

EXPERIMENTAL STUDY

Warm liver ischemia in experiment and lysosomal markers

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Abstract: *Background:* The aim of the study is to perform a morphological analysis of certain lysosomal enzymes and parenchymal alterations during warm ischemia in the pig liver.

Methods: Standard hepatectomies were performed in a set of 24 pigs. Intra-operative intravenous (portal vein) Pentoxiphylline and hydroxyl radical scavenger Stobadine was administered. Tissue specimens were removed from the margo acutum in 10 minutes interval.

Result: In normal pig liver, the acid phosphatase (ACP) activity is in not numerous Kupffer cells and on the biliary pole of hepatocytes, diffusely in the whole parenchyma. One hour after the beginning of warm ischemia, there was an increase in ACP activity in the cytoplasm of hepatocytes. The activity in Kupffer cells could not be detected. Lactate dehydrogenase (LDH) is localized exclusively in the cytoplasmic matrix of liver cells, so only cytoplasmic enzymes leak into the blood plasma. LDH activity has remained low in areas around portal and central veins.

Conclusion: Morphological findings of enzyme activities showed that zone 2 and 3 of the liver lobule are essential for the organ survival and signs of diffusion of lysosomal enzymes into the cytoplasm of hepatocytes indicate one of the possible explanations for the findings after liver reperfusion. The study showed that intravenous administration of Pentoxiphylline and Stobadine protects the liver from warm ischemia injury (Tab. 2, Fig. 2, Ref. 37). Full Text (Free, PDF) www.bmj.sk.

Key words: warm ischemia, pig liver, acid phosphatase, lactate dehydrogenase, Pentoxiphylline, antioxidant.

Warm ischemia of the liver occurs when blood perfusion of the liver is reduced or stopped in an unchanged body temperature. This situation intentionally occurs in extensive liver resections, trying to reduce blood losses. Liver perfusion is reduced by clamping the hepatic artery and the portal vein as well as the infra- and supra-hepatic segment of the vena cava inferior. Using such procedures a complete elimination of liver blood circulation is reached, generally known as the *hepatic vascular exclusion* (1). Issuable remains the problem of determining the time interval causing no serious and irreversible damage of liver function. The maximal recommended blood perfusion occlusion interval is 60 minutes in healthy liver and 30 minutes in cirrhotic liver respectively (2, 3). Some authors preconditionally adapt the liver for ischemia using the interrupted blood flow technique, allowing the ischemia interval to be doubled (4, 5). The warm ischemia method is also associated with specific phases of the liver transplant and in circulation disorder following a hemorrhagic shock (6).

The aim of the experimental study was to clarify the nature and principles of liver parenchyma changes after blood perfusion stop with no hypothermic protection.

Methods

In our experiment in pigs, the applied methods of morphological analysis were concentrated on studying the activity of enzymes, which are considered to be the markers of lysosomal system function (acid phosphatase – AP, orthophosphoric-monoester phosphohydrolase 3.1.3.2), as well as markers of cellular energetic metabolism state (lactate dehydrogenase – LDH, (S)-lactate:NAD⁺ oxidoreductase 1.1.1.27) (7, 8, 9). Detected changes in the control group were to the changes in the next two groups. In the first group antioxidant pyridoindol (Stobadin.2 HCl, m.w: 275.2224, batch: STB/II – DH1011) was used for hepatic tissue protection, because of its significant effect as a scavenger of hydroxyl, peroxy and alcoxyl radicals and the singlet oxygen quencher effect (10, 11). In the second group the hepatic tissue was protected from ischemic injury by administering with vasodilating and rheologic effect – a methylxanthine-theobromine derivate Pentoxiphylline (Agapurinr, Zentiva). Its effect is generally explained as intracellular cyclic adenosin monophosphate phosphodiesterase decrease, followed by intracellular cyclic adenosin monophosphate levels increase (12, 13). Pentoxiphylline detectably inhibits the synthesis and secretion of tumor necrotizing factor alpha in several organs (14). Rudiger and

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Clavien (15) prove the inhibitive effect of Pentoxiphylline in laboratory mice livers. The protective effect of Pentoxiphylline in the cascade of pathological changes during the warm liver ischemia has been described by more authors (16, 17, 18).

In our study we analyzed morphologically liver tissue of 24 pigs (hybrid of Slovak White Meat Pig and German Landrasse, SBM x LS), average weight 36 kg (26–48 kg), in which standard hepatectomy was performed.

In group A hepatic tissue samples were taken without any tissue vitality influence. We applied 500 ml of normal saline through a gravitational infusion cannula inserted into the portal vein immediately after the hepatectomy. The saline was applied continually during the interval 0–10 min after hepatectomy. We took the first sample in the interval 0 minutes of warm ischemia, before the saline application. Next samples were taken in time intervals 10, 60, 80 and 100 minutes after blood flow interruption. Between the samplings livers were stored in a vessel with saline kept in thermostat with a stable temperature of 37.6 °C

In group B (Stobadine) we applied the antioxidant Stobadine using a portal vein inserted gravitational infusion set. Dissolving 4.2 mg of the substance in 500 ml saline a 30 µmol.l⁻¹ otherwise 30 µM solution was made. The sample was taken in interval 0 of the warm ischemia, before the infusion. Next samples were taken analogically with the timetable in control group – group A.

Correspondingly we applied 100 mg Pentoxiphylline in 500 ml of saline using a gravitational infusion set through a portal vein inserted cannula immediately after hepatectomy in group C. Next samples were taken analogically with the previous groups.

Acid phosphatase activity was detected using naphthol AS-BI and hexose-p-rosaniline.

Standard method of tetrazolium salt detection of phormasane deposits was used in lactate dehydrogenase activity detection (8, 9).

We evaluated each sample using Image Forge Version 1.1 software (PROVER LTD, Bratislava). Applying this software we measured percentage of pre-measured activity levels of both enzymes. We used the method of thresholding based on a presumption that different objects of the image differentiate from the background by brightness of single points in the scanned object. All same coloration intensity points were marked by preselected color spectrum threshold level. The number of marked points was compared to the total viewing field points number. Each analyzed sample was assigned with numeric value, expressing the activity of studied enzymes. Afterwards, the results were statistically analyzed. Our aim was to find out whether there were any differences in enzymatic activities between the groups in defined time intervals. To answer this question we used a generalized linear model for repeated measurements which models the bearing of a dependent variable in all groups during the whole course.

In the next part of the analysis we compared 3 groups in defined time periods. We used the simple dispersion analysis at first; afterwards we applied the Bonferoni (19) otherwise Tamhan test for multiple comparisons (20).

In the third part we investigated the dependence of component values on the time interval in the three groups. We prima-

Tab. 1. Average values of AP activity in defined time intervals.

Time (min)	Control (%)	Stobadine (%)	Pentoxiphylline (%)
0	34.08	34.32	34.45
10	23.25	32.73	33.17
60	23.26	27.38	21.66
80	20.86	22.64	14.74
100	21.85	19.41	13.64

rily applied linear regression. We determined the curve of a function which optimally fits this dependence. The significance level of our measured values was higher than the standard value $\alpha=0.05$, generally accepted in biological research.

Results

Acid Phosphatase

Group A: Acid phosphatase – control (Tab. 1)

AP activity in normal pig liver was uniform in not numerous Kupffer cells, on the biliary poles of the hepatocytes and diffusely in the whole parenchyma. The number of Kupffer cells with AP activity increased after the 10 minutes warm ischemia interval in the uninfluenced samples. We detected no changes at the biliary poles of the hepatocytes. As the warm ischemia advanced, reduction of AP activity in zone 3 occurred. After 100 minutes of warm ischemia increase of AP activity in hepatic cells cytoplasm was detected. Focal AP activity remained in Kupffer cells, however we observed it also in whole lobules. Quantitatively, in AP activity rapidly sunk in the 10 minutes interval, later remained around the same levels.

Group B: Acid phosphatase – Stobadín (Tab. 1)

After 10 minutes warm ischemia interval the number of Kupffer cells with AP activity increased in Stobadine influenced liver samples. No AP activity changes were detected at the biliary pole of the hepatocytes. AP activity decreased in zone 3 of the lobules in the later phases of warm ischemia. Focal AP activity remained in Kupffer cells, but it was sporadically detected in whole lobules. Quantitatively, Stobadine application during warm ischemia resulted in slower AP activity decrease in comparison to the uninfluenced liver.

Group C: Acid phosphatase – Pentoxiphylline (Tab. 1)

Number of Kupffer cells with AP activity increased after the 10 minutes warm ischemia interval in Pentoxiphylline influenced liver samples. No AP activity changes occurred at biliary poles.

Tab. 2. Average LDH activity levels in the studied time intervals.

Time (min)	Control (%)	Stobadine (%)	Pentoxiphylline (%)
0	24.17	24.27	24.41
10	26.25	26.09	18.48
60	29.37	24.74	24.58
80	30.50	25.34	24.65
100	32.41	26.18	26.36

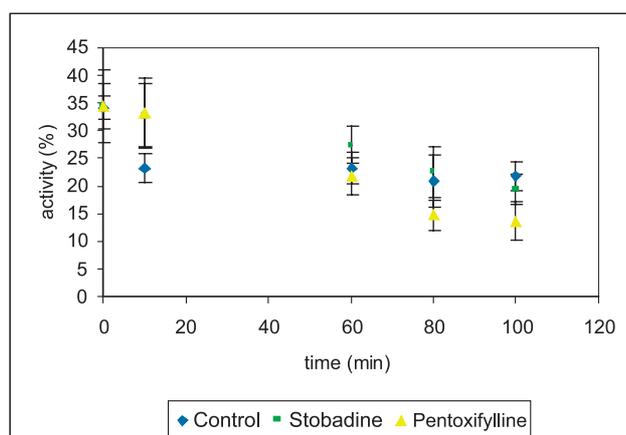


Fig. 1. Correlation between AP activity and warm ischemia duration interval.

As the warm ischemia time advanced, AP activity decrease in zone 3 of the hepatic lobules was detected. They were minor in the beginning, later rapidly sinking. AP was not focally active in Kupffer cells; however it sporadically remained active in whole lobules. In 10 minutes AP activity rapidly sunk, afterwards remained about the same levels. Quantitatively, more significant AP activity decrease occurred after Pentoxifylline application compared to the uninfluenced as well as Stobadine protected liver.

Lactate Dehydrogenase

Group A: Lactate dehydrogenase – control (Tab. 2)

In comparison to the 0 min. interval, in 10 min. interval the LDH activity in hepatocytic cytoplasm increased, more in zone 1 of the hepatic lobules. In the following intervals, focal, lobular zone independent changes developed. Quantitatively LDH levels rose.

Group B: Lactate dehydrogenase – Stobadin (Tab. 2)

In the 10 minutes interval LDH activity in the liver cells cytoplasm slightly increased, afterwards it descended almost to earlier values, particularly in zone 3 of the hepatic lobules. Only focal, zone independent changes were detected in later phases. Quantitatively, LDH activity was steady.

Group C: Lactate dehydrogenase – Pentoxifylline (Tab. 2)

In the 10 minutes interval LDH activity decreased, later returned to original values. It was found positive in cytoplasm, more in zone 3. Only focal and zone independent changes occurred in the following intervals. In subjective evaluation LDH reaction intensity after Pentoxifylline application was lower than in uninfluenced and Stobadine influenced samples. Quantitatively, LDH levels showed practically no changes.

Statistical analysis

Acid Phosphatase (Fig. 1)

1. AP values decreased as the warm ischemia time advanced. Comparing changes of activity values between the measurements all three groups showed statistically significant differences in each time interval ($p < 0.001$).

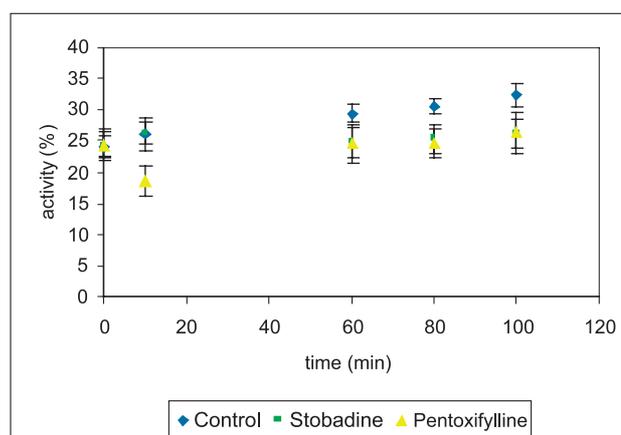


Fig. 2. Correlation between LDH activity and warm ischemia duration interval.

2. AP activities at 10 minutes time in uninfluenced control group were significantly lower than the ones in Stobadine and Pentoxifylline influenced groups ($p < 0.001$). In 60, 80 a 100 min warm ischemia time AP activity values in Pentoxifylline influenced group were significantly lower than in the next two groups ($p = 0.006$).

3. Linear regression was used to determine the trend of AP activity progression in component groups. This model fitted the values detected in all three groups, however it fitted the Pentoxifylline influenced liver group best ($R^2 = 0.808$), in contrast to the Stobadine and control group ($R^2 = 0.582$ a $R^2 = 0.379$ otherwise).

Lactate Dehydrogenase (Fig. 2)

1. As the warm ischemia progressed LDH activity values increased and there were statistically significant differences between all 3 groups in 0–10 minutes interval and 10–60 minutes intervals in two after each other subsequent measurements ($p < 0.001$).

2. We found that LDH activity levels were significantly lower in Pentoxifylline group than in Stobadine and control group after 10 minutes warm ischemia interval ($p < 0.001$). In 60, 80 and 100 minutes time LDH values in control group were significantly higher than in the next two groups ($p < 0.001$).

3. Interesting is the trend of LDH activity values progression in component groups. The applied model of linear regression suited all three groups, mostly the control group ($R^2 = 0.756$), in contrast to Stobadine influenced group with function $R^2 = 0.015$ and Pentoxifylline influenced group with function $R^2 = 0.208$.

Discussion

Acid Phosphatase

Group A: Acid phosphatase – control (Tab. 1, Fig. 1)

AP activity is generally accepted as a marker of lysosomal stability (21, 22). This is why we used it in our experiment with warm ischemia of liver. Already in 10 minutes time was the number of Kupffer cells with AP activity increased. That indicates

the possibility of ischemia induced Kupffer cells activation. However, image analysis results suggested AP activity reduction. AP activity rose diffusely in hepatocytic cytoplasm only from the 100 minutes warm ischemia time. Quantitatively, in 10 minutes interval AP activity rapidly sunk and remained about the same level during the next intervals. Decomposition of Kupffer cells lysosomal membranes as well as AP diffusion to cytoplasm of surrounding hepatocytes might be the cause of diffuse AP activity, likewise *Henell* or *Vokurka* (23, 24) published.

Group B: AP – Stobadine (Tab. 1, Fig. 1)

Detected AP activities in Stobadine protected liver were similar to normal pig liver findings. Image analysis showed lower AP activity decrease after Stobadine application. Considering the membrane stabilizing effect of Stobadine, our finding is acceptable. *Voss* and *Štolc* (25, 26) explain the effect by its antioxidative activity. We did not recognize any lysosomal membrane stability differences in pig liver however we should admit this possibility. That is the reason of quantitative evaluation of findings requires cautious procedures *Leader* (27).

Group C: AP – Pentoxiphylline (Tab. 1, Fig. 1)

Compared to control, more significant AP activity decrease occurred in Pentoxiphylline protected liver than after Stobadine application. That indicates that antioxidant Stobadine was more efficient on lysosomal membranes protection in liver during warm ischemia than Pentoxiphylline. The advantage of Pentoxiphylline is reduction of synthesis and secretion of TNF-alpha and above all reduction of its release from Kupffer cells (28). Moreover, other authors present findings of Pentoxiphylline mediated effective inhibition of Kupffer cells activation; these findings were confirmed in a cold ischemia model (18).

Lactate Dehydrogenase

Group A: Lactate dehydrogenase – control (Tab. 2, Fig. 2)

Biochemical and histochemical proof of LDH activity is often used for studying ischemia in miscellaneous tissues and organs (29, 30). That is why its localisation is described in our experiments as well.

In contrast to findings from ischemic-reperfusion injury of rat liver (31), in our experiment of simple warm ischemia in pig liver cytoplasmic LDH activity quantitatively rose, although focal activity losses occurred. The exploration might be found in interspecies differences (rat, pig), or in the fact, that LDH levels in cytoplasm and plasma do not correlate (32). Under normal circumstances, LDH presence in rat liver lobules is typically most concentrated in zone 1 (periportal area), less in the around central vein located zone 3 (33). To our knowledge there is no information in PubMed database about its distribution in pig liver. LDH activity after 10 minutes rose universally in our experiment. It is present in cytoplasm, more in zone 3 of the lobules. Only focal, lobule zone independent changes were detected in the next time intervals.

Group B: LDH – Stobadine (Tab. 2, Fig. 2)

Comparison between uninfluenced warm ischemic liver and Stobadine influenced warm ischemic liver shows a situation in which LDH levels stagnate. *Guzy* (34) refer about a cardiopro-

TECTIVE effect of Stobadine on isoproterenole damaged myocardium accompanied by serum LDH level decrease. In our case LDH level in liver slightly increases or does not change. That indicates that different organs and tissues may show different reactions to Stobadine. Findings of LDH level increase in rat hypothalamus sections after Stobadine application (10, 35) affirm this theory.

Group C: LDH – Pentoxiphylline (Tab. 2, Fig. 2)

The effects of Pentoxiphylline and Stobadine are similar. This is supported, apart from others, by the information of *Terry* (36) and *Yildirim* (37) who refer about hepatocytes viability improvement from Pentoxiphylline protection and reduction of LDH release in rats and human.

Conclusion

Having studied morphological changes of liver parenchyma after blood flow interruption without hypothermic protection of the organ we conclude these results:

1) Detected enzymatic activity values showed statistically significance in component groups compared to control group.

2) We prove that antioxidant Stobadine and methylxanthine derivate Pentoxiphylline can protect pig liver during warm ischemia, especially in metabolic processes in which the studied enzymes participate.

3) Values of studied variables can be considered as linear time dependent. Application of other usual mathematical function is more exact in individual cases. It is usually a cubic function.

4) The results allow us to suggest that single administration of an antioxidant – Stobadine and methylxanthine derivate Pentoxiphylline into the portal circulation of warm ischemia exposed liver may provide protective effect also in human medicine during extensive liver resections, liver transplantations or in cases of organ hypoperfusion caused by other circulation disorders, such as haemorrhagic shock.

References

- Dixon E, Vollmer CM, Bathe OF, Sutherland F.** Vascular occlusion to decrease blood loss during hepatic resection. *Amer J Surg* 2005; 190: 75–86.
- Habib NA, Černý J, Danninger F.** Total vascular exclusion of the liver—an advance in hepatic surgery. *Rozhl Chir* 1994; 73 (5): 221–222.
- Monbaliu D, Crabbe T, Roskams T, Fevery J, Verwaest C, Pirenne J.** Livers from non-heart-beating donors tolerate short periods of warm ischemia. *Transplantation* 2005; 79 (9): 1226–1230.
- Nuzzo G, Giuliante F, Vellone M et al.** Pedicle clamping with ischemic preconditioning in liver resection. *Liver Transplant* 2004; 10 (2, Suppl. 1): S53–S57.
- Banga NR, Homer VS, Graham A, Al-Mukhtar A, White SA, Prasad KR.** Ischaemic preconditioning in transplantation and major resection of the liver. *Brit J Surg* 2005; 92 (5): 528–538.
- Serracino-Ingloft F, Habib NA, Mathie RT.** Hepatic ischemia — reperfusion injury. *Amer J Surg* 2001; 181: 160–166.

7. **Kiszely G, Pósalaky Z (Eds).** Mikrotechnische und Histochemische Untersuchungsmethoden. Budapest; Akadémiai kiadó, 1964: 135—142.
8. **Lojda, Z., Papoušek, F (Eds).** Basics of the enzyme histochemical detection. Brno; ÚDV - ZSZ, 1970: 68—74.
9. **Bancroft JD.** Theory and practice of histochemical techniques. 5th ed. Edinburgh, London; Churchill Livingstone, 2002.
10. **Horáková L, Štolc S, Chromíková Z, Pekárová A, Derková L.** Mechanisms of hippocampal reoxygenation injury. Treatment with antioxidants. *Neuropharmacology* 1997; 36 (2): 177—184.
11. **Horáková L, Štolc S.** Antioxidant and pharmacodynamic effects of pyridoinol Stobadine. *Gen Pharmac* 1998b; 30 (5): 627—638.
12. **Semmler J, Gebert U, Eisenhut T et al.** Xantine derivatives: comparison between suppression of tumor necrosis factor-alpha production and inhibition of cAMP phosphodiesterase activity. *Immunology* 1993; 78: 520—525.
13. **Sinha B, Semmler J, Eisenhut T, Eigler A, Endres S.** Enhanced tumor necrosis factor suppression and cyclic adenosine monophosphate accumulation by combination of phosphodiesterase inhibitors and prostanoids. *Eur J Immunol* 1995; 25: 147—153.
14. **Zabel P, Schade FU, Schlaak M.** Inhibition of endogenous TNF formation by pentoxifylline. *Immunobiology* 1993; 187: 447—463.
15. **Rudiger HA, Clavien PA.** Tumor necrosis factor-alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. *Gastroenterology* 2002; 122 (1): 202—210.
16. **Lemasters JJ, Peng XX, Bachmann S, Currin RT, Gao W, Thurman RG.** Dual role of Kupffer cell activation and endothelial cell damage in reperfusion injury to livers stored for transplantation surgery. *J Gastroenterol Hepatol* 1995; 10 (Suppl 1): 84—87.
17. **Nishizawa H, Egawa H, Inomata Y, Uemoto S, Asonuma K, Kiuuchi T.** Efficiency of pentoxifylline in donor pretreatment in rat liver transplantation. *J Surg Res* 1997; 72: 170—176.
18. **Vajdová K, Smreková R, Kukan M et al.** Endotoxin-induced aggravation of preservation-reperfusion injury of rat liver and its modulation. *J Hepatol* 2000; 32: 112—120.
19. **Keselman HJ, Leventhal L.** Concerning the statistical procedures enumerated by Gentile et al. Another perspective. *Journal of applied behavior analysis* 1974; 7 (4): 643—645.
20. <http://www.cs.cmu.edu/afs/cs/project/jair/pub/volume24/ortizboyer05a-html/node14.html>.
21. **De Duve C.** The lysosome. *Sci Amer* 1963; 208 (1): 64—72.
22. **Novikoff AB.** Lysosomes: a personal account. 1—41. In: Hers G, Van Hoof F (Eds). *Lysosomes and storage disease*. New York; Academic press, 1973.
23. **Henell F, Ericsson JL, Glaumann H.** Degradation of phagocytosed lysosomes by Kupffer cell lysosomes. *Lab Invest* 1983; 48 (5): 556—564.
24. **Vokurka J.** Influence of electric current on the liver parenchyma of the rat. *Acta Vet Brno* 2003; 72: 547—551.
25. **Voss P, Horáková L, Jakstadt M, Kiekebusch D, Grune T.** Ferritin oxidation and proteasomal degradation: protection by antioxidants. *Free Radic Res* 2006; 40 (7): 673—683.
26. **Štolc S, Snirc V, Májeková M, Gašparová Z, Gajdošíková A, Štvrtina S.** Development of the new group of Indole-derived neuroprotective drugs affecting oxidative stress. *Cell Mol Neurobiol* 2006; 26 (7—8): 1493—1502.
27. **Leader RW.** Toxicologic pathology: an introduction. 1—8. In: Haschek WM, Rousseaux CG (Eds). *Handbook of toxicologic pathology*. London; Academic press, 1991.
28. **Selzner N, Rudiger H, Graf R, Clavien PA.** Protective strategies against ischemic injury of the liver. *Gastroenterology* 2003; 125 (3): 917—936.
29. **Liu W, Pitcher DE, Morris SL et al.** Differential effects of heparin on the early and late phases of hepatic ischemia and reperfusion injury in the pig. *Shock* 1999; 12 (2): 134—138.
30. **Kamiya T, Kwon AH, Kanemaki T et al.** A simplified model of hypoxic injury in primary cultured rat hepatocytes. *In Vitro Cell Dev Biol Anim* 1998; 34 (2): 131—137.
31. **Frederiks WM, Marx F.** Changes in cytoplasmic and mitochondrial enzymes in rat liver after ischemia followed by reperfusion. *Exp Mol Pathol* 1987; 47 (3): 291—299.
32. **Nakae Y, Stoward PJ.** Kinetic parameters of lactate dehydrogenase in liver and gastrocnemius determined by three quantitative histochemical methods. *J Histochem Cytochem* 1997; 45 (10): 1427—1431.
33. **Griffini P, Freitas I, Vigorelli E, Van Noorden CJ.** Changes in the zonation of lactate dehydrogenase activity in lobules of rat liver after experimentally induced colon carcinoma metastases. *Anticancer Res* 1994; 14 (6): 2537—2540.
34. **Guzy J, Barnova E, Benes L.** Stobadine and heart mitochondria. *Life Sci* 1999; 65 (18—19): 1935—1937.
35. **Horáková L, Štolc S, Chromíková Z, Pekárová A, Derková L.** Mechanisms of hippocampal reoxygenation injury. *Mol Chem Neuro-pathol* 1998 a; 33 (3): 223—236.
36. **Terry C, Dhawan A, Mitry RR, Lehec SC, Hughes RD.** Preincubation of rat and human hepatocytes with cytoprotectants prior to cryopreservation can improve viability and function upon thawing. *Liver Transplant* 2006; 12 (1): 165—177.
37. **Yildirim S, Tok H, Koksall H, Erdem L, Baykan A.** Allopurinol plus pentoxifylline in hepatic ischemia/reperfusion injury. *Asian J Surg* 2002; 25 (2): 149—153.

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