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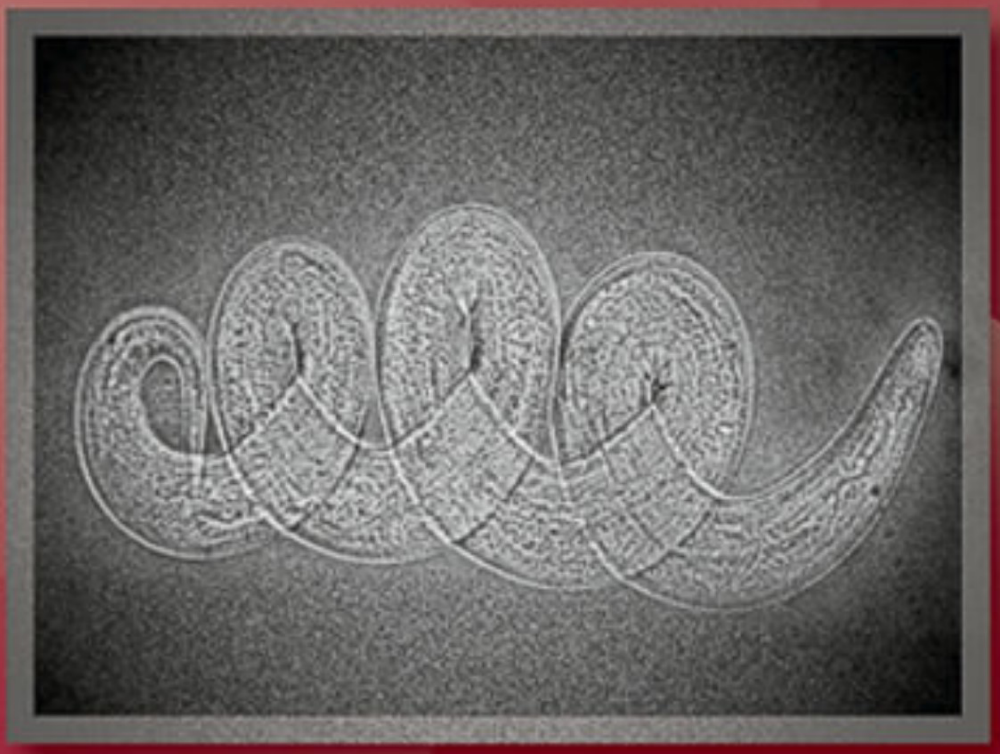

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REVIEW ARTICLE

Health risks associated with benzene formation in health supplements – An appraisal

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ABSTRACT

Benzene, classified as class-1 human carcinogen by International Agency for Research on Cancer (IARC), is a well-studied chemical over the century and is directly associated with acute and chronic health effects. Evidences reflect that benzene exposure leads to Acute Myeloid Leukemia and Acute Non-Lymphocytic Leukemia. Benzene may enter food and formulations through various environmental factors and due to inferior manufacturing techniques. Moreover, the formulations containing sodium benzoate and ascorbic acid/citric acid as preservative/constituent of herbal ingredient are at greater risks of benzene contamination by oxidative decarboxylation reaction. Although FDA has set a limit for benzene content in products yet cases with high level have been reported. At the same time the long-term use of formulations, even with permissible limits of benzene, may increase risk of carcinogenicity. Harmful health effects due to environmental and occupational exposures to benzene have been sufficiently reported, however, no such reports for generation of benzene in food and pharmaceutical products exist. There is a need to make the scientific fraternity involved in food products, formulations, and food supplements and to be aware of the undesirable effects of multiple and indiscriminate use of preservatives leading to benzene generation. So, the present manuscript highlights the mechanism of benzene formation in food products/formulations, factors affecting benzene formation, metabolism, toxicity and other health effects.

KEY WORDS: sodium benzoate; ascorbic acid; decarboxylation; benzene; ayurvedic formulation

Introduction

Benzene, an aromatic organic compound, is extensively used solvent in various chemical industries as well as laboratories (Costa & Costa, 2002). It was classified as class-1 human carcinogen by International Agency for Research on Cancer (IARC) (Cogliano *et al.,* 2008; Santos *et al.,* 2015). The study report of IARC in 1987 gives the effective evidences that benzene exposure leads to Acute Myeloid Leukemia and Acute Non-Lymphocytic Leukemia (IARC, 2012; Yoon, 2018). Benzene is commonly employed in production process of various chemicals, plastics and paints and also its presence detected in environment due to various sources of air pollution like vehicular emission, burning of fossil fuels and wood and the human activities like cigarette smoking (Vinci *et al.,* 2012). Addition to this, benzene contamination is also reported in foods and beverages (Corriher, 2009; McNeal *et al.,* 1993; Heikes *et al.,* 1995, Fleming-Jones & Smith, 2003; Lachenmeier *et al.,* 2008; Nyman *et al.,* 2007; Poucke *et al.,* 2008). Apart from its existence in food and beverages, it is also expected to be present in different marketed Ayurvedic formulation available over the counter (Nishna *et al.,* 2012). Benzene contamination in formulations occurs through preservatives, water, packaging materials and inferior manufacturing techniques (Varner *et al.,* 1991). Formulations containing benzoate salts and ascorbic acid or citric acid as preservative or as the constituent of herbal ingredient are at greater risks of benzene contamination (Nishna *et al.,* 2012). In 1990, benzene formation in the beverages and foods with added preservatives such as salts of benzoates and ascorbic acid or its isomeric forms was observed (Nyman *et al.,* 2007; Poucke *et al.,* 2008; Nishna *et al.,* 2012; Varner *et al.,* 1991; Richardson, 2006).

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Later, the study in 1993 concluded that decarboxylation of benzoate in the presence of ascorbic acid and transition metal like Cu^{2+} and Fe^{3+} lead to the formation of benzene and the reaction is accelerated by heat and light (Gardner & Lawrence, 1993).

The study conducted on Dasamoolarishta, an Ayurvedic formulation, found that level of sodium benzoate was above the permitted level which could result in the formation benzene in the presence of ascorbic acid present as a constituent of herbal ingredient (Nishna *et al.,* 2012). The discovery regarding the production of benzene from the salts of benzoates in the various products demanded manufacturers to re-evaluate their formulations containing benzoate salts and ascorbic acid with the view to reduce benzene content (Azab *et al.,* 2019; Prnová *et al.,* 2019; Richardson, 2006). According to International Council for Harmonization (ICH) the residual solvent limit for benzene is kept as 2 parts per million (ppm) (ICH, 2018). Although maximum exposure to benzene occurs through inhalation from environment, the amount of benzene ingested plays a crucial role and interfere in biological processes (Maithani *et al.,* 2019; Dos *et al.,* 2015). So, the product with the benzene concentration above 2ppm is regarded as unsafe for human consumption. Looking at the risk, Margin of Exposure (MOE) value has been set, which ranges from 400,000 to 2,000,000. In general, a MOE ≥10,000 is considered to be protective. MOE value is regarded as the ratio between specific point on the dose- response graph that is responsible for tumor formation in various human and animal intake experiments (Benford *et al.,* 2010; Smith *et al.,* 2010).

Mechanism of benzene formation

Sodium benzoate or the other salts of benzoates react with ascorbic acid in the presence of transition metal like Fe^{2+} and Cu^{3+} form benzene as volatile organic compound (Gardner & Lawrence, 1993). Sodium benzoate gets converted to benzoic acid and finally, by the process of decarboxylation, benzene is produced. The chemical reaction of benzene formation is given in Figure 1 (Totally Wild, 2017). In this reaction, when the concentration of ascorbic acid is increased, the production of benzoic acid rises along with the formation of sodium L-ascorbate (Gardner & Lawrence, 1993). Further benzoic acid gets reduced to benzene by decarboxylation mechanism as illustrated in Figure 2 (Commons.wikimedia.org., 2015). In this mechanism benzoic acid reacts with hydroxyl group of sodium L-ascorbate forming water molecule and unstable benzoate ion as reaction intermediate. The unstable benzoate ion dissociates into carbon dioxide (CO_2) and positively charged benzene. From the positively charged benzene, H^+ ion reacts with another hydroxyl group and form water molecule. Hence, benzene molecule gets stabilised (Anon, 2018).

Factors affecting/influencing the benzene formation

Benzene formation in various food and beverages containing ascorbic acid and sodium benzoate depends upon various extrinsic and intrinsic factors. Concentration of sodium benzoate, ascorbic acid, pH, various raw materials, metal ions, chelating agent and artificial sweeteners/sugars are some of the intrinsic factors whereas temperature, UV radiation and storage time are extrinsic factors. Both intrinsic and extrinsic factors play an effective role in benzene formation in foods and health supplements.

Temperature

A study was conducted by Apera *et al.* (2008) on the influence of temperature on benzene formation using aqueous solution with similar concentration of sodium benzoate and ascorbic acid. They concluded that the benzene formation process enhances with the increase in storage temperature. The similar study was carried out by Morsi et al. (2012) on the soft drink samples. The samples were stored at 3 different temperatures (20 °C, 45 °C, and 90 °C)

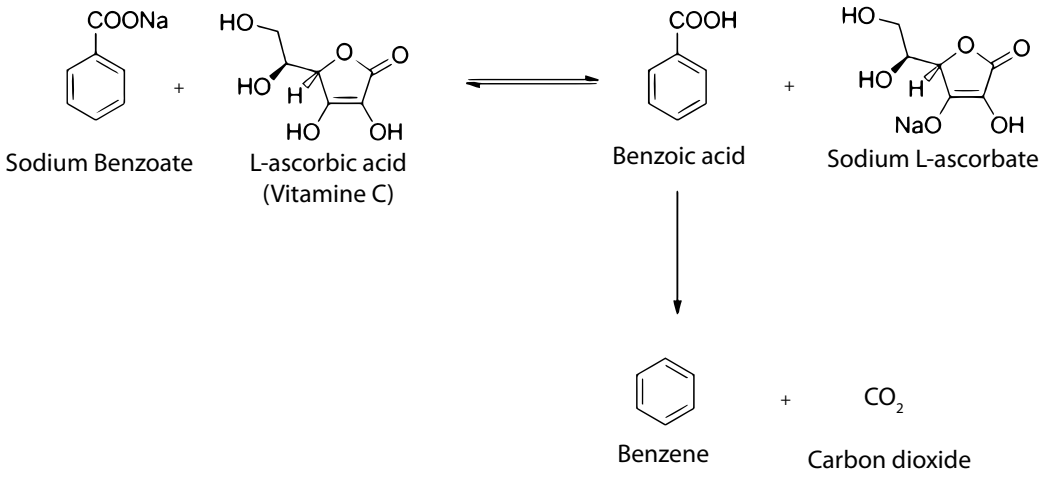

Figure 1. Chemical reaction for the formation of benzene.

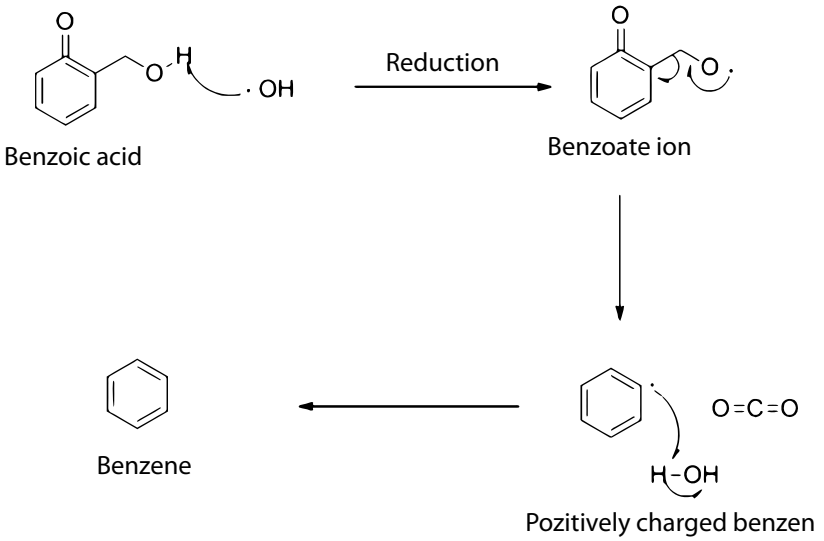

Figure 2. Decarboxylation mechanism of benzoic acid.

for 21 days. The samples stored at temperature 90 °C resulted in maximum benzene formation compared to samples stored at other two temperatures.

Sugar concentration

In the same study Aprea et al. (2008) discussed the effect of sugar on the formation of benzene at three different concentrations of sucrose, 0.1, 0.25 and 0.5. The study reflected that with the increase in sugar concentration the benzene formation was reduced.

Ultraviolet light

McNeal *et al.* (1993) studied the effect of temperature and UV on benzene formation. In order to conduct the study aqueous models containing sodium benzoate and ascorbic acid were stored at two different environmental conditions for 20 hours. One of them was storing at 45 °C or under intense UV light for 20 hours and the other condition was storing in dark and room temperature. Finally, it was discovered that benzene concentration in UV exposed sample was comparatively much higher than the samples stored in dark rooms.

Effect of copper and iron ions

Gardner & Lawrence (1993) studied the effect of transition metal catalyst in process of benzene production. According to the study Cu ion catalysis the electron reduction of oxygen molecule (O_2) by ascorbic acid or vitamin C and forms superoxide anion radical that further produce hydrogen peroxide by disproportionate reaction. Further, hydrogen peroxide (H_2O_2) get reduce to hydroxyl radical that attacks benzoic acid to produce benzene.

$Cu^{2+} + H_2Asc \rightarrow Cu^{+} + HAsc^{*}$

$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-}$

$2O_2^{-} + 2H^{+} \rightarrow O_2 + H_2O_2$

$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{*}$

Chang & Ku (1993) conducted similar study as McNeal *et al.* to determine the role of copper and iron ions in the benzene formation process. When the aqueous solution is stored in dark for eight days, the benzene concentration was found to increase more than McNeal *et al.* study. Hence, it was concluded that presence of these metal ions in water was enough to catalyze the reaction.

Effect of EDTA

Mahoney & Graf (1986) conducted the study on EDTA as oxidant in model system. In this study it was observed that EDTA complexation deactivates the catalytic activity of transition metal ions by utilizing their coordinating positions. Therefore, chelating agents like EDTA prevents the benzene formation when metallic ions are present in the solution (Codex Alimentarius commission, 2009).

Buffer system and antioxidants

Vinci *et al.* (2011) studied factors responsible for benzene formation. In this study, it was concluded that presence of buffer system and various sources of hydroxyl radical formation along with the presence of metal ions enhance the process of benzene formation. The factors like antioxidants, as comparative to EDTA, were more potent in inhibiting the benzene formation at concentration of 1M.

Incidents related to detection of benzene in Ayurvedic formulations

The presence of sodium benzoate is detected in various ayurvedic formulations like asava and arishta (Nishana *et al.,* 2012). These liquid formulations do not require any artificial preservative as in such formulations self-generated alcohol itself act as preservative. Nishana *et al.* study on the commercial samples of dasmoolarishta clearly indicated the presence of excessive amount of sodium benzoate as a preservative. This ayurvedic formulation contains vitamin C as a constituent of Amla. As per the reaction between ascorbic acid and sodium benzoate, benzene formation could occur in dasmoolarishta (Anon, 2017). Regular use of formulations containing high level of

benzene could lead to acute and long-term health effects. Apart from the dasmoolarishta, various other asava and arishta marketed formulations contain sodium benzoate (Nishana *et al.,* 2012). There is need of conducting more research studies on Ayurvedic formulations in order to quantify benzene formation in them.

Benzene metabolism

According to animal data, ingested benzene gets completely (100%) absorbed in the gastrointestinal tract and gets freely distributed throughout the body (ATSDR, 1993). Adipose tissues contain high levels of benzene metabolites on uptake. The metabolism of absorbed benzene follows the same pathway in both laboratory animals and humans. Benzene is converted mainly to phenol by the multi-function oxidase system, primarily in the liver followed by bone marrow. Low amount of phenol is metabolized to hydroquinone and catechol. Further, a very little amount gets converted to phenylmercapturic or *trans*-muconic acid. About 14% is excreted unchanged in expired air and a small unchanged part is excreted via urine (WHO, 2003; Cooper & Snyder, 2017).

Procedure of benzene metabolism

Biotransformation

Metabolic pathway for biotransformation begins with the formation of phenol. The metabolic process, carried out by Cytochrome P4502E1 and Cytochrome P450 in the liver, generates hydrogen peroxide. Hydrogen peroxide forms hydroxyl radical, which hydroxylate benzene to yield phenol (Snyder & Hedli, 1996). An alternative pathway for the formation of phenol involves the benzene oxide-oxepin system (Rappaport *et al.,* 2009). When metabolism of benzene forms benzene oxide, it rearranges itself nonenzymatically to form phenol (Rappaport *et al.,* 2010). Benzene oxide, so formed, yields 1,2-benzene dihydrodiol by hydration in the presence of epoxide hydrolase (Ross, 1996). Further, 1,2-benzene dihydrodiol gets oxidized in the presence of dihydrodiol dehydrogenase to form catechol (Smith, 1996). Phenol can also form hydroquinone by the process of hydroxylation (Bleasdale *et al.,* 1996).

Urinary metabolites

The metabolites of benzene found in urine are categorized as urinary metabolites. These includes phenolic metabolites–L-phenylmercapturic acid, 6-N-acetylcysteinyl-S-2,3-cyclohexadienol and 2,5-diOHphenylmercapturic acid (Nerland & Pierce, 1990); opened ring metabolites–trans-trans-muconic acid and 6-OH-t,t-2,4-hexadienoic acid (Kline *et al.,* 1993); and covalently bound DNA adduct – N^7-phenylguanine (Qu *et al.,* 2000).

Microsomal metabolism

In this process, hydroquinone is formed in much larger amount at lower benzene concentration. Addition of microsomal epoxide hydrolase also yields hydroquinone instead phenol (Snyder *et al.,* 1993).

Benzene metabolism with metabolites is summarized in Figure 3.

Benzene toxicity and health effects

Benzene toxicity causes damage to multiple classes of hematopoietic cells and a variety of hematopoietic cell functions resulting in both bone marrow depression and

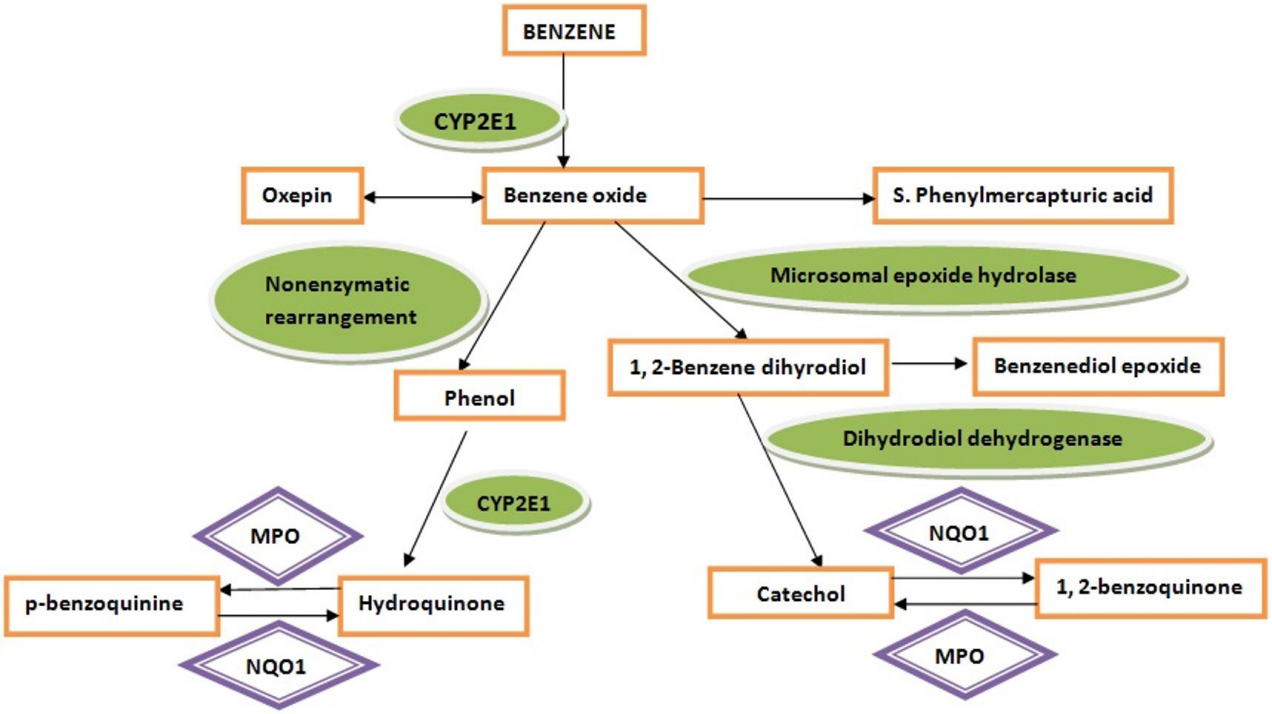

Figure 3. Schematic diagram of benzene metabolism.

leukemogenesis. Benzene metabolites damage cellular macromolecules from two ways, either by covalent binding of reactive metabolites of benzene or by inducing oxidative damage (Chen *et al.,* 2017). Relative contributions of these mechanisms to toxicity remains unrecognized hence it is clear that different mechanisms contribute to the toxicities associated with different metabolites (Snyder & Hedli, 1996). The general scheme of benzene metabolism, its metabolites and various adverse health effects is illustrated in given Figure 4 (D'Andrea & Reddy, 2018).

Benzene induced leukemia and hematotoxicity

Various national and international agencies conducted studies to determine the environmental substances that can cause cancer (a substance that causes cancer or multiplies its growth is called a carcinogen). The American Cancer Society collects data from these agencies to evaluate the risk of cancer based on evidence from animal and human research studies. From such animal and human evidence, various expert agencies have concluded that benzene has enough cancer-causing potential (Cancer.org, 2016).

The International Agency for Research on Cancer (IARC) is part of the World Health Organization (WHO) (Grosse *et al.,* 2009). Among its various functions, its major function is to detect causes of cancer. IARC classifies benzene as "carcinogenic to humans," based on enough evidence that benzene causes acute myeloid leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, multiple myeloma, and non-Hodgkin lymphoma (Grosse *et al.,* 2009). Several different US government agencies, including the National Institutes of Health, the Centres for Disease Control and Prevention, and the United State Food and Drug Administration (USFDA) jointly established National Toxicology Program (NTP). NTP classified benzene as "known to be a human carcinogen" (Cancer.org, 2019). Apart from this, the US Environmental Protection Agency (EPA) also classified benzene as human carcinogen (Cancer.org, 2016). US EPA maintains the Integrated Risk Information System, an electronic database that contains information on human health effects from exposure to various substances in the environment (IPCS, 1999).

Quantitative risk extrapolation was used to estimate lifetime cancer risks due to clear evidences of carcinogenicity and chromosomal effects of benzene on laboratory animals and humans (WHO, 1993). It was calculated from different concentrations in drinking water using data on leukemia from epidemiological studies involving inhalation exposure. Increased lifetime cancer risk with various concentration of benzene in water is given in Table 1.

Due to lack of data on carcinogenic risk to humans following the ingestion of benzene, risk assessment was also carried out on the basis of a 2-year gavage study in rats and mice. The robust linear extrapolation model was used. The estimated range of concentrations in drinking-water corresponding to excess lifetime cancer risks of 10^{-4}, 10^{-5} and 10^{-6} are 100–800, 10–80, and 1–8 µg/l, respectively. This was calculated based on leukemia and lymphomas in female mice and oral cavity squamous cell carcinomas in male rats (WHO, 2003). The results were similar to those derived from epidemiological data that formed the basis for the previous guideline value of 10 µg/l associated with a 10^{-5} excess lifetime cancer risk.

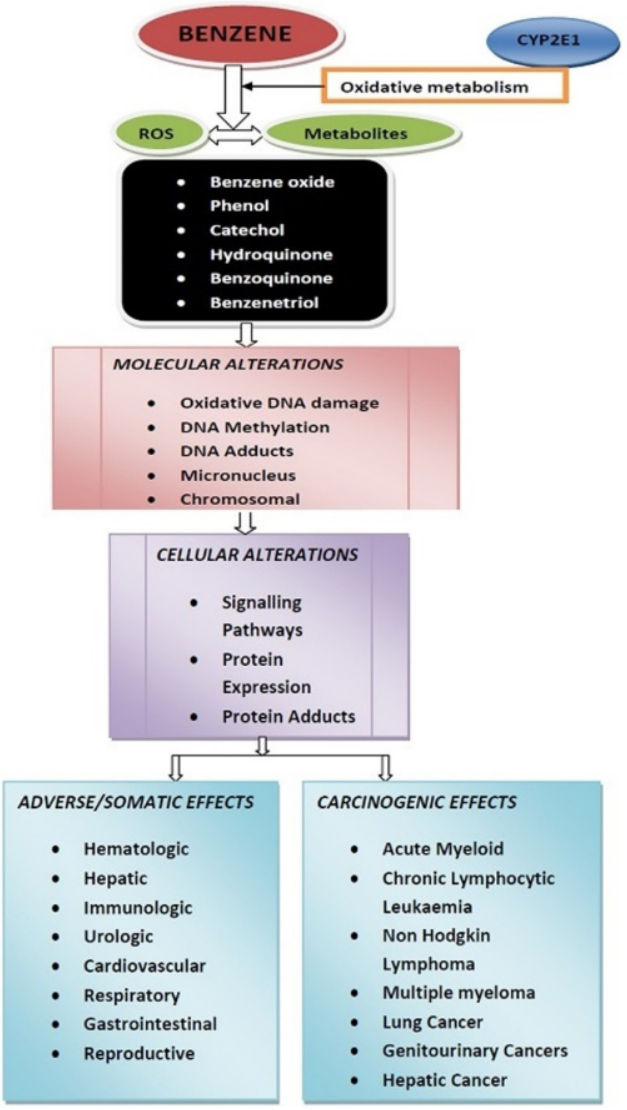

Figure 4. Schematic representation of benzene toxicity and health effects.

Table 1. Increased risk associated with benzene concentration.

Concentration (µg/l)	Increased risk
1	10^{-6}
10	10^{-5}
100	10^{-4}

Mechanism of benzene induced carcinogenicity

The metabolites produced in liver undergo secondary metabolism in bone marrow to produce benzoquinone (Rappaport *et al.,* 2009; Linhart *et al.,* 2011). Benzene induced leukemia begins when these metabolites targets genes that are unfavorable to hematopoiesis in

hematopoietic stem cells (HSCs) (Schoch *et al.,* 2005). Benzoquinone forms adduct with protein and DNA, which results in myelotoxicity (Linhart *et al.,* 2011; Loomis *et al.,* 2017). This adducts damages hematopoietic cells along with chromosomal aberrations, oxidative stress, alteration in gene expression, error prone DNA repair, apoptosis, and epigenetic regulations.

Damage to DNA causes bone marrow depression resulting in aplastic anemia, which could further leads to dysplasia and finally to acute myeloid leukemia (Snyder & Kali, 1994).

Along with adducts of protein and DNA free radicals are also generated. These free radicals cause oxidative stress and dysregulation of immune system (Kolachana *et al.,* 1993; Wiemels & Smith, 1993).

The transcription factor aryl hydrocarbon receptor (AhR) works as a negative regulator of HSCs. The AhR plays an effective role in benzene-induced hematotoxicity as it induces HSCs cycling from quiescence (Hirabayashi & Inoue, 2009; Singh *et al.,* 2009; Westphal *et al.,* 2009). The damage caused by benzene in HSCs results in apoptosis that is observed as hematotoxicity (McHale *et al.* 2011).

HSCs in bone marrow interact with mature lymphocytes in the stem cell niche. Hematotoxic damage to stem cell microenvironment results in abnormal hematopoiesis and finally leads to clonal expansion of leukemic stem cells (LSCs) as illustrated in Figure 5 (LSCs) (McHale *et al.,* 2011).

Benzene metabolites leading to reproductive failures

Benzene is colorless, volatile, and highly flammable liquid (ATSDR, 2007). Several epidemiological studies showed that the long-term exposure to benzene results in adverse impact on both male and female reproductive system (Mihaic & Borgert, 2019). It has been proven to cause high rates of functional disturbances in the system. Benzene is believed to cause negative impact on gonadotrophin hormones. Disturbances in such hormones, directly or indirectly, lead to reproductive failures (Reutman *et al.,* 2002).

Female reproductive system

Brown *et al.* (1998) disclosed in his study that women metabolize 23%–26% more benzene than men under similar exposure conditions. It was stated in research study conducted by Reutman *et al.* (2002) that regular benzene exposures for longer period results in abnormal menstruation and reduction in the size of ovaries. Continuous exposure to the benzene causes hormonal disturbances, which in turn are responsible for irregular menstruation among women and also effects size of ovaries at an adolescence age (Reutman *et al.* 2002). The study conducted by Alviggi *et al.* (2014) concluded that with increase in the concentration of benzene in the ovaries disturbs the gonadal functions and ovaries respond negatively to gonadotrophin hormones (FSH, LH). As a result, the effected ovarian sensitivity to FSH reduces the oocyte quality, which in turn effects pregnancy and increases the chances of frequent abortion. Apart from

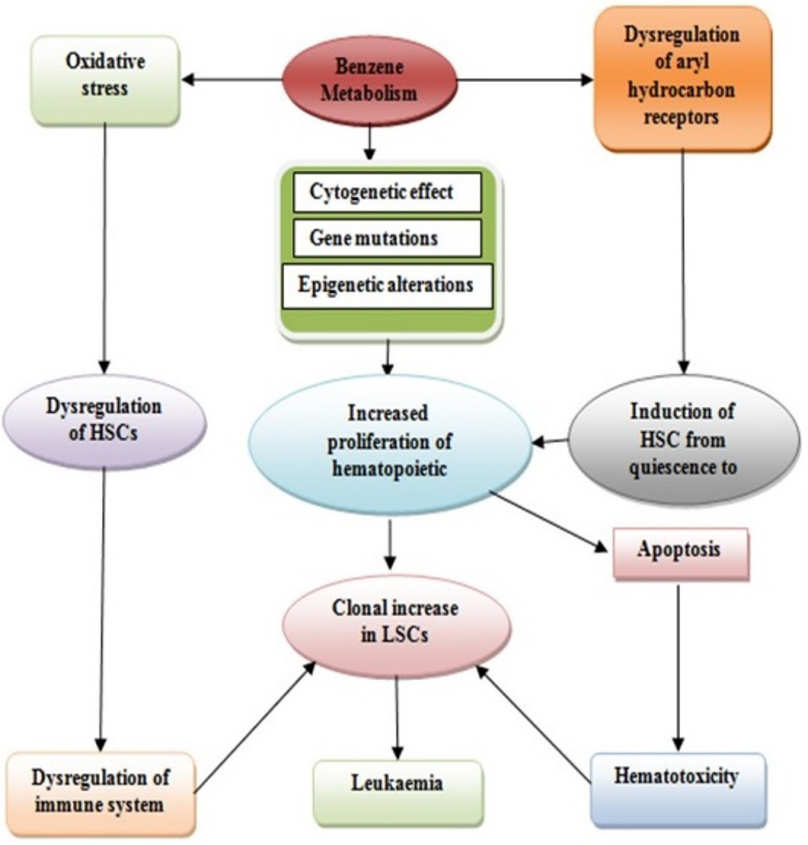

Figure 5. Illustration of benzene induce leukaemia and hematotoxicity.

this, various benzene metabolites cause damage to ova, decreases fertility, and increase the risk of tumor formation among young women (Alviggi *et al.,* 2014). In NTP bioassay various reproductive tract tumors (uterus, ovary) were reported (Cal/EPA, 1997).

Benzene also passes into umbilical cord of pregnant mother. From umbilical cord it reaches to fetus and causes abnormal development (Cal/EPA, 1997). The various adverse effects on fetal growth includes decreased birth weight, prematurity, growth retardation, spontaneous abortion, congenital diseases, and even childhood leukemia. Benzene exposure at particular stage of pregnancy affects the immune system of fetus, which becomes a major cause of childhood leukemia. Various other factors like maternal anemia lead to abnormal organogenesis in fetal development. Benzene also causes reduction in platelets (thrombocytopenia), as a result, there occur excessive bleeding (hemorrhage) during delivery. This condition could be life threatening to both baby and mother (Cal/EPA, 1997).

Male reproductive system

Recent experimental verifications reveal that males might be more susceptible than females to benzene toxicity. Long term benzene exposure among men is responsible for abnormal chromosomal number in sperm, which in turn affects fertility and fetal development (Cal/EPA, 1997). Benzene metabolites, phenol-hydroquinone and catechol, affects sperm motility, sperm viability, sperm nuclear DNA integrity and effective sperm count (Mandani *et al.,* 2013).

Sperm motility

In study conducted by Mandani *et al.* (2013), when the semen sample is exposed to phenol- hydroquinone significant decrease in sperm motility was observed than in normal sample. Another semen sample was exposed to catechol, again decrease in sperm motility was observed but less than by phenol-hydroquinone.

Sperm viability

Similarly, sperm viability was tested in two phases. In phase 1 spermatozoa were exposed to phenol-hydroquinone and in phase 2 spermatozoa were exposed to catechol (Mandani *et al.,* 2013). At lower concentration catechol reduces sperm viability greater than phenol hydroquinone.

Sperm nuclear DNA integrity

In the same research study, it was found that the number of spermatozoa with intact double stranded DNA was significantly decreased when exposed to phenol-hydroquinone and catechol (Mandani *et al.,* 2013). Hence, benzene exposure significantly reduces sperm DNA integrity.

Effective sperm count

On benzene exposure, it was also found that there was decrease in effective sperm count as compared to sperm density (Mandani *et al.,* 2013).

In all the above ways, benzene decreases fertility and also causes developmental effects. Reduce in quality of sperm lead to abnormal fetal development and various birth defects.

Cardiovascular disease risk associated to benzene exposure

Urinary metabolites like trans, trans-muconic acid and acrolein increase with increase of benzene level in blood. These metabolites were found to be responsible for causing cardiovascular effects. With the increase in these metabolite level circulating angiogenic cells (CACs) decrease and as result cardiovascular disease risk increases (DeJarnett *et al.,* 2014; O'toole *et al.,* 2010).

Research study conducted by Abplanalp *et al.* (2017) reported that every 20 (parts per billion) ppb increase in benzene increases cardiovascular mortality by 33%. Increase in benzene exposure marks the onset of acute myocardial infarction due to the suppression of CACs (Abplanalp *et al.,* 2017).

Hepatotoxicity due to benzene metabolism

Liver is primary organ for metabolism of benzene and toxic effect of these metabolites on liver is reported in initial pilot study. Higher levels of liver functioning enzymes alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase were observed in this study. Increased level of these enzymes in serum indicate the impairment of liver functions (D'Andrea & Reddy, 2014; 2016). The toxicity profile of benzene over the various parts of body is summarized in the Table 2 given (ATSDR, 2014).

Current legislative of benzene

Several government agencies regulate benzene levels and exposures. The Occupational Safety & Health Administration (OSHA) is the federal agency responsible for health and safety regulations in most workplaces. OSHA limits exposure to benzene in the air in most

Table 2. Benzene exposure and Health effects.

S.N.	Health effects	Description	Concentration of exposure	Time
1	Leukemia & hematoxicity	Aplastic anemia, pancytopenia, dysplasia, AML	2 ml	Years
2	Reproductivity failures	Irregular menstruation, damage to ova, reproductive tract cancer, decreased sperm count, viability and motility	–	–
3	Cardiovascular	Ventricular fibrillation, artherosclerosis	1000 ppm	Hours
4	Hepatotoxicity	Impairement of liver functions	–	–
5	Central nervous system	Vomiting, dizziness, sleepiness, convulsions, rapid heart rate and coma	300 ppm	60 minutes
6	Death	–	10 ml (8.8 g)	Minutes

workplaces to 1 ppm during an average workday and a maximum of 5 ppm over any 15-minute period. When working at potentially higher exposure levels, OSHA requires employers to provide personal protective equipment such as respirators. The Environmental Protection Agency (EPA) limits the percentage of benzene allowed in gasoline to an average of 0.62% by volume (with a maximum of 1.3%). The EPA limits concentrations of benzene in drinking water to 5 ppb. Some states may have lower limits. Likewise, the USFDA sets a limit of 5 ppb in bottled water. The Consumer Product Safety Commission (CPSC) considers any product containing 5% or more by weight of benzene to be hazardous, requiring special labeling.

Future perspective

Looking through the doors of present research studies on the various hazardous affects caused by benzene, it becomes an essential need to carry out extensive research in the field of Ayurvedic sciences to detect its presence. Research in these areas provides a better understanding about benzene formation and its level in various Ayurvedic formulations. Although number of studies has been carried in food sciences, where the preserved foods consist of sodium benzoate along with ascorbic acid, similar studies are also required in Ayurvedic sciences. Most of the nonalcoholic syrups are added with salts of benzoate to enhance its shelf life ignoring the drastic step that take place when such syrups already consist of ascorbic acid.

An oral reference dose (RfD) for benzene set by EPA is 0.004 mg/kg/d based on hematological effects in humans. The RfD is an estimate of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious noncancer effects during a lifetime. At exposures above RfD, the potential for adverse health effects increases. Hence, the formulations that are at risk of benzene contamination should be necessarily investigated.

Various research studies are conducted on the factors that play crucial role in benzene formation by the process of decarboxylation. As even the presence of sodium benzoate and ascorbic acid do not necessarily lead to the formation until the various intrinsic and extrinsic factors favor the formation. In 2006, American beverage association recommended certain guidelines for benzene reduction. It includes removing, reducing, or replacing benzoates with other microbial growth inhibitors like potassium sorbate and checking the product storage conditions since strong light and high temperatures speed up the formation of free radicals. In case of formulations containing transition metals, the addition of chelators such as EDTA or sodium polyphosphates was recommended to reduce benzene formation.

Owing to the various serious health issues related to benzene exposure and increasing degree of relying on herbal drugs or formulations in the people for both, the purpose of health and personal needs, it becomes necessary to carry out preclinical trials and, whenever necessary, clinical trials should be carried out before the various formulations are made available over the counter.

Conclusion

Present manuscript highlights the mechanism of benzene formation in food products/formulations, factors affecting benzene formation, metabolism, toxicity and other health effects. In light of oral exposure to benzene, study reports are scarce but to clarify the risk of oral exposure and for establishing more appropriate limit for benzene in foods and drugs more experimental studies are required to be conducted and also the cumulative effect of oral and environmental exposure should also be studied for finding out the health risk increment. Presence of higher level of benzene in food can further impact their acceptability at international level and cause loss to export revenues of our country. There is a need to assess the level of benzene formation in marketed formulations so that alternative preservatives can be sorted out and regulatory authorities may issue new directions for use of alternative and safe preservatives. It is high time that regulatory authorities recognize the problem of noncompliance of GMP for ASU drugs and take steps to enforce the provisions of Drug and Cosmetics Act in letter and spirit of the law.

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ORIGINAL ARTICLE

Toxicity study of graphene-coated Poly(methyl methacrylate) membranes on the brain cortex of rats

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ABSTRACT
Graphene is a material, which has attracted great attention of the scientific community in several fields of biomedicine, neurosciences being one of the fields holding great interest for the application of graphene-based materials and devices. Our study aimed to determine the *in vivo* brain tissue reaction and to study the possible impairment of memory in a long-term Graphene exposure. Towards this aim we tested the toxicity of graphene membrane in the form of few layers (few layers graphene, FLG) implanted on the frontal brain cortex of adult Wistar rats after careful durotomy. The results from this study advance our knowledge on graphene *in vivo* toxicity in CNS and suggest that the application of FLG as a patch on the brain cortex seems to be quite safe under the experimental conditions tested, herein as no change in locomotor activity of the rats or major histopathological reactions of the brain to the material were observed.

KEY WORDS: few layer graphene; toxicity; brain; rats

Introduction

Graphene, a nanosized material of carbon allotrope has attracted much attention in the fields of physics, chemistry, biology and medicine, and their related interdisciplinarities with applications ranging from biosensors to diagnosis and from tissue engineering to drug delivery and cancer therapy. Transparency, durability, elasticity and high conductivity make graphene an attractive material for applications also in the field of neurosciences (Bitounis *et al.*, 2013; Kuzum *et al.,* 2014). Generally, graphene-based nanomaterials are classified into several types such as graphene oxide, reduced graphene oxide and graphene with varied layers (Yao *et al.,* 2012), which types also show variability in their physicochemical and biological properties.

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The majority of the biomedical studies using graphene-based nanomaterials have focused on graphene oxide, while there are only a limited number of biological studies on graphene itself (Orecchioni *et al.,* 2016). The number of layers of graphene is important because it determines the characteristics of the bioactive surface. It is expected that the adsorption capacity of biological molecules decreases significantly as the number of layers increases. For Few Layers Graphene (FLG), the thickness of every single layer is 0.34 nm. FLG consists of 2 to 10 graphene layers. Characteristics determining its biological activity are chemical purity, the bio-exposed surface, the number of layers, the cross-section and the chemical characteristics of the surface. Generally, nanoparticles sized less than 35 nm cross the blood-brain barrier (BBB), less than 40 nm can enter the nucleus and less than 100 nm can enter the cell (Mytych & Wnuk, 2013).

A crucial concern for biomedical applications is the assessment of the toxicity of the materials to be used. Despite their tremendous applications and potential in biomedicine, these materials are not devoid of toxicity.

Pristine graphene (pG) for example has been shown to exhibit toxic effects in chicken embryos and impair DNA synthesis especially in the brain of the embryos (Sawosz *et al.,* 2014). The toxicity of graphene has been extensively studied and debated in the literature. Results show that the toxicity of graphene is dependent on several factors such as shape, size, purity, post-production processing steps, oxidative state, functional groups, dispersion state, synthesis methods, route and dose of administration, and exposure times (Lalwani *et al.,* 2016).

Regarding FLG *in vitro* exposure of nerve cells to 10 μg/ml FLG, has been shown to increase the levels of reactive oxygen species (ROS) and mitochondrial lesions after 4 and 24 hours (Zhang *et al.,* 2010). Creation of intracellular ROS is the predominant mechanism of Graphene materials cytotoxicity. Other *in vitro* studies saw unaffected electrical activity and neuronal viability after exposure to Graphene (Li *et al.,* 2011, Fabbro *et al.* 2016, Bramini *et al.* 2016). Furthermore, the exposure of healthy neurons to a Graphene scaffold resulted in their development and the creation of synapses (Fabbro *et al.,* 2016). A short term (7 weeks) *in vivo* study, using colloidal Graphene coated on electrospun microfiber scaffolds, saw suppression of glial process in rat brain, which is very promising in brain injury cases (Zhou *et al.,* 2016). There are several studies focusing on *in vitro* study of Graphene but *in vivo* study is still deficient.

However, as it is clear from discussed above, very few studies are known for the toxicity of graphene placed directly in the brain of animal models for use *e.g.* in tissue engineering for brain injuries or depot formulations for the local administration and release of drugs. In this context we herein aimed to study the *in vivo* brain tissue reaction and the possible impairment of memory in long-term exposure to graphene in the form of FLG. Towards this purpose we performed durotomy and directly placed an FLG membrane on PMMA in the brains of adult Wistar rats.

Materials and methods

Materials

The chemical vapor deposition method was chosen to produce Graphene because it allows the easy transfer of material to different substrates, while produced FLG has not many defects (Miao *et al.,* 2011; Yi *et al.,* 2013). In our study, FLG was transferred on Poly (methyl methacrylate) (PMMA 495K dissolved in anisole at a thickness of 170 nm, Microchem, Newton, USA)), which is already used in different neurosurgical procedures (*e.g.* cranioplasty). FLG was produced by the Epitaxy and Surface Science (ESSL) laboratory, National Center for Scientific Research "Demokritos" (Athens, Greece). FLG was sterilized by putting the material into Phosphate buffer saline (PBS, Thermo Fisher Scientific, Waltham, USA), which was further heated at 120 °C under 1 bar pressure for 30 minutes, conditions that did not result in any changes of the FLG properties.

Durotomy and FLG placement on the brain of Wistat rats

Male 3-month-old albino Wistar rats (Hellenic Pasteur Institute, Athens, Greece) that weighted 250–300 g were used in this study. The animals were housed in Makrolon cages (47.5 cm length × 20.5 cm height × 27 cm width) three per cage, in a climate-regulated environment (21±1 °C; 50–55% relative humidity) under a 12 h/12 h (lights on at 7:00 AM) light/dark cycle with free access to food and water. All experiments were approved by the IACUC of the Faculty of Medicine, University of Thessaly and experimental procedures and handling of animals were conducted in accordance with the Greek laws (PD 56/2013 and Circular 2215/117550/2013) and to the guidelines of the European Union (2013/63/EU).

Two groups of rats were formed. In Group A we made all the procedures without using Graphene (control/sham group). In Group B we used the Graphene. Every group included 3 rats. All rats went under pre-surgical medical control and measurement of different parameters (age and weight). Only healthy animals were included in our study. One hour before surgery, 0.05% buprenorphine as an analgesic was administered.

Anesthesia was achieved by administration of mixture Ketamine (100 mg/ml)/Xylazine (20 mg/ml) at a dose of 1500 μl/200 gr of animal's weight. Animals were in complete anesthesia after about 3 minutes and anesthesia had a duration of 1–2 hours. Craniotomy took place according to the guidelines of Comparative Medicine and Animal Resources Center – SOP 202.01. (steps 4.2.2, 4.2.5–4.2.6, 4.2.8–4.3.9, 4.3.11 and 4.3.17) (Gourdon & Jimenez, 2016). The material was placed on the frontal brain cortex, after careful durotomy. Post-operative management was indicated, including subcutaneous Buprenorphine 0.05 mg/kg 2 times daily. Animals were observed for signs of pain or anxiety until they returned to their normal preoperative routine and behavior.

Locomotor activity

Spontaneous locomotor activity took place three months after the placement and was assessed in an activity cage (Ugo Basile, Varese, Italy). The apparatus consisted of a box made of Plexiglass (41 cm length × 33 cm height × 41 cm width). Both horizontal and vertical activities were recorded. The test was performed as described by Grivas *et al.* (Grivas *et al.,* 2016). On the test day, rats were transported to the test room and left in their home cages for 2 h. Thereafter, each animal was placed into the locomotor activity arena and spontaneous locomotion was recorded for 5 min. Experiments were conducted between 9:00 AM and 3:30 PM during the light phase of the light/dark cycle. To avoid the presence of olfactory cues, the apparatus was thoroughly cleaned with 20% ethanol and then after each trial wiped with dry paper.

Histopathological analysis

Three and a half months after the procedure, animals were euthanized using CO_2 and their brains were removed, [following a standard protocol (Paul *et al.,* 2008)] for further study. Tissue sections from sampled brains were fixed in

10% buffered formalin for 24 hours and were processed to paraffin blocks in an automate tissue processor (Shandon Citadel 2000, THERMOELECTRON corporation, UK). Three μm thick sections were obtained using a Leica TP1020 microtome and they were routinely stained with Hematoxylin and Eosin stain. Stained slides were subsequently examined under a light microscope.

Statistics

Data are expressed as mean ± S.E.M. and were evaluated by the Student's t-test. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

We studied FLG on PMMA substrate delivered directly on the frontal lobe cortex of the rats' brain. Post-surgical period was normal for all studied animals with no indications of toxicity or other pathological signs.

Spontaneous locomotor activity test results indicate that graphene did not induce any change in horizontal or vertical motor activity of rats (Table 1).

Total histological findings are summarized in Table 2. Histologically, the examined tissue specimens regarding Group A showed no particular changes. Among specimens from the graphene group (Group B) only one sample (rat 3) showed slight mononuclear infiltration in the subarachnoid region (Figure 1a). This sample also showed marked inflammatory infiltration with foreign-body tissue reaction most probably to suture (Figure 1b). There have been detailed descriptions of suture reactions in different studies related with the biocompatibility of various sutures materials (Anderson *et al.,* 2008; Selvi *et al.,* 2016). In our study, we used polypropylene 3-0 suture for monolayer skin closure. For that kind of material, foreign body reactions have been described previously (Anderson *et al*., 2008) and the secondary histological findings, such as subarachnoid oedema and fibrin deposition observed in this animal, might be explained in the context of this particular tissue reaction. There was an amorphous substance remaining focally in the area where the Graphene was administered in rat 1. This specimen showed an oedematous subarachnoid region with fibrin deposition without inflammatory infiltration (Figure 2). These are typical findings for traumatic subarachnoid hemorrhage caused during placement procedure (Suzuki *et al.,* 1977; Gaberel *et al.,* 2014). The cerebrum of the third rat of group B (rat 6) showed no remarkable changes (Figure 3).

Graphene [single graphite layer, consisting of a hexagonally arranged, sp2 bonded, stable two-dimensional allotrope of carbon with a plethora of unique properties (Novoselov *et al.,* 2004)] and graphene oxide have been extensively studied as some of the most promising biomaterials for biomedical applications due to their unique properties: two-dimensional planar structure, large surface area, chemical and mechanical stability, higher conductivity and good biocompatibility. Due to their advantageous properties, graphene and GO have recently emerged as new and competitive drug delivery systems with the potential to be applied for systemic targeting and local drug delivery systems. Before any clinical studies can be designed and begin, the successful design of graphene and graphene oxide-based systems requires to address some important issues for a biomedical application such as the modifications required to build an efficient carrier able to release drugs in controlable manner, but at the same time, it is required to address their biocompatibility and toxicity**.**

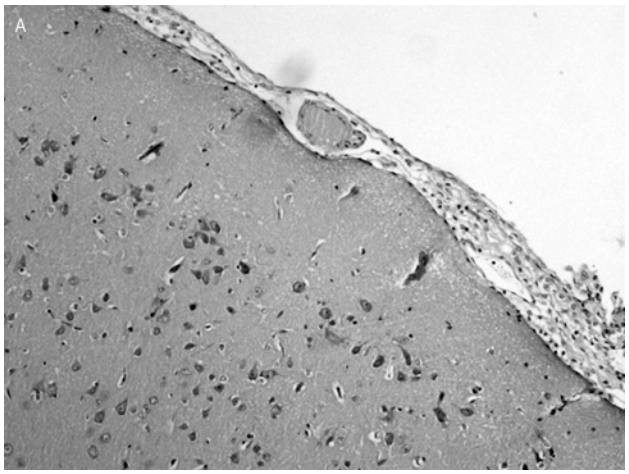
Graphene nanosheets' biocompatibility is still controversial. This is due to the high heterogeneity of materials present on the market and the large variety of synthesis methods that affect, to various extents, its interaction with the biological systems. Two studies have already pointed out that graphene nanosheets may be harmful for the environment and human health, resulting thus to a debate about their use in biomedical applications (Bramini *et al.,* 2016; Reina *et al.,* 2017). According to Amrollahi-Sharifabadi *et al*. (Amrollahi-Sharifabadi *et al*. 2018) GO nanoplatelets (GONs) in particular, induce inflammation and granulomatous reaction in various

Table 1. Effects of Graphene on rats' performance in a locomotor activity test. Group A, sham rats; Group B, rats that received graphene membrane.

Group	Horizontal activity (Counts/5 min)	Vertical activity (episodes/5 min)
A	1270.7±158.4	30.7±1.3
B	1359±106.3	32±2.1

Table 2. Detailed histopathological findings for every rat of the two groups A and B. Group A, sham rats; Group B, rats that received graphene membrane.

Group	Rats	Post-surgical observation	Histopathological findings	Conclusion
A	2	Normal	No remarkable changes.	No pathological findings
A	4	Normal	No remarkable changes.	No pathological findings
A	5	Normal	No remarkable changes.	No pathological findings
B	1	Normal	Amorphous substance remaining focally where FLG was administered. Oedema and fibrin deposition in the suburachnoid region	Chronic traumatic subarachnoid hemorrhage
B	3	Normal	Mononuclear infiltration in the subarachnoid region	Suture reaction
B	6	Normal	No remarkable changes.	No pathological findings



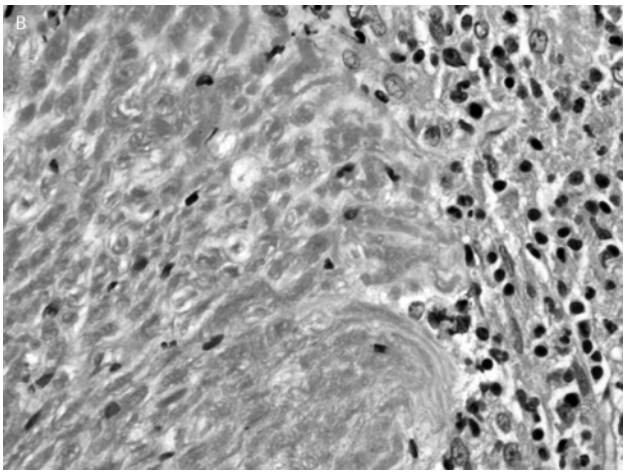


Figure 1 (a). Histology shows mononuclear inflammatory infiltration in subarachnoid region. Hematoxylin and Eosin stain, original magnification ×10. **(b).** Histology shows foreign body reaction to suture. Hematoxylin and Eosin stain, original magnification ×40. Images are representative of rat no 3, group B.

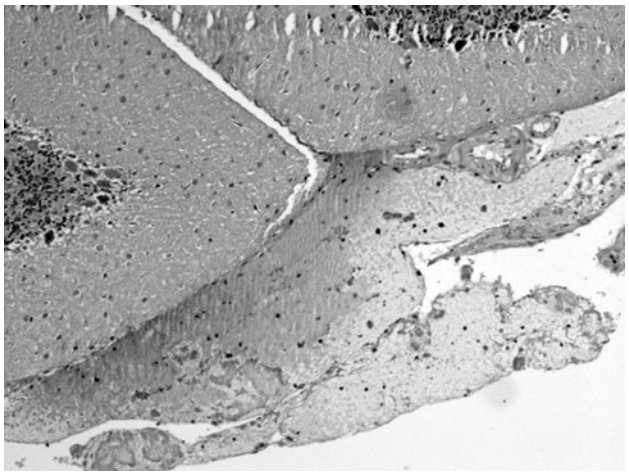


Figure 2. Histology shows oedema and fibrin deposition in an area with amorphous substance in the surface of brain. Hematoxylin and Eosin stain, original magnification ×10. Images are representative of rat no 1, group B.

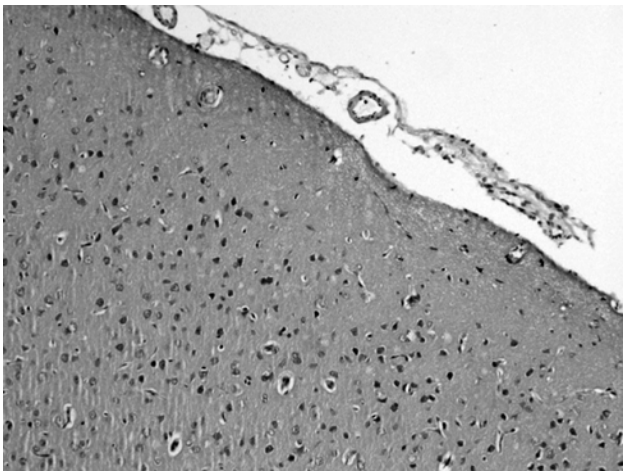


Figure 3. Histology shows normal brain tissue without inflammation. Hematoxylin and Eosin stain, original magnification ×10. Images are representative of rat no 6, group B.

organs, depending on the given dose, because of accumulation in the organism after intraperitoneal injections of the material. Based on various studies, GO appears to have the most severe inflammatory effect for the lungs among different graphene-based materials in respiratory exposure (*in vivo*) studies, from which FLG appears to have the safest profile (Duch *et al*. 2011, Schinwald *et al*. 2014, Park *et al*. 2015, Roberts *et al*. 2016, Mao *et al*. 2016, Ema *et al*. 2017, Lee *et al*. 2017, Bengtson *et al*. 2017).

In this study using the chemical vapor deposition we have produced FLG on a PMMA substrate. This procedure is considered inexpensive and produces high-quality monolayer and few-layer graphene with a low number of defects (Miao *et al.,* 2011; Yi *et al.,* 2013).

FLG is considered a very promising material in the field of biomedicine. Recently in a nice study Russier *et al.* (Russier *et al.* 2017) showed that FLG dispersions could kill specifically the monocytes in a myelomonocytic leukemia model, displaying neither toxic nor activation effects on the other cells of the immune system. Importantly the authors tested the toxicity of this FLG dispersion in C57BL/6 mice after intraocular delivery and found it safe.

Obviously, any further exploitation in the central nervous system of biomedical devices requires deep knowledge of their interactions with the neuronal tissue, toxicity being among the most important. I*n vitro* and *in vivo* toxicological studies have evaluated the interactions of graphene-based nanomaterials with various living systems and have examined the short- and long-term *in vivo* toxicity and biodistribution of various types of graphene (for a detailed review on the toxicology of graphene see Lalwani *et al.,* 2016). Toxicity of graphene derivatives has been tested in small and large animal models (mice and rats) under various modes of administration: intravenous, oral, pulmonary, intraperitoneal and even pharyngeal and intravitreal (Lalwani *et al.* 2016), though, with controversial results. Another form of the graphene materials (high biocompatible graphene quantum dots, HGQDs) has been administered *in vivo* showing a safe profile, even though it can cross the blood-brain barrier, promising

various interesting biomedical applications (Yan *et al*. 2020). However, the toxicity of graphene on the brain and especially in the form of FLG has not been reported so far. To our knowledge, only two studies have so far addressed the issue of graphene toxicity in the neural system but again not directly in the brain. One study tested reduced graphene oxide (rGO), seeing the safety of the material using it under the form of injectable dispersion in phosphate buffer saline, in a period of 30 days. The target for the injections was the adult mouse olfactory bulb (Deferal *et al.,* 2016). Other one studied graphene-polyelectrolyte multilayer onto electrospun poly-ε-caprolactone and the target was striatum of adult rats (Zhou *et al.,* 2016). Both studies showed no potential tissue toxicity. In this context, we next sought to study the toxicity of this device on the brain of living animals, in this case adult Wistar rats. The placement of FLG/PMMA membrane, after careful durotomy, was not found to result in any toxicity after more than 100 days showed by spontaneous locomotor activity tests and post mortem histological examination. A relevant study using ultra-trace concentrations of GO in the environment of zebrafish, shown significantly disturbed locomotor activity inducing Parkinson's-like symptoms (Ren *et al.* 2016). That study supports the idea of possible dose-related toxicity of graphene materials, as potential biohazards are based on the accumulation in the organism. At this point, we must underline the huge differences in the biological activity between the different types of graphene-based materials (Mytych & Wnuk, 2013) and that the toxicity of graphene is dependent on several factors such as shape, size, purity, post-production processing steps, oxidative state, functional groups, dispersion state, synthesis methods, route and dose of administration, and exposure times (Lalwani *et al*. 2016).

Concluding, our *in vivo* study is the first where the application of FLG as a patch on the brain cortex is studied regarding its safety. The results were indeed encouraging as the material was very well tolerated by the animals for more than 3 months. Extending studies, using larger cohorts and more specific applications such as the use for local drug release, would be of great interest in order of safety of FLG for use in CNS to be further certified, but its functionality as well to be tested.

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ORIGINAL ARTICLE

Effects of long term antiepileptic therapy on serum trace element levels in epileptic children

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ABSTRACT

Conventional anticonvulsants including valproate (VPA) and phenytoin (PHT) have been shown to influence the trace element homeostasis in patients with epilepsy. Even though the newer agents such as levetiracetam (LEV) are considered to have a better tolerability profile, given the lack of long-term data this confidence is somewhat guarded. In this study, we evaluated the trace element profiles of epileptic children receiving conventional agents *i.e.*, PHT and VPA and newer agent – LEV and compared them with healthy controls. The study groups based on drug treatment were as follows: Group-I (n=35) children received PHT, Group-II (n=30) received VPA, Group-III (n=27) were treated with VPA plus LEV while Group-IV (n=28) included healthy children. Serum levels of zinc, copper, selenium, strontium, magnesium, manganese, and iron were estimated using inductively coupled plasma-atomic emission spectrometry (ICP-AES). As compared to healthy controls, monotherapy with phenytoin was associated with increased serum levels of copper (1568.8 vs. 1053.6 ng/ml; p=0.002) as well as strontium (37.0 vs. 30.7 ng/ml; p=0.014). On the contrary, epileptic children treated with valproate monotherapy had decreased serum selenium (67.0 vs. 84.7 ng/ml; p=0.02) and zinc (1010.5 vs. 1242.9 ng/ml; p=0.003) concentrations. However, in the valproate plus levetiracetam therapy group, no significant alterations in the trace element status were observed. Our findings suggest a potential association between treatment with PHT and VPA and trace element changes in children diagnosed with epilepsy. However, newer agent LEV when used with VPA was not associated with these alterations. Further prospective studies are warranted to confirm our findings and to assess the impact of drug treatment on trace element homeostasis in epileptic children.

KEY WORDS: antiepileptic drugs; trace element status; phenytoin; valproate; levetiracetam

Introduction

Epilepsy and epileptic syndromes are among the most common neurological conditions in childhood and adolescence. Based on epidemiological data, the overall prevalence of childhood onset epilepsy is upto 5.5 per 1000 in developed countries and upto 44 per 1000 in developing countries (Camfield and Camfield, 2015). Higher incidence of childhood epilepsy in developing world is possibly linked to greater incidence of trauma and central nervous system (CNS) infections (De Bittencourt *et al.*, 1996; Peter & Carol, 2006). Conventional antiepileptic drugs (AEDs) are generally considered as first line drugs as they are more affordable and their long term safety profile is well characterized. The newer AEDs are indicated when patients fail to respond to or are intolerant to conventional drugs (Indian Epilepsy Society, 2008; Roy & Das, 2013). However, pharmacoepidemiological studies have indicated a progressive increase in prescription of newer AEDs to children and adolescents (Ackers *et al.*, 2007; Kwong *et al.*, 2012; Nicholas *et al.*, 2012).

Trace elements are critical building blocks in various organ systems including central nervous system(CNS) and several of them viz. zinc, copper and selenium are essential elements of enzymes involved in various metabolic processes (Ashrafi *et al.*, 2007; Frederickson *et al.*, 2012; Hunt, 1980). Alteration in the levels of trace elements and consequent deficient functioning of antioxidant defense mechanisms have been implicated in neuronal excitotoxicity, seizure recurrence as well as

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intractability (Ashrafi *et al.*, 2007; Savaskan *et al.*, 2003; Seven *et al.*, 2013). Antiepileptic drugs can possibly alter the levels of trace elements, however, the relationship has been controversial and never convincingly documented (Nazıroğlu & Mürekli, 2013). Several adverse reactions associated with AED therapy have also been attributed to alteration of these crucial elements (Armutcu *et al.*, 2004; Hurd *et al.*, 1984; Palm *et al.*, 1986; Yuen *et al.*, 1988). Studies of copper, manganese, selenium, and zinc, status in patients receiving conventional antiepileptics viz. valproate(VPA), phenytoin (PHT), carbamazepine (CBZ), and phenobarbitone have demonstrated considerable alterations in trace element levels, however, the results have been contradictory and uncertainty prevails (Armutcu *et al.*, 2004; Castilla-Guerra *et al.*, 2006; Hamed *et al.*, 2004; Kürekçi *et al.*, 1995; Kuzuya *et al.*, 1993; Suzuki *et al.*, 1992; Verrotti *et al.*, 2002). Few studies till date have evaluated the impact of newer antiepileptic drug, levetiracetam on serum trace element status in pediatric epilepsy patients. Therefore, this study was conducted to evaluate the level of a panel of nine serum trace elements namely zinc , copper , selenium , magnesium , manganese , iron, strontium (Sr), lead (Pb) and cadmium (Cd)] in epileptic children receiving conventional (phenytoin/ valproate) and newer AEDs (levetiracetam as an add-on therapy) and compare them with healthy controls.

Methods

Study settings and participants

This was a cross-sectional study that included children diagnosed with epilepsy, of either sex, aged 6–16 years, and receiving antiepileptic drug therapy for a minimum of six months, from child neurology clinic at a tertiary care institute – All India Institute of Medical Sciences – Delhi, located in northern part of India. The drugs considered were VPA and PHT – as monotherapy, and combination therapy with valproate plus levetiracetam (VPA+LEV). Subjects unwilling to provide informed consent/ assent, receiving other medications or dietary/trace element supplements, having signs of malnutrition, dietary restrictions due to a concomitant diagnosis, abnormal neurologic examination findings, intellectual disability and progressive brain disease or serious co-morbidities including severe cardiac, renal, pulmonary, hepatic or other systemic disease or cancer were excluded.

Apparently healthy children who were first degree cousins of study patients were recruited as controls. This was done to correct for socioeconomic and geographical differences that might influence the trace element levels. The Institute Ethics Committee, AIIMS approved the study protocol. The details of the study were explained prior to enrollment, with the consent form signed by all parents and assent taken from pediatric patients as well as healthy controls. The study was conducted in accordance with Declaration of Helsinki and Good Clinical Practice(ICH-GCP) guidelines.

For children with epilepsy the following information were obtained: complete medical history and clinical examination, age, gender, age of onset of seizures, prescribed antiepileptic drugs and compliance to therapy. Geographical, sociodemographic background; dietary habits; use of dietary supplements; results of biochemical and radiological investigations were also recorded. For each child, height and weight were recorded. In addition, they were evaluated for the clinical signs of malnutrition. All the participant children were seizure free for a minimum of 24 hours in the period preceding the sampling of blood. The latter was done as per the guidelines of International Union of Pure and Applied Chemistry (Cornelis *et al.*, 1996).

Estimation of trace element levels

To begin, dissolution of study samples was performed as described previously (Sarangi *et al.* 2014) using microwave assisted digestion system Following the digestion procedure clear solutions were obtained that were analyzed using Inductively Coupled PlasmaAtomic Emission Spectrometry (ICP-AES) to determine serum levels of zinc, copper, selenium , manganese , magnesium , iron , strontium , lead and cadmium. (The study samples were analyzed using equipment JY 2000-2 from HORIBA Jobin Yvon, France).

A multi-element standard solution that contained 1000 mg/L of 23 elements in 1 mol/L nitric acid was used as reference standard (Merck Chemicals, Germany). Selenium estimation was carried out using selenium standard (Sigma Chemicals, USA), separately along with concomitant metal analyzer. ICP-AES as described above and fitted with a quartz spray chamber and a cross flow nebulizer was employed throughout the estimation with the following conditions: forward power = 1200W; nebulizer flow rate = 0.76 L per min dual detector and sweep/ reading = 3, replicates = 3, dwell time = 5 seconds, and an integration time = 10 seconds. Wavelengths for each trace element were chosen from a pre-defined set using the ICP proprietary software v. 5.2. Background correction was done using a blank solution. The same batch of samples were run in duplicate for precision. The limit of detection, as detailed in the instrument manual was used for study calculations. For calibration curves, the standard mixture solution was diluted stepwise with five percent nitric acid in the concentration range of 5 to 100 parts per billion. The calibration curves had a good linearity with a correlation coefficient of ≥0.993.

Statistical analysis

Sample size was estimated using G*Power v. 3.1.3 statistical software. The mean serum levels of zinc in epileptic children receiving 6 months of valproate therapy formed the basis for calculation (Yilmaz *et al.*, 2009). For alpha equal to 0.05 and power of 90%, a sample size of 26 children per group was required for the study.

Data were analyzed using software SPSS v.16 – (Statistical Package for the Social Sciences Chicago, IL, USA). Distribution of study data was checked using

the Shapiro-Wilks method. Comparisons between study groups were made for variables following normal distribution using analysis of variance (ANOVA) test, while Kruskal Wallis test was used for variables not demonstrating a normal distribution. Appropriate post hoc analysis were carried out for statistically significant differences. Categorical variables were analyzed using Chi square test. Since the trace element levels followed a non-normal distribution, statistical comparisons were made using median and range and the level of significance was considered as <0.05.

Results

Study subjects and demographics

A total of 126 epileptic children and 36 healthy controls were screened for enrolment into the study. Out of them 92 patients and 28 healthy controls were found eligible and agreed to participate in the study (Figure 1).

Based on the antiepileptic drug therapy received by these children, they were grouped as:

- Group I: Phenytoin monotherapy (PHT, n=35);
- Group II: Valproate monotherapy (VPA, n=30);
- Group III: Levetiracetam add on therapy to valproate (LEV+VPA, n=27);
- Group IV: Healthy controls (n=28).

There were no significant differences between the four study groups with respect to age, sex distribution, age in years at onset of epilepsy, seizure-free rate and place of residence (rural/urban) (Table 1). The mean duration of therapy with study drugs was 17.97±9.26 months in phenytoin treated group, 19.20±10.23 months in valproate treated group and 12.85±6.13 months in children receiving levetiracetam treatment. The latter group also received valproate for a mean duration of 22.44±12.59 months. Analysis of Body Mass Index (BMI) of study participants in different study groups revealed a significantly higher BMI in children treated with valproate monotherapy (p<0.001) when compared with other patients as well as healthy controls (Table 1).

Serum trace element levels

The Limits of Detection (LoD) for all trace elements reported in the study were below 1 µg/L (except lead). The serum trace elements levels for children with epilepsy in the three study groups and controls are summarized in Table 2.

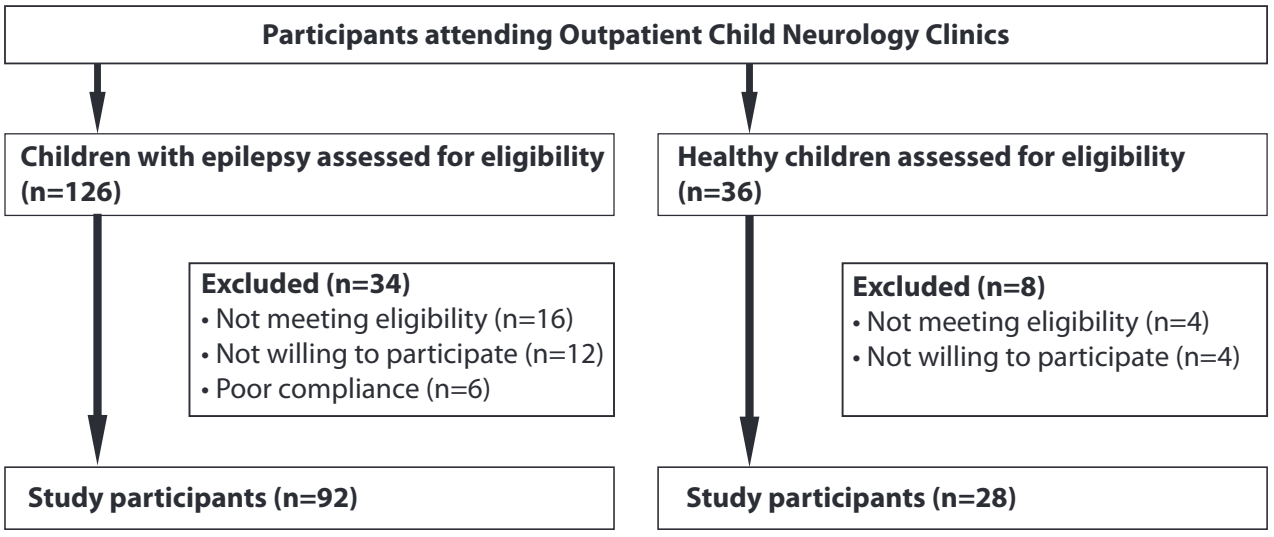

Figure 1. Flow of participants into the study.

Table 1. Demographic and clinical characteristics of study participants.

Parameter	Group I (PHT) (n=35)	Group II (VPA) (n=30)	Group III (LEV+VPA) (n=27)	Group IV (Controls) (n=28)	P value
Age(yrs)	9.43±2.48	9.9±2.74	8.78±2.75	10.02±2.61	0.24
Gender (M/F)	25/10	25/5	22/5	19/9	0.43
BMI (kg/m^2)	15.03±2.69	18.58±3.82	16.28±3.43	15.38±2.27	<0.001
Age at onset (yrs)	6.96±3.1	6.16±3.09	5.1±3.42	–	0.08
Seizure free rate (%)	71.4	60	48.1	–	0.18
Residence (Urban/Rural)	21/14	20/10	16/11	12/16	0.3

Data expressed as mean±S.D except when indicated; PHT=Phenytoin, VPA=Valproate, LEV= Levetiracetam, M=Male, F=Female, BMI= Body mass index.

Table 2. Comparison of trace elements levels among children receiving conventional and new AEDs

Elements	Group I (PHT)		Group II (VPA)		Group III (LEV+VPA)		Group IV (Control)		*p*-value (overall)	*p*-value (I vs. IV)	*p*-value (II vs. IV)	*p*-value (III vs. IV)
	Mean±SD	Median and Range	Mean±SD	Median and Range	Mean±SD	Median and Range	Mean±SD	Median and Range				
Zn	1332.9±638.1	1135.5 (537.7, 2992.2)	937.7±246.2	1010.5 (465.1, 1464.5)	1066.8±403.9	1067 (460.9, 2258.4)	1354.8±531	1242.9 (644.5, 2686.6)	0.014	0.5	0.003	0.05
Cu	1451.3±495.8	1568.8 (603.8, 2197.0)	1111.2±520.2	1100.1 (416.3, 2278.0)	1317.7±527.1	1321.2 (565.3, 1954.4)	1092.1±365.1	1053.6 (575.6, 1886.0)	0.007	0.002	0.97	0.12
Se	78.2±21.3	76.2 (36.7, 126.4)	67.9±22.7	67.0 (29.6,118.6)	81.6±21.2	78.1 (48.9, 121.9)	87.3±30.3	84.7 (35.9, 145.8)	0.04	0.25	0.02	0.52
Mn	1.6±0.6	1.5 (0.5, 3.0)	2.0±0.7	2.1 (0.5, 3.3)	2.0±0.7	2.04 (0.7, 3.61)	1.9±0.9	1.9 (0.4, 3.5)	0.03	0.15	0.29	0.43
Mg (mg/L)	17.7±4.0	18.0 (9.3, 28.4)	19.4±4.6	19.8 (12.7, 31.3)	19.1±3.5	18.4 (13.7, 27.5)	18.2±2.7	18.2 (14.1, 23.7)	0.31	0.53	0.29	0.47
Fe (mg/L)	1.6±0.5	1.5 (1.1, 2.9)	1.7±0.4	1.7 (1.1, 2.6)	1.6±0.4	1.6 (1.1, 2.3)	1.5±0.4	1.4 (1.1, 2.5)	0.10	0.95	0.06	0.27
Sr	39.5±10.6	37.0 (21.9, 75.7)	28.3±11.0	25.0 (14.9, 55.9)	25.9±11.4	24.1 (11.1, 51.4)	31.9±15.1	30.7 (11.3, 65.7)	<0.001	0.014	0.53	0.11

PHT-Phenytoin , VPA-Valproic acid, LEV- Levetiracetam, Zn- Zinc, Cu- Copper, Se- Selenium, Mn- Manganese, Mg- Magnesium, Fe- Iron, Sr- Strontium. All levels expressed as (ng/ml) except when indicated.

Serum zinc levels in the control group and in the phenytoin treated group were comparable. However, when compared to control group, the median serum zinc levels in the valproate treatment group were significantly lower. The serum levels of copper in phenytoin treated participants were significantly higher as compared to the children in the control group (p=0.002). Among the valproate treated children i.e. VPA and VPA+LEV treatment groups, copper levels were not significantly different from the healthy control group (Figure 2).

Serum selenium concentrations were unaltered among the PHT and VPA+LEV groups in comparison to controls, while the VPA monotherapy group exhibited significantly lowered selenium levels (p=0.02) (Table 2). No differences were observed in the levels of manganese, magnesium and iron in all the patient groups in comparison to the controls. Serum strontium levels in the phenytoin treated subjects were found to be higher (p=0.014) as compared to controls. However, no alterations were observed in the strontium levels in the VPA and VPA+LEV groups (Figure 2).

Toxic trace elements, lead and cadmium were below the limit of detection in most study participants. Cadmium could be detected in only two patients and one healthy control. Lack of sufficient number of valid cases precluded any statistical comparisons. However, lead was detected in all the patient groups but not among healthy controls. The differences in the mean serum lead levels in the patient groups were not significant. The correlation between duration of treatment and serum levels of trace elements was not found to be significant.

Discussion

Several studies in the past have demonstrated abnormalities in serum trace elements concentrations in epileptic patients, adults as well as children (Ashraf *et al.*, 1995; Hamed *et al.*, 2004; Sarangi *et al.*, 2014; Verrotti *et al.*, 2002; Wojciak *et al.*, 2013) and significant body of data suggests the role of trace elements in the pathophysiology of epilepsy (Ashrafi *et al.*, 2007; Dudek, 2001; Hamed *et al.*, 2004; Ikeda, 2001; Smith & Bone, 1982). More recent reports have even linked alterations in level of trace elements especially selenium and zinc with treatment response in epilepsy (Ashrafi *et al.*, 2007; Saad *et al.*, 2014; Seven *et al.*, 2013). As noted above data regarding effects of AED therapy on trace element status has been contradictory. There is limited data available evaluating the effect of newer AEDs like levetiracetam on trace element levels and the impact of classic AEDs on trace element levels other than those of zinc, copper and selenium in children with epilepsy receiving long term therapy. Due to the unavailability of standard reference ranges for trace element levels in Indian children, serum levels of these elements in study patients were compared with that of healthy children taken as controls. While alterations were noted in trace element levels in the AED therapy groups, there were important differences in between the study groups. Children treated with valproate alone had significantly higher BMI when compared with other patients as well

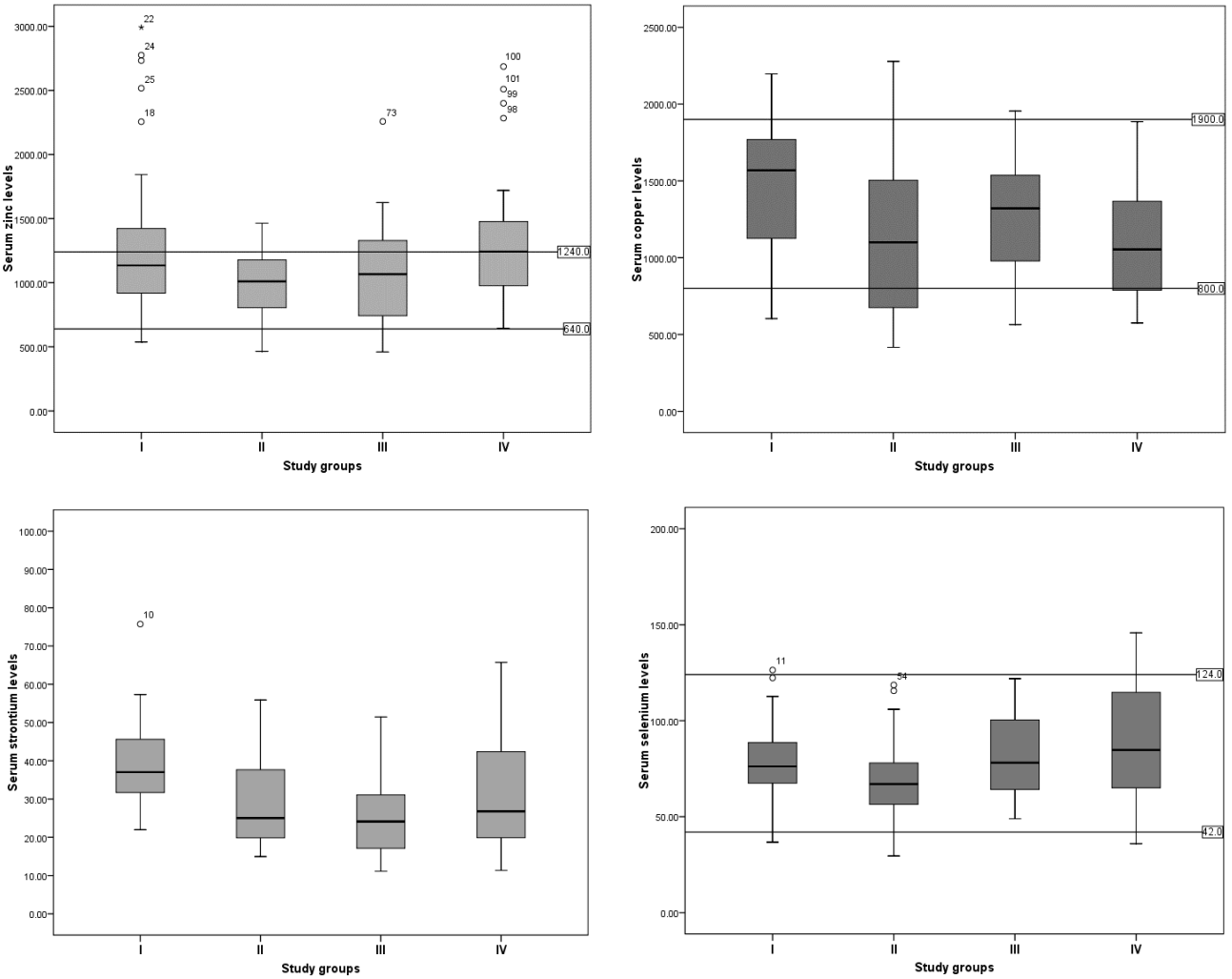

Figure 2. Serum levels of (a) zinc (µg/l) (b) copper (µg/l) (c) strontium (µg/l) (d) selenium (µg/l) among the patient and control groups. (Study groups; Group I=PHT, Group II=VPA, Group III=VPA+LEV, Group IV=Controls).

as healthy controls. This finding was expected since weight gain is a common adverse consequence linked with valproate therapy. On the contrary, both phenytoin and levetiracetam have been classified as weight neutral AEDs (Ben-Menachem, 2007; Sarangi *et al.*, 2016). In support, no significant differences were noted in the BMI of phenytoin treated children when compared to the control group. Similarly, the BMI of children treated with combination of VPA and LEV was also not significantly different from that of controls. A study by Gelisse *et al.* has even reported significant weight loss associated with levetiracetam treatment (Gelisse *et al.*, 2008).

In the present study serum zinc levels in the valproate treatment group were found to be significantly lower when compared to the healthy controls. Our results are in agreement with several other studies that have reported decreased serum zinc concentrations in adult (Kuzuya *et al.*, 1993; Steidl *et al.*, 1987; Suzuki *et al.*, 1992) as well as pediatric (Armutcu *et al.*, 2004; Deniz *et al.*, 2008; Yilmaz *et al.*, 2009) patients treated with VPA. On the contrary, few studies (Doneray *et al.*, 2012; Kaji *et al.*, 1992; Verrotti *et al.*, 2002) have failed to demonstrate any significant effect of VPA administration on serum zinc levels in children with epilepsy. The mechanisms behind reduced serum zinc levels in patients treated with VPA have not been established although it has been postulated that valproate can bind zinc, thus preventing the inhibition of glutamic acid decarboxylase (GAD) by zinc and resulting in an increased level of gamma amino butyric acid (GABA) (Hurd *et al.*, 1981). Other possible mechanisms include reduction of the total zinc concentration via binding zinc and causing its redistribution in tissues and inducing metallothionein synthesis in liver (Keen *et al.*, 1989). Several signs of zinc deficiency such as GI disturbances, taste alterations, pancreatitis, hepatotoxicity, hair loss, stupor, lethargy, tremor and anorexia resemble adverse effects reported with VPA therapy (Lerman-Sagie *et al.*, 1987). In addition, reported association between maternal valproate intake and neural tube defects in newborns could be possibly ascribed to zinc deficiency since zinc deprivation has been shown to be teratogenic in animals (Robert *et al.*, 1984). In contrast, in children receiving levetiracetam add on therapy, serum zinc levels were maintained despite concurrent treatment with VPA.

No significant differences were noted between serum copper levels of epileptic children receiving VPA monotherapy or VPA+LEV therapy when compared to healthy controls. In epilepsy patients treated with VPA, all kinds of alterations in serum copper levels – increase, decrease, and no change, have been previously reported (Doneray *et al.*, 2012; Kaji *et al.* 1992; Kürekçi *et al.*, 1995; Kuzuya *et al.*, 1993; Verrotti *et al.*, 2002). Our results are in agreement with those of Verrotti *et al.*, Kurekci *et al.*, Smith and Bone and Hamed *et al.* Experimental studies in rodents have reported VPA induced increased biliary flow and enhanced biliary excretion of copper as possible causes for decrease in serum copper levels (Kuzuya *et al.*, 2002). However, it has been argued that chronic treatment with VPA despite increasing biliary excretion of copper, may not induce copper deficiency in epileptic patients and that specific oxidase activity of ceruloplasmin may be a more useful biomarker of copper status in such individuals (Tutor-Crespo *et al.*, 2003). A significant increase in serum copper levels in epileptic children treated with phenytoin was observed as compared to healthy controls. An increase in the liver ceruloplasmin synthesis through enzyme-induction by phenytoin has been suggested as a possible mechanism (Palm *et al.*, 1986; Tutor *et al.*, 1982).

Serum selenium levels were reduced in valproate monotherapy group when compared to healthy children but not in the PHT and VPA plusLEV treated children. Few studies in the past have reported unchanged serum selenium levels in patients receiving AED treatment (Kürekçi *et al.*, 1995; Verrotti *et al.*, 2002). However, Hurd *et al.* reported reduced plasma selenium levels in rats treated with VPA and replicated these results in epilepsy patients as well (Hurd *et al.*, 1984). They proposed that valproate associated hepatotoxicity occurring in children could be due to decreased selenium concentrations and consequently reduced glutathione peroxidase (GSH-Px) activity. In our study VPA monotherapy was associated with reduced selenium levels, however none of the participants reported hepatotoxicity.

Strontium levels were significantly higher in the PHT treated children as compared to controls. It has been postulated that since strontium accumulates significantlyin bone, it can interfere with the normal bone development when present in high concentrations. Several studies have shown strontium to be associated with osteomalacia (Cohen-Solal, 2002; Oste *et al.*, 2005). Experimental studies in rats have demonstrated defective mineralization reflected by a decreased bone mineral density on exposure to high doses of strontium (Morohashi *et al.*, 1994). Phenytoin has been associated with osteomalacia as well as osteoporosis. The mechanisms for adverse consequences on bone health are unclear and several plausible mechanisms have been proposed (Pack *et al.*, 2004). Our results demonstrate increased strontium levels associated with phenytoin therapy which may contribute to the bone disease seen with long term phenytoin therapy.

The use of levetiracetam as an add-on treatment for epilepsy in children in our study is in sync with routine clinical practice at the time study was conducted. Treatment with LEV was not associated with any significant trace element alterations in epileptic children as compared to the control group. Possible mechanisms of alteration of element levels include enhanced absorption of metals from gut and/or their reduced elimination from body. LEV by virtue of having minimal metabolism, minimal protein binding, lack of dependence on and interaction with CYP P450 system is less likely to affect trace element status on account of its pharmacokinetics (Patsalos, 2000).

Our study has few limitations including a cross sectional design and lack of previous data on various trace elements in epileptic children which precluded accurate sample size calculation. Precise clinical interpretation of our study results depends, to a large extent, on the use of reference intervals. For various reasons including relatively smaller blood volumes and parental reluctance to allow blood to be drawn from healthy children, there has been a paucity of data for determining trace element reference intervals in children. Serum levels of trace elements across populations may vary widely on account of nutritional factors, industrial and environmental exposure, geographical differences and factors affecting soil and water conditions. Since these crucial elements play a vital role in child health, any unwanted alterations with AED therapy can not only affect the treatment response but may also contribute to significant adverse effects. Well-designed large scale prospective studies comparing levels of the trace elements before and after initiation of treatment in children with epilepsy are needed to provide conclusive evidence of impact of AED therapy on trace element status.

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ORIGINAL ARTICLE

Do the somatic parametres in slovak newborn on the 1st day of life overlap the Fenton curve?

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ABSTRACT
Gestational age, birth weight and birth length are basic somatic characteristics of each newborn. To compare the obtained data with the weight and growth curves according to Fenton 2013 (Fenton, 2013) and to analyze the occurrence of some risk factors (nicotinism, hypertension, gestational diabetes and diabetes mellitus) in mothers. The monitored group of newborns consisted of 2331 newborns admitted to the Neonatal Department of Intensive Medicine in 2012-2016 (n=2331). The number of premature newborns was 1061 and term newborns were 1270 (mean birth weight: boys – 2645.2 g, girls – 2443.79g; average birth length: boys – 45.82 cm, girls – 44.71 cm; spontaneous delivery: 1282 newborns, delivery of caesarean section: 1049 newborns). We found that both graphical representations overlap each other. We did not notice significant differences in the values of the monitored parameters. In our group of prenatally hypertrophic newborns above 97th percentile in 72 newborns were confirmed 8 cases of diabetes mellitus or gestational diabetes in the mother. In the analysis of the prenatal hypotrophic newborns below the 3rd percentile we found 71 hypotrophic newborns (6 cases with severe hypertension, 7 cases with multifetal pregnancy, 3 mothers during pregnancy smoked more than 20 cigarettes daily). The obtained result is based on the fact that data from the European population of newborns were also the basis for drawing up the Fenton graphs. We have confirmed the difference in birth weight and gender.

KEY WORDS: newborn; birth weight; birth length; charts

Introduction

The development of an individual, since its prenatal age, has been studied for many years. Somatic factors are influenced by the development of humanity and by the individual's living conditions. Gestational age, birth weight and birth length are basic characteristics of each newborn.

The term newborn weighs 3 200 grams on average, newborns weighing less than 2 500 grams are referred to as newborns with low birth weight and neonates over 4 000 grams are referred to as hypertrophic. After birth, a physiological weight loss occurs between 100 and 300 grams (5–10%). The weight gain occurs within 2 weeks.

The length of newborn is 45 to 50 cm with a median of 50.5 cm for boys and 49.9 cm for girls. During the first year, the height will increase by about half the birth length, in the second year it will increase by 12 cm per year, up to five years about 7 cm per year and later 5 to 6 cm per year until puberty.

We can use different physical methods for measurement of the length and weight. To assess individual growth and development we use standards, the average values obtained by examining a larger set of healthy children. Norms are obtained using two mathematical-physical methods: obtaining average values and standard deviation or percentile method. Based on the data obtained from the statistical evaluation of large groups of children, tables and diagrams are produced. Tables with the so-called the standard deviation score (standard deviation, SDS or Z score) uses the average and standard deviation method. The deviation is a measure of the scatter of the examined character in the array around the diameter, and indicates how much the values may differ from the mean to still be considered normal. Values outside this variance are

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considered to be below or above average. We compare the value of the tracer with the average value for the age in the tables and divide the difference between them by the standard deviation given in the tables for that age. We use growth/weight charts in the percentile network for long-term growth tracking (Košťalová & Kovács, 2005).

The percentile method is a method for assessing somatic indicators. The given dimension is expressed in percentages appropriate for a certain age (value 3rd, 16th, 25th, 50th, 75th, 84th, 97th percentiles, or integer 10 and 90 instead of 16 and 84). The percentile method is based on the evaluation of the set of children. On the one side the graph/table starts with the lowest values of the character and on the other side it ends with the highest values of the monitored character. The interval of the 3rd to the 97th percentile corresponds to the value of the mean ± 2 sigma. Every gender has its own tables.

A gestational week affects the development of a newborn and his/her somatic factors. Scientists have long been comparing the results of an individual's development at different locations in the world (Schack-Nielsen *et al.*, 2006; Odlind *et al.*, 2003; Skjaerven *et al.*, 2003). Generally accepted standards comprehensively list charts according to Fenton (Fenton, 2003; Fenton, 2013). The time update, which data were collected from 1991–2007, was published in April 2013. The group of children consisted of 3 986 456 newborns ranging from the 20th week of gestation to the term individuals. This sample was composed only of healthy newborns from Germany, Australia, Canada, the United States of America, Italy and Scotland (Fenton, 2013). The goal of creating Fenton charts was to unify the growth and weight curves with World Health Organization (WHO) growth standards and for better expression of the gestational week (Fenton, 2013).

Material and methods

The monitored group of newborns consisted of 2331 newborns admitted to the Neonatal Department of Intensive Medicine at the Faculty of Medicine of Comenius University in Bratislava (NKIM LF UK) in 2012–2016 (n=2331).

Selected somatic signs (birth weight in grams, birth length in centimetres, gestational age in weeks) were obtained by retrospective analysis of newborn redundancy reports. We used the pivot tables and charts for the analysis and summary. The aim of our work was to compare the obtained data with the weight and growth curves according to Fenton 2013 (Fenton, 2013). Statistically, we processed the data file in Microsoft Excel.

Results

The monitored set consisted of 2331 newborns, of which premature newborns were 1061 and term newborns were 1270 (mean birth weight: boys- 2645.2 g, girls- 2443.79 g, average birth length: boys- 45.82 cm, girls- 44.71 cm; spontaneous delivery: 1282 newborns, delivery of caesarean section: 1049 newborns) (Figures 1, 2). The division of newborns according to their maturity and gender is shown in Table 1.

In a detailed analysis of the hypertrophic newborns over the 97th percentile for age and gender according to Fenton we confirmed 72 hypertrophic newborns (in 8 cases, diabetes mellitus or maternal gestational diabetes was confirmed).

In a detailed analysis of prenatal hypotrophic infants below the 3rd percentile for age and gender according to Fenton, we found 71 hypotrophic newborns (6 cases had severe hypertension in 7 cases- multifoetal pregnancy, in 3 cases- nicotinism in mother).

Discussion

The prenatal development is a significant period of human life. Development of the newborn may be accompanied by various complications reflected on its health and selected somatic parameters such as birth weight and birth height.

Fenton charts express the interdependence between birth weight and height and gestational age of the newborn, and are then divided by gender (Fenton, 2013).

We compared the data on the birth weight and the birth length with the weight and growth curves according to Fenton from 2013. We found that both graphical representations overlap each other. We did not notice significant differences in the values of the monitored parameters. We assume that the obtained result is based on the fact that data from the European population of newborns were also the basis for drawing up the Fenton graphs. We have confirmed the difference in birth weight and gender.

Table 1. Division of newborns according to maturity.

	Premature newborns				Term newborns	
	late term (≥36+6 – <37 g.w.)	late preterm (≥31 – <36 g.w.)	very premature (≥27 – <31 g.w.)	extremely premature (<27 g.w.)	term delivery (≥37 – <41 g.w.)	postterm delivery (≥41 g.w.)
boys	93	255	155	88	691	77
girls	65	226	113	66	451	51
total	158	481	268	154	1142	128

abbreviations: g. t. – gestational week

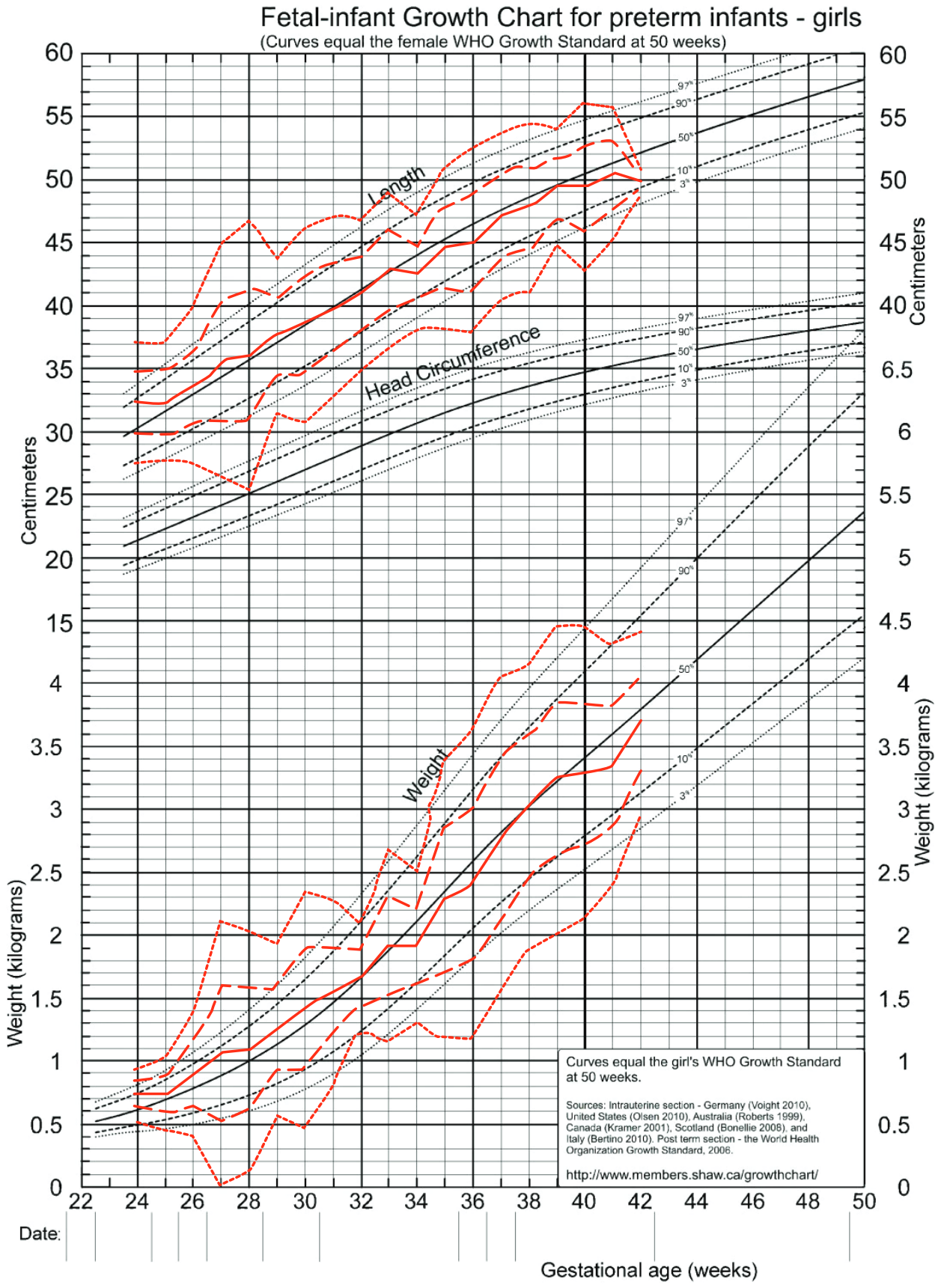

Figure 1. Comparison of results with Fenton's chart for girls

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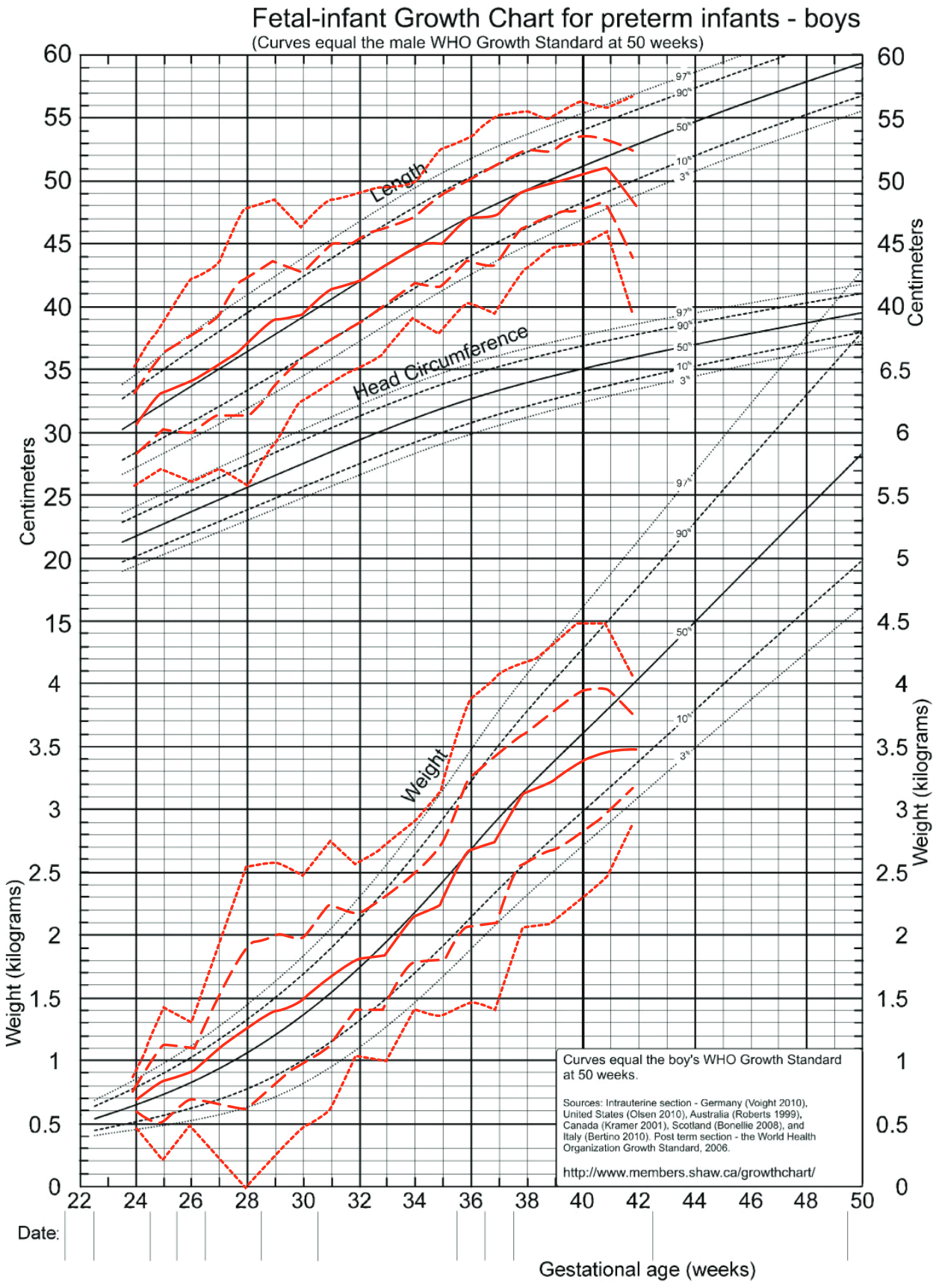

Figure 2. Comparison of results with Fenton's chart for boys

Risk factors can significantly affect an individual's development. Fetal growth during intrauterine development is affected by a number of factors: genetic features of each individual, racial and ethnicity, external factors of the environment (radiation, social conditions), maternal diet (nutrition, diet), maternal disease (diabetes mellitus, chromosome anomalies, multifetal pregnancy, infection, hormones, different blood groups and Rh factor), placenta (too small placenta, insufficient function, reduced oxygen, vitamins and nutrients supply).

In our work we analyzed the occurrence of some risk factors: nicotinism, hypertension, gestational diabetes and diabetes of mothers, which could be the cause of severe prenatal hypertrophy or hypotrophy. Our findings of the incidence of risk factors for prenatal hypertrophy are identified with results of Brucknerová (Brucknerová, 2014). In our group of prenatally hypertrophic newborns above 97th percentile for age and gender according to Fenton, in 72 newborns were confirmed 8 cases of diabetes mellitus or gestational diabetes in the mother. Gestational diabetes mellitus occurs most often at the intersection of the second and third trimesters. A normal amount of insulin is present in the blood and with a pregnancy course it is more difficult for insulin to pass into the cells. It is caused by the increased levels of pregnancy hormones produced by the placenta. Diabetes mellitus belongs into other risk factors. The condition of maternal metabolic compensation during pregnancy affects fetal growth.

In the detailed analysis of the prenatal hypotrophic newborns below the 3rd percentile for age and gender according to Fenton, we found 71 hypotrophic newborns, 6 cases with severe hypertension, 7 cases with multifetal pregnancy and 3 mothers during pregnancy smoked more than 20 cigarettes daily. Smoking of a pregnant woman negatively affects the development and health of the fetus directly, through the transplacental transfer of chemical substance nicotine, as well as indirectly through adverse changes in the physiological functions of the future mother, mainly by the decreased supply of oxygen to the placenta with the possibility of fetal hypoxia. This is due to the tight binding of CO to hemoglobin, which prevents the transfer of oxygen (Pregnancy and Postpartum Smoking Cessation, webpage). Maternal hypertension affects the size of blood flow through the placenta.

Several studies have shown that smoking in pregnant woman increases the risk of spontaneous abortion by up to 28%, as well as of premature birth. Children of smoker mothers have a lower birth weight (Pregnancy and Postpartum Smoking Cessation, webpage), with a negative correlation with smoking intensity. On average, one cigarette daily reduces the weight of the newborn by about 12 grams, i.e., smoking of 16 cigarettes a day leads to a reduction in the child's birth weight by about 200 grams (Wald & Hackshaw, 1996). In this respect, smoking is the most harmful in the second and third trimesters indicating the importance of ending smoking at the beginning of pregnancy. Congenital birth defects are more common compared to non-smokers, proportionately to the amount of mother's smoked cigarettes during pregnancy.

The most accurate comparison would be based on diagrams and curves for a given country, given many differences among countries in nutrition as well as between races and continents. We are currently using charts based on data processing of over 3.5 million healthy newborns from 3 continents and 6 countries. In the future, we would like to develop Slovak standards that best reflect the real characteristics of born newborns.

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