + 3%	110 + 7%
0.25 μM Malathion — 2 weeks	130 + 3.1%	110 + 4.2%	90 + 2.7%
1 μM Malathion — 1 week	98 + 7.4%	90 + 4.8%	90 + 7.1%
1 μM Malathion — 2 weeks	140 + 9.2%	120 + 8.1%	110 + 7.7%
10 μM Malathion — 1 week	100 + 6.3%	85 + 3.9%	100 + 3.4%
10 μM Malathion — 2 weeks	120 + 8.6%	100 + 3.8%	100 + 4.2%

All measurements are percentage compared to control group of similar period.

Table 3
% of AchE in the three treated groups compared to control.

	AchE assay
Control	100%
0.25 μM Malathion	34 + 2.1%**
1 μM Malathion	28 + 3.4%**
10 μM Malathion	17 + 4.8%**

Statistically significant ($p < 0.05$) compared to control.
** Highly significant ($p < 0.001$) compared to control.

physiological cascades (Schetinger et al., 2000). The activity of the cholinergic system is important in maintaining normal behavior and muscular function (Payne et al., 1996). In several neurological disorders, activity of AChE has been altered, for instance, Acker et al. (2011), showed that exposure to different doses of Malathion inhibited AChE activity in brains of rats' pups. In their study high dose of Malathion (200 mg/kg) caused more AChE inhibition and more behavioral alterations than at the dose of 100 mg/kg, suggesting a link between AChE inhibition and behavioral impairment. Since cholinergic system has important functions in brain development, OP pesticides exposure during the developmental period can interfere with neurotransmitter function leading to neurodevelopmental abnormalities (Slotkin, 2004). Animals exposed to AChE inhibitors during the postnatal period of active synaptogenesis are vulnerable to develop several behavioral impairments, including motor development/coordination deficits (Dam et al., 2000). This is because, during brain development, acetylcholine and cholinergic projections play major roles in proliferation, migration, and synaptogenesis and in the development of normal neural cytoarchitecture (Hohmann, 2003).

6. Conclusions and clinical application

In humans, the developing nervous system is highly vulnerable to the deleterious effects of environmental toxins e.g. etiology of schizophrenia and autism are assumed to result from the deleterious effects of environmental factors during normal brain growth spurt (Rice and Barone, 2006). Consequently, to prevent harm, there is an increasing need for developmental neurotoxicity (DNT) to test chemical compounds in general use and determines which one is considered a developmental neurotoxin (Coecke et al., 2007; Goldman and Korduru, 2000; Hass, 2006; Needleman et al., 2002). There is an increasing concern about exposure of pregnant women, infants and children to OPs. Children in agricultural communities, like Egypt, carry the risk of continuous exposure to acetylcholinesterase inhibitors through hand to mouth behavior (Eskenazi et al., 1999). Children are considered to be more vulnerable due to the significant anatomical and maturational changes occurring in the brain during developmental periods (Andersen, 2003). Moreover, Acker et al. (2011) showed that repeated exposure to small doses of Malathion is capable of producing behavioral impairment in rat pups. This was proved by significant decrease in motor coordination, vestibular function and muscular strength/coordination, negative geotaxis and forelimb support tests, respectively. More interestingly, the degree of behavioral impairments was significantly correlated to the activity of AChE in brains of rat pups that was demonstrated.

The findings of the present study confirm the negative impact of Malathion on proliferation, differentiation and viability of NPCs, which reflects possible developmental disorders that can affect different endpoints for the growing brains. This is in accordance with Selmi et al. (2012), Vidair (2004), and da Silva et al. (2006). These studies, however, showed the neurotoxic effects of early postnatal exposure in rodent models. Although they reflected the abnormal neurobehavioral performance of children exposed to pesticides like Malathion in early life (Ostrea et al., 2012), they needed to be verified by more detailed study as in neurosphere system. To the best of our knowledge, we are the first team to confirm the DNT of Malathion on 3D neurosphere model. In addition the present work confirmed the critical role played by the AchE decreased activity in developing such effects. On the other hand there is no observed role for oxidative stress in Malathion induced DNT in a neurosphere system.

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