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Effect of homeopathic drug phosphorus 15ch on diethylnitrosamine induced liver injury in rats

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ABSTRACT

In this study, the hepatoprotective and antioxidant effect of the homeopathic drug Phosphorus 15CH on liver injury induced by diethylnitrosamine (DEN) was investigated. Thirty-two Wistar rats were separated into 4 groups, control group (K), DEN group, DEN+ Phosphorus 15CH group, and Phosphorus 15CH group. In the DEN group, rats were administered with DEN in a single dose of 70 mg/kg once a week for 4 months. For hepatoprotective studies, Phosphorus 15CH was administered daily for 2.5 months. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), total bilirubin (TB), lactate dehydrogenase (LDH) and superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) were estimated in serum samples. Administration of DEN caused a significant increase in AST, ALT, GGT, total bilirubin, and LDH. Oxidative stress parameters in DEN treated group showed a significant decrease in their levels. MDA levels were significantly increased by lipid peroxidation in DEN treated group. Based on these results, it was concluded that Phosphorus 15CH has a protective effect on DEN-induced liver injury in rats.

Keywords: diethylnitrosamine, phosphorus 15CH, homeopathy, liver injury.

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Introduction

The liver plays a very important role for the body that maintain and function the homeostasis. Among the most important functions of the liver is the elimination of toxic substances and drugs from the body. This organ is also involved in killing pathogens that enter the body. (Zaahkouk et al., 2019). Due to these functions, the liver is vulnerable to many metabolic and ischemic attacks and this vulnerability increases the risk of liver toxicity in many organisms (Higuchi and Gores; 2003; Robinson et al., 2016). Various chemicals cause liver toxicity (Bischoff et al., 2018). The most important of these hepatotoxic chemicals are DENs, which are nitrozo compounds. Nitrosamines are reduced to nitrites in the organism and show hepatotoxic, mutagenic and carcinogenic effects in humans and many animal species (Jagan et al., 2008; Tolba et al., 2015). DENs are found in processed meat and its products, alcohol products, tobacco products, various pesticides, pharmaceutical agents and cosmetics (Salau et al., 2016; Ahmed et al., 2022). DEN causes damage to the liver enzymes and this damage leads to increase in reactive oxygen species in liver hepatocytes (Jayakumar et al., 2012).

Liver diseases can be treated medically in a limited way. In addition to that these treatments have multiple side effects, they are also among the economically very expensive methods (Henson et al., 2017). Therefore, alternative and complementary treatment methods can be used in liver diseases in addition to medical treatment (Guan et al., 2013; Henson et al., 2017). Homeopathic treatment, which is one of the alternative treatment methods, is used in many diseases by various

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researchers (Ullman and Frass, 2010; Da Silva et al., 2015). For this purpose, it has been stated by various researchers that the homeopathic drug named Phosphorus 15CH is used as an adjuvant agent in oncology, liver damage, epilepsy, tuberculosis and chemotherapy (Chiaramida, 2017; Bagot, 2020). Therefore, the present study was carried out to evaluate the hepatoprotective effects of Phosphorus 15CH on DEN-induced liver injury.

Materials and Methods

Animals

Male Wistar rats weighing 200 ±20 g acquired from Center of Laboratory Animal Research, Van Yuzuncu Yil University. Animals were kept in properly aerated cages with light dark cycle of 12/12h. Rats were fed standard pellets and water ad libitum. The experimental protocol was approved on the 30th of May 2019 by the instructions of the Animal Ethics Committee in Van Yuzuncu Yil University.

Chemicals

DEN (CAS No: N0258) were purchased from Sigma-Aldrich (ISOPAC®, Germany) and the homeopathic drug Phosphorus 15CH was purchased from Boiron®, France.

Experimental design

Thirty-two rats were randomly divided into four groups, each group consisting of eight animals.

The control group (K): Normal rats subjected to standard diet and free water for 16 weeks. Animals did not receive any medication in this group.

In DEN group (DEN): Rats were administered with DEN, 70 mg/kg, intraperitoneally (IP) once a week for 16 weeks.

DEN + Phosphorus 15CH group: In this group, rats were administered DEN 70 mg/kg IP once a week for 10 consecutive weeks and 0.6 ml Phosphorus 15CH was administered orogastrically from day 1 till 16 consecutive weeks.

Phosphorus 15CH group: Rats were administered 0.6 ml of Phosphorus 15CH orogastrically from 1 to 16 consecutive weeks.

Blood and tissue samples

At the end of experimental period, animals were sacrified by xylazine (10 mg/kg, % 2 Rompun®, BAYER) and ketamine injectable anesthesia (5 mg/kg, % 10 Ketasol®, İNTERHAS). The blood samples of each animal were taken by cardiac puncture. Blood was collected in EDTA and dry tubes. Serum was separated by centrifugation (3000 rpm for 15 minutes). The livers were rapidly removed, washed in 0.9% NaCl and cold chain. The materials were stored at -20 ◦C until they were analyzed.

Biochemical analysis

Serum samples were taken out of the deep freezer 30 minutes before the biochemical analyses and brought to room temperature. AST, ALT, GGT, LDH and TB parameters were measured by chemiluminescence microparticle immunoassay using a calibrator, control and kit suitable for each parameter on an Abbott Architect I6200 SR biochemistry device in the laboratories of Van Yuzuncu Yil University Department of Internal Medicine.

Oxidative stress parameters

GSH, SOD and MDA values were measured with the Sinogenecolon and Shangai Korain Biotech brand enzyme linked immunosorbent assay (ELISA) test kits at 450 nanometer (nm) wavelength using the ELISA reader® (DAS, Italy), in accordance with the kit procedures, in the laboratories of Van Yuzuncu Yil University Department of Internal Medicine.

Statistical analysis

SPSS 25 statistical package program was used to evaluate the data. Variables mean ± standard error values were used. The Shapiro-Wilk test was used to check whether the data were normally distributed and assumption for homogenity of variance. Kruskal Wallis test was used to analyze the statistical difference between the groups. $P<0.05$ value was accepted for the significance level of the tests. Results with a P value of 0.05 and less were considered significant.

Results

Biochemical findings

The results of the analysis of biochemical parameters of the control, DEN, DEN+Phosphorus 15CH and Phosphorus 15CH groups are given in Table 1. Oxidative stress parameters of control, DEN, DEN+Phosphorus 15CH and Phosphorus 15CH groups are given in Table 2. In this study, serum ALT, AST, LDH, TB and GGT concentrations of DEN group were significantly higher than control, DEN+Phosphorus 15CH and Phosphorus 15CH groups ($p<0.05$). Serum GSH and SOD concentration levels of DEN group were significantly lower than control, DEN+Phosphorus 15CH and Phosphorus 15CH groups, while serum MDA concentration levels of DEN group were significantly higher than control, DEN+Phosphorus 15CH and Phosphorus 15CH groups ($p< 0.05$).

Discussion

The liver is one of the largest organs in mammals and this organ is located between the gastrointestinal tract and circulatory system. Due to this location, it is the organ most exposed to endogenous and exogenous

Table 1. Biochemical parameters of control, DEN, DEN+Phosphorus 15CH and Phosphorus 15CH groups. (x ± standard error).

Parameters	Control	DEN	DEN+ Phosphorus 15CH	Phosphorus 15CH
ALT (U/L)	35.8 ± 0.78 [d]	56.5 ± 2.16 [a]	47.5 ± 1.01 [b]	40.8 ± 0.58
AST (U/L)	85.0 ± 2.82 [d]	141.1 ± 6.69 [a]	119.0 ± 1.33 [b]	107.1 ± 1.65 [c]
LDH (U/L)	786.5 ± 65.93 [d]	1663.7 ± 48.14 [a]	1371.3 ± 21.48 [b]	1140.2 ± 32.70c
GGT (U/L)	1.2 ± 0.32[d]	12.5 ± 3.27[a]	1.4 ± 0.24[b]	1.3 ± 0.28[d]
TB (U/L)	0.287 ± 0.02	0.687 ± 0.03[a]	0.475 ± 0.03[b]	0.350 ± 0.04[c]

n=8*, a, b, c, d; The difference between the mean values of the same parameter displayed with different letters on the same line is important (p<0.05). There was no significant difference between groups containing the same letter (p > 0.05). DEN: Diethylnitrosamine, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, LDH: lactate dehydrogenase, GGT: gamma glutamyl transferase, TB: Total bilirubin.

chemicals (Higuchi and Gores, 2003; Xu et al., 2014; Nagy et al., 2020). Various diseases affecting the liver cause damage and resulting in inflammation and necrosis. Liver damage can occur acutely and chronically (Malhi and Gores, 2008; Xu et al., 2014). Acute injuries are seen as a result of viral agents, alcohol consumption, acetaminophen toxicity, ischemic and some other metabolic changes, while chronic injuries occur when acute injury is prolonged due to various agents (Sherlock and Dooley, 2002; Chow and Chow, 2006).

DEN is one of the substances that cause liver damage and it has been reported to have hepatotoxic effects in studies (Horng et al., 2017; Adebayo et al., 2020). DEN shows its hepatotoxic and carcinogenic effects by disrupting the structure of enzymes that provide DNA replication in the cell and by generating reactive oxygen products. In DEN-induced liver damage, hepatic cytolysis syndrome occurs. This syndrome causes elevation of cellular enzymes in the liver (Jayakumar et al., 2012; Kalaiselvan et al., 2013; N'DO et al., 2018).

Changes in the levels of parameters such as AST, ALT, LDH, GGT and total bilirubin are used to evaluate liver function (Center, 2007). These enzymes are specific indicators of liver failure and are reported to be elevated in liver diseases (Pradeep et al., 2007; Amin et al., 2017; Khan et al., 2017). At least two of ALT, AST, SDH, GDH or total bile acid (TSA) levels should be determined to determine liver damage (Boone et al., 2005).

ALT and AST are aminotransferase enzymes, also called transaminases. ALT is primarily a liver-specific enzyme and is found in the cytoplasm of hepatocytes (Liu et al., 2014). ALT is a more specific and sensitive parameter in determining hepatocellular damage in rats than AST. The increase in ALT during liver injury is usually more severe than AST because ALT has a longer half-life and the majority of AST is bound to mitochondria in cells (Kim et al., 2008; Lioudaki et al., 2011; Cui et al., 2012; Bahar et al., 2019). AST is both a cytosolic and mitochondrial enzyme and is found in liver and striated muscle, brain, pancreas and blood cells. Increases in serum AST levels indicate hepatocyte damage and its amount increases in liver diseases (Khan et al., 2017; Alsahli et al., 2021). ALT and AST levels were measured in studies in which liver damage was induced by DEN, and many researchers found

Table 2. Oxidative stress parameters of control, DEN, DEN+Phosphorus 15CH and Phosphorus 15CH groups.

Parameters	Control	DEN	DEN+ Phosphorus 15CH	Phosphorus 15CH
GSH (mg/L)	183.8 ± 2.06[a]	142.9 ± 2.450[d]	162.5 ± 1.282[c]	169.8 ± 0.743[b]
SOD (pg/ml)	2013.4 ± 10.66[a]	1929.1 ± 5.12[c]	1936.6 ± 0.145	1980 ± 1.096[a]
MDA (mmol/l)	8.69 ± 0.15[d]	10.653 ± 0.142[a]	9.798 ± 0.074[b]	9.432 ± 0.032[c]

n=8*, a, b, c, d; The difference between the mean values of the same parameter displayed with different letters on the same line is important (p<0.05). There was no significant difference between groups containing the same letter (p> 0.05). GSH: glutathione, SOD: superoxide dismutase, MDA: malondialdehyde.

elevated levels of this enzyme in the damaged groups (Bingul et al., 2013; Kalaiselvan et al., 2013; Amin et al., 2017; Sharma et al., 2021). In our study, AST and ALT levels were found to be higher in the DEN-treated group compared to the control group and this increase was found to be statistically significant. These data support the data of the related researchers' studies in which liver damage was induced by DEN (Atakisi et al., 2013; Yi et al., 2014; Kim et al., 2016; Khan et al., 2017). Similarly, the serum ALT level of DEN + Phosphorus 15CH group was lower than DEN group and this increase was statistically significant. It is thought that the increase in ALT and AST levels in rats in the DEN group indicates that DEN induced liver damage in this group. In addition, the ALT level in the DEN + Phosphorus 15CH group was lower than that in the DEN group, indicating that Phosphorus 15CH had a partially protective effect. The serum ALT level determined in the rats in the group given only Phosphorus 15CH was found to be lower than in the DEN group and this lower level was found to be statistically significant.

LDH is an enzyme found in five molecular forms in the organism and used to determine tissue damage in the organism (Ceyhan et al., 2017). It has been reported that LDH levels are elevated in diffuse hepatic necrosis and inflammation, ischemic liver diseases; and in myositis and muscle damage, hemolytic anemias and lymphosarcomas other than liver diseases (Kotoh et al., 2008; Forkasiewicz et al., 2020). In various studies in which DEN induced liver damage, an increase in serum LDH levels was detected in the damaged group (Naura et al., 2007; Pradeep et al., 2007; Jagan et al., 2008; Jayakumar et al., 2012; Kalaiselvan et al., 2013). Researchers have reported that this increase in LDH enzyme may be due to cellular damage caused by DEN damage to hepatocyte membranes and as a result, this enzyme exits the cell cytoplasm and enters the circulation. As a result of cellular damage, the enzyme leaks out of the cell and enters the circulation and its amount increases (Pradeep et al., 2007; Jayakumar et al., 2012). In our study, LDH levels in the DEN group were found to be higher in the DEN-treated group compared to the control group. These increases were also statistically significant. The data obtained were found to be consistent with the studies of the above researchers. In our study, similar to the above findings, the serum LDH level in the DEN + Phosphorus 15CH group was lower than in the DEN group and this increase was statistically significant. The fact that the LDH level in the DEN + Phosphorus 15CH group was lower than in the DEN group suggests that Phosphorus 15CH has a protective effect on the liver. In support of these data, the serum LDH level of the rats in the group given only Phosphorus 15CH was also found to be lower than the rats in the DEN group and this decrease was statistically significant.

GGT is an enzyme found in the cell membrane in the liver. This enzyme is increased in various physiological and pathological conditions, especially in carcinogenesis. GGT is used as an important marker in hepatobiliary diseases, liver damage and liver cancers (Center, 2007; Zhao et al., 2014; Kozat and Sepehrizadeh, 2017). Serum GGT levels are determined at low levels or not detected at all in healthy rats. Although it has been stated that the evaluation of serum GGT activities in rats is not very meaningful, it has been reported that increases in serum GGT activity in rats are an indicator of bile duct necrosis and GGT levels can be used as an indicator in the differentiation of bile duct necrosis from hepatocyte necrosis and cholestasis in rats (Leonard et al., 1984). It has been reported that serum GGT levels increased in diethylnitrosamine-induced liver injury studies (Jayakumar et al., 2012; Kalaiselvan et al., 2013; Liu et al., 2016; Uzunhisarcikli et al., 2016). In the present study, serum GGT levels of the DEN group were found to be higher than the control group. In our study, the increases in serum GGT levels in DEN-treated rats are thought to be due to cancer and related damage in the hepatocellular and hepatobiliary system as stated by the researchers. The findings obtained are similar to the findings of the above researchers.

Bilirubin is a yellow-green pigment released by the breakdown of erythrocytes. Bilirubin is used to determine the secretory ability of the liver and serves as an important indicator in liver diseases (Vítek and Tribelli, 2021). Bilirubin measurement is one of the markers used in liver damage (Fathy et al., 2017; Latief et al., 2019).

Serum total bilirubin levels increase in liver injury and hepatocellular carcinomas (Liu et al., 2016; Fathy et al., 2017; Latief et al., 2019). One of the reasons for the increase in bilirubin in liver injury is that the carcinogenic agent damages hepatocytes and sinusoidal cells in the liver and disrupts the reticulin network around the central vein, resulting in bleeding and increasing bilirubin (Pradeep et al., 2007; Zhao et al., 2014; Liu et al., 2016). In our study, the total bilirubin level of the DEN group was found to be higher in the DEN-treated group compared to the control group. This increase was statistically significant. The data obtained were found to be consistent with the studies of the above researchers. Similarly, total bilirubin level in DEN + Phosphorus 15CH group was lower than DEN group and this increase was statistically significant. It is thought that the increase in total bilirubin level in the DEN group is related to the

damage caused by DEN in the liver. The fact that the total bilirubin level in the DEN+ Phosphorus 15CH group was lower than that in the DEN group can be interpreted that Phosphorus 15CH may have a hepatoprotective effect. In addition, the total bilirubin level in the group given only Phosphorus 15CH was lower than that in the DEN group and this decrease was statistically significant.

Oxidative stress is shaped by the increase in the amount of reactive oxygen species produced in the body and the oxidant substances produced exceed the antioxidant capacity of the cells. The organism must keep these oxidant substances at a certain level in the body in order to maintain normal cellular functions and health (Betteridge, 2000; Buyukuslu and Yigitbasi, 2015). The defense system of the organism against free radicals and oxidant substances is provided by antioxidant substances. Antioxidant substances neutralize harmful oxygen species by preventing their reactive oxidation in the organism (Husain and Kumar, 2012). Among the antioxidants capable of destroying these free radicals are GSH and SOD. GSH is one of the antioxidant substances found in the cells of all mammals and protects the organism against oxidative stress caused by endogenous and exogenous free radicals (Aguilar et al, 2016). SOD is an enzyme that works against oxidative damage in the organism and protects the organism against the effects of free radicals and shows antioxidant activity by converting superoxide radicals into hydrogen peroxide (Pradeep et al., 2007; Kalaiselvan et al., 2013; Adelani et al., 2020). In various studies in which liver damage was induced by DEN, GSH and SOD values, which are important endogenous enzymes of the antioxidant system, were examined and these values were found to be low in the damaged group (Aly et al., 2018; Alsahli et al., 2021).

In our study, GSH and SOD concentrations were found to be lower in the DEN-treated group compared to the control group and this decrease was statistically significant. In addition, GSH concentrations were lower in the DEN+ Phosphorus 15CH group compared to the control group and this decrease was statistically significant. The GSH value in the DEN+ Phosphorus 15CH group was higher than that in the DEN group and this increase was statistically significant. In our study, the decreases in GSH and SOD concentrations are thought to be due to increased lipid peroxidation and the resulting oxidative stress. The findings obtained are consistent with the findings of researchers (Jayakumar et al., 2012; Salau et al., 2016; Aly et al., 2018; Adelani et al., 2020).

MDA is one of the free radicals whose level increases in the organism in case of oxidative stress, leading to lipid peroxidation and DNA damage. This free radical disrupts the structure of lipids, proteins and nucleic acids in the cell and causes carcinogenic effects (Uzunhisarcikli et al., 2016; Salau et al., 2016; Aly et al., 2018). MDA is an oxidative stress parameter used to determine tissue damage and it has been reported that MDA increase plays an important role in the development of liver fibrosis (Kim et al., 2016; Salau et al., 2016). The increase in MDA level due to DEN administration is caused by an increase in free radicals and impairment of antioxidant mechanisms as a result of DEN inducing oxidative stress and lipid peroxidation (Salau et al., 2016; Fathy et al., 2017; Aly et al., 2018). In various studies in which liver damage was induced by DEN, MDA values were examined and this value was found to be high in the damaged groups (Kalaiselvan et al., 2013; Latief et al., 2019; Seriner et al., 2022). In our study, the MDA value was found to be higher in the DEN-treated group compared to the control group. This elevation was statistically significant. In addition, in this study, the MDA value was found to be higher in the DEN+ Phosphorus 15CH treated group compared to the control group and this increase was statistically significant. The MDA value in the DEN+ Phosphorus 15CH group was lower than in the DEN group and this decrease was statistically significant. It is thought that the reason for the high MDA level in this study may be due to lipid peroxidation as stated by the researchers, and it is also thought that increased MDA concentrations may trigger the formation of liver fibrosis. The data obtained in our study are consistent with the findings of researchers (Salau et al., 2016; Fathy et al., 2017; Aly et al., 2018).

Conclusion

In this study, a partial protective effect of Phosphorus15C on liver injury was demonstrated. It is thought that the reason for the fact that the damage was not completely reduced in the group in which DEN and Phosphorus15CH and only Phosphorus15CH were given together and the reason for the damage in the healthy liver may be related to the degree of dilution, dose and duration of administration of the homeopathic preparation given. Homeopathic treatment can be used as an adjunct to medical treatment. It is thought that the protective efficacy of phosphorus 15CH in liver damage can be increased with different doses alone or with additional adjunctive treatments and more detailed studies on this subject would be useful.

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