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The Effect of Benzene on the Activity of Adenosine Deaminase in Tissues of Rats

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Swiss Albino (*Rat rattus norvegicus*) rats were intraperitoneally injected with a 100 mg kg^{-1} dosage of benzene, a toxic and carcinogenic agent widely used for industrial purposes. Changes in the adenosine deaminase (ADA) activity in the liver, kidney and serum of rats were investigated at 0, 2, 4, 8, 16, 32 and 64 h following injection. Serum physiological was administered to each control group. Enzyme activities were measured spectrophotometrically. Our purpose was to further investigations of some diseases caused by benzene, and present evidence of variations in the activity of ADA enzyme effected by benzene. While benzene caused significant inhibitions in ADA activity in the liver at 16 and 32 h and at 0.05 probability level, no significant inhibition or activation occurred at other test periods (hours). ADA activity did not present any significant variation in the kidneys. It was observed that ADA activity displayed similar patterns in the control groups. Comparisons of ADA activities in the two groups showed a statistically significant decrease between 4th and 64th hours ($p < 0.05$), demonstrating a direct correlation between benzene and its effects on ADA enzymes.

Keywords: Adenosine deaminase, Benzene, Kidney, Liver, Serum

Introduction

Benzene is a volatile aromatic hydrocarbon. It is a ubiquitous environmental pollutant known to cause hematotoxicity and leukemia in humans. However, because it is an organic solvent, it is being widely used for industrial purposes. Found in paint, varnish, paint-thinner, plastic and degreaser, it is currently being monitored in many European cities and industrial centers with high-traffic densities. Risk of exposure to benzene in western European cities was recently found to be greatest indoors, where levels of benzene were reported to range from 4.5 to 12.3 μg/m^3 (0.001-0.003 ppm). Populations with direct, personal exposure range from 6.6 to 23.1 μg/m^3 (0.002-0.007 ppm). Outdoor levels were found to be between 4.4 and 20.7 μg/m^3 (0.001-0.006 ppm) (Cocheo *et al*., 2000).

The major metabolites of benzene metabolized in the liver are: phenol, hydroquinone, and catechol. They and other intermediate products are transported to target tissues by the hepatic portal veins, causing severe direct and indirect damage (US EPA, 1998). Recent studies conclude that intermediate products appear because of benzene metabolism, which produce greater toxicity than benzene itself (Snyder *et al*., 1993; Albertini *et al*., 2003).

Humans with acute inhalation exposure to benzene may experience drowsiness, dizziness, headaches and a host of eye, skin, and respiratory tract irritations. At higher levels, people may even fall unconsciousness. In occupational settings, chronic inhalation exposure has caused various blood disorders, including aplastic anemia and a reduction of red blood cells. Effects on reproduction systems have been reported in women exposed to high levels via inhalation. In animal tests, adverse effects on the developing fetus have also been observed (US EPA, 1998). An important health concern is the development of haematological disorders, resulting from exposure to ambient air pollution from motor vehicle exhaust (Wester *et al*., 1986; Kaneko *et al*., 1997). Benzene is a hepatotoxin causing changes to membrane phospholipids bi-layer properties (Engelke *et al*., 1993). The age of the animals and the level of dosage may result in either an increase in the synthesis of cytochrome P-450 (Arinc *et al*., 1991) or suppression of same (Plewka *et al*., 1993). Benzene microsomal metabolism plays a critical role in benzene toxicity (Sukhodub and Padalko, 1999). Benzene, being a lipid soluble, is transported through the blood and absorbed by red cell membranes. It tends to accumulate in tissues with high lipid content and about 50% of the absorbed dose is eliminated, unchanged, through exhalation. The remainder metabolizes in the liver, primarily by the cythochrome P-450 2E1 systems (Snyder and Hedli, 1996).

Adenosine deaminase (ADA, EC 3.5.4.4) is a key enzyme

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involved in the metabolism of adenosine. Purine nucleotides are degraded by a pathway in which the phosphate group is lost by the action of 5'-nucleotidase. Adenosine is then deaminated to inosine by ADA. Inosine is hydrolyzed to yield its purine base hypoxanthine.

Various diseases characterized by the alteration of cell-mediated immunity, such as rheumatoid arthritis, systemic lupus erythematosus and tuberculosis, are effected by the serum activity of ADA, making ADA a non-specific marker of cell-mediated immunity (Ungerer *et al*., 1992). Its main physiologic activity is related to lymphocytic proliferation and differentiation. As a marker of cellular immunity, its plasma activity is found to be elevated in diseases in which there is a cell-mediated immune response (Galanti *et al*., 1981; Piras *et al*., 1982). High serum ADA activities were observed in patients with acute hepatitis, alcoholic hepatic fibrosis, chronic active hepatitis, liver cirrhosis and hepatoma (Kobayashi *et al*., 1993). It has been suggested that monocyte-macrophage cell system or lymphocytes contribute to changes in serum ADA activity (Zuckerman *et al*., 1980; Ungerer *et al*., 1992). The human immune system's ability to function properly is impaired by the deficiency of ADA, resulting in severe combined immunodeficiency (SCID) characterized by severe T lymphocyte dysfunction and agammaglobulinemia (Giblett *et al*., 1972; Hirschhorn, 1990).

ADA plays an important role in controlling adenosine levels. However, it is known to be an important enzyme in the maturation and function of T lymphocytes. Its main physiological activity is related to lymphocytic proliferation and differentiation. The enzyme activity increases substantially during mitogenic and antigenic responses of lymphocytes, and conversely, ADA inhibitors inhibit lymphocyte blastogenesis. It is also known that ADA activity is higher in T cells than in B lymphocytes (Galanti *et al*., 1981).

The deficiency of ADA causes a build-up of adenosine and 2 deoxyadenosine in lymphocytes. This build-up of 2 deoxyadenosine changes d ATP stored in lymphocytes. In this case, DNA synthesis is inhibited by the ribonucleotide reductase effected by allosteric. A correlation was found between hemolytic anemia and an increase of enzyme activity. Some leukemia diseases and Acquired Immunodeficiency Syndrome (AIDS) may cause the development of abnormalities of ADA, as well (Oteyza *et al*., 1996; Chan *et al*., 2005). It has been suggested that changes in activity of ADA may be an indicator for chemotherapy (Cristalli *et al*., 1994; Maggirwar *et al*., 1994).

Our study investigated the effects of benzene on activity of adenosine deaminase, which is an important enzyme of purine metabolism, in serum, liver, and kidney of rats. ADA activity can change and interact with different molecules. In an effort to assist studies investigating benzene, we identified variations in the activity of ADA enzyme, its role on the differentiation and maturation of lymphoid system cells, and its side-effects, such as its possible inhibition of DNA synthesis.

Materials and Methods

Wistar rats (*Rat rattus norvegicus*), weighing 200-250 g, were used. Animals were obtained from the laboratory of experimental animals at Uludag University. For each trial period, four rats from the control group and eight from the experimental group (being a total of 84 rats for the entire experiment), were used. Control groups were treated with serum physiological while experimental groups were injected intraperitoneally with a dosage of 100 mg kg^{-1} of benzene using 1 ml sterile injectors. The rats were left without food and water for 24 h before injection, ensuring the start of metabolism of animals in both groups at the same time. Following injection, food and water were regularly given to the animals until the trial periods were completed. Animals were killed, via cervical dislocation, at 0, 2, 4, 8, 16, 32 and 64 h after injection. Livers and kidneys were quickly removed. Blood, obtained from the heart, was centrifuged in a Nüve NT 201 at 12000 rpm for 15 minutes. Both liver and kidneys perfused in ice-cold 0.15 M KCl. The homogenates were prepared after the addition of ice-cold 0.15 M KCl (1/3 mass/volume) in a glass homogenizer with a Teflon pestle, and were homogenized with 4 shots at 1500 rpm in a T-line laboratory stirrer (model No: 136-2) type homogenizer. Homogenization was made in ice. Each homogenate was centrifuged in a Dupont Instruments Sorval "RC-5 super speed refrigerated centrifuge" at 48000 g for 30 minutes. The enzymatic reaction rates were determined with these freshly made preparations. Centrifuging and homogenizing at 0-4°C were done with great care. ADA activities were estimated spectrophotometrically using the Bohringer Manheim (1973) method. Protein concentration was determined using the biuret method (Varley *et al*., 1980). Bovine serum albumin was used as a protein standard. Statistical analysis was performed using the SPSS for Windows V11 computer statistics program. All data displayed a normal distribution. Results are presented as mean ± standard error. p values < 0.05 were regarded as statistically significant. Pearson correlation analyses were used to determine relationships between variables.

Results

The effects of benzene on ADA activity in liver, kidney and serum are given in Fig. 1. The statistical and specific activity data are presented in Table 1.

Similar ADA levels in the liver tissue were seen in both control and experiment groups. When the effect of benzene on ADA enzyme in liver tissue in the experimental groups was compared with the control groups, there was little variation in values at 0, 2 and 4 h. A statistically insignificant inhibition started at the 8^{th} h. However, this inhibition was statistically significant at 16 and 32 h ($p < 0.005$). Even though ADA levels at the 64^{th} h indicated some inhibition, with regard to control groups, this inhibition was found to be statistically insignificant ($p > 0.05$). When the effect of benzene on ADA enzyme in kidney tissue was monitored, more or less the same activation values were observed at 0, 2, 4, 8 and 16 h.

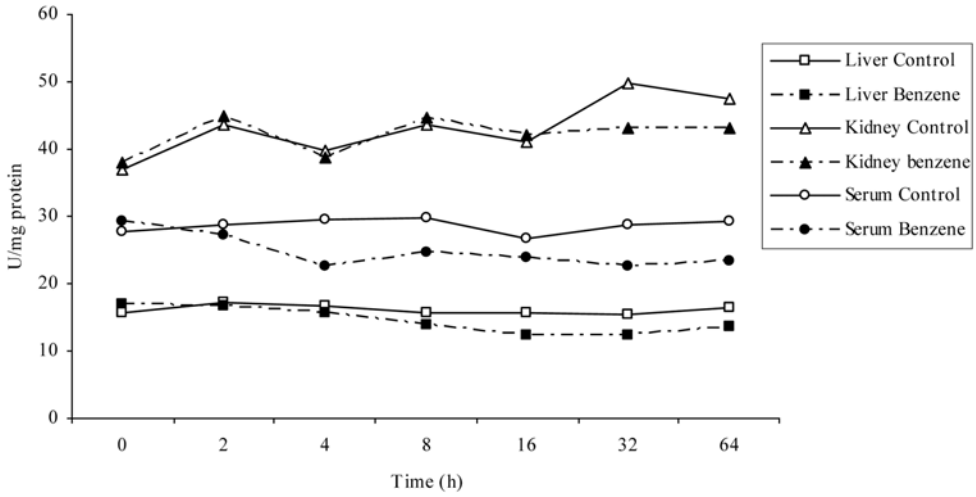

Fig. 1. The effect of benzene on the ADA activity in the tissues of liver, kidney and serum.

Despite the fact that some inhibition was observed at 32 and 64 h test periods, they were found out to be statistically insignificant ($p > 0.05$). Conversely, no significant difference was detected at 0 and 2 h for serum ADA activation. Statistically significant inhibitions were seen in other periods ($p < 0.05$) (Table 1). ADA enzyme's activity was similar in control groups, although they were activated or inhibited when compared with the control groups in all periods.

In groups where serum physiologic was applied, liver and kidney ADA activity values were compared at the same hours. Kidney activity values were observed to be 2 to 3 times higher than those in the liver. Similar results were noticed in groups where benzene was applied. However, the variations between the ADA activity values of both tissues gradually increased at 8, 16 and 32 h (Table 1). Kidney ADA activity values were much higher than those of liver's.

Discussion

In developing countries, problems occur as a result of ignorant usage of natural resources, rapid industrialization, upsurge in population and ill-shaped urbanization. Rapid development causes excessive accumulation of substances harmful to organisms in nature; reaching humans through the food chain, and magnifiying their effects. Research into understanding the magnitude of the situation threatening our health and lives has accelerated in the last few years and studies to produce preventive measures have begun.

Although benzene has documented harmful effects, numerous countries still endorse its use, as it is frequently needed in technological, industrial, and scientific areas. Past studies indicate that, rather than the benzene itself, it is the by-products, and their inter-relationships, which cause toxicity

Table 1. The change in ADA activities in liver, kidney tissues and serum of control and benzene-treated group animals with respect to time

Time Hours[r]		0 Mean ± SE	2 Mean ± SE	4 Mean ± SE	8 Mean ± SE	16 Mean ± SE	32 Mean ± SE	64 Mean ± SE
Liver	Control	15.69 ± 0.38a*x**	17.27 ± 0,95ax	16.55 ± 0.88ax	15.74 ± 0.83ax	15.68 ± 1.28ax	15.42 ± 0.23ax	16.31 ± 1.19ax
	Benzene	16.96 ± 0.50ax	16.72 ± 1.12abx	15.63 ± 0.45abcx	13.95 ± 0.46bcdx	12.24 ± 0.78dy	12.30 ± 0.46dy	13.70 ± 0.35cdx
Kidney	Control	36.95 ± 1.60ax	43.54 ± 0.45abx	39.84 ± 2.0abx	43.58 ± 2.50abx	41.08 ± 0.70abx	49.85 ± 1.20bx	47.52 ± 3.30abx
	Benzene	38.07 ± 1.0ax	44.88 ± 1.8ax	38.59 ± 1.40ax	44.74 ± 1.50ax	42.08 ± 0.70ax	43.07 ± 0.75ax	42.98 ± 1.80ax
Serum	Control	27.60 ± 2.7ax	28.81 ± 1.5ax	29.52 ± 1.0ax	29.86 ± 0.9ax	26.73 ± 2.5ax	28.72 ± 1.7ax	29.35 ± 2.2ax
	Benzene	29.20 ± 0.8ax	27.23 ± 2.4ax	22.51 ± 1.2by	24.49 ± 2.0by	23.81 ± 1.4by	22.56 ± 0.9by	23.44 ± 1.7by

*Data shown with the same symbols in the horizontal column are not different from each other at 0.05 statistical levels (abc)
**Data shown with the same symbols in the vertical column are not different from each other at 0.05 statistical levels (x.y)
r: All data in the table showed enzyme activities as U · (mg protein)$^{-1}$
SE: Standard Error
Control group (n = 28)
Benzene group (n = 56)

and carcinogenity (WHO, 1993; Chen and Eastmond, 1995). It was reported that the conjugated metabolites of benzene, which are formed through Phase 2 metabolic reactions, are not generally based on toxicity and they are discarded by the kidney (Henderson *et al.*, 1989). The first metabolite of benzene is benzene oxide. Furthermore, benzene oxide morphs into various compounds, such as phenol, hydroqinone, benzoqinone and pyrocatechol during metabolic oxidation of benzene (Sabourin *et al*., 1989).

The effect of benzene on ADA activity in liver tissue has similar values in both the control groups' and experimental groups' values; activations and inhibitions at 0, 2, 4 and 8 h were not statistically significant. Furthermore, liver ADA activity values were almost identical between the two groups. Beginning at the 8th h, inhibition increased and was found statistically significant ($p < 0.05$) at the 16th and 32th hours. At the 64th h, inhibition still continued, but it became insignificant with a slight drop-off (Fig. 1). Inhibition can occur with the binding of benzene or its metabolites to enzyme. In one of the studies, it was discovered that ADA can be connected to various molecules and its activity can also be effected (Koch *et al.*, 1992). There are indications that benzene, or its metabolites, can metabolize during transportion to target tissues and again in the tissue themselves, causing a reaction with nucleophilic parts of cell macromolecules. Metabolites perform their reactions with enzymes with the aid of reactive groups in them, such as epoxcite, oxepin, quinone and aldehyde. The metabolism of quinone compounds includes the reduction of one or two electrons. The reduction of a single electron leads to the appearance of semiquinone radical. This radical reduces the molecular oxygen immediately, and superoxide radicals form, followed by the creation of other reactive oxygen products (Arfellini *et al*., 1985; Smith *et al.*, 1989; Witz *et al.*, 1996). Benzene causes some changes (first increases then decreases) in LDH, ALP, ALT, AST, PK enzyme activities and estradiol, testosterone levels in different tissues of rats (Dere *et al*., 2003).

Initially, there was no significant change in the liver ADA activity; possibly because benzene detoxifies in rats very quickly. While most of the benzene is converted into conjugated metabolites, namely: phenylsulphate, phenylglucronite, phenylmercapturic acid, and discharged as a result of phase 2 metabolic reactions, small amounts of toxic metabolite are formed.

One possible reason for fluctuations in liver ADA activity, and the inhibitions at 6th and 32nd hours, is significant rates of accumulation of benzene metabolites and the direct interaction of these metabolites with ADA or their influence on ADA synthesis effecting DNA or RNA. Previous tests showed that the toxic metabolites of benzene formed covalent bonds on the proteins of liver, kidney and stomach organs, as well as binding with DNA and RNA (Snyder and Hedli, 1996; Lindstrom *et al*., 1997). Snyder *et al.*, (1987) demonstrated that ^{3}H labeled benzene metabolites were irreversibly bonded to rat liver and bone morrow proteins. Many mutagens and carcinogens acted via covalent interaction of metabolic intermediates with DNA in the target cell. Moreover, they noted that covalent bonding got stronger with an increased dose, evidenced by microsomal enzymes. While toxic metabolites - which were initially formed in small quantities - had no effect on ADA activity, the interaction between ADA and benzene metabolites could have increased as the hours passed, and they might have been the reason for a statistically significant inhibition at the 16th h. In a study to determine where benzene binds to tissues, and applied a 3.35 $\mu mol \cdot kg^{-1}$ dosage of radioactive-tagged benzene on mice and rats *in-vivo* and *in vitro* (Arfellini *et al.,* 1985). Even though the dosage applied was approximately 1/6000 of LD_{50} dosage of rats, and constituted 1/200 of the dosage we applied in our study, 22 h after the intraperitoneally injection it was identified that benzene bonded to DNA, RNA, and in tissue proteins in the kidney, lung and spleen for both mice and rats. Benzene bonded to liver tissue proteins more than DNA and RNA and its bonding to liver tissue proteins was 70% higher than in the kidneys. Covalent bond index (CBI) was approximately 10. Results of this study show that, even with nominal dosages, benzene can have an interaction with cell macromolecules in rats. When dosages are increased, it would be natural to expect a resulting emergence of this interaction at earlier hours and the occurrence of harmful effects. Another study tried to explain the interaction of benzene on the level of replication by noting that benzene metabolites inhibit topoizomerase II enzyme *in vitro* (Frantz *et al.*, 1996). Moreover, the formation of these inhibitions may be interrelated with the metabolization of benzene in liver. In our study, the fact that we were unable to see any effects of benzene on kidney seems to support this claim.

It was reported that many of these intermediate metabolites, which emerge as a consequence of the metabolism of benzene, are bonded to microsomal fractions, proteins, enzymes and they effect many hepatocides functions (Brodfuehrer *et al.*, 1990). Another study demonstrated that the metabolites forming inhibitions seen in liver ADA activity could be benzene oxide or trans, trans-muconaldehit, rather than phenolic metabolites of benzene. Researchers reported that, although the phenolic metabolites of benzene cause bone marrow damage, they are not responsible for hepatotoxiticity. They showed that conversion of the phenolic metabolites of benzene to reactive quinone forms is reduced by DT-diaphoras and carbonyl reductase enzymes. Scientists indicated that these enzymes were greater in liver than in bone marrow, and the lack of hepatotoxiticity formation was explained by the abundance of these enzymes in liver (Smart and Zannoni, 1985; Wermuth *et al.*, 1986). It should be emphasized that the inhibitions, which are seen at the 16th and 32nd hours in ADA activities, are "very limited" inhibitions. Hence, it was seen that statistically significant results surfaced as a result of miniscule differences.

At the 64th h, inhibition in liver ADA activity was statistically insignificant. For both rat and mouse specimens, no matter how radioactive labeled benzene is applied (ie: inhalation, oral

gagave, intraperitoneally injection), more than 95% of the benzene was discharged within 40-48 h. While 90% of the metabolized benzene was discharged with excreted urine, the small remainder was fecally discharged (Sabourine *et. al.,* 1987; Henderson *et al*., 1989; WHO, 1993). Therefore, we contend the reason for no statistically significant change at 64th h is the degradation of benzene. In one study, where 100 mg · kg^{-1} dosage of benzene was injected to rats it was discovered that as the level of glycogen decreased, the level of glucose became insignificant in both liver and muscle tissues of male and female rats (Ozdikicioglu and Dere, 2004). They stated that this glycogen drop off disappeared at the 64th h.

Within the control groups, benzene's effects on ADA enzyme and ADA activity values on kidney tissue were compared and no statistically significant inhibition or activation was observed. Benzene initially metabolized in liver, and later established itself in blood, bone marrow and kidney tissues as shown by the binding of intermediate metabolites with the tissues (Subrahmanyam *et al.*, 1990). Additionally, researchers explained that these metabolites have interactions among themselves in the tissues where they bonded. Thus, they play a key role in the occurrence of other, serious diseases. The binding of benzene to liver proteins was 70% higher than to kidney proteins (Arfellini *et al*., 1985). However, a contradictory situation emerges when binding to DNA and RNA is considered. This explains why no inhibition or activation occurred. It was seen that kidney ADA activity values were considerably higher than the liver values. Another study reported that kidney ADA activity was higher than liver ADA activity (Centelles *et al.*, 1987). Moreover, this difference increased a bit in groups where benzene was applied, due to the fact that benzene mainly metabolized in liver (Sabourin *et al*., 1987; Henderson *et al*., 1996). It was determined that serum ADA activity underwent inhibition after the first few hours ($p < 0.05$) (Table 1).

The effect of benzene on living organisms depends on the dosage. In a study conduced on humans, rabbits, mice and rats, with each group receiving different dosages of benzene, it was demonstrated that the quantitative differences resulted in hepatic and pulmoner metabolism (Powley and Carlson, 1999). While 9.8% muconic acid occurred when 0.5 mg kg^{-1} was intraperitonally injected into mice, this ratio decreased to 0.4% when 880 mg kg^{-1} was applied (Weisel *et al*., 1996). Nevertheless, and regardless of whether it was metabolized and/or discharged without being metabolized, the benzene ratios varied significantly depending on the application method and species type (WHO, 1993). While the discharge of 880 mg kg^{-1} dosage benzene without being metabolized in rats was 50%, this ratio was found to be 69% in mice. When 150 mg kg^{-1} dosage of benzene was intraperitoneally injected into rats, it was seen that approximately 50% metabolized. Furthermore, it was reported that the metabolized benzene ratio was high with low dosages, but, as the dosage increased, the ratio of benzene eliminated - without being metabolized - also increased (Sabourin *et. al*. 1987, Henderson *et. al*., 1989). Rickert *et. al.* (1979) showed that the elimination of benzene without being metabolized is biphasic when they applied a 1600 mg/m^3 dosage to rats for 6 h, via inhalation.

As a result, when we applied a 100 mg kg^{-1} dosage - which is 1/200 of LD_{50} dosage - we found that benzene creates a statistically significant inhibition in liver ADA activity at the 16th and 32nd hours, and does not result in any changes in kidney ADA activity. However, a statistically significant decrease was observed with serum from the 4th through 64th hours ($p < 0.05$). Benzene effects the ADA activity just like it effects many other enzymes. Workers, who work with and/or are exposed to benzene, must take precautions to prevent themselves from the harmful effects of this toxic substance. Perhaps, however, it would be more appropriate for industries to work with less toxic solvents as alternatives to benzene.

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