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EXPERIMENTAL STUDY

Methylene blue as an antioxidant agent in experimentally-induced injury in rat liver

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Abstract: *Background:* We aimed to test the methylene blue (MB) as a dye and also to test its antioxidant activities in devascularization-induced liver injury.
Methods: Twenty rats weighing 240–280 g were randomly divided into two groups, each containing 10 rats. High-grade liver injury was induced by using a pair of long pliers with blades. MB was injected into portal vein of the rats with no hepatic injury (Group 1; control group) and those with injured livers (Group 2; injury group). Liver and hepatic function tests, paraoxonase, stimulated paraoxonase, arylesterase activity, total antioxidant, and oxidant status were evaluated before and 24 h after MB injection.
Results: MB did not stain the non-perfused area. Total antioxidant status decreased significantly in Group 2 at hour 24 compared to Group 1. In Group 2, total antioxidant status was lower at hour 24 compared to hour 0. Total oxidant status in Group 2 at hour 0 increased significantly compared to Group 1. Total oxidant status in Group 2 at hour 24 was lower compared to that at hour 0. Lipid peroxidation parameters did not alter due to devascularization.
Conclusion: MB is useful in defining the devascularization area. Moreover, it showed to have a beneficial effect on oxidant status *(Tab. 3, Fig. 3, Ref. 25)*. Text in PDF *www.elis.sk.*
Key words: devascularization, liver, injury, methylene blue, rat.

Introduction

The liver is the most commonly injured organ during abdominal trauma. Grade IV to V of liver injury leads to mortality in range of 35–80 % (1, 2). Treatment approach includes surgical or non-surgical interventions. Non-surgical intervention is becoming popular for high-grade liver injuries since surgery is associated with high mortality rates (3).

The major determining factor for treatment of liver injury is the hemodynamic status of the patient. Non-operative treatment is indicated in patients with stable hemodynamic status and isolated organ injury (4, 5). In instable patients with uncontrolled bleeding, the requirement of surgery remains to be a controversial issue. Surgical techniques include packing, anatomic liver resection, selective vessel ligation, resectional debridement, parenchymal sutures, and hepatectomy with liver transplantation (6, 7).

Devascularization injury is a life-threatening form of liver injury resulting from disrupted vascular inflow to one or more hepatic segments (5). It is important to be able to diagnose this type of injury, since stable patients may develop decompensation due to septic complications. It has been suggested that surgical resection is necessary in patients with extensive liver necrosis before the development of secondary complications (2).

Hepatic injury score is determined by parenchymal disruption as seen on admission computed tomography (CT). However, the grade of injury alone does not determine the need for surgical intervention since parenchymal disruption may be due to hemorrhage or devascularization injury (8, 2). If the patient is stable and has hemorrhage, non-surgical treatment is likely to be successful (9). Conversely, if the parenchymal disruption is secondary to devascularization, non-surgical management is run at risk of septic complications. Moreover, there is no method to detect the non-perfused area precisely during intraoperative procedure to help with the decision-making. Therefore, it is crucially important to indentify the devascularized area precisely. (2).

Methylene blue (MB) is a liposoluble dye marking the tissue, and thus its injection allows to localize a tissue such as tumor or lymph nodes (10, 11). In addition, MB has been shown effective against oxidative stress (12, 13). It is also known that hepatic devascularization increases oxidative stress and lipid peroxidation parameters (14, 15).

In the present study, MB was tested for identification of non-perfused tissue in rat liver with experimentally induced devascularization injury. Additionally, antioxidant properties of MB were also examined for oxidative stress and lipid peroxidation parameters in serum.

Materials and methods

Study design

The present study is a prospective, randomized, and controlled

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experimental study. It was approved by Ankara Hospital Ethics Committee for Animal Experiments. All animal experiments were carried out in accordance with the rules issued by European Community Council.

Animals and experimental procedure

Female Wistar albino rats weighing 240–280 g were used in this study. They were maintained under standard conditions of room temperature and humidity. The rats were fed with standard rat chow and fasted with free access to water for 12 hours before the experiment

The rats were randomly divided into two groups, each containing 10 rats as follows:

Group 1 (control group); MB was injected into portal vein of the rats with no hepatic injury.

Group 2 (injury group); MB was injected into the portal vein of the rats with injured liver.

Surgical procedure

The rats were anesthetized with intramuscular ketamine hydrochloride (75 mg/kg, Ketalar, Parke Davis, Eczacibasi, Istanbul, Turkey) and intraperitoneal xylazine (5 mg/kg, Alfazyne 2 %, Ege Vet, Izmir, Turkey). The abdomen was opened through an upper mid-line incision 3 cm in length and the liver was exposed. High grade liver injury was induced by using a pair of long pliers with blades 1.5 cm in length. "Liver Injury Scale of the American Association for the Surgery of Trauma" was accepted as the guide for inducing the injury (16). The portal vein was exposed following injury, and a disposable 22 G catheter was inserted 1–2 cm into the proximal vein toward the liver. Injection of MB was performed through the catheter (1.5 mg/kg–0.2 mL in 2 ml of saline, 2 % MB, Lexi–Comp. Inc., Hudson, Ohio, USA). Following MB injection, stained and unstained areas were observed and recorded. Discoloration times were also recorded. Additionally, the times of staining and discoloration onsets were noted. Catheters were removed after discoloration. Liver samples of 1–3 cm^3 were taken from the unstained areas and fixed with 4 % buffered formaldehyde for histopathological evaluation. Liver injury was repaired by simple sutures in the presence of uncontrollable bleeding from borders. The surgical procedure consisting of repairing the liver injury and abdominal closure was completed within 10 min (16).

Laboratory and pathological evaluation

Blood samples were taken from tail veins of rats before (at hour 0) and 24 hours after MB injection to evaluate the serum levels of albumin, alanine transferase (ALT), aspartate aminotransferase (AST), bilirubin, creatinine, urea, paraoxonase (PON), stimulated paraoxanase (SPON), and arylesterase. Hematocrit and total oxidant status were also examined.

Measurement of total antioxidant status

A fully automated calorimetric measurement method was used to assess the total antioxidant status (17). In this method, e2+-o-dianisidine complex reacts with hydrogen peroxide in Fenton type reaction, producing hydroxyl radical. This strong reactive oxygen type reacts with colorless o-dianisidine molecule. As a result, bright yellowish-brown dianisyl radicals are produced, and participate in further oxidation reactions increasing the color production. This reaction is inhibited and the coloration is stopped by antioxidants in test tube to a degree that is proportional to their concentrations. The assay is performed by the measurement with automatic spectrophotometric analyzer and the results are expressed as millimolar Trolox equivalent per liter (mmol Trolox eq/L).

Measurement of total oxidant status

The total oxidant status was determined using a fully automated colorimetric measurement (18). Ferrous ion-o-dianisidine complex is oxidised to ferric ion by oxidants in the sample. The glycerol speeds up this reaction approximately three times. A colorful complex with "xylenol orange" is produced by the ferric ions in acidic media. The color intensity is measured spectrophotometrically and is proportional to the amount of oxidants. Results were expressed as micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 eq/L).

Measurement of PON, SPON, and arylesterase activities

Commercial Rel assay kits were used for the measurement of PON and arylesterase activity (19). PON activity was measured in absence and presence of NaCl (SPON). PON1 activity was determined with the increase in absorbance at 412 nm resulting from the formation of 4-nitrophenol in the presence of PON as substrate. The absorptivity coefficient of 18290M^{-1} was used for calculation of enzymatic activity. Phenylacetate was used as substrate for determination of arylesterase activity. Enzymatic activity was calculated by using molar absorptivity coefficient of 1301 M^{-1} cm^{-1} of phenol. The enzymatic activity was expressed as U/L.

Statistical analysis

All data in the text and tables are presented as mean ± standard deviation. Within group comparisons were performed using the paired sample t test. The student t test was used to compare variables between groups. Statistical analyses were performed using SPSS 11 software. The level of statistical significance was set at $p < 0.05$.

Results

Staining and discoloration times

In Group 1 (control group), liver parenchyma was stained homogeneously. Non-perfused areas of the liver in Group 2 (injury group) were not stained. As shown in Table 1, the mean onset time of staining in perfused parenchyma was 3.89 ± 0.18 sec in controls and 3.31 ± 0.27 sec in cases ($p > 0.05$). The mean dis-

Tab. 1. Staining and discoloration times between groups.

	Group 1 (Control, n=10)	Group 2 (Liver injury, n=10)	p
Staining onset time (sec)	3.89±0.18	3.31±0.27	< 0.001
Discoloration time (sec)	22.11±1.02	19.92±0.99	< 0.001

Data are given as mean±standard deviation.

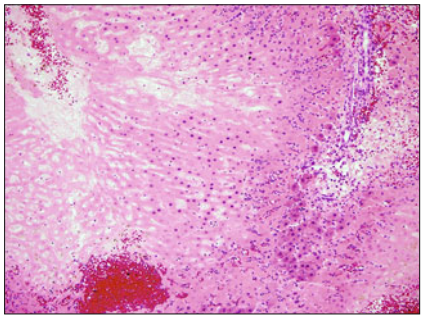

Fig. 1. Hepatocytes undergoing necrosis and polymorphonuclear leukocytes are seen. (HE x200)

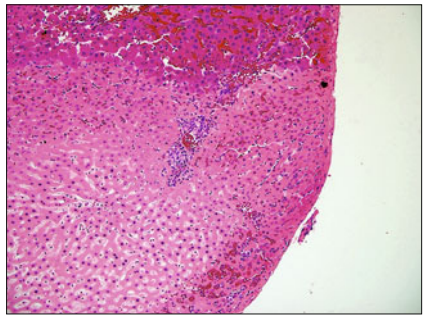

Fig. 2. Subcapsular bleeding, necrosis and polymorphonuclear leukocytes are seen in liver. (HE x200)

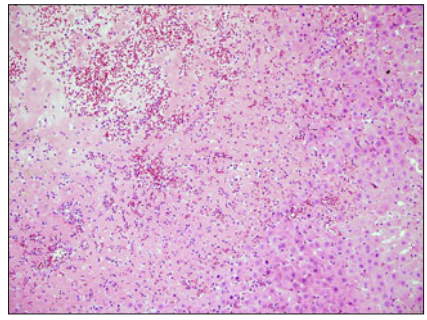

Fig. 3. Geographic necrosis is seen in liver. (HE x40)

coloration time was 22.11 ± 1.02 sec in controls and 19.92 ± 0.99 sec in cases ($p > 0.05$).

Histopathology of liver specimens

Hypoperfusion and devascularization–induced necrosis was confirmed by histopathological evaluation in biopsy specimens (Figs 1, 2, 3).

Kidney and liver function tests

Renal function tests did not differ between control and liver injury groups at hours 0 to 24 (Tab. 2).

ALT and AST values significantly increased in each group at hour 24 compared to hour 0. In addition, ALT values at hour 24 were lower in Group 2 than in Group 1. Direct bilirubin values were significantly higher in each group at hour 24. In Group 2, direct bilirubin values decreased significantly at hour 0 in comparison with Group 1 (Tab. 2).

Oxidative stress and lipid peroxidation parameters

Total antioxidant status decreased significantly at hour 24 after MB injection in Group 2 ($p = 0.002$). Additionally, antioxidant status was also significantly reduced in Group 2 when compared to Group 1 at hour 24^t ($p = 0.009$) (Tab. 3).

Total oxidant status was significantly higher in Group 2 (liver injury) when compared to Group 1 (control) at hour 0 ($p < 0.001$). In Group 2, total oxidant status decreased at hour 24 compared to hour 0 (Tab. 3).

The levels of PON, SPON and arylesterase did not differ between Groups 1 and 2 at hours 0 and 24 (Tab. 3).

Discussion

In the present study, we evaluated the use of MB for identification of non–perfused tissue in experimentally induced devascularization injury in rat liver. As a result, MB did not stain the non–perfused area. The antioxidant properties of MB were also tested for oxidative stress and lipid peroxidation parameters in this experimental animal model. MB did not exhibit any beneficial effect on antioxidant status. However, MB showed to have a useful effect on oxidant status. Lipid peroxidation parameters did not alter due to devascularization or due to MB treatment. ALT and AST values have been found higher in each group at hour 24. These results may be attributed to traumatic injury due to surgical intervention. MB led to a decrease in ALT value in Group 2 at hour 24 compared to Group 1. Moreover, direct bilirubin at hour 0 was lower in Group 2 than in Group 1. These results may be interpreted as a suggestion that MB may have protective properties for the liver.

Tab. 2. Kidney and liver function tests between groups.

Parameters	Group 1 (Control, n=10)	Group 2 (Liver injury, n=10)	p^a
Hematocrit (%)			
Preoperative	40.68±1.33	42.96±1.69	0.003
Postoperative hour 24	38.78±1.13	42.57±1.92	<0.001
p^b	0.005	0.441	
Albumin			
Preoperative	1.82±0.49	2.21±0.28	0.039
Postoperative hour 24	2.58±0.75	2.18±1.11	0.364
p^b	0.047	0.941	
Urea (mmol/L)			
Preoperative	46.5±6.76	49.9±4.68	0.207
Postoperative hour 24	69.4±14.63	92±49.81	0.185
p^b	0.001	0.024	
ALT (IU/L)			
Preoperative	65.9±16.22	68.6±11.82	0.675
Postoperative hour 24	668.8±411.19	290.9±138.48	0.013
p^b	<0.001	<0.001	
AST (IU/L)			
Preoperative	170.9±27.69	181±41.92	0.533
Postoperative hour 24	1261.2±456.35	941.1±297.2	0.079
p^b	<0.001	<0.001	
Creatinine (µmol/L)			
Preoperative	0.42±0.08	0.43±0.09	0.785
Postoperative hour 24	0.46±0.06	0.48±0.14	0.676
p^b	0.223	0.213	
Total bilirubin (µmol/L)			
Preoperative	0.1±0	0.1±0	1.000
Postoperative hour 24	0.11±0.04	0.11±0.04	1.000
p^b	0.343	0.343	
Direct bilirubin (µmol/L)			
Preoperative	0.04±0.01	0.03±0.01	0.035
Postoperative hour 24	0.07±0.02	0.07±0.02	0.931
p^b	<0.001	<0.001	

Data are given as mean±standard deviation.
a^p values for between groups comparisons; b^p values for in–group comparisons of pre– vs. postoperative values.

Tab. 3. Oxidative stress and lipid peroxidation parameters.

Parameters	Group 1 (Control, n=10)	Group 2 (Liver injury, n=10)	p[a]
Total antioxidant status (mmol Trolox equiv./L)			
Preoperative	0.45±0.19	0.53±0.09	0.226
Postoperative hour 24	0.5±0.21	0.25±0.18	0.009
p[b]	0.670	0.002	
Total oxidant status (μ mol H_2O_2 Equiv./L)			
Preoperative	7.15±3.22	13.81±3.21	<0.001
Postoperative hour 24	4.29±1.58	2.82±2.39	0.12
p[b]	0.066	<0.001	
Paraoxonase (U/L)			
Preoperative	127.47±149.58	103.43±108.88	0.686
Postoperative hour 24	60.54±63.37	43.9±62.84	0.563
p[b]	0.071	0.068	
Stimulated paraxonase (U/L)			
Preoperative	22.99±22.22	22.84±17.31	0.987
Postoperative hour 24	16.56±14.77	11.4±15.43	0.455
p[b]	0.151	0.0037	
Arylesterase (U/L)			
Preoperative	57.46±56.62	56.38±46.64	0.963
Postoperative hour 24	27.92±23.19	25.39±22.73	0.808
p[b]	0.043	0.038	

Data are given as mean±standard deviation.
[a]p values for between groups comparisons; [b]p values for in–group comparisons of pre– vs. postoperative values.

Liver injury may lead to death as a result of uncontrolled bleeding or sepsis (5, 20). Non–operative management of stable liver trauma patients is associated with reduced mortality (21, 22). However, it is important to recognize traumatic liver devascularization since an initially stable patient may decompensate due to septic complications. Life–threatening liver necrosis resulting from devascularization may be diagnosed either intraoperatively or at autopsy (23, 24). It is not possible to detect the extent of liver necrosis exactly during surgery. Therefore, surgeons overestimate the borders and extend the resection area. The patients with extensive liver necrosis should be brought for definitive surgical resection (25). In a previous study, the importance of resecting areas of devascularized liver has been stressed (24).

It has been shown that it is possible to differentiate the ischemic area from the viable liver tissue by using MB in the evaluation of traumatic necrosis in a rabbit liver injury model (16). Since no perfusion occurred in devascularized area, only viable tissue retained the MB. Additionally, no adverse effect was observed due to MB usage. In accordance with this study, we confirmed that non-perfused areas of the liver remain unstained whereas the color of viable liver tissue changed from purple to dark blue in rat livers with experimentally induced devascularization injury. It was also safe as shown before. This study was performed in rats for the first time. Since CT scanning does not provide exact information about the difference between intrahepatic hemorrhage and devascularization injury in the liver, it seems feasible to inject MB into the portal vein for anatomically precise resection of non-perfused liver tissue due to devascularization in patients. Further studies are required to confirm the effectiveness and safety of MB to be able to use it in humans.

A few studies show that oxidative stress and lipid peroxidation increases due to liver necrosis resulting from devascularization (14, 15). Exact mechanisms have not been clearly demonstrated. In this study, we also tested the antioxidant activity. Our hypothesis was that both oxidative stress and lipid peroxidation play a role in the pathophysiology of devascularization injury in the liver and the use of MB may be useful against these factors. To test this hypothesis, total oxidant and antioxidant status, PON and arylesterase were measured in blood samples. There were two experimental animal groups each including 10 rats, (control and liver injury group). Blood samples were collected after injection of MB into animals (before and thereafter at hour 24 of experiment).

In the present study, total antioxidant status decreased significantly in Group 2 at hour 24 in comparison with that at hour 0 as well as with Group 1 at hour 24[t]. Our results show that antioxidant activity impaired at hour 24 due to liver injury and injection of MB does not produce any beneficial effect on antioxidant status.

In respect of oxidant status, it was found to be increased significantly in Group 2 (liver injury) at hour 0 when compared to Group1 (control). In addition, in Group 2, total oxidant status was lower at hour 24 than that at hour 0. It is likely that MB has a useful effect on oxidant status.

Our results suggest that oxidative stress may play a role in the pathophysiology of liver necrosis due to devascularization injury. MB exhibited beneficial effect on oxidant status.

PON, SPON and aryl esterase activities were also determined in two experimental animal groups. No significant change was found between groups. Our results suggest that lipid peroxidation has no role in the pathophysiology of devascularization injury in the liver.

Due to technical reasons it was not possible to evaluate the oxidative/antioxidative stress and lipid peroxidation parameters in liver tissue. Therefore, it is not possible to comment on the contribution of tissue activity of these parameters to pathophysiology. On the other hand, this is an animal study which makes it difficult to apply the results to human beings. These factors are limitations for the present study. However, the application of MB during surgery promises a new method for identification of non-perfused areas due to liver devascularization.

In conclusion, MB seems useful for identification of non-perfused tissue in devascularization-induced liver injury. Since MB showed a beneficial effect on oxidant status in this study, it may be feasible to use MB as an antioxidant agent in liver injury due to devascularization. Further studies are required to confirm these results.

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