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Early Protective Effect of Trimetazidine on Heart, Liver and Kidneys Tissues of Wistar Rats Subjected to Hemorrhagic Shock. The Fractal Dimension Technique Utility

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Abstract

Introduction: Hemorrhagic Shock (HS) is a condition of reduced tissue perfusion. Trimetazidine (TMZ) is an antianginal drug that promotes a more efficient glucose oxidation and ATP production under ischemic conditions by inhibition of free fatty acid oxidation. Kidney and heart function are affected by the HS; we hypothesized that TMZ could be protect these tissues. The objective of the current study is analyzed, by fractal dimension, the early organ damages observed in a HS animal model and the eventually protective effect after TMZ administration.

Material and Methods: Wistar rats were randomly divided into 2 groups: Control (n=6) and TMZ (n=7) and submitted to HS. Shock was induced by controlled arterial bleeding; mean arterial blood pressure was maintained between 37 mmHg and 42 mmHg for 60 min. After HS, shed blood was reinfused and Ringer lactate was injected over a Resuscitation Period (RP) of 30 min. Arterial blood samples were obtained for baseline and at the end of HS and RP. Animals were sacrificed immediately after RP and samples of kidneys and left ventricle of heart were obtained. Data are expressed as mean ± SD. Statistical comparisons between different groups were made by Student t-test. Histological scores were analyzed by the Mann-Whitney test. A $p<0.05$ was considered statistically significant.

Results: At the end of HSP, compared with Controls, TMZ group showed less increase of serum Cr (Control: 0.77 ± 0.08 mg/dl; TMZ: 0.64 ± 0.05 mg/dl; p=0.011), lactate (Control: 7.88 mmol/L ± 1.39 mmol/L; TMZ: 5.58 mmol/L ± 1.11 mmol/L, p=0.020) and higher bicarbonate (Control: 13.34 mmol/L ± 3.19 mmol/L; TMZ: 17.08 mmol/L ± 1.50 mmol/L; p=0.045). Fractal Dimension analysis showed TMZ protection in heart (FD: Control: 1.732 ± 0.041; TMZ: 1.759 ± 0.043; p<0.0001 and L: Control: 0.33 ± 0.075; TMZ: 0.29 ± 0.066; p=0.01), liver (FD: Control: 1.776 ± 0.039; TMZ: 1.786 ± 0.037; p=0.0021 and L: Control: 0.28 ± 0.057; TMZ: 0.29 ± 0.058; p=ns) and kidneys (FD: Control: 1.742 ± 0.040; TMZ: 1.796 ± 0.042; p<0.0001 and L: Control: 0.33 ± 0.075; TMZ: 0.29 ± 0.066; p=0.01)

Conclusion: In an experimental model of HS, TMZ administration attenuated cell and tissue damage in kidneys and heart. Thus, TMZ might be a viable adjunct in the treatment of severe hypovolemia.

Keywords: Hypotension; Ischemia reperfusion injury; Acute kidney injury; Shock therapy; Drug effects

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Introduction

Despite advances in critical care, Hemorrhagic Shock (HS) is still a major cause of death, with an estimated 1.9 million deaths per-year worldwide [1]. It is a high mortality condition in which reduced tissue perfusion limits oxygen delivery and can induce multiple organ dysfunction and failure [1]. Small intestine, kidneys, liver and heart are typically affected by HS, conditioning survival [2]. Trimetazidine (TMZ) is a well-tolerated antianginal drug that protects cardiomyocytes from ischemic damage in stable coronary artery disease [3,4]. TMZ acts on mitochondria reducing ischemic effects and improving metabolic disturbances in low-flow ischemia. Through selective

inhibition of long chain3-ketoacyl coenzyme A thiolase, TMZ promotes glucose metabolism through switching energy substrate preference from fatty acid oxidation to glucose oxidation [4,5]. The produced switch in energy substrate results in greater ATP production with the same amount of oxygen [6].

In models of individual organ ischemia and reperfusion (*in vivo* arterial transient clamping) TMZ has shown to be protective in different parenchyma (mostly kidney and heart). However, it is not known whether this effect can be reproduced when whole body hypoperfusion of different organs occurs simultaneously during HS.

The fractal dimension was originally used in the field of mathematics, architecture, and industry, while in the biological sciences it was useful for the analysis of the structural organization, growth, form of infiltration of neoplasms on neighboring tissues, etc. Also is a useful tool in neuroscience studies. These considerations make of the fractal dimension a useful method, because it applies a validated mathematical algorithm, which only requires histological sections stained with H&E. It is sensitive, since it detects various structural modifications of the tissues, which escape direct microscopic observation. Furthermore, being a structural analysis methodology, it remedies a frequent problem in this type of experiment, which is the "n" to be used in statistics.

Finally, we hypothesized that TMZ could protect the target tissues during severe HS. The objective of the current study is to compare, in a HS animal model, organ damage parameters between a group with and a group without the administration of TMZ.

Material and Methods

Animals

Eighteen male Wistar rats (weight, 400 g to 500 g; 12 to 15 weeks old) were kept under standardized conditions of temperature (21 ± 2ºC), and 12: 12-h light: Dark cycles with free access to food and water. Animals received humane care according to the standards of the Canadian Council on Animal Care on the protection of animals used for scientific purposes [5,7]. The study was approved by the local Ethics Committee.

Hemorrhagic shock model

Shock model was performed as previously described [8,9]. Rats were anesthetized with intraperitoneal sodium pentobarbital, 50 mg/kg for induction and 10 mg/kg for maintenance, given as needed (interdigital reflex).

An incision was made along the ventral cervical skin, a tracheal tube was placed to maintain an open airway and polyethylene catheters were placed within both carotid arteries and the jugular vein. After catheter insertions, rats were allowed to stabilize for 20 min. To compensate for fluid loss over surgical areas and respiration, Ringer lactate (RL, 3 ml/h) was infused through the jugular vein catheter throughout the experiment.

Hemorrhagic shock period (HSP): A controlled fixed pressure hemorrhagic shock was induced by controlled arterial bleeding; 2 mL of blood were removed every 3 min through the left carotid artery catheter. Bleeding was continued until Mean Arterial Blood Pressure (MAP) dropped under 42 mmHg; MAP was maintained between 38 and 42 mmHg for 60 min (Figure 1). To keep MAP within the desired range, in some cases, small amounts (0.1 mL to 0.5 mL aliquots) of 0.9% NaCl solution were administered, or additional small blood samples (0.1 mL to 0.5 mL aliquots) withdrawn. Drawn blood was stored in sterile plastic conical tubes prefilled with heparin in a heater at 37°C for subsequent reinfusion.

Resuscitation period (RP): After HSP, drawn blood was reinfused and, during the next 30 min, RL was injected through the jugular vein, in a volume equal to twice that of the removed blood [9]. Animals were sacrificed immediately after RP (Figure 1).

Treatment protocol: Rats were randomized to receive either TMZ (10 mg/kg administered orally by gavage just before anesthesia, n=9) or placebo (0.9% NaCl solution administered in same conditions and equal volume as TMZ, n=9) A 10 ml/kg TMZ dose was selected considering previous studies (reported dosage between 2.5 mg/kg and 15 mg/kg [10,11] and a single dose was chosen due to the acute and usually unplanned nature of HS [12].

Monitoring

Arterial pressure was measured (and MAP calculated) continuously by using the right carotid artery catheter which was connected to a pressure transducer and displayed on a monitor. Rectal temperature was monitored *via* a rectal sensor and maintained at 37 ± 1°C by a heating pad. All animals were euthanized at the end of the RP by pentobarbital overdose.

Assessment of blood parameters

Arterial blood samples were obtained at baseline (just before HSP, referred to as T0) and at the end of both HSP (referred to as T1) and RP (referred to as T2). Hematocrit, pH, arterial oxygen and carbon dioxide partial pressures (pO2, pCO2), oxygen saturation, plasma bicarbonate, base excess, lactate, Creatinine (Cr), Lactic Dehydrogenase (LDH) activity, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Creatine Phosphokinase (CPK), were assessed [8,9,13].

Fractal dimension and lacunarity

Fractal analysis was performed as previously described [14,15]. Fractal Dimension (FD) of binarized micrographs was determined using box-counting method, based on so called Richardson–Mandelbrot Plot. This technique calculates the box fractal dimension of the micrograph, which is also known as capacity dimension or Kolmogorov dimension. Lacunarity (L) is a complement to fractal analysis in biology and histology and defined as the degree of structural in homogeneity. In another word, lacunarity of a biological structure is highly correlated with its heterogeneity, and the cells/tissues with higher number and/or size of gaps in their structure are expected to have higher value of this parameter. Fractal dimension and lacunarity are sometimes in negative correlation [16]. Moreover, complete mapping of semi serial tissue sections colored by H&E, was performed in each animal of the Control (C) and Trimetazidine (TMZ) groups. Then, were obtaining digital images with a magnification of 100x, using a Nikon Eclipse 50i optical microscope, equipped with a Coolpix DS-Fi1 camera and connected to a desktop PC with specialized software for analysis, capture and photo-documentation (NIS-Elements Basic Research, Nikon Instruments Inc., USA). Were obtained between 5 to 7 digital images per animal and in tiff format, with the following properties: Dimensions of 1280 pixels × 960 pixels; horizontal and vertical resolution of 5254 dpi; unit of resolution of 3 bits and depth of 24 bits. Next, we proceeded to the processing of each of them prior to the fractal analysis, using the ImageJ image analysis software that included the FracLac plugin (NIH, Bethesda, Maryland, USA). They were transformed into binary images and then applied the outline filter. In this way, series of dimensionless values of fractal

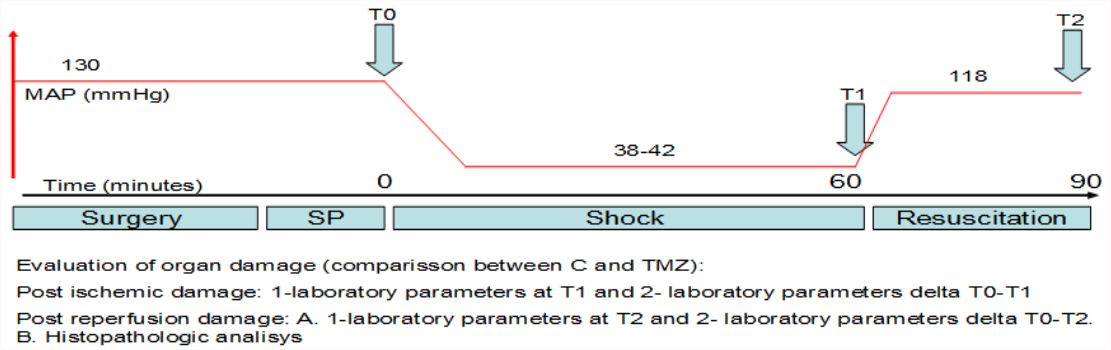

Figure 1: Shock Model and organ damage evaluation. Schedule of the experimental procedure.
T0: Start of shock induction; T1: End of shock period and beginning of resuscitation period; T2: End of resuscitation period; SP: stabilization Period; MAP: Mean Arterial Pressure; C: Control group; TMZ: Trimetazidine

dimension and lacunarity were obtained.

Exclusion criteria: Unintentional bleeding due to surgical procedure complications plus a hematocrit descend greater than 15%; measured MAP below 80 mmHg during the whole RP (inadequate resuscitation). Acid-base protocols of rats with serum lactate greater than 3 mmol/L or oxygen saturation below 84% before shock induction were also excluded (oxygen delivery diminished before HSP).

Comparisons between groups

Hemodynamic parameters: MAP during the whole experiment was compared between both groups.

Organ damage: A- Pre-shock (baseline) status was assessed by comparing laboratory parameters measured from blood samples collected at T0 (pre-HSP) between both groups (C *vs.* TMZ); B- Post ischemic damage was assessed by comparing between groups (C *vs.* TMZ): 1-laboratory parameters at T1 (post-HSP, i.e., creatinine at T1) and 2-laboratory parameters difference T0 to T1 (i.e., creatinine increase or delta from T0 to T1); C-Post reperfusion damage was assessed by comparing between groups (C *vs.* TMZ) both laboratory parameters and histopathology as follows: 1-laboratory parameters at T2 (post RP); 2-laboratory parameters difference T0 to T2; histopathologic evaluation of organs by conventional and fractal (fractal dimension and fractal lacunarity) analysis (Figure 1).

Statistical analysis

Data are expressed as mean ± SD. Statistical comparisons between different groups were made by Student t-test. Histological scores were analyzed by the Mann-Whitney test. A $p<0.05$ was considered statistically significant.

Results

Eighteen rats were subjected to HS; five were excluded (3 in the C group and 2 in the TMZ group) and thirteen included (C, n=6 and TMZ, n=7).

Hemodynamic parameters

All animals subjected to HS reached the desired level of hypotension. No significant differences were observed in mean MAP al baseline, HSP or RP between groups (mmHg, baseline: C: 132.8 ± 29.9, TMZ: 128.4 ± 25.2, p=0.77; HSP: C: 40.8 ± 0.5, TMZ: 40.5 ± 0.3, p=0.31, RP: C: 118.7 ± 15.0, TMZ: 119.1 ± 22.1, p=0.96). Time needed to reach the shock pressures was similar for both groups C: 6.5 min ± 0.9 min, TMZ: 7.1 min ± 0.4 min, p=0.23). Mean amount of withdrawn blood was similar between both groups (total removed blood: C: 11.9 ml ± 1 ml, TMZ: 11.2 ml ± 0.7 ml, p=0.19; ml/kg: C: 28.1 ± 2.6, TMZ: 25.6 ± 2, p=0.08).

Blood markers and histopathologic evaluation

Laboratory parameters of both groups measured at T0, T1 and T2 are displayed in Table 1, 2 and 3 respectively.

Similar hematocrit decrease was found in both groups (hematocrit, %; baseline, Control: 51.3 ± 4.1, TMZ: 50.7 ± 2.4, p=0.74; post-HSP, C: 40.7 ± 3.9, TMZ: 40.4 ± 1.5, p=0.88; post-RP, C: 35.5 ± 4.5, TMZ: 36.8 ± 1.6, p=0.47).

Pre HSP (T0): At baseline, pCO2 was lower (mmHg, C: 43.8 ± 2.5, TMZ: 38.5 ± 4; p=0.039) and there was a non-significant trend to a higher pH (C: 7.39 ± 0.01, TMZ 7.44.04, p=0.0596) in TMZ group. No other significant differences between groups were found before HSP.

Post HSP (T1): At the end of HSP, TMZ group showed reduced serum Cr (C: 0.76 mg/dl ± 0.09 mg/dl; TMZ: 0.65 mg/dl ± 0.06 mg/dl; p=0.011), reduced serum lactate (C: 7.88 mmol/L ± 1.39 mmol/L; TMZ: 5.58 mmol/L ± 1.2 mmol/L, p=0.020) and increased serum bicarbonate (C: 13.34 mmol/L ± 3.19 mmol/L; TMZ: 17.08 mmol/L ± 1.50 mmol/L; p=0.045). Also significant differences favoring the TMZ group were observed in bicarbonate decrease (mean T0 to T1 difference, C: 13.02 mmol/L ± 2.97 mmol/L; TMZ: 8.72 mmol/L ± 2.08 mmol/L, p=0.029), lactate increase (mean T1 to T0 difference, C: 5.78 mmol/L ± 1.61 mmol/L; TMZ: 3.42 mmol/L ± 0.80 mmol/L), creatinine increase (mean T1 to T0 difference, C: 0.47 mmol/L ± 0.09 mmol/L; TMZ: 0.37 mmol/L ± 0.06 mmol/L) and LDH increase (mean T1to T0 difference, C: 671.6 U/l ± 276.2 U/l; TMZ: 280.2 U/l ± 325.3 U/l; p=0.041). Non-significant trends also favoring the TMZ group were observed in base excess (C: -9.3 mmol/L ± 2.7 mmol/L; TMZ: -5.8 mmol/L ± 2.4 mmol/L, p=0.066), ALT increase (T1 to T0 difference, Control: 21.6 mmol/L ± 15.3 mmol/L; TMZ: 10 mmol/L ± 5.6 mmol/L) and AST increase (T1 to T0 difference, Control: 103.4 mmol/L ± 14.2 mmol/L; TMZ: 54.4 mmol/L ± 46.9 mmol/L). No other significant differences or trends were observed in the rest of the blood parameters after HSP.

Post RP (T2): No significant differences were found in blood chemistry after RP. A non-significant trend toward a lower increase in LDH (T2 to T0 difference, C: 983 mmol/L ± 248 mmol/L; TMZ: 636 mmol/L ± 315 mmol/L) was observed in the TMZ group.

Organ changes: Fractal analysis: TMZ decreased the structural

Table 1: Laboratory parameters of both groups measured at baseline (before shock induction, T0).

T0 (Pre-shock, before HSP)	TMZ Group (mean ± SD)	Control Group (mean ± SD)
pH [7.35-7.45]	7.44 ± 0.2 **	7.391 ± 0.02
pCO2 (mmHg) [35.0-43.0]	38.5 ± 4 *	43.8 ± 2.5
pO2 (mmHg) [80.0–90.0]	80.8 ± 3.8	83.4 ± 8.2
HCO3- (mmol/L) [22.0-26.0]	25.8 ± 1.2	26.4 ± 0.9
BE (mmol/L) [-2.0–2.0]	2.2 ± 1.5	1.6 ± 1
Lactate (mmol/L) [3-.,4]	2.2 ± 0.4	2.1 ± 0.2
AST (U/L) [74–143]	120.1 ± 33.5	127 ± 54.5
ALT (U/L) [18–45]	45.4 ± 12.7	41.2 ± 7.5
Cr (mg/dL) [0.2–0.5]	0.27 ± 0.02	0.29 ± 0.06
CK (U/L) [0-190]	1411.8 ± 1276	1735.4 ± 1171
LDH (U/L) [240–480]	596.8 ± 250	498 ± 213
Hematocrit (%) [39-52]	50.7 ± 2.4	51.3 ± 4.1

HSP: Hemorrhagic Shock Period; TMZ: Trimetazidine; T0: Start of Shock Induction; pO2: Arterial Oxygen Partial Pressures; pCO2: Carbon Dioxide Partial Pressures. Oxygen Saturation; HCO3- Plasma Bicarbonate; BE: Base Excess; Cr: Creatinine; LDH: Lactic Dehydrogenase Activity; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; CK: Creatine Phosphokinase

* = p ≤ 0.05

** = p=0.05-0.1

damage in heart (FD: C: 1.732 ± 0.041; TMZ: 1.759 ± 0.043; p<0.0001 and L: C: 0.33 ± 0.075; TMZ: 0.29 ± 0.066; p=0.01), liver (FD: C: 1.776 ± 0.039; TMZ: 1.786 ± 0.037; p=0.0021 and L: C: 0.28 ± 0.057; TMZ: 0.29 ± 0.058; p=ns) and kidney (FD: C: 1.742 ± 0.04; TMZ: 1.79 ± 0.04; p<0.0001 and L: Control: 0.25 ± 0.04; TMZ: 0.22 ± 0.05; p<0.01) (Figure 2, panels A, B, C and D, E and F).

Discussion

According to our results, TMZ attenuates cellular damage in different peripheral tissues after severe HS. Within each group decrease in hematocrit and blood pressure was marked, showing the severity of the shock model; however no significant differences were found between groups, thus reinforcing the similarity of hemodynamic condition in both groups. Furthermore, hemodynamic parameters remained similar between both groups, irrespective of treatment, consistent with the notion that TMZ does not affect arterial pressure or heart rate.

In HS, because of the reduced blood flow, oxygen delivery is impaired and mitochondrial capability to synthesize ATP is reduced. This in turn results in ATP depletion, reduction in the activity of cell ATPases, intracellular accumulation of sodium and cellular edema [17,18]. When mitochondrial glucose oxidation collapses, anaerobic glycolysis produces lactate accumulation and intracellular acidosis. High rates of FFA oxidation during ischemia inhibit glucose oxidation (through a direct inhibitory action on pyruvate dehydrogenase), thus increasing lactate and H+ accumulation.

Trimetazidine inhibits β-oxidation and increases the metabolic rate of glucose utilization [6] by selectively inhibiting the enzyme long-chain 3-ketoacyl coenzyme A thiolase [4], which is the final enzyme in the FFA β-oxidation pathway [19]. Importantly, FFA oxidation requires more oxygen than glucose to produce the same amount of ATP [20]. Therefore, considering that in terms of oxygen consumption, glucose is a better energy source than FFA, TMZ administration is expected to result in greater ATP production for the

Table 2: Laboratory parameters of both groups measured after hemorrhagic shock period (T1).

T1 (Post HSP)	TMZ Group (mean ± SD)	Control Group (mean ± SD)
pH [7.35 - 7.45]	7.41 ± 0.07	7.42 ± 0.08
pCO2 (mmHg) [35.0–43.0]	27.8 ± 3.8	21.7 ± 8.6
pO2 (mmHg) [80.0–90.0]	98.3 ± 9.1	103.5 ± 7.4
HCO3- (mmol/L) [22.0-26.0]	17,1 ± 1,5 *	13.3 ± 3.2
BE (mmol/L) [-2.0–2.0]	-5.8 ± 2.4 **	-9.3 ± 2.7
Lactate (mmol/L) [1.4–3]	5.6 ± 1.2 *	7.9 ± 1.4
AST (U/L) [74–143]	174.6 ± 43.3	212.3 ± 73.5
ALT (U/L) [18–45]	55.4 ± 11.9	62.8 ± 19.2
Cr (mg/dL) [0.2–0.5]	0.65 ± 0.04 *	0.76 ± 0,09
CK (U/L) [0-190]	2678 ± 1791	3898.7 ± 2844
LDH (U/L) [240–480]	877.14 ± 245	1086.5 ± 522
Hematocrit (%) [39-52]	40.4 ± 1.5	40.7 ± 3.9
T1-T0 difference		
pH	0.03 ± 0.09	0.02 ± 0.07
pCO2 (mmHg)	10.8 ± 6.5 *	22 ± 8.4
pO2 (mmHg)	17.5 ± 10.6	20.1 ± 6.6
HCO3- (mmol/L)	8.7 ± 2.1 *	13 ± 2.9
BE (mmol/L)	8 ± 3.3	10.9 ± 2.4
Lactate (mmol/L)]	3.4 ± 0.8 *	5.8 ± 1.6
AST (U/L)	54.4 ± 46.9 **	103 ± 31.8
ALT (U/L)	10 ± 5.6 **	21.6 ± 15.3
Cr (mg/dL)	0.37 ± 0.06 *	0.47 ± 0.09
CK (U/L)	1266 ± 1984	2452 ± 2017
LDH (U/L)	280.2 ± 325.3 *	671.6 ± 276.2
Haematocrit	10.3 ± 1.9	10.7 ± 2.9

HSP: Hemorrhagic Shock Period; TMZ: Trimetazidine; T1: End of shock period and beginning of resuscitation period. pO2: arterial oxygen partial pressures; pCO2: Carbon Dioxide Partial Pressures. Oxygen Saturation; HCO3: Plasma Bicarbonate; BE: Base Excess; Cr: Creatinine; LDH: Lactic Dehydrogenase Activity. ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; CK: Creatine Phosphokinase

* = p ≤ 0.05

** = p=0.05-0.1.

same amount of oxygen [19]. This is an advantage under conditions of reduced oxygen availability, such as severe under perfusion. TMZ also increases pyruvate dehydrogenase activity, which starts restoring homeostasis between glucose oxidation and glycolysis (imbalanced during ischemia) and decreases H+ accumulation [20]. Also, in principle, relative FFA inhibition with concomitant metabolic switch over to glycolysis could be beneficial as ATP produced from glycolysis is closer to cytoplasmatic membrane and therefore more easily used by membrane ionic pumps [21]. TMZ has been shown to attenuate changes in intracellular sodium (with the resulting decrease in cellular edema) and ph during ischemia and IRI [22-25]. TMZ protects mitochondrial function and integrity from IRI since it prevents the opening of the mitochondrial permeability transition pore [25,26]. TMZ also reduces lipid peroxidation [27-30], enhances natural antioxidants defenses [29,31], acts as a free radical scavenger, reduces ROS production [31,32], and inhibits neutrophil accumulation in tissues after ischemia and reperfusion. A great congruence exists between TMZ mechanism of action and

Table 3: Laboratory parameters of both groups measured after resuscitation period (T2).

T2 (Post RP)	TMZ Group (mean ± SD)	Control Group (mean ± SD)
pH [7.35-7.45]	7.44 ± 0.02	7.461 ± 0.015
pCO2 (mmHg) [35.0–43.0]	33.4 ± 3.1	31.6 ± 2.4
pO2 (mmHg) [80.0–90.0]	78.6 ± 5.4	87.1 ± 3
HCO3- (mmol/L) [22.0-26.0]	21.9 ± 5.3	22.1 ± 1
BE (mmol/L) [-2.0–2.0]	-0.48 ± 0.86	-0.58 ± 0.72
Lactate (mmol/L) [1.4–3]	3.7 ± 0.4	4 ± 0.6
AST (U/L) [74–143]	230.6 ± 26.2	219.8 ± 44.1
ALT (U/L) [18-45]	88.1 ± 14.8	78.3 ± 14.4
Cr (mg/dL) [0.2–0.5]	0.4 ± 0.13	0.44 ± 0.03
CK (U/L) [0-190]	3464 ± 777	4591 ± 1033
LDH (U/L) [240-480]	1233.6 ± 158.6	1398.5 ± 198
Haematocrit	36.8 ± 0.6	35.5 ± 1.8
T2-T0 difference		
pH	0.01 ± 0.09	0.06 ± 0.05
pCO2 (mmHg)	3.9 ± 10.9	9 ± 6.9
pO2 (mmHg)	0.2 ± 17.4	3.8 ± 5
HCO3- (mmol/L)	2.5 ± 2.8	3.6 ± 1.2
BE (mmol/L)	2.5 ± 1.3	1.7 ± 1.3
Lactate (mmol/L)]	0.9 ± 0.4	1.4 ± 0.7
AST (U/L)	110.4 ± 48.6	136.6 ± 46.7
ALT (U/L)	42.7 ± 32.4	37.2 ± 32.3
Cr (mg/dL)	0.13 ± 0.03	0.16 ± 0.05
CK (U/L)	2052 ± 2126	3145 ± 1668
LDH (U/L)	636.7 ± 315.7 *	983.5 ± 248.5
Haematocrit	13.8 ± 3.5	15.8 ± 6.8

HSP: Hemorrhagic Shock Period; TMZ: Trimetazidine; T2: End of resuscitation period; pO2: Arterial Oxygen Partial Pressures; pCO2: Carbon Dioxide Partial Pressures. Oxygen Saturation; HCO3: Plasma Bicarbonate; BE: Base Excess; Cr: Creatinine; LDH: Lactic Dehydrogenase Activity; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; CK: Creatine Phosphokinase

* = p ≤ 0.05

** = p=0.05-0.1

pathophysiologic mechanism of cellular damage during HS.

TMZ has shown cytoprotective effects in many different organs: heart, kidney, liver, lung, brain, and intestine (22,23,25,31,33,34). Although there is already a large amount of data pertaining to the effect of TMZ on isolated organ IRI (mostly in animal models of either transient clamping of one organ's nutritious artery or one organ transplantation), there is almost no information about the effect of TMZ as part of shock treatment: no animal studies are available. To our knowledge, there is only one study approaching the effect of TMZ in shock: Authors compared renal function in patients with shock and standard treatment with and without the addition of TMZ and found that TMZ improves renal function [35]. Zhang et al. did not report any other organ damage parameter or any perfusion index [35].

Our finding shows that the protective effect of TMZ, previously proved on isolated organ IRI, is reproduced in HS. The laboratory results observed in TMZ group, suggest an enhances peripheral tissue tolerance to ischemia and protective aerobic cellular metabolism after HSP. After RP there were no significant differences between groups in blood chemistry; however, histopathological analysis (both conventional and fractal analysis, both better ways of assessing organ injury than blood markers) showed beneficial effects of TMZ. Fractal analysis is essentially a mathematical algorithm applied to an image evaluation, and it is a well-established method that can detect subtle changes that are not detectable using conventional microscopy methods [36,37]. Fractal analysis is based on an algorithm which is objective and, therefore, independent of the researchers potential bias or level of expertise [36,38]. Two main parameters are calculated through this method: Fractal dimension and fractal lacunarity, which are indicators of cellular complexity and structure heterogeneity, respectively [36]. Phenomena that occur during reperfusion injury (such as cellular vacuolization and fading of internal tissue boundaries related to membrane destruction) influence fractal parameters. In injured tissue there is a reduction of cellular complexity and fractal dimension and an increase in cellular heterogeneity and fractal lacunarity [36,39]. Microscopically, dilatation of the coronary microcirculation and interstitial edema were observed, both in C and TMZ groups. On the other hand, the mean value of the fractal dimension of the left ventricle in the animals of C group was significantly lower than TMZ group. Likewise, the mean value of left ventricular lacunarity in the animals of Control group was significantly higher than that of the remaining group.

These findings suggest that the dilatation of the coronary microcirculation and interstitial edema were more pronounced in C group, thus reducing the structural complexity of the left ventricle and, at the same time, increasing its heterogeneity with respect to the TMZ group. Both parameters did not show significant differences between the treated groups. In conclusion, the analysis carried out suggests that TMZ exert an early protective effect on the left ventricle of rats subjected to hemorrhagic shock.

In kidneys, the vacuolization of the epithelial cells lining the renal tubules, the disruption of the proximal convoluted tubules brush border, the damage to their basement membranes and the dilation of tubular lumens represents factors that favoring the reduction of structural complexity of kidney tissue, conducting to the reduction of fractal dimension and increase of lacunarity. These changes are producing by the reperfusion of the renal tissue after hemorrhagic shock, which appear as early as 30 min of restoration of blood flow. In this sense, it is interesting to note that certain drugs can preserve the renal structure during the reperfusion period, and, probably, the functional reserve of the tissue against future injuries that may affect it. The findings of the presented experiment suggest that TMZ maintains renal structural complexity after reperfusion for 30 min during the hemorrhagic shock. Also, TMZ improves the complexity and structural homogeneity of the renal parenchyma. This implies less cytoplasmic vacuolization of the tubular epithelium, less dilation of its lumens, less interstitial edema and, ultimately, preservation of the renal parenchyma.

The mean value of the fractal dimension of the liver parenchyma in the control group is significantly lower than TMZ group, while no differences were observed in the lacunarity value. These findings suggest that, in the control group, the structural complexity of the liver parenchyma was lower, attributable to greater cytoplasmic vacuolization of hepatocytes, greater vascular dilation (congestion), and parenchyma edema compared to animals treated with TMZ. On the other hand, both groups did not show differences regarding the texture or appearance of the tissue (lacunarity). In other words, microscopically the liver parenchyma of the C and TMZ have a

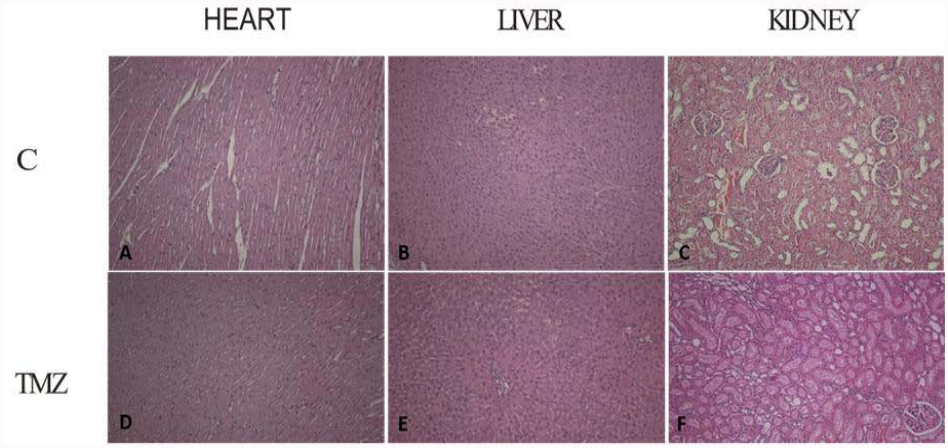

Figure 2: TMZ decreased histopathological damage in the heart (D), liver (E), and kidney (F) compared with C group (A, B and C). The renal parenchyma showed disruption of the brush border of proximal convoluted tubules, necrosis of the lining epithelium and intraluminal detached epithelial cells (C). The treatment with TMZ prevented the changes previously described (E) (H&E, 200x).

similar appearance, but vary in their fractal dimension, demonstrated the usefulness of the method employed.

Some limitations of our study should be addressed. The RP of 30 min could possibly be too short to let the reperfusion injury be fully observable by light microscopy. In our experiment, shed blood was stored with heparin; although heparin can reduce organ damage in hemorrhagic shock, both control and treatment group were exposed to the same conditions to avoid this bias. TMZ was administered prior to shock and pre-treatment in HS might not seem clinically relevant. However, beneficial effects of TMZ administered either pre or post ischemia have been shown [40,41]. On the other, critical patient's hemodynamic deterioration can many times be foreseen and scenarios like surgery are programmed hypovolemic states. All of the previous make our model clinically relevant.

Although hypothetical, it seems logical to infer that results in HS could be reproduced in other types of shock (cardiogenic, septic) since, from the cellular perspective, all types of shock have some degree of peripheral tissue hypoperfusion and IRI and TMZ protects from both.

Conclusion

According to the results of our study, TMZ enhances aerobic metabolism of peripheral tissues, acting as a cytoprotective agent and attenuating damage on the kidney, heart and small intestine after severe HS. Because of this, TMZ might be a viable complementary option (associated to standard resuscitation therapy) in the treatment of severe hypovolemia.

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