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Original Article

ISCHEMIA/REPERFUSION INJURY AFTER CONTINUOUS OR INTERMITTENT HEPATIC PEDICLE CLAMPING IN RABBITS

Lesão de isquemia e reperfusão após clampagem contínua ou intermitente do pedículo hepático em coelhos

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ABSTRACT - ***Background*** - The control of bleeding in hepatectomy is a challenge for surgeons. The hepatic pedicle clamping is a surgical maneuver that can provide reduction in bleeding, but it provokes a hepatocellular suffering. This, along with reperfusion after the clamping finishes, leads to an injury known as ischemia/reperfusion injury. ***Aim*** - To examine the effects of the ischemia/reperfusion injury on the liver after continuous and intermittent hepatic pedicle clamping in an animal model, using the quantification of apoptosis for evaluation. ***Method*** - Twenty New Zealand rabbits were assigned to groups 1 (control), 2 (60 minutes of continuous ischemia) and 3 (60 minutes of intermittent ischemia alternating 12 minutes of ischemia and three minutes of reperfusion). Liver biopsies were collected before ischemia, at its end and after six hours of reperfusion, when the animals were killed. The liver fragments were subjected to histological analysis (paraffinization and hematoxilin-eosin staining) and histochemical (Tunel reaction). Microscope fields of view were scanned for characterization and quantification of apoptosis. ***Results*** - Ischemia led to an increased apoptotic index in both experimental groups in comparison to controls, but similarly between them. After the reperfusion, the indexes returned to baseline values. ***Conclusion*** - Clamping of the hepatic pedicle, either continuous or intermittent, induces apoptosis in liver cells in a similar way.

HEADINGS - Ischemia/surgery. Reperfusion injury. Liver/surgery. Apoptosis

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RESUMO - ***Racional*** - O controle do sangramento na hepatectomia é um desafio para os cirurgiões. A clampagem do pedículo hepático é manobra cirúrgica que pode promover redução do sangramento, mas provoca isquemia hepatocelular. Isso, junto com a reperfusão depois que a clampagem termina, leva à lesão de isquemia e reperfusão. ***Objetivo*** - Examinar os efeitos da lesão de isquemia e reperfusão no fígado após clampagem contínua e intermitente do pedículo hepático, usando a quantificação de apoptose como ferramenta. ***Método*** - Vinte coelhos New Zealand foram divididos em grupos 1 (controle), 2 (60 minutos de isquemia contínua) e 3 (60 minutos de isquemia intermitente alternando 12 minutos de isquemia e três minutos de reperfusão). Biópsias hepáticas foram colhidas antes e ao fim da isquemia e após seis horas de reperfusão, quando os animais eram sacrificados. Os fragmentos obtidos foram submetidos à análise histológica e histoquímica (reação de Tunel). Campos microscópicos foram analisados para caracterização e quantificação de apoptose. ***Resultados*** - A isquemia levou à elevação do índice apoptótico em ambos os grupos experimentais em relação aos controles, mas similar entre eles. Depois da reperfusão os índices voltaram aos valores iniciais. ***Conclusão*** - A clampagem do pedículo hepático, tanto contínua quanto intermitente, induz a apoptose em células hepáticas de modo igual.

DESCRITORES - Isquemia/cirurgia. Traumatismo por reperfusão. Fígado/cirurgia. Apoptose.

INTRODUCTION

Bleeding control in liver surgery remains a major challenge for surgeons. The control techniques include the hepatic pedicle clamping proposed by Pringle[24] and total vascular exclusion, among others. The common goal is to reduce hemorrhage of the hepatic parenchyma, allowing the surgeon to perform the procedure without bleeding that could interfere in the morbidity and mortality.

An adverse effect of hepatic pedicle clamping is ischaemia of the

organ, which together with its reperfusion determines ischemia-reperfusion (I/R) injury. To a certain threshold, the patients will better respond to injuries caused by liver I/R injury than to the damage caused by extensive hemorrhages and multiple transfusions. The I/R injury results in microcirculatory failure, followed by cell death, thus protecting the hepatocytes of this event is important for liver surgery. Several mechanisms have been proposed to explain the I/R injury, but its pathophysiology is still not entirely clear[7,20,27,29,30].

Apoptosis is a special type of cell death, where the cell is stimulated to trigger mechanisms that will culminate with his own death. Therefore, it is also known as cell suicide[8]. It seems to be important in situations of cellular oxidative stress and as a mechanism of cell death in I/R injury[12,25].

The intermittent hepatic clamping, in which short periods of clamping are interspersed with reperfusion, may be a way to minimize cellular damage in the body because it prevents prolonged warm ischemia[1,4,28].

This study aimed to analyze the I/R injury after continuous or intermittent hepatic pedicle clamping, using the quantification of liver apoptosis as a marker. This way, different levels of apoptosis induction could demonstrate the superiority of one method over the other, ensuring greater protection to the liver parenchyma.

METHODS

Twenty adult male albino rabbits (Oryctolagus cuniculus) with average weight of 2218 ± 404 g (Veterinary Farm in Igarapé, MG, Brazil) were subjected to quarantine, clinical assessment, individual accommodation with provision of water and food ad libitum. The animals were randomly allocated into three groups: control (n = 5), continuous ischemia (n = 8) and intermittent ischemia (n = 7). All procedures were previously approved by the Ethics Committee on Animal Experiments of Minas Gerais Federal University (Approval Certificate nº 170/2006) and obey international standards.

The preoperative fasting was 12 hours for solids. There was no fasting for liquids. The animals underwent two surgical procedures, defined as initial operation and final operation, carried out with an interval of six hours. Anesthesia, operations and euthanasia (performed immediately after the second operation) were performed following the protocol published in our institution[22].

The animals in the experimental groups of ischemia underwent laparotomy, liver biopsy, continuous or intermittent ischemia for 60 minutes and a new liver biopsy immediately after reperfusion. They were accommodated in cages for postoperative recovery. Six hours after the initial operation, they were anesthetized and underwent another laparotomy with liver biopsy and euthanasia. Biopsy samples were obtained by wedge incision in the right lobe and puncture using commercial kit Hepafix®. The moments in which biopsies were collected were nominated in sequences as T1, T2 and T3. The clamping of the hepatic pedicle was done as described by Pringle [1] using a lace and a latex ring and intermittence was done with periods of 12 minutes of ischemia and three minutes of reperfusion.

Morphometry

The liver biopsies were placed into individual identified vials containing 10% buffered formaldehyde. They were processed routinely with paraffin embedding, cutting and staining with hematoxylin-eosin (HE) or Tunel14 to detect genome fragmentation in situ / characterization of apoptosis.

Was used a commercial kit for in situ detection of genome fragmentation (FragEl Klenow DNA fragmentation Detection Kit - QIA21 in the Catalog, Calbiochem / Oncogene, available on the website http://calbiochem.com/), following the protocol specified by the manufacturer. The tissue was deparaffinized by immersion in xylene and alcohol in decreasing dilutions, and then washed twice with distilled water. Endogenous peroxidase was inactivated by covering the sections with 3% hydrogen peroxide for five minutes. The tissue was then washed in PBS and immersed in equilibrium buffer solution. The sections were covered with dTT (deoxinucleotide terminal transferase), UTP and digoxigenin and placed in humid atmosphere at 37ºC for two hours, and then incubated for 10 minutes in stop and wash buffer. Then the slides were placed in a humidified chamber at 37º C for 18 hours (overnight incubation) with an anti-digoxigenin peroxidase conjugate. The slides were immersed in PBS again, then immersed for six minutes in diaminobenzin (revelation of the reaction), washed with distilled water, stained with the dye Methyl Green and mounted for microscopic evaluation. The slides were examined under optical microscope and images of histological fields of view were digitized and analyzed with a morphometry software (UTHSCSA Image Tool for Windows®) to obtain the apoptotic index.

Each histological section with HE staining had random fields of view captured in digital format at 400X magnification. A same observer counted total and apoptotic hepatocytes in each field of view. Cells were considered apoptotic when they showed at least three of the following findings: 1) shrunken anoykic cells (cell shrinkage and loss of adhesion with neighboring cells), 2) cytoplasmic condensation; 3) nuclear condensation (condensation of nuclear chromatin, sometimes permeating the upper part of the nuclear membrane and displaying pictures of "crescent moon", 4) nuclear fragmentation, 5) formation of apoptotic bodies.

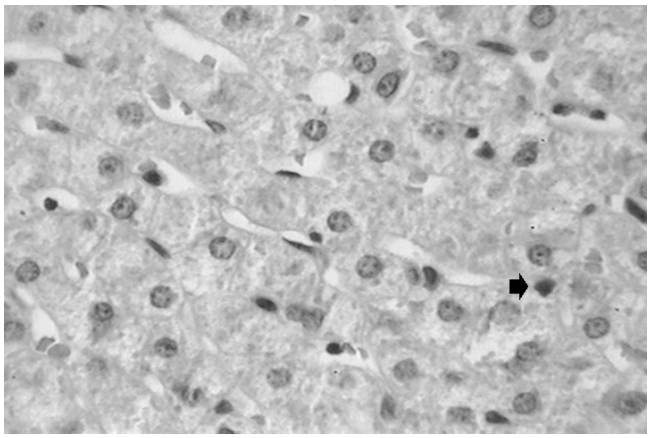

FIGURE 1 - Identification of cell apoptosis in a scanned field of view. The arrow points an apoptotic cell, with nuclear condensation and shrunken anoykic. Hematoxylin-eosin, 400x

The quantification of apoptotic index was performed by blind test by a single observer. The apoptotic index (AI) was calculated by the following formula:

AI = (apoptotic hepatocytes / total hepatocytes) x 100

The coefficient of variation (CV) was used to determine the minimum number of fields of view necessarily analyzed in order to obtain statistical significance. The method consisted in obtaining sub-groups of a smaller sample among 100 different fields of one slide stained with HE randomly chosen in the sample. Sub-groups were randomly selected with an increasing number of fields and the variation coefficients obtained for each sub-group were plotted in a graph, where a curve was obtained. Thus, the minimum number of representative fields was found when increasing the number of fields did not result in considerable reduction in the value of CV, which occurred after counting 12 fields, as noted in Figure 2.

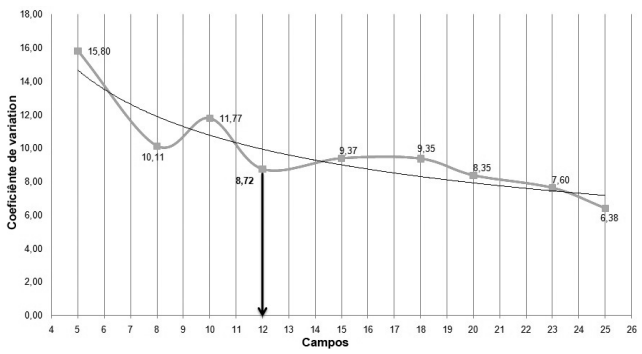

FIGURE 2 - Result of analysis determining the minimum number of representative fields of view (with the trend line in black)

The validation of the results obtained with the of HE-stained slides morphometric analysis was done with Tunel-reaction slides randomly selected in the proportion of 20% of all blades, uniformly distributed in the different groups. These slides were submitted to Tunel reaction as described above. Fields with 400X magnification were digitized in the same way as described for the slides stained with HE.

Statistical analysis

The data obtained in this study was classified as continuous parametric variables and was presented as mean ± standard deviation. The Kolmogorov-Smirnov test was used to verify the normality of the sample and the Student t test was used with independent samples (between groups) and paired samples (same group analysis). Significancy was considered when p values were below 0.05. SPSS® and GraphPad Prism® for Windows® were the software used.

RESULTS

All animals demonstrated normal behavior during assessment of the study, without clinical manifestations of disease. There was no need for replacement of any animal. There were no pre, trans or postoperative complications. About three to four hours after the initial operation, the animals were awake and moving.

Apoptosis quantification

Was obtained AI from different groups and times were normally distributed according to the Kolmogorov-Smirnov test, which presented the distante = 0.1746 (p>0.10, alpha=0.05).

Its distribution occurred as shown in Table 1, with uniformity in the control group and increased after intervention in groups of ischemia, which returned to levels close to baseline after six hours of reperfusion.

TABLE 1 - Mean apoptotic indexes in different groups, according to the moment of the measurement

Group Means	T1 (0)	T2 (1h)	T3 (6h)
Group 1 (Control)	7,32	7,36	7,32
Group 2 (Continuous ischemia)	7,77	8,78	7,83
Group 3 (Intermittent ischemia)	7,93	8,64	7,79

In the control group, there was no difference between the AI at any time of the protocol. In the ischemia groups, difference was found at the end of ischemia compared to baseline, and reperfusion reestablished the AI to initial values before ischemia. The result of this analysis is summarized in Table 2.

The distribution positivity obtained by the Tunel method for the same animal before and after ischemia showed patterns ranging from mild to more dense (Figure 3).

TABLE 2 - Paired analysis of the apoptotic indexes

Group	Pair	Paired differences			p value
		Mean	Standard deviation	Standard error mean	
Group 1 Control	IA1 – IA2	-0,38	0,18	0,81	0,666
	IA2 – IA3	0,46	0,22	0,98	0,664
	IA1 – IA3	0,08	0,17	0,74	0,920
Group 2 Continuous ischemia	IA1 – IA2	-1,01	0,56	0,20	0,001*
	IA2 – IA3	0,95	0,53	0,19	0,002*
	IA1 – IA3	-0,57	0,59	0,21	0,794
Group 3 Intermittent ischemia	IA1 – IA2	-0,71	0,47	0,18	0,007*
	IA2 – IA3	0,85	0,79	0,30	0,030*
	IA1 – IA3	0,14	0,39	0,15	0,375

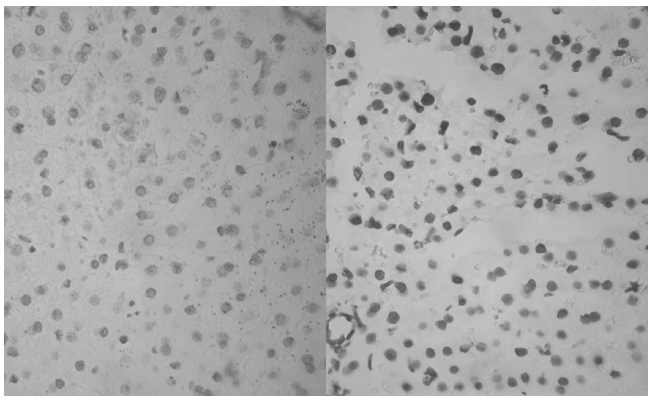

FIGURE 3 - Microscopic field of view with Tunel technique before and after ischemia, showing differences in staining pattern, more intense after ischemia (right image), confirming the results of apoptotic cell counts. Tunel, 400x

The inter-group analysis showed that AI was equal in all groups before ischemia. After ischemia there were different rates in both experimental groups, when compared to controls. The results shown in Table 3 also show there was no difference in AI between groups after the reperfusion.

DISCUSSION

Available papers in literature that study I/R injury in the liver with animal models are numerous and focus on different variables including the animal model, method and duration of ischemia, intermittent ischemia, ischemic preconditioning and remote ischemic preconditioning[18,19]. This study aimed to identify, between two methods of clamping the hepatic pedicle (methods that present technical differences, with advantages and disadvantages in both sides), which one produces less liver damage.

It is known that I/R injury is a consequence of liver normothermic or hypothermic ischemia followed by reperfusion of the liver. Tissue dysfunction is secondary to all these events. Duration of ischemia influences the degree of injury, although many of the injuries appear in the reperfusion phase[5,7,18,20,29].

There are some tools to assess the degree of lesion in I/R injury, including serum markers with variables degrees of specificity, tissue-specific markers, morphometry and immunohistochemistry[11,28]. There is not a consensus approach of which method is the best for clamping the hepatic pedicle and many authors present different results[4,21,23]. The Cochrane Institute for Systematic Reviews published a paper in 2007 showing the evidence that intermittent clamping is safe but it does not decrease the morbidity of the procedure without any clinical evidence appearing until that date[15].

The use of rabbits allows the study of I/R injury in a model of total hepatic ischemia, unlike other rodents in which models can be used only with hemi-hepatic clamping (which do not reflect the situations that occur the clinic in many cases) or with the addition of technical complications. Was observed that these animals tolerate ischemia for 60 minutes with low mortality and submitting them to intermittent ischemia produces no mortality[18]. Different models of intermittent ischemia have been described and was chosen one of them in this study, considering that after 10 to 12 minutes of ischemia, three to five minutes of reperfusion are sufficient to restore the intra-hepatic tissue oxygenation[6,23,28]. In this study, the difference between total ischemic time of the ischemia in the continuous and intermittent groups (60 versus 48 minutes) occurred to mimic the real clinical situations experienced during the surgical procedures[4].

Apoptosis is provoked by ischemia not only in liver, but also in other organs. Different papers showed an increase in the intensity of apoptosis secondary to I/R injury and discovered that its activation occurs during early reperfusion, that is, just after ischemia end in the liver. Finally, apoptosis seems to be a predominant mode of cell death in I/R injury during hepatic warm ischemia[12,13].

The use of Tunel to assess apoptosis in liver cells and in situations of ischemia of various organs is a consensus in the literature[3,17,26]. In an experimental model in Sprague-Dawley mice, Baier et al.[3] found that after 15 minutes of ischemia there was induction of apoptosis detected by Tunel method in reperfusion times ranging from 30 minutes to two hours.

The blockade of apoptosis was proposed[10] to cellular protection during hepatic ischemia and reperfusion. Crenesse et al.[9] provoked intermittent ischemia on an experimental model, resulting in reduction of apoptosis with a significant difference in Tunel expression between that type of ischemia, continuous ischemia and controls, and presenting the AI as an efficient method to quantify, in continuous values, apoptosis in the liver.

There are variable results in quantitative studies show that 5% to 80% of apoptosis after ischemia[10,16,17]. Isolated studies, however, did not show apoptosis[2]or found necrosis as the dominant finding[20]. In those papers, however, ischemic time seemed to be excessive.

The AI assessed by paired analysis, in continuous and intermittent ischemia groups, rose after the period of ischemia and returned to initial values after six hours of reperfusion. Independently, the AI between the groups of ischemia did not change from one group to another, so the AI in this study could not determinate difference between the methods of clamping. That has not happened with other authors who reported a difference between AI in the continuous or intermittent ischemia or cited difference, but not quantified[9].

Finally, the last aspect to discuss is the usage of standard HE staining, without usage of histochemical reaction and immunohistochemistry to quantify apoptosis, that find subsidies in the literature[17,26]. Its ease of use, after appropriate training, provides low cost as it reduces the amount of histochemical reactions required.

CONCLUSION

Apoptosis develops after continuous or intermittent clamping of the hepatic pedicle, but no difference was showed between the methods of clamping used here.

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