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Hormetic Neurobehavioral effects of low dose toxic chemical mixtures in real-life risk simulation (RLRS) in rats



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ABSTRACT

The current study aims to assess the long-term effects of very low dose exposures to a complex chemical mixture on motor performance and behavioural changes in rats. For twelve months (equivalent to thirty years in human terms), four groups of Sprague Dawley rats (five males and five females per group) were exposed to a thirteen chemical mixture (in drinking water) in doses of 0, 0.25, 1 and 5xADI/TDI (acceptable daily intake/tolerable daily intake) (mg/kg body weight/day). After twelve month exposure, the rats' motor performances were assessed by rotarod test, and their behavioural changes were assessed by open field exploratory test and elevated plus maze test. Exposure to the chemical mixture resulted in a statistically significant increase in the locomotor activity quantified by the number of crossings over external squares and in the spatial orientation activity quantified as the number of rearings in the lower dose group (0.25xADI/TDI) compared with the control group ($p < 0.05$). No significant changes were observed in the two higher dose groups (1xADI/TDI, 5xADI/TDI) compared with the control group. The administration of a very low doses of a cocktail of 13 chemicals led to a dose-dependent stimulation of the nervous system, rather than its inhibition.

1. Introduction

Since the dawn of the industrial age, and especially since the latter part of the 20th century, new technologies have been introduced into our environment, workplace, and personal life. While undoubtedly beneficial, these technologies have been accompanied by other anthropogenic effects on our environment and health (Kostoff, 2015).

To control the adverse impacts of these technologies, a number of regulatory agencies have been established. Through a combination of 1) tests on different species (including humans) and 2) epidemiological studies, these agencies have identified levels of exposures to potentially toxic substances that may result in harm to both the general population

and especially susceptible populations. Based on this information, regulatory agencies have set limits on exposures to these harmful technology products that should not be exceeded if adverse effects are to be minimized (OSHA; EPA).

Unfortunately, the tests used most widely for setting regulatory exposure limits are incomplete and flawed simulations of real-world exposures experienced by human beings. As a result, the degree of real-world protection afforded by the regulatory exposure limits is unknown, and could (in theory) be essentially non-existent (Kostoff, 2018a).

Humans are exposed to myriad potential disease contributing factors in parallel (e.g., lifestyle, iatrogenic, biotoxic, occupational/

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Abbreviations

AD	Alzheimer's disease
ADI	acceptable daily intake
ALS	Amyotrophic lateral sclerosis
ANOVA	analysis of variance
EDTA	calcium disodium ethylene diamine tetra-acetate
EquRay	equipartition ray
HD	Huntington's disease
MS	Multiple sclerosis

NOAELS	no-observed-adverse-effect level
OECD	Organization for Economic Co-Operation and Development
OSHA PEL	Occupational Safety and Health Administration permissible exposure limits
PD	Parkinson's disease
PEL	permissible exposure limits
RLRS	Real-Life Risk Simulation
TDI	tolerable daily intake

environmental, psychosocial/socioeconomic). Additionally, their genetic makeup influences how they will respond to these potential disease contributing factors, and whether these toxic stimuli exposures will translate into adverse health effects.

Prevalent animal experiments typically involve exposure to one potential toxicant (aka single stressor experiments). Exposures to multiple toxicants are rarely used in these experiments, and, even when they are, most are limited to two-toxicant exposures for a limited period of time. How relevant 1) these single stressor experiment-derived regulatory exposure limits for a given toxicant are 2) to exposure limits that would result from the toxicant operating in combination with myriad other potential toxicants remains unknown.

One of the main reasons for the predominance of single stressor laboratory experiments in determining exposure limits is resource constraints (time and money). Increasing the size of combinations of potential toxicants for exposure limit determinations increases the complexity and numbers of experiments required drastically (Kostoff, 2018b; Kostoff et al., 2018d). There are tens of thousands of potential toxicants to which the public might be exposed.

The full-scale problem of testing potential toxicants in all relevant combinations is prohibitive. The present study takes a small step forward by examining the effects of one combination of toxicants *at very low doses* on one broad type of impact (neurological disorders). The exposure limits determined from the combination can then be compared with the exposure limits that were obtained from single stressor tests performed on each member of the combination, and the levels of differences can be analysed. Definitive conclusions about the effects of combinations on determining exposure limits cannot be obtained from one combination, given the multiple potential combinations that would have to be analysed.

Neurological disorders as a class have seen substantial increases in the past few decades (Alzheimer's Association, 2016). Because of extensive caretaking requirements for diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and multiple sclerosis (MS), to name a few, efforts to mitigate these diseases have received national attention from myriad countries. Many studies have been performed focusing on potential contributing factors (Kostoff et al., 2018c; Hakansson et al., 2003; Lin et al., 2016; Bondy, 2014; Chen et al., 2012; Helou and Jaeger, 2014), as well as potential treatments for ameliorating symptoms and possibly reversing these diseases (Kostoff et al., 2018c; Bredeesen, 2014; Misik et al., 2016; Joshi et al., 2013; Dobarro et al., 2013).

Interactions between genetic, lifestyle, iatrogenic, biotoxic, occupational and environmental risk factors are key triggers of aetiology and progression of the diseases (Olsson et al., 2016; Baltazar et al., 2014; Dardiotis et al., 2013; Campdelacreu, 2014; Dardiotis et al., 2018). These risk factors can influence the progression of neurological diseases by acting during particular time and exposure level windows (Olsson et al., 2016). Exposures to different types of pesticides, heavy metals, food additives or compounds generated during food processing, or to endocrine disrupting chemicals (even at doses near the regulatory limits) can influence the development of different neurological

disorders as shown in different epidemiological studies (Baltazar et al., 2014; Giacoppo et al., 2014; Taheri, 2017; Petrakis et al., 2017; Ruszkiewicz et al., 2017).

These exposure combinations can lead to a 'cocktail' effect. Here, exposure to several chemicals from different sources in doses near or well below regulatory-permitted levels can lead to additive, synergistic, antagonistic, or potentiative effects determined by toxicodynamic or toxicokinetic interactions (Hernandez et al., 2013). Investigation of the interactions among the different mixture chemicals that can lead to additive, synergistic, antagonistic, or potentiative effects is missing from the classical approaches. These classical experiments assess one single chemical exposure and simulate poorly the real-life myriad exposure scenarios.

To address this real-life simulation deficiency, recent toxicological studies have focused on simulation of real-life exposures, where humans are exposed to myriad toxic stimuli in parallel or in tandem. These toxic stimuli can include: 1) various types of radiation (ionizing, non-ionizing); 2) multiple chemicals from different sources (e.g., pesticides, food additives, lifestyle-associated chemicals, etc) in doses near or well below the regulatory limits; 3) multiple biotoxins, etc. The purpose of potentially toxic stimuli combination experiments is to understand better the real threat to human health (Docea et al., 2016; Hernandez and Tsatsakis, 2017; Tsatsakis et al., 2016; Tsatsakis and Lash, 2017a; Tsatsakis et al., 2018).

More recent real-life simulations of exposures to mixtures of chemicals attempt to answer several outstanding questions: i) are consumers protected by regulatory limits derived from single stressor animal experiments?, ii) are the regulatory limits determined by testing individual chemicals sufficiently protective to avoid unexpected negative effects on humans caused by the multi-chemical exposures from different sources?, iii) can non-linear dose-response effects appear during the chemical mixture exposures in doses considered safe for individual chemicals?

Taking into account these questions, Tsatsakis et al. proposed a new methodology for mixture testing (Tsatsakis et al., 2017b). It would involve animal exposures to mixtures of multiple chemicals and other possible toxicants. It would then ascertain the effects of these exposures on 1) myriad biomarkers, 2) myriad motor and behavioural performance metrics, and 3) other relevant metrics.

In this paper, we present the results of one segment of the more comprehensive study proposed by Tsatsakis et al. (Tsatsakis et al., 2017b). Our focused study aims to assess the long-term effects (twelve months, equivalent to human exposures of thirty years) of 1) very low dose exposures (near or well below regulatory limits) to 2) a complex chemical mixture on 3) motor performance and behavioural changes in rats.

2. Material and methods

2.1. Animals and study design

For test subjects, our study utilized a total of forty Sprague Dawley rats (twenty males and twenty females) divided into four groups (ten

animals per group) from the University of Medicine and Pharmacy Craiova Animal House. All procedures regarding the animal study were approved by the Ethical Committee of the University of Medicine and Pharmacy Craiova, Romania, and were conducted in accordance with the European directive for the animal experiments (EU Directive, 2010/63/EU). The methodology regarding the animal treatment procedures and administration of the test mixtures was based on the methodology proposed by Tsatsakis et al. (Tsatsakis et al., 2017b). Our methodology complied fully with the Organization for Economic Co-Operation and Development (OECD) TG 452 protocol for chronic toxicity studies (OECD, 2009). The methodology details are presented in another article (Docea et al., 2018).

Briefly, the animals were acclimatized for 2 weeks at the new conditions before the beginning of the study. When the experimental data gathering commenced, the animals were eight weeks of age, had body weights ranging from 241 to 362 g for males and 157–235 g for females, and (as stated above) were divided randomly in four groups (five males and five females per group). They were held at the University of Medicine and Pharmacy Craiova Animal House Facility, and were subjected to a 12-h light/dark cycle, at 19–23 °C controlled room temperature, and with humidity controlled between 35 and 55%. The animals received access to animal food (Cantacuzino Institute, Bucharest) and filtered water with test substances *ad libitum*.

The following test mixture components were administered in the drinking water for a twelve month period: 1) carbaryl (ADI/TDI = 0.0075 mg/kg body weight/day (EFSA, 2006)); 2) dimethoate (ADI/TDI = 0.001 mg/kg body weight/day (EFSA, 2013a)); 3) glyphosate (ADI/TDI = 0.5 mg/kg body weight/day (EFSA, 2015b)); 4) methomyl (ADI/TDI = 0.0025 mg/kg body weight/day (EFSA, 2009a)); 5) methyl parathion (ADI/TDI = 0.003 mg/kg body weight/day (WHO, 2004)); 6) triadimefon (ADI/TDI = 0.03 mg/kg body weight/day (EFSA, 2009b)); 7) aspartame (ADI/TDI = 40 mg/kg body weight/day (EFSA, 2013b)); 8) sodium benzoate (ADI/TDI = 5 mg/kg body weight/day (EFSA, 2016b)); 9) calcium disodium ethylene diamine tetra-acetate (EDTA) (ADI/TDI = 2.5 mg/kg body weight/day (FAO Nutrition Meetings, 1974)); 10) ethylparaben (ADI/TDI = 10 mg/kg body weight/day (EFSA, 2004)); 11) butylparaben (ADI/TDI = 0.5 mg/kg body weight/day (EFSA, 2004)); bisphenol A (ADI/TDI = 0.004 mg/kg body weight/day (EFSA, 2017a)); and, 13) acacia gum (ADI/TDI = 34 mg/kg body weight/day (FDA, 2013; EFSA, 2017b)). Four dose levels of the mixture were used for the experiment: 1) 0 x ADI/TDI (mg/kgbw/day); 2) 0.25 x ADI/TDI (mg/kgbw/day); 3) 1.0 x ADI/TDI (mg/kgbw/day); and, 4) 5 x ADI/TDI (mg/kgbw/day). All the chemicals were purchased from Sigma-Aldrich (USA).

2.2. Functional and behavioural tests

One goal of the study was long-term testing, to insure that any latency effects would be captured. The 12 months exposure period used for rats in the present study reflects up to 30 years of exposure in humans (Sengupta, 2013), and is a reasonable simulation of the long-term. Nonetheless, it should be recognized that it might not be adequate for exploring long latency effects, such as linkages between early childhood exposures to toxic mixtures and diseases of late adulthood (Alzheimer's disease, etc.). In order to assess the changes in neurological function associated with long-term (twelve months) exposure to the test mixture solutions, the rats were tested on different tasks for: i) motor performance, and ii) behavioural changes for anxiety and depression. The motor performance assessment used a Rotarod-based test, and the behavioural changes assessment used 1) an open-field exploratory test and 2) an elevated plus-maze test.

2.2.1. Assessment of motor performance

2.2.1.1. Rotarod. The motor performance (as reflected by the coordination and sensorimotor function of the rats) was measured using an accelerating rotarod (Whishaw et al., 2003). The rotarod

apparatus is a horizontal rotating rod thirty cm long and six cm diameter. It is attached to a motor with two speed settings: i) one constant at five rpm, and ii) one adjustable from five rpm to forty rpm in 300s. The rod is 27 cm above the landing table. The animals were taken to the testing room for at least 1 h before testing for acclimatization. All the rotarod procedures were carried out at the same hour of the day for all the animals from each group, starting at 2:00 p.m.

The rotarod experiment consisted of an initial training phase followed by a testing phase. The training phase consisted of three trials separated by 10 min intervals between each trial. That allowed the animal to walk forward on the rotating road at five rpm for 60 s. Each animal was required to stay on the rotating rod at five rpm speed for 60 s before proceeding to the testing.

The testing procedure consisted of recording the time that animals stayed on the rotating rod at an accelerated speed from four to forty rpm in 300s. Testing ended when the animal either 1) fell off the rod or 2) clung to the rod and completed full passive rotation. Testing was terminated after three consecutive trials (separated by 15 min intervals) were completed. After the third trial, the animals were weighed. For each animal, the latency to fall from the rotating rod expressed as seconds was analysed.

2.2.2. Assessment of behavioural changes

2.2.2.1. Open field exploratory test. It is widely used to assess novel environmental exploration, general locomotor activity and emotional behaviour in rodents [Prut and Belzung, 2003]. An open field test area of 100 × 100 cm divided into 25 squares was used. The squares were defined as “internal squares” – the nine squares located in the centre of the test area and the “external squares” – the other sixteen outer squares adjacent to the boundary. Each rat was placed in the centre square of the open field area 20 × 20 cm wide. The rats movements were recorded for 5 min by a video camera placed above the area. The area was cleaned with disinfecting solution, rinsed several times with fresh water, and dried after use by each animal. The recorded videos were examined separately (in blind) by two experts, who analysed and noted the exploratory behaviour responses from three perspectives: i) spatial orientation activity, quantified as the number of rearings; ii) locomotor activity, quantified as 1) the number of crossings over internal and external squares and 2) latency of leaving the internal square; and iii) emotional state associated with the rat's stress level during the experiment, quantified by the number of grooming acts and the numbers of boluses (Grigor'ev et al., 2008; Sayapina et al., 2017). When there were differences between the two video analysts, they watched the videos again and arrived at a consensus.

2.2.2.2. Elevated plus-maze test. It assesses anxiety in rodents (Grigor'ev et al., 2008). The apparatus used for the test had a cross form with two open arms and two closed dark arms connected to a central platform. The arms were located fifty cm above the floor and had a length of ninety cm from the centre. The rats were placed in the centre of the apparatus and their movements were recorded by a video camera. The testing time was 5 min for each animal. After each animal test, the area was cleaned one time with a disinfecting solution, rinsed several times with fresh water, and then dried. The recorded videos were analysed separately by two experts (in blind), who noted the level of individual stress by calculating 1) the time spent in the closed sleeves and 2) the number of groomings. The experts quantified the exploratory activity by calculating 1) the time spent in the open sleeves, 2) time spent in the centre, 3) number of rearings and 4) number of bendings over the edge (Grigor'ev et al., 2008; Sayapina et al., 2017). When there were differences between the two video analysts, they watched the videos again and reached a consensus.

2.3. Data analysis

Numerical data were expressed as mean \pm standard error ($x \pm$ s.e.). The differences among all groups' means were assessed by ANalysis Of VAriance test (ANOVA), and the differences between the means of test groups and the control group were assessed by Student's *t*-test. The data on open cross and maze test were cumulated over 5 min. For the rotarod tests, the mean of the last three measurements of the time-to-fall was used. The data for every minute of open field tests and the data of rotarod tests were analysed using repeated measures ANOVA to detect the differences among the groups.

3. Results

3.1. The effect of different mixture doses on coordination and sensorimotor function

In the accelerating rotating pole test (rotarod) for differences in the coordination ability and motor performance, the mixture treated groups showed a non-significant decreased trend compared with the control group. All the groups showed increased time on the rotarod before falling for consecutive trials. However, the low dose group had indistinguishable time to fall at the second and third trials. The differences did not reach statistical significance (Fig. 1). No differences were observed in the decrease in body weight between the three trials (Fig. 1).

3.2. The effect of different mixture doses on behavioural changes

The exploratory behaviour responses in the open field test were different in test groups relative to the control group. There was an increasing trend in locomotor activity, quantified as: i) number of crossings over internal and external squares, ii) latency of leaving the internal square. The difference between the low dose group and control group was significant in the number of crossing over external squares at 1 min and 5 min ($p < 0.05$) (Table 1, Fig. 2, Fig. 3).

The spatial orientation activity, quantified as number of rearings, was significantly increased in the low-dose compare with the control rats at 5 min ($p < 0.05$) and did not reach statistical significance at 1 min (Table 1, Figs. 2, Figure 3).

The rats' anxiety levels during the experiment (quantified as the number of grooming acts and the numbers of boluses) were moderately increased, and did not reach statistical significance (Table 1, Figs. 2, Figure 3).

The elevated plus maze tests didn't show any statistically significant differences between the test groups and the control group. A moderate increase in the anxiety level of the rats from the treated groups, quantified as the number of groomings and the time spent in closed sleeves (Table 2, Fig. 4) was found.

4. Discussion

The present section is divided into two parts: discussion of results obtained from the study, and relevance of chronic low-dose mixture testing on animals to real-life human exposures.

4.1. Discussion of results

In this study, our objective was to evaluate the possible combinatorial effects on behavioural and neurological functions in rats after chronic (twelve months) exposure to a chemical mixture. The chemical doses used (in drinking water) were well below the health-based reference values for each individual component (specific pesticides and additives). The lowest dose group was exposed to 0.25x ADI/TDI levels of each chemical, the medium dose group was exposed to 1xADI/TDI levels of each chemical, and the highest dose group to 5xADI/TDI levels of each chemical. We posited that such combination may affect the neurological outcome upon chronic exposure in a dose-dependent manner.

4.1.1. Potential hormetic effects

Interestingly, we found that the animals from the lowest dose mixture group (0.25x ADI/TDI) were more active in the open field than the control group. This difference was attenuated in the medium (ADI/TDI) and highest dose (5xADI/TDI) groups, while preserving the trend without reaching statistical significance. Coordination and locomotor activity were increased in the lowest dose group when compared with the control group, and were decreased when the mixture dose was increased, showing an inverted pattern. One explanation for these results

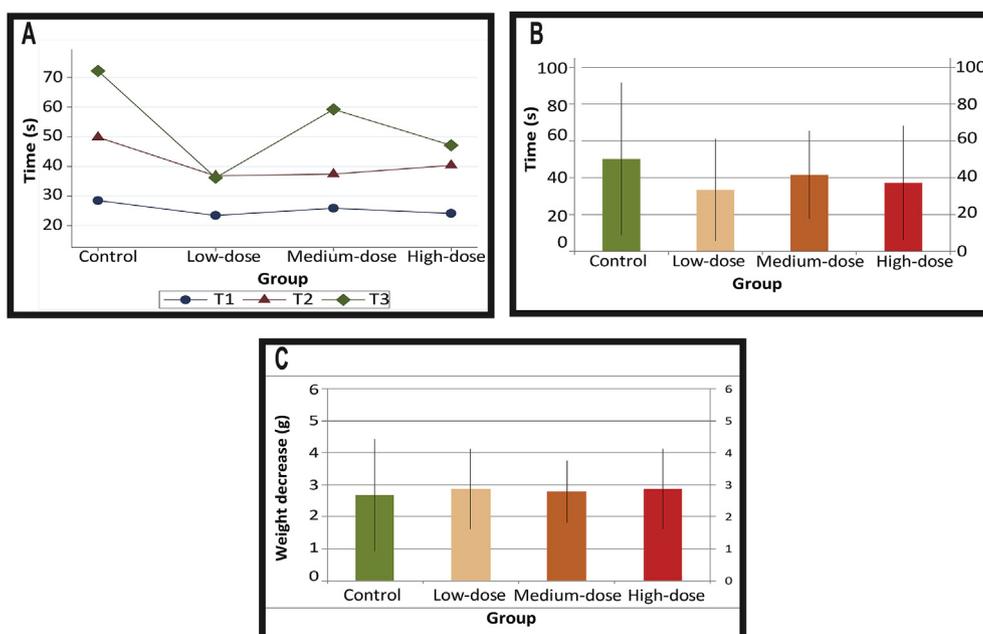


Fig. 1. Rotarod test: A. Time-to-fall at trial 1 (T1), trial 2 (T2), and trial 3 (T3), for each group. B. Mean time-to-fall in the three trials. C. Weight difference between the level before testing and the level after third test.

Table 1
Open field test results.

Parameter	Control Group	Low Dose Group	Medium Dose Group	High Dose Group	ANOVA p	Contrast Low vs. Control	Contrast Medium vs. Control	Contrast High vs. Control
Exploratory behaviour								
Latency	3.80 ± 0.88	1.70 ± 0.52	3.30 ± 0.86	4.20 ± 0.90	0.154	0.176	0.947	0.971
Int. cross 5 min.	6.20 ± 1.76	7.50 ± 1.61	8.40 ± 1.86	6.50 ± 1.97	0.819	0.921	0.723	0.999
Ext. cross 5 min.	13.70 ± 6.15	46.80 ± 11.57†	34.10 ± 10.02	19.30 ± 7.63	0.060	0.038*	0.277	0.948
Rearing 5 min.	6.90 ± 1.61	22.20 ± 4.77†	21.70 ± 3.94	14.50 ± 3.63	0.017*	0.015*	0.020*	0.340
Int. cross 2 min.	4.40 ± 1.39	4.11 ± 1.02	5.44 ± 1.13	3.30 ± 0.76	0.594	0.996	0.843	0.812
Ext. cross 2 min.	5.70 ± 3.14	17.44 ± 5.57	11.78 ± 4.22	7.70 ± 2.46	0.161	0.104	0.564	0.967
Rearing 2 min.	4.10 ± 1.14	9.56 ± 2.54	8.33 ± 1.61	6.50 ± 1.97	0.144	0.079	0.213	0.619
Int. cross min 1	2.80 ± 1.04	3.50 ± 0.79	3.00 ± 0.61	2.40 ± 0.56	0.791	0.860	0.996	0.968
Ext. cross min 1	2.60 ± 2.07	10.10 ± 2.32†	7.50 ± 2.60	2.40 ± 0.67	0.027*	0.037*	0.234	0.999
Rearing min 1	2.10 ± 0.57	5.80 ± 1.29	5.90 ± 1.75	1.90 ± 0.60	0.021*	0.078	0.068	0.999
Emotional state								
Grooming 5 min.	1.30 ± 0.47	1.60 ± 0.31	2.20 ± 0.44	2.20 ± 0.51	0.384	0.932	0.348	0.348
Boluses 5 min.	1.20 ± 0.63	2.20 ± 0.63	1.20 ± 0.49	0.70 ± 0.37	0.271	0.426	0.999	0.851
Grooming 2 min.	0.20 ± 1.13	0.44 ± 0.24	0.56 ± 0.29	0.10 ± 0.10	0.359	0.723	0.461	0.969
Boluses 2 min.	1.00 ± 0.52	1.60 ± 0.56	0.40 ± 0.31	0.70 ± 0.37	0.290	0.668	0.668	0.935
Grooming min 1	0.10 ± 0.10	0.20 ± 0.13	0.60 ± 0.31	0.10 ± 0.10	0.178	0.961	0.143	0.999
Boluses min 1	0.40 ± 0.22	0.90 ± 0.31	0.20 ± 0.20	0.50 ± 0.27	0.279	0.381	0.901	0.985

Note: *p < 0.05.

may be hormetic behaviour: there could be a two-phase dose-dependent effect - the chronic use of a very low doses of potentially toxic chemicals having a stimulating effect on the organism, with the higher doses eliminating/masking the stimulatory effects of the lower doses. These

effects are mediated via an adaptive response secondary to the activation of genes involved in homeostatic adaptation, which protect it from external and internal stressors (e.g. ischemic preconditioning, food restriction). The administration of a very low dose of the chemical

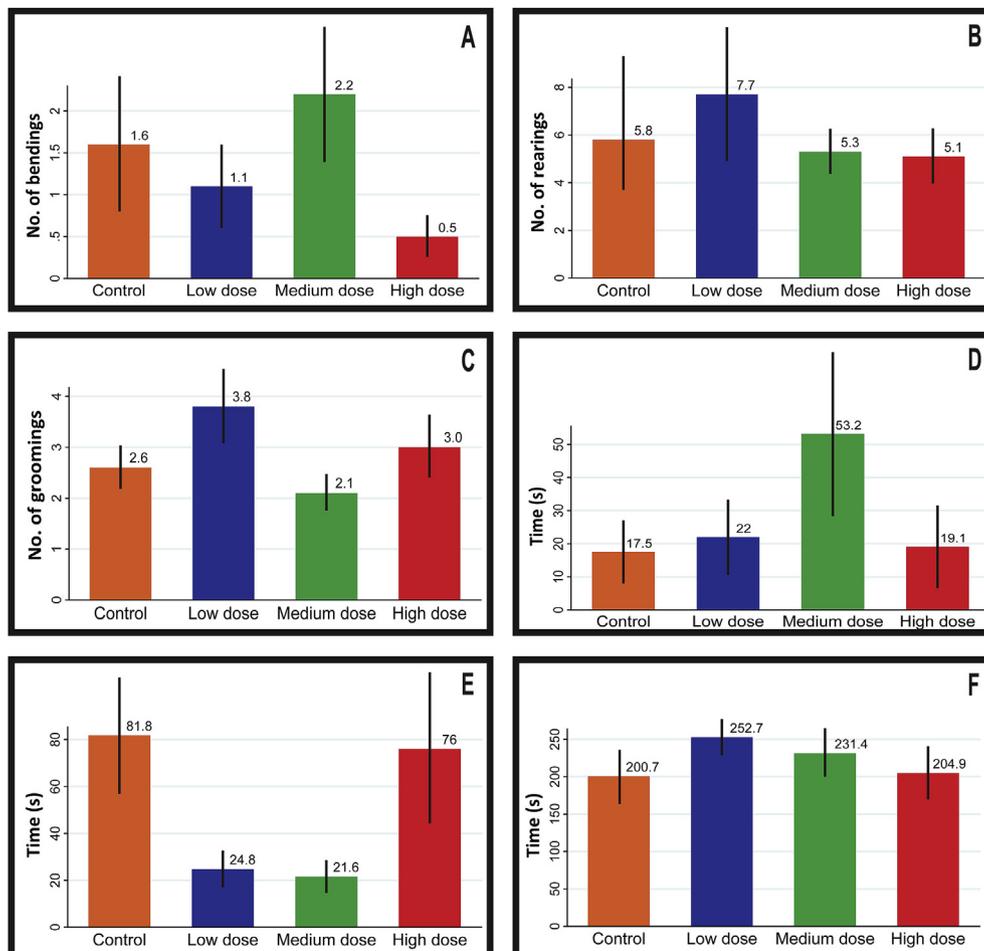


Fig. 2. Open field test results at 5 min in rats. A. Crosses over internal squares. B. Crosses over external squares. C. Groomings. D. Rearings. E. Boluses. F. Latency. Note: *p < 0.05.

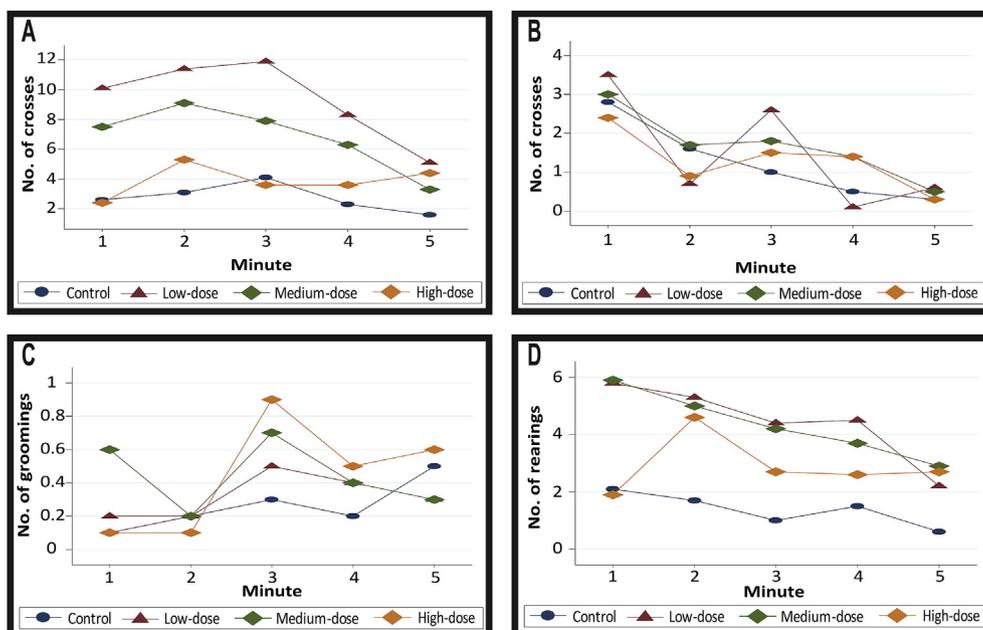


Fig. 3. Open field test results per minutes A. Crosses over external squares. B. Crosses over internal squares. C. Groomings. D. Rearings.

Table 2
Elevated plus maze test results.

Parameter	Control group	Low Dose Group	Medium Dose Group	High Dose group	ANOVA p	Contrast Low vs. Control	Contrast Medium vs. Control	Contrast High vs. Control
Maze bending	1.60 ± 0.85	1.10 ± 0.46	2.20 ± 0.89	0.50 ± 0.27	0.336	0.913	0.862	0.520
Maze rearing	5.80 ± 1.38	7.70 ± 1.76	5.30 ± 0.84	5.10 ± 1.06	0.162	0.611	0.986	0.965
Maze grooming	2.60 ± 0.37	3.80 ± 0.76	2.10 ± 0.28	3.00 ± 0.71	0.209	0.325	0.869	0.926
Maze open sleeves	17.50 ± 9.64	22.00 ± 10.82	53.20 ± 28.41	19.10 ± 11.45	0.404	0.995	0.325	0.999
Maze centre	81.80 ± 23.23	24.80 ± 6.50	21.60 ± 7.63	76.00 ± 32.82	0.081*	0.145	0.117	0.995
Maze closed sleeves	200.70 ± 24.04	252.70 ± 14.55	231.40 ± 31.09	204.90 ± 37.68	0.532	0.429	0.782	0.999

Note: *p < 0.05.

mixture (for each constituent, the dose was one to three orders of magnitude below the original single constituent NOAELs) showed dose-dependent stimulation of the nervous system, rather than inhibition. In addition, in the elevated plus maze (anxiety) and rotarod (muscle weakness and discoordination) experiments, the performance was not affected. A trend for less risky behaviour was observed in the elevated plus maze in the lowest dose group compared with control group assessed by less bending and more time spent on the closed sleeves. The differences are considered negligible for interpretative purposes.

4.1.2. Larger hormetic context

Our findings corroborate other reports, showing neuroprotective effects of environmental neurotoxins at very low doses, well below the doses that produce toxic effects as is also the case in the current study (Marini et al., 2007; Calabrese and Rubio-Casillas, 2018). Otani et al. showed that low-dose-rate radiation presented a protective effect in mouse models of retinitis pigmentosa. The utilization of low-dose-rate radiation prevented apoptosis of rod photoreceptor cells and the functional and morphological cone photoreceptor cell degeneration (Otani et al., 2012). Calabrese et al. in a review article on inorganic agents and hormesis emphasize the need for new testing protocols for chemicals, taking into consideration the biphasic responses that may appear upon exposure to low-compared to high-dose. The authors surmise that responses may not be credibly predicted when extrapolating the data from protocols that test high-doses of chemicals (Calabrese and Baldwin, 2003). Different inorganic agents, such as aluminium, mercury, chromium and lead, exhibit biphasic effects on the central

nervous system level. Aluminium, a well known neurotoxicant, has been shown to have biphasic effects on a large spectrum of neural processes. Rabbits exposed to low doses of aluminium showed increased brain protein synthesis and stimulated phosphorylation of neurofilament subunits by neurofilament-associated protein kinase, effect that was decreased or inhibited at higher doses (Nicholls et al., 1990; Nicholls et al., 1991; Letierrier et al., 1992). Mercuric ions exhibited a hormetic effect with stimulation of inositol phosphate accumulation at low doses and decreased formation at high doses in an *in vitro* study on synaptoneurosomes prepared from 8-day-old rats (Vignes et al., 1993). The hexavalent form of chromium (Cr) exerted a biphasic effect on norepinephrine release from stimulated chromaffin cells. An increased secretion of norepinephrine at low doses of Cr (below 10 µM) likely reflected the penetration of Cr into the cytosol affecting intracellular function of calcium and an inhibition; doses of Cr above 100 µM likely blocked nicotine receptors on the plasma membrane exerting an inhibitory effect on voltage-gated calcium channels (Liu and Lin, 1997). Leasure et al. showed that low-level human equivalent gestational lead exposure induced non-monotonic dose-dependent responses, with increased effects in groups exposed to low-compared to groups exposed to high-doses concomitant with decreased spontaneous motor activity, increased amphetamine-induced motor activity and decreased rotarod performance (Leasure et al., 2008). Acrylonitrile also showed a hormetic effect on acetylcholinesterase activity in both blood and brain in mice (Yuanqing et al., 2013).

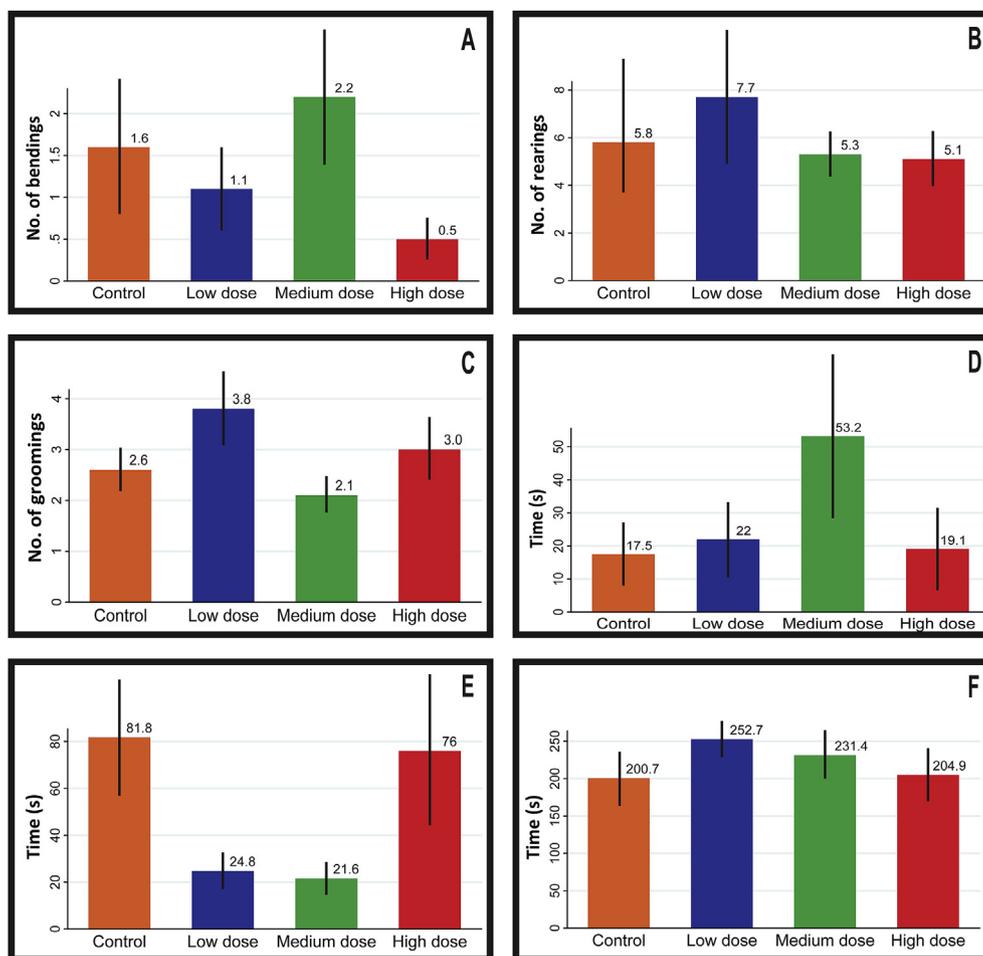


Fig. 4. Elevated plus Maze test results. A. Bendings. B. Rearings. C. Groomings D. Time spent in open sleeves. E. Time spent in centre. F. Time spent in closed sleeves.

4.1.3. Extrapolation of results

The results represent one data point from an infinite universe of possible data points, where each data represents a specific combination of chemicals. We cannot state how representative these results are of the larger universe, or how likely such combinations of very low dose toxic substances are to produce hormetic effects. Many more combinations would have to be tested to derive more definitive and generalizable conclusions.

In addition, for complex mixtures of chemicals at very low doses, there could be multiple hormetic regions, analogous to resonances in mechanical or electrical systems. Consider the simple example of a two toxic substance mixture where each component is present far below its NOAEL level. If both substances have regions in which they exert hormetic effects when measured in isolation, there is no requirement that their hormetic dose levels have to be the same fraction of the NOAEL dose levels. Thus, for the two component mixture, one component could be exerting hormetic effects while the other component is exerting no effects, and the resulting effect could be hormetic. Then, the same situation could hold when the second component is exerting hormetic effects. For the mixture, as the dose rates increased starting from extremely low levels, the trajectory could be.

no effect— > hormetic effect— > no effect— > hormetic effect— > no effect, and eventually adverse effects when the NOAELs are exceeded or even approached (synergistic effect).

4.1.4. Predicting hormetic responses

Even if computational toxicology evolved every year, prediction of the hormesis effect of a mixture is still a challenge. First, not all the chemicals present in very low doses in toxicological studies could

produce hormetic effects. Second, because all the models applied until now are based on binary mixtures and not on real life complex mixtures of multiple chemicals (Qu et al., 2018). Until now, the most used models for predicting toxicological interaction effects of a mixture from individual components are model concentration addition and independent action. Qu et al. tested a new model, IDV_{equ} (an interpolation method based on the Delaunay triangulation and Voronoi tessellation as well as the training set of direct equipartition ray design (EquRay) mixtures) that present advantages for the prediction of the hormetic effects of mixtures compared to concentration addition model, but this was successfully used only for binary mixtures (Qu et al., 2018).

4.2. Relevance of chronic low-dose mixture testing in animals to real-life human exposures

4.2.1. Bounding the problem

Humans could potentially be exposed to many thousands of toxic chemicals (and other toxic stimuli) over their lifetime, and the exposures do not necessarily have to be simultaneous in order to exert adverse additive or synergistic effects. The number of combinations of potential exposure 'signatures' for any individual is essentially infinite. This is the challenge posed by real-life human exposures to potentially toxic stimuli.

One approach to gaining greater understanding of mixture effects of chemical (and other types of toxic) combinations is to bound the problem. One would select a mixture of potentially toxic chemicals, and examine dose levels ranging from extremely low to relatively high. To limit the number of cases to a reasonable level, consider the following three cases: low-exposures, modest exposures, and high exposures.

For animals, the low-exposures case would use mixture doses (for each constituent) far below the NOAEL values for each mixture component. The possible mixture outcomes could range from hormetic effects to no effects to harmful effects.

The modest exposures case would use mixture doses (for each constituent) at the NOAEL values for each mixture component. The possible outcomes would range from 1) no effects to 2) harmful and possibly synergistic effects, with outcome 2) being more likely.

The high exposures case would use mixture doses (for each constituent) far above the NOAEL values for each mixture component. Selecting such large doses is not an academic exercise. In the USA, for example, the only Federal legally enforceable occupational exposure limits are the OSHA PELs (Permissible Exposure Limits). A recent study showed the PEL values to range from one to four orders of magnitude above the NOAELs (Kostoff, 2018a). Thus, using the equivalent of PEL values for humans as dosages for animal exposure experiments would reflect possible real-life worst-case exposures in the USA in occupational environments.

4.2.2. How the present experiment fits into the larger framework

In the experiment reported here, the doses of the chemicals in the mixture were *far below* the NOAEL values for the constituents. This is because the ADIs/TDAs are the original NOAEL values *divided by the Uncertainty Factor relevant to each experiment*. The Uncertainty Factor can be a large/very large number, ranging from single digits to three orders of magnitude higher.

How would these very low-dose exposures relate to real-life exposures? There are individuals, who either by deliberate choice and planning or by residence in selected environments and cultures, have minimal exposures to myriad toxic stimuli. This group is probably small in the industrialized segments of the world, but the results of the present experiment would be most applicable to them. Given the broader objectives of our overall study, the present results should be viewed more appropriately as the first step in the three-step process described in Section 4.2.1.

The fact that even modest statistically insignificant adverse effects were shown for these mixtures at doses *far below* the NOAELs is cause for concern. We would expect that for mixture doses at the original constituent-specific NOAELs, combination effects would result in much more severe (and possibly synergistic) adverse effects. Experiments of mixtures with doses near/at NOAEL values are needed to confirm or reject this hypothesis.

Finally, as stated in Section 4.2.1, even exposure to mixture doses at the original constituent-specific NOAELs (as suggested in the previous paragraph) are challenging but might still be insufficient to address the longer-range goal of this study, simulation of real-life exposures due to overall multifactorial components of RLRS. Experiments at the test animal exposure dose equivalents of the OSHA PELs for humans (approximately the test animals' NOAELs *times* the Uncertainty Factor for each mixture constituent) would be required to reflect the real-life worst case condition, especially for the USA. Such experiments could be run in parallel or in tandem with the NOAEL-level dosage experiments suggested in the previous paragraph.

5. Conclusions

The administration of exceedingly low doses of a thirteen chemical cocktail (one to three orders of magnitude below the single stressor NOAEL dose for each stressor in isolation) showed a dose-dependent stimulation of the nervous system, rather than its inhibition. At the doses studied herein, exposure to any of the single stressors used in the combination has been shown to have no adverse effects (WHO, 1987). This stimulatory effect could be explained by chemical mixture–induced hormesis, a phenomenon supported by several studies. Some modest adverse effects were noted (e.g., rats' increased anxiety levels), but they did not reach the level of statistical significance.

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