Effects of Copper, Zinc, and Vitamin Complex (Cernevit®) on Hepatic Healing in Rats Experimentally Subjected to Blunt Hepatic Trauma

Refik Ayten · Erhan Aygen · Yusuf Ziya Cerrahoglu ·
Cemalettin Camci · Yavuz Selim Ilhan · Mustafa Girgin ·
Necip Ilhan · Ibrahim Hanefi Ozercan

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Abstract Solid organ injuries following blunt trauma are frequently encountered. The use of non-operative approach is gradually increasing. Thus, research on the methods that could enhance healing in solid organ injuries is in progress. Agents known to have antioxidant property were used after an experimentally induced blunt hepatic trauma. Thirty-two Wistar albino rats weighing 200 g were dropped from a height of 40 cm on to the right upper abdominal quadrant to produce a grade II–III hepatic injury. Rats were divided into control, Zn-administered, Cu-administered, and vitamin complex-administered groups, with eight rats in each. Aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were measured in the blood samples. The percentage of cells displaying Ki-67 nuclear staining was estimated. The sections were stained with hematoxylin and eosin and the degree of inflammation in the samples was semi-quantitatively assessed. Treatment with zinc, copper, and Cernevit® caused varying levels of decrease in AST, ALT, and LDH levels compared to the control group. Ki-67 positivity was significantly lower in group I compared with groups II and III ($p=0.002$). Ki-67 positivity was significantly higher in group II compared to the other groups ($p<0.05$). A marked improvement was observed in inflammation in group II. Copper and zinc treatment decreased inflammation as well as

blood levels of AST and ALT, and enhanced the healing of traumatized hepatic tissue. However, Cernevit® reduced only the degree of inflammation.

Keywords Blunt hepatic trauma · Cooper · Zinc · Vitamin complex · Hepatic healing

Introduction

Trauma remains the most common cause of death for all individuals between 1 and 44 years of age, being the third most common cause of death regardless of age [1]. The liver is the largest intra-abdominal solid organ and is enclosed anteriorly and laterally by the rib cage. The large size of the liver, its friable parenchyma, its thin capsule, and its relatively fixed position in relation to the spine make it particularly prone to blunt injury. As a result of its larger size and proximity to the ribs, the right lobe is injured more commonly than the left. Injuries to the liver occur in 35–45 % of patients with significant blunt abdominal trauma [2].

Non-operative management of blunt abdominal injuries is well established, and strategies based on hemodynamic stability and computed tomography (CT) scan findings are now being widely used in the treatment of solid organ injuries, including liver, spleen, kidneys, and pancreas [3].

Currently, hepatic traumas can be clearly identified using the technological advances in imaging. By clear identification, blunt hepatic traumas can be successfully treated non-operatively. The focus now is to reach the optimum recovery time for liver. Copper (Cu), zinc (Zn), and vitamins have positive effects on wound healing [4, 5]. We have evaluated the effects of these substances on the healing of hepatic injury caused by blunt trauma.

R. Ayten (✉) · E. Aygen · Y. Z. Cerrahoglu · Y. S. Ilhan · M. Girgin
General Surgery Department, Firat University, Elazığ, Turkey
e-mail: refikayten@yahoo.com

N. Ilhan
Biochemistry Department, Firat University, Elazığ, Turkey

I. H. Ozercan
Pathology Department, Firat University, Elazığ, Turkey

C. Camci
General Surgery Department, Istanbul Bilim University, Elazığ,
Turkey

Methods

Preparation of the Rats

The work was carried out in the Experimental Medical Research Laboratory of the University Medical Faculty after the approval of the Local Ethical Committee was obtained. Thirty-two Wistar albino rats weighing 220–250 g were used. The rats were caged in groups of five, and were fed with standard pellet diet and tap water under conditions of a constant temperature and humidity. The rats were fasted for 6 h prior to the trauma, as also before the surgery. Prior to the study, 50 mg/mL ketamine HCL (Ketalar® Flakon, Eczacıbaşı Pharmaceutical Co., Istanbul, Turkey) and 20 mg/mL xylazine HCL (Rhompon® Flakon, Bayer Istanbul, Turkey) were injected intramuscularly into the right hind leg of six rats at 0.25 mL/100 g of body weight to induce general anesthesia. At this time, a 200-g constant weight was dropped from a 40-cm height with 0.784 J of kinetic energy onto the right upper abdominal quadrant of the rats fixed on a table by a custom-manufactured platform (Fig. 1) [6].

These rats were laparotomized after sterilization of the skin, and the degree of hepatic injury was recorded, usually at grade II–III. Having used six rats as a pilot, they were excluded from the experiment, which was continued with a further 32 rats subjected to trauma in exactly the same way.

Groups

The animals were divided into four groups with eight rats as follows: group I, control group, the rats were fed with standard pellet diet and tap water (standard feeding) following trauma; group II, in addition to the standard feeding, these rats were

Fig. 1 The trauma platform specifically designed for the study

given Zn at 0.68 mg/day via a lavage tube after anesthetizing them with ether; group III, in addition to the standard feeding, the rats were given Cu at 0.136 mg/day as for group II; group IV, in addition to the standard feeding, 2 mL of diluted Cernevit® Flacon (diluted with 6-mL saline Eczacıbaşı Pharmaceutical Co., Istanbul, Turkey) was administered intraperitoneally.

The rats were followed for 3 days after trauma, with alternating light and dark periods of 12-h, each group being in its own cage. Group-adjusted additional therapies were given for 3 days at the same time daily. At the end of the third day, the rats were fixed on a table in the supine position after general anesthesia, and were laparotomized through a midline incision of 2.5 cm following abdominal shaving and sterilization. A blood sample of 2 mL was obtained from the vena cava for measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels. The animals were killed, and their livers were totally resected, fixed in 10 % formaldehyde, and sent to the pathology laboratory.

Preparation of the Samples for Histopathological Examination

Sections (5 µm) were prepared from paraffin blocks of the tissue samples by a pathologist unaware of the study groups. The sections were mounted on poly-L-lysine coated slides to detect Ki-67 expression by immunohistochemistry (SP6) (NeoMarkers, Thermo Fisher Scientific Anatomical Pathology, Fremont, USA) with ready-to-use rabbit monoclonal antibody. The staining pattern was evaluated assessed by the method of Wintzer et al. [7]. One hundred and fifty to 500 cells were counted on the slides at ×400 magnification, and the percentage of cells with positive nuclear staining were measured.

Sections (5 to 6 µm) were stained with hematoxylin and eosin for the degree of inflammation to be assessed on a graded scale from 0 (+) and 3 (+) by a pathologist who was blinded to the study groups (Table 1).

Statistical Analysis

The difference between the groups in AST, ALT, LDH, and Ki-67 levels, and the degree of inflammation, were analyzed using the Mann–Whitney *U* test. The data are expressed as

Table 1 Scoring of the degree of inflammation

Degree of inflammation	
0	No inflammation
1	Minimal inflammation
2	Moderate inflammation
3	Intense inflammation

mean±standard deviation (SD), using Statistical Program for Social Sciences (SPSS Inc., Chicago, IL, USA) version 11.0. $p<0.05$ was considered as statistically significant.

Results

After being anesthetized, rats were subjected to trauma. One rat died from each group and was excluded from the study. At autopsy, these rats had grade II–III hepatic lacerations without accompanying organ damage. Hypovolemic shock due to bleeding was the cause of death of these rats. Thus, the rate of post-traumatic mortality was 12.5 % in each group.

In the surviving rats, the mean AST level was higher in group I compared with the other groups (Table 2). The mean AST level of group I was significantly higher than groups II and III ($p=0.002$, for each), whereas the difference between groups I and IV was not significant ($p=1$, Table 3). A significant decrease was observed in AST level in group II as compared with the other groups ($p<0.05$, Table 3). No significant change was observed in AST levels in group IV after the trauma ($p>0.05$, Table 3). However, the mean AST level of group IV was significantly higher than that of groups II and III ($p=0.002$ and $p=0.009$, respectively, Table 3).

The mean ALT level was higher in group IV compared with the other groups (Table 2). Group IV proved significantly higher than groups II and III (both $p=0.025$, Table 3). A significant improvement in ALT levels was seen in groups II and III compared with the other groups ($p<0.05$, Table 3); however, the degree of improvement was similar in groups II and III ($p=0.11$, Table 3).

The highest level of LDH occurred in group I. Post-traumatic elevation in LDH level showed a significant decrease only in group II (Table 2). This decrease was significant compared with the other groups ($p<0.05$, Table 3). Decrease mean LDH levels in groups III and IV were not statistically significant ($p>0.05$, Table 3).

Ki-67 positivity was significantly lower in group I compared with groups II and III (both $p=0.002$, Tables 2 and 3). Ki-67 positivity was significantly higher in group II compared with that of the other groups ($p<0.05$, Table 3). While Ki-67 positivity of group IV was similar to that of group I ($p=$

Table 3 Statistical evaluation of the groups in terms of the mean levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, Ki-67 positivity, and the mean scores of inflammation

	AST	ALT	LDH	Ki 67	Inflammation
Groups I–II	0.002	0.004	0.002	0.002	0.001
Groups I–III	0.002	0.025	0.848	0.002	0.001
Groups I–IV	1	0.338	0.565	0.371	0.002
Groups II–III	0.048	0.11	0.003	0.002	0.035
Groups II–IV	0.002	0.025	0.035	0.002	0.002
Groups III–IV	0.009	0.025	0.338	0.002	0.114

AST aspartate aminotransferase, ALT alanine aminotransferase, LDH lactate dehydrogenase

0.371), it was significantly lower than those of groups II and III (both $p=0.002$, Table 3).

The mean inflammation score was higher in group I compared with those in groups II, III, and IV ($p=0.001$, $p=0.001$, $p=0.002$, respectively, Tables 2 and 3). A remarkable improvement was observed in inflammation in group II, which was significant compared with the other groups ($p<0.05$, Table 3). There was also a significant improvement in groups III and IV compared with group I (both $p<0.05$, Table 3). Although the mean inflammation score was lower in group III than that in group IV, the difference was not statistically significant ($p=0.114$, Table 3).

Discussion

Trauma is among the leading causes of death in the developed countries throughout the world, particularly among the 0–44 years age group [1]. Of the Turkish population, 81.8 % is between the ages of 0 to 44. Traffic accidents in Turkey are the first and occupational accidents are the second leading causes of trauma-induced deaths [8]. Currently, the majority of solid organ injuries following blunt abdominal trauma can be successfully treated non-operatively [2, 3].

Using computed tomography (CT), liver is seen as the most commonly injured organ due to blunt trauma and hepatic injury which has been encountered in many unsuspected cases [9]. CT is also an important method for the grading damage,

Table 2 Mean levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, Ki-67 positivity levels, and mean scores of inflammation in the study groups are presented

AST aspartate aminotransferase, ALT alanine aminotransferase, LDH lactate dehydrogenase

	Group I (n=7)	Group II (n=7)	Group III (n=7)	Group IV (n=7)
AST	674.86±133.31	227±25.25	323.86±35.79	583.71±63.11
ALT	324.86±106.97	101.86±6.75	132.57±17.19	378.14±76.55
LDH	1,918.48±251.78	1,046±47.52	1,826.86±118.78	1,637.57±302.94
Ki-67 (%)	3.93±0.30	7.11±0.17	5.69±0.20	4.44±0.09
Inflammation	2.86±0.14	0.14±0.14	0.86±0.26	1.43±0.20

but the need for laparotomy is determined on clinical grounds. Recently, with advanced medical imaging techniques and improved conditions of intensive care, most trauma cases can be managed without surgical operation, i.e., in the absence of hollow organ perforation and hemodynamic instability [10]. These advances have led to investigations on substances that might aid hepatic healing in cases after injury [11].

During trauma, hypoxanthine accumulates in cells due to adenine nucleotide catabolism. Simultaneously, the production of some proinflammatory products (leukocyte adhesion molecules, cytokines) and bioactive agents (endothelin, thromboxane A₂) increases in the vascular endothelium, whereas the production of some other protective products (structural nitric oxide synthase, thrombomodulin) and bioactive agents (prostacyclin, nitric oxide) is suppressed. Thus, post-traumatic ischemia initiates a proinflammatory condition, which enhances the vulnerability of the tissue during the subsequent reperfusion period. Neutrophil infiltration frequently occurs in the involved tissues. Production of free oxygen radicals by parenchymal cells, endothelial cells, and leukocytes increases.

Vitamins, particularly C and E, are the most important chain-breaker antioxidants in tissues and are the first-line preventive agents against lipid peroxidation. Zinc is a trace element that also protects against oxidative stress and inhibits apoptosis. Although copper ions are involved in the structure of many antioxidant enzymes, they may also catalyze free radical damage. Vitamins, zinc, and copper also play an important role in wound healing [4, 5].

In our study, a remarkable increase occurred in the degree of inflammation following blunt hepatic trauma in the control group. Copper, zinc, and Cernevit® treatments significantly decreased the degree of inflammation. Zinc was significantly better than copper and Cernevit®.

The sensitivity of serum transaminases in determining hepatocyte damage is quite high; regardless of etiological factors, serum levels of transaminases increased in all conditions where hepatic damage persists. Serum hepatic transaminase concentration has been used to detect blunt liver injuries for over 40 years [12]. We found that elevated enzyme levels in the control group decreased after the treatment with zinc and copper, with that achieved with zinc treatment being more prominent. No notable decrease was observed after Cernevit® treatment.

Ki-67 antigen localized in cell nucleus and the monoclonal antibodies against this antigen were identified by Gerdes et al. [13] for the evaluation of hepatic healing and the degree of hepatic regeneration. Increase in Ki-67 expression is directly proportional to hepatic healing. Ki-67 positivity in our rats was highest in the group treated with zinc. Copper treatment also increased Ki-67 positivity, whereas Cernevit® treatment had no effect.

Copper may act as a catalyzer in free radical damage [14]. In addition, copper ions, together with iron, play a role in the activity of cytochrome oxidase enzyme, notably in the transfer of electrons to oxygen. Copper is found in the active site of lysyl oxidase, an enzyme which promotes crosslinking between collagen and elastin polypeptides. In addition, copper is found bound in catalase, phenoloxidase, and ascorbic acid oxidase. It is also required for the metabolism of iron in the body. Since copper is a redox active metal, it has an effect on oxidative stress. Copper-induced oxidative damage generally occurs due to the formation of a highly reactive hydroxyl radical, and these may initiate lipid peroxidation, leading to tissue damage [15]. We observed that copper had positive effects on the wound healing following hepatic trauma, reducing the inflammation in hepatic tissue.

Zinc is a trace element needed by most organisms. It binds to the active sites of the enzymes and plays a key role in catalysis. Liver is the main organ that metabolizes the zinc [16]. The expression of metallothioneins increases with the stimulation due to oxidative stress. These metallothioneins protect the tissues against various forms of oxidative stress, such as radiation, lipid peroxidation, oxidative stress induced by anti-cancer agents, and hyperoxia. Zinc-bound metallothioneins reduce the toxicity of heavy metals, such as cadmium, copper, and mercury. Intracellular metal facilitates homeostasis, protects against oxidative stress, and prevents apoptosis. The concentration of zinc increases with the induction of metallothionein [17]. Zinc deficiency increases apoptosis, whereas excess zinc inhibits apoptosis [18]. Redox-stable zinc substitutes for redox-active metals, such as iron and copper, in critical cellular and extracellular regions, and induces the synthesis of metallothioneins, thus preventing the generation of free radicals and oxidative damage [19].

We found that zinc, known for ages to have a positive effect on wound healing, assisted in hepatic healing, as expected. Zinc caused significant alterations in all parameters compared with the other therapy modalities, being more effective than copper or Cernevit®.

The vitamin complex, Cernevit®, was expected to decrease the inflammatory cell infiltration, increase tissue healing, and reduce the serum enzyme levels due to those vitamins with antioxidant properties. Vitamin E reduces superoxide and hydroxyl radicals, singlet oxygen, lipid peroxide radicals, and other radicals. Vitamin C (ascorbic acid) is also a good reducing agent. It provides a protective effect against reactive oxygen species, acting as a reducing agent and a radical scavenger [19].

We previously found that Cernevit® administered together with total parenteral nutrition reduced the levels of AST and ALT in patients with hepatic disease [18]. Although Cernevit® treatment was expected to reduce hepatic enzymes in the present study, increased AST and ALT levels following trauma were unaffected by Cernevit® treatment. Treatment with

this complex caused a reduction only in the degree of inflammation. Cernevit® treatment was not found effective on hepatic healing, nor in reducing serum enzyme levels.

Limitations

Although the platform was used to perform a standard hepatic trauma, hepatic injuries were grades II and III. The groups may be added to the study for evaluate the ideal dose of cooper, zinc, and vitamin complex in hepatic healing.

Conclusions

Treatments with copper and zinc following blunt hepatic trauma reduced the degree of inflammation in the hepatic tissue, enhanced healing, and reduced the hepatic enzyme (AST, ALT, LDH) levels. Cernevit® treatment, however, caused no significant decrease in AST, ALT, and LDH levels, but did significantly reduce the degree of inflammation.

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