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The effect of trimetazidine on intrahepatic cholestasis caused by carmustine in rats

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Abstract

This study investigated the effect of trimetazidine (TMZ), known as an anti-oxidant agent, on intrahepatic cholestasis caused by Carmustine (BCNU) in rats. Rats were assigned into four groups. The first group (Saline) consisted of 12 rats, which were injected with 2 ml/kg of saline intraperitoneally (IP) 48 h before the study. The second group (corn oil group, $n = 15$), which were injected with 2 ml/kg of corn oil IP 48 h before the study. The third group (BCNU group, $n = 16$), which were injected with 2 ml/kg of corn oil + 25 mg/kg BCNU IP 48 h before the study. The fourth group (TMZ group, $n = 12$), which were injected with 2.5 mg/kg per day of TMZ IP, administered at the same hour of the day as a single-dose. Twelve hour after the first dose of TMZ, corn oil 2 ml/kg + BCNU 25 mg/kg IP were injected, and the rats were included in the study 48 h after the administration of corn oil + BCNU. Following a pentobarbital anaesthesia, abdomen was opened with incision, a cannula was placed into the channel of choledocus, and the amount of bile was measured per hour. Then intracardiac blood sample was taken, and consequently centrifuged to obtain the plasma. Finally, the rats were killed with cervical dislocation, and their livers were removed and weighted. In addition to histopathological examination of liver, the levels of malon dialdehyde (MDA), oxidised glutation (GSSG), and reduced glutation (GSH) were detected. Also the osmolality of bile and plasma was estimated in mOsm/kg. As a result, the biliary flow was seen to decrease in BCNU group ($P < 0.005$), but to be normal in TMZ group. The serum level of conjugated biluribin was higher in BCNU group compared to other groups ($P < 0.05$ for each). Although the level of total glutation was lower ($P < 0.005$) in TMZ

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group, GSH/GSSG ratio was normal. These findings suggest that TMZ has a protective effect on intrahepatic cholestasis caused by BCNU.

Keywords: Carmustine; Trimetazidine; Intrahepatic cholestasis

1. Introduction

Trimetazidine (TMZ:1-[2,3,4-trimethoxy-benzyl] piperazine HCl) is an anti-oxidant agent that is mainly used for the treatment of coronary artery diseases. This drug that has been investigated in several studies since 1970, was noticed to be anti-ischemic substance showing a cytoprotective effect without changing coronary blood flow and systemic haemodynamics [1,2]. Therefore, it has been used alone or in a combination with other anti-anginal agents for the treatment and prophylaxis of arteriosclerotic cardiac diseases. Since its anti-anginal effects, studies with TMZ have largely focused on the cells of myocardium, and subsequently it has been used in the field of otolaryngology, e.g. for the treatment of ischemia-related diseases [1–4].

Recently TMZ has been shown to protect erythrocytes against free oxygen radicals [5], and to show a protective effect for the injuries resulted from ischemia-reperfusion in the liver of rats [6]. On the other hand, Carmustine (BCNU: 1,3-*bis*(2-chloroethyl)-1-nitrosourea), a carcinostatic agent, has been reported to cause to intrahepatic cholestasis, with a role of increased paracellular permeability in the development of such cholestasis [7–10]. It has been suggested that BCNU may weaken glutation, thus reducing the anti-oxidant effect, and enhance the oxidative stress, which may eventually lead to an increase in paracellular permeability [10,11]. In the light of these findings, we investigated the effect of TMZ, which was known as an anti-oxidant agent, on intrahepatic cholestasis caused by a single-dose BCNU in rats.

2. Materials and methods

2.1. Animals

This study included 55 Wistar Albino rats whose weight ranged from 200 to 350 g. All of the rats were male. Animals were supplied by The Center of Experimental Animals, The Medical Faculty of Ege University. Animals allowed to have free food and water were kept in the cages containing eight to ten animals, which were exposed to 12-h light: 12-h dark at 22–24°C.

2.2. Drugs

TMZ was supplied by Servier Laboratories (Istanbul), BCNU (Carmustine), by Bristol Myers Squibb. Picric acid, GSH, GSSG, NADPH, GR, 5,5′-dithiobis-(2-ni-

trobenzoic acid) (DTNB) was delivered from Sigma Chemical, 2-vinylpyridine(2-VP) from Aldrich Chemical, and corn oil from Taris (Ýzmir).

2.3. Study protocol

Individual rats were weighted prior to enter the study; their weights were recorded, and they were randomly assigned to four groups. Group I (saline group); This group consisted of 12 rats. These rats were injected with 2 ml/kg of saline intraperitoneally (IP) 48 h before the study, being included by the study 48 h later. Group II (corn oil group) consisted of 15 rats. These rats were injected with 2 ml/kg of corn oil (vehicle) IP 48 h before the study. Group III (BCNU group) consisted of 16 rats. These rats were injected with 1 ml per day of saline IP, administered at the same hour of the day as a single-dose for 3 days. Twelve hours after the first dose of saline, corn oil 2 ml/kg + BCNU 25 mg/kg IP were injected, and the rats were included in the study 48 h after the administration of corn oil + BCNU. Group IV (TMZ group) consisted of 12 rats. These rats were injected with 2.5 mg/kg per day of TMZ IP, administered at the same hour of the day as a single-dose for 3 days. 12 h after the first dose of TMZ, corn oil 2 ml/kg + BCNU 25 mg/kg IP were injected, and the rats were included in the study 48 h after the administration of corn oil + BCNU.

A Phenobarbital dose of 60 mg/kg IP was used for the anaesthesia of rats. Abdominal hairs of anaesthetised rats were shaved to be ready for the procedure. Rectal temperature was measured with a thermometer to avoid a body temperature under 37°C. Cases whose rectal temperatures tended to decrease were heated using heating devices, providing a normal body temperature. Abdomen was opened with a 5–6 cm-length incision at the median line, then the bile duct was isolated and cannulated with PE-10 tubing. Subsequently free biliary flow was followed for 60 min in all rats, and the amount of collected bile was measured either as microliters per minute per gram of liver or microliters per minute per kilogram b.wt. Surgery was performed between 8:00 h and 13:00 h. Collected bile were placed into the vials of 2 ml in containers at 0°C, and sent to related laboratory for biochemical tests. Then livers of the rats were explored and completely removed. Removed tissues were weighted, and each liver tissue was cut into three pieces. One piece was placed into formaldehyde solution of 10%, and sent to related laboratory for histopathological examination. The second piece was placed into picric acid solution of 1%, and sent to the related laboratory for malon dialdehyde (MDA) and the third piece was preserved to measure the levels of oxidised and reduced glutation. Finally animals were killed with cervical dislocation. The blood samples from rats were centrifuged at 2000 bps for 20 min to obtain the plasma. Then biochemical assessments were performed to separate the plasma using spectrophotometer. The biochemical parameters included plasma Na^+ and K^+ levels, alkaline phosphatase, GGT, conjugated bilirubin and Na^+ and K^+ concentrations in bile. The osmolality of plasma and bile was measured in mOsm/kg using Wescor 5100 Vapor Pressure Osmometers.

2.3.1. Glutation level

The levels of reduced (GSH) and oxidised glutation (GSSG) were determined enzymatically in accordance with a modification by Teare et al [12]. The liver tissues were homogenised in 0.875 mM picric acid at.% 1. A 100 μl of the supernatant, which was obtained after a centrifugation at $2500 \times g$ and $+4$°C for 15 min was used for GSH following an appropriate dilution, while an amount of 200 μl was preserved for the measurement of GSSG levels with 2-vinylpyridine.

Standard reaction media was prepared using 210 μl of NADPH (0.3 mmol/l), 30 μl of DTNB (6.0 mmol/l), 30 μl of standard solution, or sample solution, and 30 μl of Glutation Reductase (GR: 1 U/100 μl). The reaction was followed with 5 s-intervals for 3 min for GSH, with 15 s-intervals for 3 min for GSSG. The standard solutions were prepared according to the concentration of picric acid, which was contained by the last preparation of reaction in the biological samples, that was 0.175 mM for GSH, 0.875 mM for GSSG. The results from analysis were calculated in μmol/g of wet tissue.

2.3.2. Malon dialdehyde (MDA) level

It was calculated according to Modified Okhawa method [13]. Firstly, the liver tissue was homogenised with phosphate buffer. Phosphate buffer was adjusted as with a pH level of 5. The homojenates were adjusted as to contain 100 mg tissue per ml, then centrifuged for 15 min, and 0.4 ml of supernatant was taken.

According to Okhawa method, thiobutiric acid 0.67%, sodium dodecyl sulphate 8.1%, acetic acid 20% (pH:3) were prepared. Three ml of thiobutiric acid, 0.4 ml of sodium dodecyl sulphate, 3 ml of acetic acid, and 0.4 ml of supernatant were put into the separate tubes, then boiled in a water bath, which was previously heated until 100°C. After the tubes were cooled, they were read at 532 nm against the blank. The results from the samples, which were prepared from MDA standard were presented in nmol/g of tissue.

2.4. Statistical analysis

The mean values $\pm$ standard deviation (S.D.) of results were estimated for all groups. Whether there was a statistical significant difference between the values obtained from all groups was evaluated using unpaired t-test. Paired t-test was only used to assess whether there was a statistically significant difference between the baseline and pre-treatment body weights of rats. P values that are equal to and lower than 0.05 were considered significant. The correlation between various parameters was assessed using the regression test.

3. Results

In our study, when the rats were weighed just before phenobarbital anaesthesia, BCNU group showed a statistically significant weight loss compared with the period prior to the study ($P < 0.005$). However, other groups showed no significant

weight loss compared with the pre-treatment period (Table 1). There was no statistically significant difference in various parameters between corn oil and saline groups (Table 2).

The ratio of liver weight to body weight was higher in BCNU group compared with both corn oil ($P < 0.02$) and TMZ group ($P < 0.01$). Biliary flow were significantly reduced in BCNU group than CO group ($P < 0.005$). Beside this biliary flow was lower in BCNU group than TMZ group ($P < 0.05$). There was no significant difference between CO and TMZ groups according to biliary flow. When considered the volume of biliary flow per 1 *g* of liver tissue, with adjustments according to the body weights of rats, the volume of biliary flow was lower in BCNU group than that in both corn oil ($P < 0.005$) and TMZ group ($P < 0.02$). The level of plasma conjugated bilirubin was significantly higher in BCNU group compared to both corn oil group ($P < 0.05$) and TMZ group ($P < 0.05$) (Table 2).

The values of bile and plasma osmolality, Na and K levels, plasma alkaline phosphatase and GGT levels observed in corn oil, BCNU and TMZ group, and the statistical differences between these parameters are shown in Table 2.

GSSG and GSH/GSSG values were lower in BCNU group than CO group (for each of them $P < 0.02$).GSH value was also lower in BCNU group than CO group ($P < 0.005$). GSH/GSSG value was lower in BCNU group than both CO group ($P < 0.02$) and TMZ group ($P < 0.005$). The levels of liver MDA, oxidised and reduced glutation observed in all groups in the study, and the statistical differences between the groups are shown in Table 3. In addition, no statistically significant correlation was observed between the parameters such as MDA, GSSG, GSH and GSH/GSSG, and the biochemical parameters of biliary flow, bile and plasma.

Mild celluler edema and mononuclear cellular infiltrations were present in the histologic examination of the liver of the BCNU group rats. The histologic examination of the liver were normal in S and CO groups.

4. Discussion

In 1969, Thompson and Larson [7] reported the cholestatic effect of BCNU in rats. This cholestatic effect resulted from a single-dose administration of BCNU is dose-dependent, and reflected by a decreased biliary flow, increased serum conju-

Table 1
Body weights at baseline and 48 h after the intraperitoneal injections [a]

Groups	Baseline	After 48 h	*Paired t-test*
S (*n*:12)	248.8 ± 21.0	247.0 ± 20.8	NS
CO (*n*:15)	251.3 ± 23.4	249.0 ± 18.6	NS
BCNU (*n*:16)	248.2 ± 40.0	214.7 ± 30.8	$P < 0.005$
TMZ (*n*:12)	259.6 ± 46.5	246.7 ± 38.8	NS

[a] S, saline; CO, corn oil; BCNU, carmustine; TMZ, trimetazidine; NS, non-significant.

Table 2
Various parameters observed in all four groups and the statistical differences between them [a]

	S (n:12)	CO (n:15)	BCNU (n:16)	TMZ (n:12)	t-test (S-CO)	t-test (CO-BCNU)	t-test (CO-TMZ)	t-test (TMZ-BCNU)
LW/W ($\times 100$)	3.32 ± 0.40	3.38 ± 0.42	3.75 ± 0.57	3.13 ± 0.65	NS	$P<0.02$	NS	$P<0.01$
Bile flow (μl/min per g.liver)	1.14 ± 0.31	1.08 ± 0.030	0.76 ± 0.27	1.14 ± 0.52	NS	$P<0.005$	NS	$P<0.02$
Bile flow (μl/min per kg)	34.8 ± 8.7	35.2 ± 8.3	25.6 ± 8.0	33.7 ± 12.7	NS	$P<0.005$	NS	$P<0.05$
Bile osmolality (mOsm/kg)	291.5 ± 25.3	288.6 ± 23.6	311.4 ± 22.7	299.8 ± 23.6	NS	$P<0.01$	NS	NS
Plasma osmolality (mOsm/kg)	283.9 ± 31.2	289.5 ± 31.3	302.0 ± 21.9	308.2 ± 21.6	NS	NS	NS	NS
Bile/plasma osmolality ratio	1.03 ± 0.07	1.00 ± 0.11	1.03 ± 0.05	0.98 ± 0.14	NS	NS	NS	NS
Bile Na^+ (mEq/l)	151.7 ± 7.7	147.2 ± 6.0	152.3 ± 7.6	151.7 ± 10.4	NS	$P<0.05$	NS	NS
Plasma Na^+ (mEq/l)	140.1 ± 3.8	139.9 ± 3.5	139.1 ± 4.0	139.1 ± 1.8	NS	NS	NS	NS
Bile/plasma Na^+ ratio	1.08 ± 0.04	1.06 ± 0.05	1.09 ± 0.05	1.09 ± 0.07	NS	NS	NS	NS
Bile K^+ (mEq/l)	3.75 ± 0.36	3.79 ± 0.34	3.98 ± 0.47	3.88 ± 0.38	NS	NS	NS	NS
Plasma K^+ (mEq/l)	5.45 ± 0.56	5.62 ± 0.63	6.57 ± 1.62	6.94 ± 0.46	NS	$P<0.05$	$P<0.01$	NS
Bile/plasma K^+ ratio	0.69 ± 0.07	0.66 ± 0.09	0.63 ± 0.12	0.56 ± 0.07	NS	NS	$P<0.01$	NS
Conjugated bilirubin (mg/dl)	0.21 ± 0.19	0.24 ± 0.21	0.63 ± 0.85	0.17 ± 0.37	NS	$P<0.05$	NS	$P<0.05$
Alkaline Phosphatase(U/l)	446 ± 260	435 ± 234	428 ± 247	379 ± 91	NS	NS	NS	NS
GGT(U/l)	6.17 ± 1.60	6.55 ± 1.98	8.25 ± 4.50	7.95 ± 5.92	NS	NS	NS	NS

[a] S, saline; CO, corn oil, BCNU; carmustine, TMZ; trimetazidine, LW; weight of liver; W, weight of body; GGT, gamma glutamil transpeptidase; NS, non-significant.

Table 3
The levels of liver glutation and malondialdehyde observed in the study groups[a]

	S (*n*:12)	CO (*n*:15)	BCNU (*n*:16)	TMZ (*n*:12)	*t*-test (S-CO)	*t*-test (CO-BCNU)	*t*-test CO-TMZ	*t*-test (TMZ-BCNU)
MDA (nmol/g.liver)	234.8 ± 24.1	241.9 ± 27.6	273.5 ± 43.4	274.4 ± 33.6	NS	$P<0.02$	$P<0.01$	NS
TGSH (μmol/g.liver)	8.14 ± 3.61	7.72 ± 3.36	3.42 ± 2.08	4.05 ± 1.35	NS	$P<0.0005$	$P<005$	NS
GSSG (μmol/g.liver)	1.50 ± 1.14	1.24 ± 1.12	0.57 ± 0.25	0.46 ± 0.24	NS	$P<0.02$	$P<0.05$	NS
GSH (μmol/g.liver)	6.64 ± 3.66	6.48 ± 3.31	2.83 ± 1.93	3.78 ± 1.39	NS	$P<0.005$	$P<0.02$	NS
GSH/GSSG ratio	9.99 ± 11.43	12.09 ± 10.95	5.22 ± 3.63	17.15 ± 15.50	NS	$P<0.02$	NS	$P<0.005$

[a] S, saline; CO, corn oil; BCNU, carmustine; TMZ, trimetazidine; MDA, malondialdehyde; GSH, reduced glutation; GSSG, oxidised glutation; TGSH, total glutation; NS, non-significant.

gated bilirubin level, and BSP retention. The studies showed that the cholestasis due to BCNU was a canalicular event, and that was primarily associated with a bile salt-independent bile flow (BSIF) [8,14]. Moreover this early cholestasis, to some degree, was suggested to be associated with the selective inhibition of exogene organic anion secretion. However the mechanism of BCNU-induced cholestasis was not sufficiently cleared. Hoyt and Larson [9] determined the osmolalities of serum and bile and the levels of Na^+ and K^+ as well as the bile secretion in order to clarify the mechanism of BCNU-induced cholestasis in rats. The results of this study showed that the osmolality of bile increased, that serum electrolytes altered with the effect of cholestasis, and that such effect reached a maximum level at 48 h. It was stressed that these findings might be due to an increased Na^+/K^+ activity or an increased permeability of cell membrane. BCNU that has been demonstrated to lead to an increased paracellular permeability is an inhibitor of glutation reductase enzyme. Therefore, it has been reported that BCNU may weaken glutation, thus and reduce the anti-oxidant effect and enhance the oxidative stress, which may consequently lead to an increase in paracellular permeability [10,11]. Thus TMZ which normally has no effect on the biliary flow [2] is suggested to be able to effect BCNU-dependent cholestasis as it is an antioxidant agent, and to play a preventive role against BCNU-induced cholestasis. There are several findings suggesting this effect of TMZ which may be listed as given below.

1. In rats receiving BCNU, the amount of bile significantly decreased when compared to corn oil group, whereas the administration of TMZ caused to a significant increase in the amount of bile.
2. When the rats were weighted before the study, only those receiving BCNU showed a marked weight loss, whereas there was no significant weight loss in other groups. This may be caused by TMZ reducing the related effects of BCNU.
3. When considered the osmolality of bile and plasma, bile and plasma levels of Na^+ and K^+, and their serum/plasma ratios, the values obtained in TMZ group were seen to be closer to those in corn oil group despite no statistically significance was observed.
4. The level of serum conjugated bilirubin was higher in BCNU group than those in corn oil and TMZ group. However there was no significant difference in the plasma levels of conjugated bilirubin between corn oil and TMZ groups.
5. Mild celluler edema and mononuclear cellular infiltrations were present in the histologic examination of the liver of the BCNU group rats. The histologic examination of the liver were normal in S and CO groups.
6. As mentioned above, changes in the levels of oxidised and reduced glutation due to the TMZ-dependent effect of BCNU on glutation levels.

All these findings suggested that TMZ markedly prevented BCNU-induced cholestasis. This phenomenon probably results from a decreased oxidative stress due to an anti-oxidant agent. Indeed, changes in the glutation levels are clearly evidence for a decreased oxidative load. TMZ is known to have a cytoprotective effect on several tissues, predominantly on heart [3,5,6,15–19]. Moreover, it has

been shown to decrease oxidative stress, leading to a milder course of cholestasis in choledocus ligationed rats [20]. Our study showed that trimetazidine, in addition to these effects, had an effect on intrahepatic cholestasis caused by BCNU.

In rats treated with BCNU, MDA levels of liver increased as a natural consequence of an elevated lipid peroxidation. That MDA levels of liver were higher in BCNU group than those in corn oil group is an evidence for this [21–23]. TMZ known to be effective by eliminating toxic oxygen radicals did not change the raising-effect of BCNU on lipid peroxidation. However, TMZ was seen to effect the glutation levels in liver. As oxidised glutation levels (GSSG) were lower in rats receiving BCNU + TMZ than those receiving BCNU alone, but reduced glutation levels were higher, GSH/GSSG ratio was found much higher than even the levels obtained in corn oil group although total glutation level did not apparently change. This is a clear evidence for the effect of TMZ, an anti-oxidant compound, on glutation levels in liver. It is not clear that by which mechanism TMZ changes the effect of BCNU on gluthatione level. In another recent study, it was observed that in BCNU-dependent intrahepatic cholestasis TMZ has affected gluthation and had cytoprotective effect. In this study, in BCNU and BCNU + TMZ groups GSH and GSH/GSSG levels were lower in respect to control group [24]. Data obtained for GSH was similar to our study. However, in our study GSH/GSSG ratio was not different in both TMZ and control groups. We commented this situation as the antioxidant effect of the TMZ. Also in our study in addition to gluthation; bile flow, serum bilirubin, bile and plasma osmolalities and and biochemical parameters in rats were evaluated. That is why our study demostrating effect of TMZ in intrhepatic cholestasis had more broad spectrum. Unchanged MDA levels offered that this drug had no effect on lipid peroxidation. TMZ has been reported to decrease ischemia-dependent liver injury, especially by eliminating free oxygen radicals [6,19].

By which mechanisms does TMZ show its effect? Today the action mechanism of TMZ is not known. Studies with this drug representing cytoprotective properties for many cells have shown that it decreased the severity of oxidative stress by eliminating free oxygen radicals [3,5,6,15–19,25]. The effects of TMZ may be listed as follows.

- To prevent the decrease in cellular ATP levels.
- To decrease intracellular deposition of inorganic phosphate.
- To lower intracellular acidosis.
- To decrease oxidative injury in mithocondriums, thus showing a protective effect against ischemia-reperfusion injury.
- To prevent the formation of free oxygen radicals due to the injury, thus eliminating the deleterious effects of free oxygen radicals on cell membrane.
- To inhibit neutrophil infiltration to the damaged area.

Recent studies reported that trimetazidine changed the permeability of outer membranes in platelets and erythrocytes, and to effect even spontaneous transportation of several ions along the membrane [5,17]. This suggested that this drug may show similar effects to alter paracellular permeability in liver so that it may be

effective on BCNU-induced cholestasis. Also it has been shown to decrease intracellular acidosis by preventing the intracellular deposition of hydrogen. More recently it has been shown to be effective by blocking Na^+-K^+-ATPase enzyme in guinea pigs [3,15]. This enzyme plays an important role to form a bile acid-independent biliary secretion [2,9]. Hoyt highlighted that Na^+-K^+-ATPase might be predominantly affected by BCNU-induced cholestasis, and that electrolyte changes in bile might be due to this affection [9]. TMZ is also effective to prevent intracellular ATP loss [3,5,15]. Another study conducted in our institue has demonstrated the preventive effect of TMZ in an experimental model of acute pancreatitis [26].

At the end of this study, we observed that intrahepatic cholestasis experimentally formed by an intraperitoneal administration of BCNU 25 mg/kg in rats was significantly prevented by TMZ. Therefore, our study suggests that TMZ has a preventive effect on hepatocellular diseases such as intrahepatic cholestasis due to increased paracellular permeability, in which oxidative stress is a predominant aspect, especial. Thus further studies are needed to investigate the effects of this drug on various hepatobilier pathologies.

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