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Full Length Research Paper

The *in vivo* effect of N-nitrosomorpholine on the activity of enzymes in rat blood serums and liver

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Neoplasm antigens outnumber the enzymes which are utilized to determine the cancer. Cancer development in the living organisms chronologically follows the cytotoxic, organotoxic and mutagenic alterations. Generally, the first symptom for chemical carcinogens is a metabolical response in connection with the detoxification phenomenon and for the infective agents the first symptom is often an immune response. Many nitrosamines similar to N-nitrosomorpholine have been considered as carcinogens. The cancerogenic effect of N-nitrosomorpholine (NMOR) on different animal species has been confirmed experimentally. The aim was to analyse the acute toxic effect of the N-nitrosomorpholine on the *Rattus norvegicus* race rats in this study. The administration of N-nitrosomorpholine causes alteration of some enzymes. The enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were determined for all the samples of blood serum and liver tissue. The results demonstrated that there was an increase in the levels of the ALP, ALT, AST and LDH enzyme activities regarding to the *in vivo* effect of the N-nitrosomorpholine and the increases were evaluated as the metabolic response of liver to hepatotoxic action. NMOR results in the modifications on the biological macromolecules owing to its alkylating characteristic. The degradation and turn over of the protein gains speed gradually till alkylating factor disappear. This case in the circulation appears as the increase of the enzyme activity. These alterations are responsible for carcinogenicity and happen as liver cancer observation in the liver.

Key words: N-nitrosomorpholine, alanine aminotranspherase, aspartate aminotranspherase, alkaline phosphatase, lactate dehydrogenase, neoplasm.

INTRODUCTION

N-Nitrosomorpholine (NMOR) belongs to the group of N-nitrosamines and represents a known hepatocarcinogen. N-nitrosomorpholine (NMOR) produces in toxic and tumoric phenomenon different animal species. The different types of injections with NMOR such as intravenous, intra-abdominal or gastrointestinal injection does not alter the result and when the tumoric phenomenon like first metabolic alterations happen, hepatocellular carcinoma (HCC) are primarily seen on the liver (IARC, 1982; Jarman and Mason, 1986; Vermeer et al., 2004; Slamenova et al., 2008).

Heterocyclic N-nitrosomorpholine is an endogenously formed N-nitroso compound. Nitrates and amine morpholines, its precursors, are found in many common foods and can react in peptic ulcer (Newsholm and Leech, 1986; Tietz, 1987; Leaf et al., 1991).

The toxic effects of NMOR were examined on various types of animal models and LD_{50} doses were detected. Lower doses (40-80ppm) of NMOR have been observed to raise the survival rate of the hepatocytes. Survival rates in the 80 ppm group and in the 40 ppm group were 57 and 81%, respectively, and these values were signify-cantly higher than that in 120 ppm. Its high concen-trations such as 10.2 mM led to increase in the number of necrotic cells pointing out to cytotoxicity. For this reason, carcinogenic effect was proven occur when morpholine and $NaNO_2$ which were the precursors of NMOR were given together through the planned controlled experiment systems and in this way the morpholine may be nitro-zoned *in vivo* conditions (Reddy and Mori 1981; Mayer et al., 1983; Yoshino et al., 2005).

NMOR metabolized by rat liver microsome system is

composed of acetaldehyde, formaldehyde, glyoxal and N-nitroso-2-hydroxymorpholine (Apel, 1980; ShengXue et al., 2008). Hecht and Young (1981) isolated the α-hydroxylation and 2-hydroxyl-1,4-dioxan. This aldehyde is interesting due to being a derivative of the dioxin as hepatocarcinogen. The NMOR was considered to be the responsible of the destructive effect on the cell structures (Hecht and Young 1981; Atalay, 1981). Necrosis and tumour in livers and pancreas were observed in the cats and dogs which were treated with glioxan.

In previous studies, carcinogenic effect of NMOR was obtained after the interaction with DNA. NMOR was observed to induce DNA damage as dependent on the concentration in the hepatocyte cultures of human and rat. NMOR results in site-specific hypometilation of the c -myconcogene in the nodules of the liver. Genotoxic lesion induced by NMOR is inherited to the coming generations (Gmez et al., 1974; Robichova and Slamenova, 2001; Lazarova et al., 2006). The antioxidants such as Vitamins C and E were considered to inhibit mutagenicity by promoting the repair of DNA damage induced by N -nitroso compounds (Robichova and Slamenova, 2001).

Since the recognition of the cancer still requires histopathological findings, *in vivo* experiments are generally monitored on a morphological basis. The acute hepatoxic effect of the NMOR on rats was assesed. The activity changes of the enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in the blood serums and liver tissue extracts was evaluated as a result of this effect in this study.

MATERIALS AND METHODS

The preparation of the rats

Rattus hovegicus and 70 different rats were taken to two big cages according to their genders. 5 distinct experimental groups were formed by picking 4 rats randomly from each cage. Five animals from opposite gender that will be utilized with each group as control were taken to a different cage. They were fed with pellet forage provided from the animal laboratory in the occupational environment.

Preliminary studies

Two males and females have been separated from the rats allocated to the preliminary studies. The rats which were left to starve were weighted in the practice. 1 µl physiological serum for each of 100 g of their body weights was injected to the control group. The blood was drawn from the heart of one of them every day at the same hour after the injection. The liver was perfused by opening of the abdomen after the ether anesthesia. The enzyme activities of the ALP, AST, ALT and LDH of the blood sample and liver tissue homogenates were measured spectrophotometrically. Different doses of NMOR were administered to the other rats and enzyme activities were measured as from the sixth hour.

Experimental groups

Due to the fact that the dose concentration of the NMOR is 160 mg/ kg body weight, it was dissolved to 1.6 mg/ ml. The rats which were left to starve for 24 h were weighted during the practice and they were injected into blood vessel 1 µl NMOR solution for each of 100 g of their body weights as well as the same-volume- physiological serum were injected to the control group. Measurements were individually carried out through the auto-analyser RA- 1000 on the male and female rats of the 6, 12, 18, 24 and 30 -time point-groups. The heart blood drawn from the experimental and control animals which were fainted by ether after the fixed *in vivo* time periods was left to coagulate. The serums were obtained through centrifugation for 5 min at 3000 rpm after the coagulation. The liver tissue of the related rats were extracted through physiological serum following the drawing after their being perfused. The tissue samples which weigh approximately 1 g were homogenized by physiological serum at the nine times more volume than their body weights. These homogenates were centrifuged for 20 min at 25000 g and the measurements of enzyme activity were carried out at the supernatant through the auto-analyser.

Biochemical analysis

The transformation of the NADH+H into NAD+ during the enzyme activity of the Alanine Amino Transferase in the blood serum samples and the liver tissue extracts were measured at 340 nm wavelength. NADH+H oxidation at the enzyme reaction of the Aspartate Amino Transferase (AST) was measured at 340 nm. P-nitrophenol produced at the reaction of Alanine Phosphatase (ALP) in the samples was measured 405 nm and the results of the activity were calculated according to the unit/ litre (u/ l) that is the international unit. All the Application were performed at 37°C.

Statistical analysis

Statistical analysis of the difference between ALP, AST, ALT and LDH levels of the control and groups with NMOR treatment was carried out using analysis of variance (ANOVA) and t test at the programme of SPSS 13 version. The results were given as average ± standard deviation. $P < 0.05$, the difference between two groups, was regarded significant.

RESULTS

ALP, AST, ALT and ALT enzyme activity values of the blood serum and liver extract of the experimental and control rats were shown in Tables 1, 2, 3 and 4 according to the effect time period of NMOR.

There were statistically significant alterations ($p < 0.05$) on the ALP enzyme level in the blood serum and liver extract between groups with NMOR treatment and control groups at the 6^{th}, 12^{th}, 18^{th}, 24^{th} and 30^{th} h (Figures 1 and 2). There was a significant increase in the ALP enzyme activity in the liver as at the 12^{th} h (Table 1).

The AST enzyme activity level went up in the blood serum as at the 6^{th} h although it declined in the liver (Figures 3 and 4).

A significant increase in the ALT enzyme activity was seen in the blood serum in comparison to the control group (Table 3). It was observed that the average activity declined in the liver extract compared to the control group (Figures 5 and 6).

It was seen that LDH enzyme activities had suddenly

Table 1. ALP activities in the blood serum and liver extract of control group and the rats with NMOR treatment.

	Groups with NMOR treatment	X (blood serum) P = 0.02		X (liver tissue) P = 0.006	
		Male	Female	Male	Female
ALP Enzyme activities (U/L)	6^{th} hour	271 ± 22	239 ± 24	75 ± 6.57	105 ± 9.12
	12^{th} hour	318 ± 12	289 ± 18	83 ± 9.48	110 ± 11.03
	18^{th} hour	338 ± 12	312 ± 15	101 ± 9.03	119 ± 11.35
	24^{th} hour	362 ± 10	293 ± 13	135 ± 19.13	149 ± 20.54
	30^{th} hour	350 ± 15	252 ± 49	157 ± 10.51	167 ± 9.73
	Control groups				
	6^{th} hour	175 ± 19	112 ± 12	79 ± 7	128 ± 12
	12^{th} hour	159 ± 18	196 ± 14	88 ± 10	102 ± 9
	18^{th} hour	281 ± 25	232 ± 15	77 ± 5	95 ± 10
	24^{th} hour	310 ± 9	195 ± 11	107 ± 9	125 ± 11
	30^{th} hour	273 ± 12	247 ± 23	94 ± 8	105 ± 9

Table 2. AST activities in the blood serum and liver extract of control group and the rats with NMOR treatment.

	Groups with NMOR treatment	X (blood serum) P = 0.02		X (liver tissue) P = 0.05	
		Male	Female	Male	Female
AST Enzyme activities (U/L)	6^{th} h	80 ± 9	125 ± 14	357 ± 7.28	369 ± 10
	12^{th} h	97 ± 10	155 ± 16	363 ± 15.1	359 ± 8
	18^{th} h	124 ± 28	178 ± 79	330 ± 22.75	354 ± 6
	24^{th} h	169 ± 11	191 ± 23	310 ± 18	343 ± 21
	30^{th} h	195 ± 24	199 ± 12	288 ± 11.87	339 ± 12
	Control groups				
	6^{th} h	59 ± 6	131 ± 27	351 ± 28	458 ± 19
	12^{th} h	78 ± 8	98 ± 11	357 ± 18	418 ± 14
	18^{th} h	112 ± 17	65 ± 8	380 ± 18	332 ± 9
	24^{th} h	67 ± 7	134 ± 13	409 ± 24	427 ± 15
	30^{th} h	73 ± 9	175 ± 34	388 ± 27	340 ± 5

Table 3. ALT activities in the blood serum and liver extract of control group and the rats with NMOR treatment.

	Groups with NMOR treatment	X (blood serum) P = 0.004		X (liver tissue) P = 0.001	
		Male	Female	Male	Female
ALT Enzyme activities (U/L)	6^{th} hour	191 ± 13	225 ± 25	387 ± 9.35	357 ± 6
	12^{th} hour	206 ± 7.5	246 ± 31	380 ± 23.51	351 ± 18
	18^{th} hour	227 ± 31	282 ± 39	390 ± 16.69	356 ± 15
	24^{th} hour	306 ± 6.3	352 ± 22	398 ± 16.28	361 ± 11
	30^{th} hour	375 ± 9	391 ± 31	404 ± 21.13	370 ± 22
	Control groups				
	6^{th} hour	158 ± 8	174 ± 6	399 ± 18	402 ± 21
	12^{th} hour	117 ± 5	187 ± 4	391 ± 17	371 ± 22
	18^{th} hour	303 ± 14	176 ± 7	385 ± 14	363 ± 10
	24^{th} hour	154 ± 11	259 ± 26	423 ± 26	397 ± 24
	30^{th} hour	183 ± 7	260 ± 25	457 ± 28	352 ± 18

Table 4. LDH activities in the blood serum and liver extract of control group and the rats with NMOR treatment.

	Groups with NMOR treatment	X (blood serum) P = 0.05		X (liver tissue) P = 0.09	
		Male	Female	Male	Female
LDH Enzyme activities (U/L)	6th hour	699 ± 52	764 ± 123	1590 ± 70.32	1591 ± 68
	12th hour	1038 ± 85	964 ± 128	1503 ± 76.02	1529 ± 64
	18th hour	1057 ± 94	950 ± 167	1460 ± 47.9	1495 ± 52
	24th hour	1028 ± 90	928 ± 265	1413 ± 71.1	1505 ± 81
	30th hour	989 ± 126	909 ± 133	1437 ± 71	1453 ± 42
	Control groups				
	6th hour	587 ± 43	837 ± 69	1719 ± 67	1792 ± 74
	12th hour	653 ± 58	613 ± 51	1744 ± 54	1703 ± 50
	18th hour	689 ± 69	567 ± 24	1721 ± 32	1661 ± 38
	24th hour	720 ± 99	696 ± 50	1606 ± 21	1768 ± 65
	30th hour	543 ± 34	734 ± 112	1583 ± 42	1711 ± 46

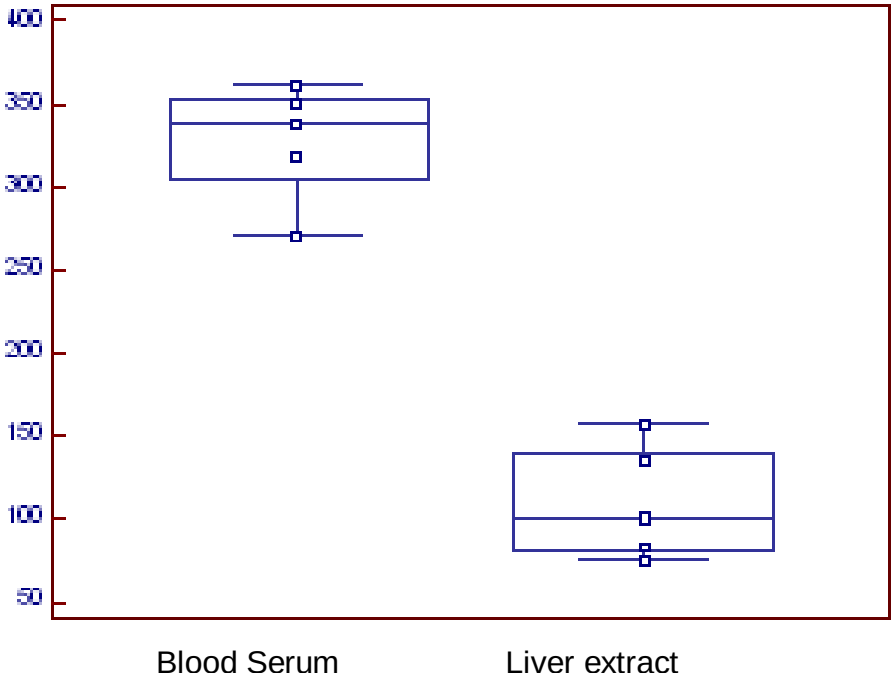

Figure 1. ALP enzyme activities observed in the blood serums and liver extracts of the male rats of the 6, 12, 18, 24 and 30-hour-experimental groups

increased after the NMOR treatment on the rats (Table 4; Figures 7 and 8).

The data on the tables showed that all the enzyme activity values of the male and female rats had increased due to the hepatotoxic effect of NMOR compared to the control group.

The alkylation of the enzyme proteins with NMOR injections causes directly alteration of enzyme activities. That this alteration had appeared within a short time period was a drastic proof of the active sensibility shown by the target cells against the cytotoxic effect of NMOR (Pegg and Hui, 1978).

DISCUSSION

N-Nitrosomorpholine (NMOR), the *in vivo* effect of which was assesed on ALP, ALT, AST and LDH enzyme activity in *R. norvegicus* blood serums and liver homogenates, is a cyclic nitrosamine.

Nitrosamines have toxic effect especially on the mammals and microorganisms (Lijinsky and Taylor, 1975; Swann

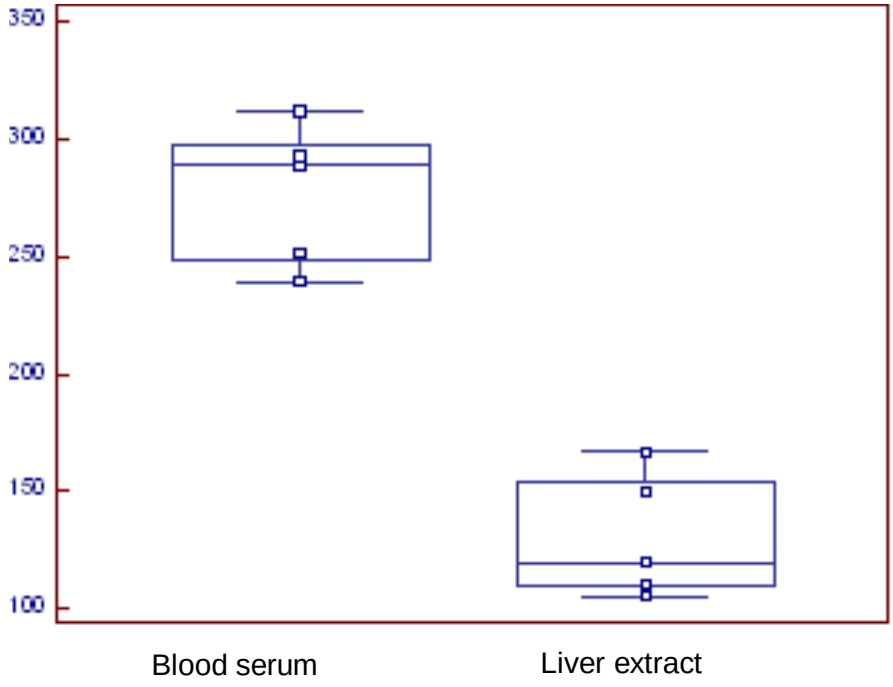

Figure 2. ALP enzyme activities observed in the blood serums and liver extracts of the female rats of the 6, 12, 18, 24 and 30-hour-experimental groups.

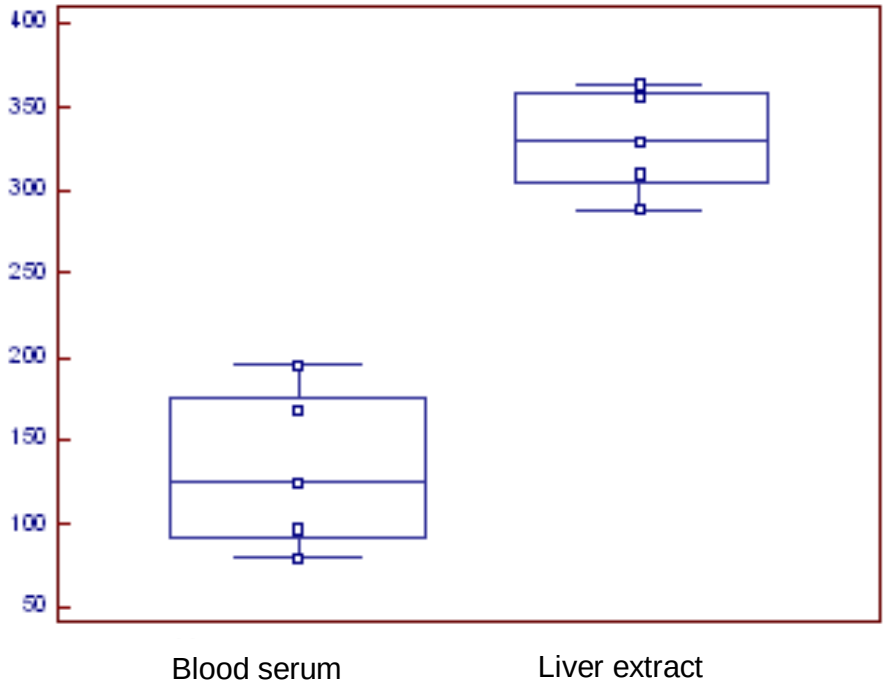

Figure 3. AST enzyme activities observed in the blood serums and liver extracts of the male rats of the 6, 12, 18, 24 and 30-hour-experimental groups.

and Magee, 1986). NMOR was reported to attach to the liver DNAs of the rats with very low doses. The *in vivo* genotoxicity studies have demostrated that it results in induction of uncontrolled DNA synthesis, and formation of hepatocytes with micronuclei, in the liver (Robichova et al., 2004).

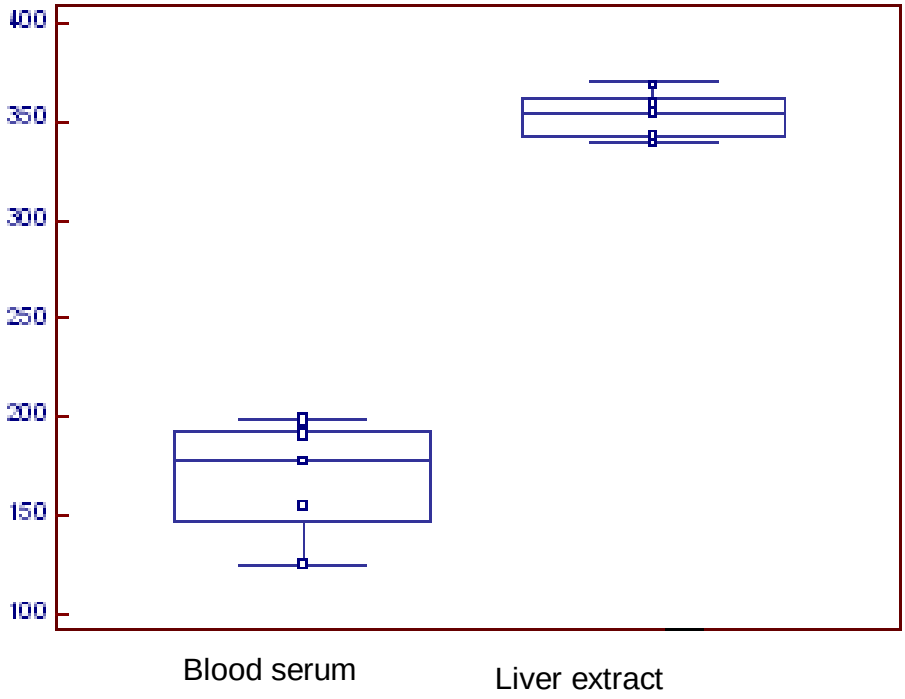

Figure 4. AST enzyme activities observed in the blood serums and liver extracts of the female rats of the 6, 12, 18, 24 and 30-hour-experimental groups.

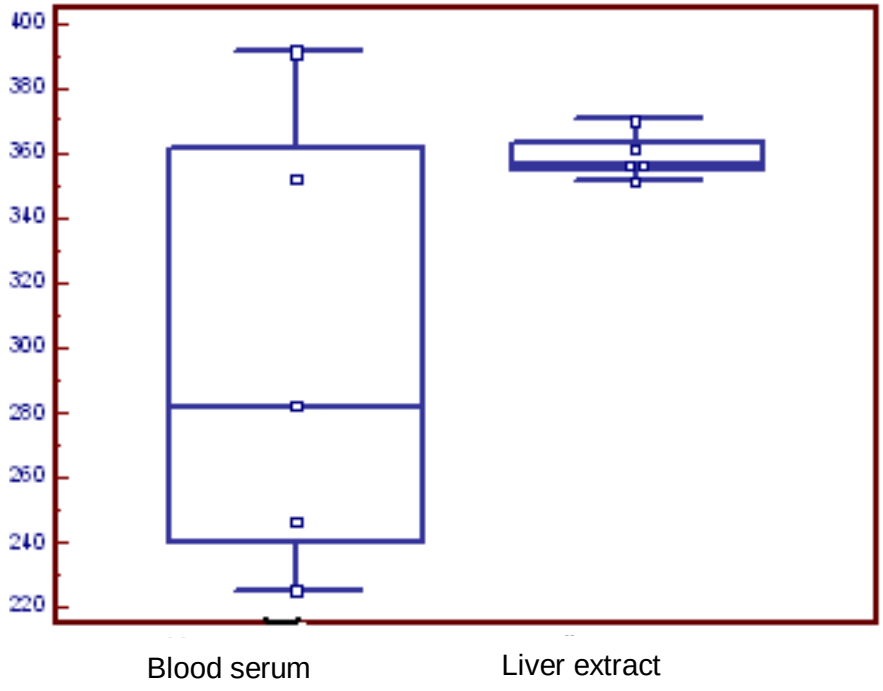

Figure 5. ALT enzyme activities observed in the liver extracts and weights of the female rats of the 6, 12, 18, 24 and 30-hour-experimental groups.

Cytotoxicity of NMOR was evaluated by Caco-2 cells. NMOR caused very slight dose-dependent inhibition of DNA synthesis in Caco-2 cells. The higher concentrations of NMOR (1.7 mmol) stimulated DNA synthesis (Robichova and Slamenova, 2002). Alkylating characteristic of the NMOR leading to modifications on the biological macro-molecules was the main factor of determining the results of this study. The proteins undergo a structural modification

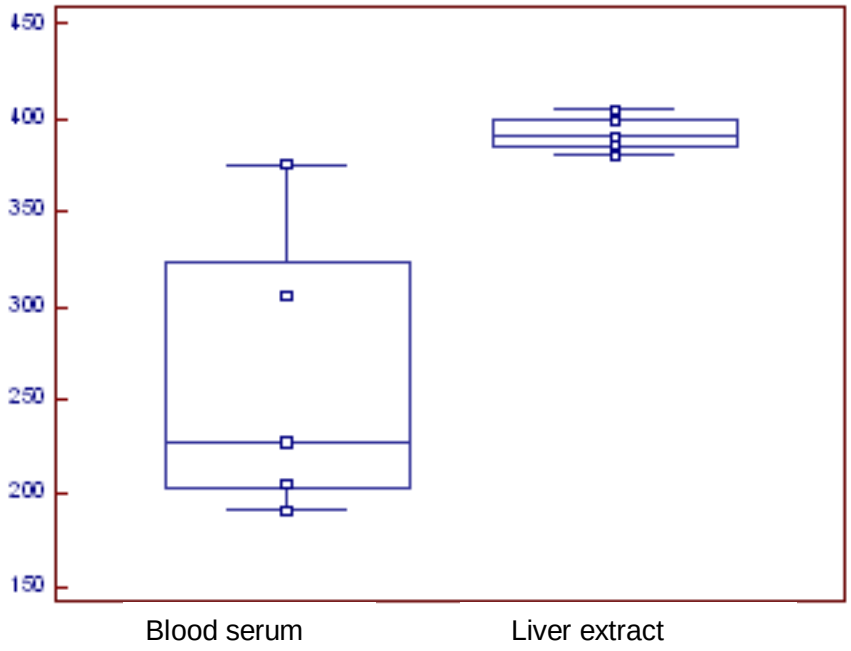

Figure 6. ALT enzyme activities observed in the blood serums and liver extracts of the male rats of the 6, 12, 18, 24 and 30-hour-experimental groups.

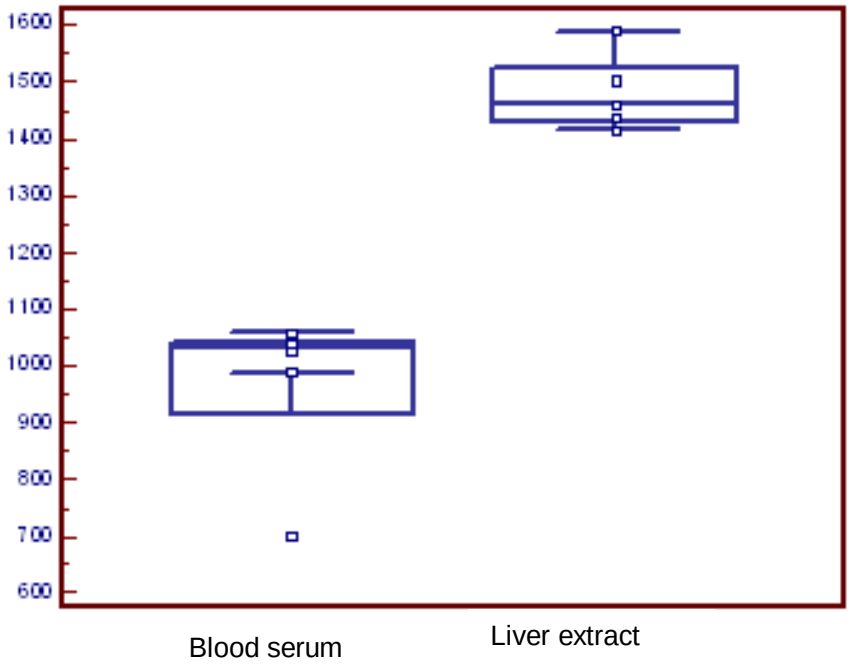

Figure 7. LDH enzyme activities observed in the blood serums and liver extracts of the male rats of the 6, 12, 18, 24 and 30-hour-experimental groups.

in cellular compartments before joining to serum or circulation (Yoshino et al., 2005). Therefore the enzyme activities were expected to decline in this study. Although a modification appearing at the far point of the active centre of a protein with an enzymatic function is always considered to result in activity losses, it is unlikely to further activate the protein regarding its natural and functional structure. NMOR and the other nitrosamines are known to have *in vitro* inhibition effects over these enzymes (Atalay, 1981; Zubay, 1983). Whether the

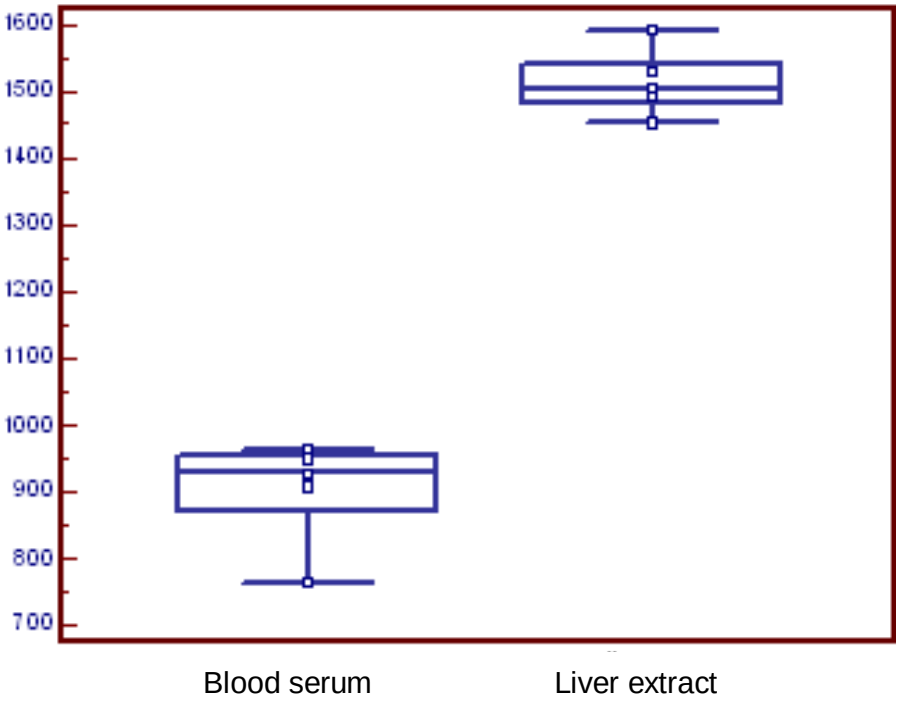

Figure 8. LDH enzyme activities observed in the blood serums and liver extracts of the female rats of the 6, 12, 18, 24 and 30-hour-experimental groups.

catalytic loss of the molecule appears as alkylation in the active centre area or as a modification giving rise to stereochemical alteration, it is a specifically activity loss and it does not reflect on the activity only if the velocity of the synthesis increases. A modified protein is destroyed by the living system. This destruction induces the metabolic control ways thus raises the velocity of the protein synthesis (Zubay, 1983). So long as the probability of the alkylation increases during the accelerating synthesis, the degradation and turn over of the protein gains speed gradually till alkylating factor disappear. As the extracellular proteins free themselves from cellular destruction, this case in the circulation appears as the increase of the enzyme activity. The cell membrane may lose the differential permeability through the alkylation of the macromolecules in its structure (Newsholm and Leech, 1986). This modification brings about proteins in the cytoplasm leaking to the extracellular matrix thus the enzymes accelerating their turn over appear in the extracellular matrix. This case forces the mitochondrial enzymes to leak to the cytoplasm and then to the extracellular matrix thus increases the AST activity (Table 2). According to Ozcan and Mengi (1998), transaminases (also called aminotransferases) are released into circulation from their compartment (the liver) when there is damage to hepatocytes and this leads to increase in their activities in the blood. The increase in the serum AST at the 18th hour stems from the fact that AST is a mitochondrial enzyme, whereas ALT is a cytoplasmic enzyme. Early start of the serum ALP at the 6th h was assesed as it is the membrane enzyme (Table 1). The sudden and linear increase of the LDH is only found in cytoplasm (Table 4). Their concentrations may alter fast depending on the modifications whose half-life is short like some biomolecules having a regulatory duty on the metabolic pathways and affecting the speed of the synthesis or destruction of the proteins whose turn-over is rapid. The alteration results in changing rapidly the enzyme concentrations regulating the metabolic pathway in a certain way. The circle speed of functional protein molecules provides the control, the regulation or the sensibility of the detoxification function like the liver (Newsholm and Leech, 1986; Özcan and Mengi, 1998; Loeppky et al., 2005).

Many animal models have been developed to elucidate the mechanism underlying metastasis (Yoshino et al., 2005). Although the tumoral development or mutagenic changes could begin in a short time period, this can only make fast scanning of chemical cancerogenic easier; it is not enough for cancer recognition. The most effective tools which would be useful in early recognition might be the biomolecules in circulation or other body fluids.

This study demostrated that N-nitrosomorpholine (NMOR) has toxic effect on enzyme activity levels. NMOR results in the modifications on the biological macromolecules like protein and nucleic acid owing to its alkylating characteristic. Hence, it is quite normal for it to rapidly alter the functions of the liver. The rat model presented here provides a good tool for the assessment of the effect of the nitrosomorpholine for metastasis

formation and for analysis of the individual steps in the metastatic process.

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