Effects of gastric ischemia-reperfusion on gastric mucosal cellular apoptosis and proliferation in rats

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Abstract: The effect of gastric ischemia-reperfusion (GI-R) on gastric mucosal cellular apoptosis and proliferation was investigated using histological, immunohistochemical methods in Sprague-Dawley rats. The GI-R model was established by clamping the celiac artery for 30 min and reperfusing for 0, 0.5, 1, 3, 6, 24, 48, 72 h, respectively. Mild gastric mucosal injury was induced by ischemia alone. However, the injury worsened and reached the maximum at 1 h after reperfusion, almost simultaneously with the gastric mucosal cellular apoptosis increase and cellular proliferation decrease in gastric mucosa. Then, gastric mucosal cells began to repair by increasing gastric cellular proliferation, which achieved the maximum at 24 h after reperfusion. The mucosal lesions were almost completely repaired at about 72 h after reperfusion. These results indicate that the gastric mucosal injury after GI-R is mainly induced by reperfusion. The damaged gastric mucosa could initiate its repairing mechanism immediately through inhibiting cellular apoptosis and increasing the number of proliferative cells, which substitute the damaged cells gradually. The plerosis almost completes in three days after reperfusion showing a strong self-repair ability of gastric mucosa.

Key words: stomach; ischemia-reperfusion; apoptosis; cellular proliferation; rats

胃缺血 - 再灌注对大鼠胃黏膜细胞凋亡和增殖的影响

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摘 要：本研究采用大鼠胃缺血 - 再灌注（gastric ischemia-reperfusion, GI-R）模型（夹闭腹腔动脉30 min后再灌注），通过组织学、免疫组化等方法，研究GI-R不同时间（0、0.5、1、3、6、24、48、72 h）对胃黏膜细胞凋亡和增殖的影响。结果发现，单纯缺血30 min胃黏膜损伤较轻，再灌注后损伤逐渐加重，胃黏膜的凋亡细胞迅速增加，而增殖细胞迅速减少；至再灌注后1 h达高峰；之后胃黏膜开始修复，凋亡细胞逐渐减少，增殖细胞逐渐增加；至再灌注后24 h胃黏膜细胞增殖达高峰；再灌注后72 h胃黏膜基本恢复正常。上述结果提示，在GI-R 中，胃黏膜损伤主要由再灌注引起，凋亡细胞增加；然后胃黏膜启动自我修复机制，增殖细胞逐渐取代凋亡细胞，3 d 左右就可基本修复，表明胃黏膜细胞具有很强的自我修复能力。

关键词：胃；缺血 - 再灌注；凋亡；细胞增殖；大鼠
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tric mucosal cells have a rapid renewal rate, which suggests that the stomach might have a strong ability of regenerative injury and repair.

It is well known that the gastric mucosal integrity depends on a balance between the cellular renewal and the death of damaged or aged cells. Cellular proliferation is essential in normal tissue life survival and turnover. The death of damaged or aged cells includes cellular necrosis and apoptosis. In contrast to necrosis, the apoptosis, characterized by morphological changes, is an active process of directional cellular self-destruction and plays an opposite role in cell populations. Apoptosis has been reported under many conditions in the rat gastric mucosal cells [7-9], including that induced by Helicobacter pylori infection [10-11]. However, the apoptosis and proliferation of gastric mucosal cells induced by ischemia-reperfusion in rats haven’t yet been fully understood. Little information is available concerning gastric mucosal cellular apoptosis and proliferation induced by GI-R, and related reports were contradictory and unsystematic [12-14]. Fukuyama et al. reported that apoptosis was not induced in the gastric mucosa after GI-R [12]. Moreover, Wada et al. demonstrated that GI-R induced significant apoptosis in the gastric mucosa [13]. So, it is interesting to make clear the process of gastric mucosal cellular apoptosis and proliferation after GI-R. Any progress in this field may help better understand the pathological mechanism of GI-R injury.

On the basis of these observations, in the present study, we systematically investigated the effect of GI-R on gastric mucosal cellular apoptosis and proliferation at different reperfusion time after ischemia using histological, immunohistochemical methods in rats.

1 MATERIALS AND METHODS

1.1 Drugs and reagents
M30 CytoDEATH monoclonal antibody was from Roche (Basel, Switzerland); mouse anti-rat proliferative nucleolus monoclonal antibody (proliferative cell nuclear antigen, PCNA), PowerVision™ two-step immunohistochemistry detection kits were from Zhongshan Biotech. Co. (Beijing, China).

1.2 Preparation of GI-R model and animal groups
GI-R models were built according to the method of Wada et al. [15]. Adult Sprague-Dawley rats (Xuzhou Medical College Experimental Animal Center, Xuzhou, China) weighing (250 ± 25) g of either sex were used in all studies. Before the experiment, all rats were fasted for 24 h and free access to water. Animals were randomly divided into nine groups, 6 rats for each: sham-operated group (the same surgical procedure without clamping the celiac artery), GI-R groups (reperfusion 0, 0.5, 1, 3, 6, 24, 48 and 72 h, respectively, after 30 min of ischemia). The randomly grouped rats were all anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally). The abdominal cavity was cut open, the celiac artery and its adjacent tissues were carefully isolated. The celiac artery was clamped with a small non-traumatic vascular clamp for 30 min to induce ischemia and then released to allow reperfusion. After reperfusion, the rats were sacrificed to remove the stomach immediately.

1.3 Measurement of gastric mucosal injury
As described previously by Wada et al. [16-18], rat’s stomach was incised along the greater curvature and flushed by ice-cold PBS (0.1 mol/L). When it had been spread out on a cold plate, a paned counting slab (with 1 mm² of each pane) was used to measure the injury area. The gastric injury index was expressed as the percentage of damaged area with dotted erosion, ulcer, and bleeding which limited epithelium in the total mucosal area in the gland region.

1.4 Observation of gastric mucosal injury and repair after GI-R by light microscope and transmission electron microscope
After gastric injury index was measured, four to five pieces of tissue of 1.0 mm×2.0 mm at the greater curvature from the gastric mucosa were immersed in 2.5% glutaraldehyde solution at 4 °C for transmission electron microscopy. The remaining gastric mucosal tissue was fixed in Bouin’s fixative for paraffin tissue slice. The paraffin tissue slices (5 µm) were pasted discontinuously to glass pretreated with poly-lysine. The part of paraffin tissue slices was stained with hematoxylin-eosin. Gastric mucosal injury and repair after GI-R was observed by light microscope and transmission electron microscope.

1.5 Observation of gastric mucosal apoptosis and proliferation after GI-R by immunohistochemical assay
For immunohistochemistry, gastric mucosal cellular apoptosis was visualized by using the M30 CytoDEATH apoptosis detection kit of Roche. PCNA was used as a marker for gastric mucosal cellular proliferation, and the immunohistochemical staining was performed with PowerVision™ two-step immunohistochemistry detection kit of Zhongshan. The sections were stained with 3,3’-diaminobenzidine (DAB) and were counterstained using hematoxylin, and then the sections were examined with a light microscope (Olympus, Japan). The presence of brown granules in cytoplasm or nucleus was defined as positive.
cell. The number of positive cells per section was counted in ten random high-power (×40) or lower-power (×10) fields, and the percentage of positive cells (positive cells/total cells×100%) was calculated. Three discontinuous sections were selected from every specimen and those indexes were averaged.

1.6 Statistical analysis
All results were expressed as mean ± SD. Statistical comparisons were made using Student’s t-test; multiple-group analyses were made by the one-way analysis of variance (ANOVA). In all cases, P<0.05 was taken to indicate statistical difference.

2 RESULTS

2.1 The changes of gastric mucosal injury at different reperfusion time after ischemia
As shown in Fig.1, there were occasionally a few pinpoint hemorrhages of gastric mucosa in sham-operated group. In the GI-R groups, 30-minitue ischemia alone (reperfusion 0 h) only induced mild injury, and the percentage of damaged gastric mucosal area in the total mucosal region was (3.77 ± 0.93)%; then the injury was gradually aggravated and the percentage of damaged area achieved the maximum at 1 h after reperfusion, reaching (39.67 ± 13.56)% of the total gastric mucosa region. Then the gastric mucosal injury degree began to lessen, declining to the basal level at 72 h after reperfusion. There was significant difference in damaged gastric mucosal area between the GI-R groups (i.e. reperfusion 0–48 h) and sham-operated group (P<0.05). These results suggest that the gastric mucosal injury induced by ischemia alone were slight and the gastric mucosal injury after GI-R was mainly induced by reperfusion.

2.2 Identification of gastric mucosal injury at different reperfusion time after ischemia by light microscope
In sham-operated group, the gastric mucosal structure remained integrity with tidily arrayed cells; there were a few pinpoint hemorrhages, and there were no mucosal edema and desquamation (Fig.2A). Mild edema in submucosal layer and epithelial cell desquamation in mucosal layer were observed at 0 h after reperfusion (Fig.2B). Furthermore, severe mucosal and submucosal hyperemia, edema, and deep erosion appeared after 1-hour reperfusion (Fig.2C), and then gastric mucosal deep ulcer was found at 24-hour reperfusion (Fig.2D). At 72-hour reperfusion, the mucosal lesions were almost completely repaired (Fig.2E).

2.3 Ultrastructural changes at different reperfusion time after ischemia by transmission electron microscope
In sham-operated group, gastric parietal cells had round nuclei in the center and plenty of large mitochondria and secretion tubule which had large and flossy cavity in cytoplasm (Fig.3A); the chief cellular nuclei in fundus of gastric glands were located in the base of the cell, and the chief cells had abundant rough endoplasmic reticulums, zymogen granules, and mitochondria in cytoplasm (Fig.3B). In the GI-R groups, there were plenty of necrotic and apoptotic cells and intra-capillary microthrombosis after 1-hour reperfusion in gastric mucosa (Fig.3C, D); undifferentiated cells (i.e. proliferating cells) were found with large and anomalous nuclei, small organelles and less cytoplasm at 6-hour reperfusion. The number of proliferating cells were further increased at 24-hour reperfusion (Fig.3E). When a few zymogen granules emerged, the proliferating cells developed eventually into chief cells at 48-hour reperfusion (Fig.3F).

2.4 The quantitative changes of apoptotic cells in gastric mucosa at different reperfusion time after ischemia by immunohistochemical assay
As shown in Fig.4, the M30 CytoDEATH-positive cells (i.e. apoptotic positive cells) were predominantly in the surface layer of the gastric glands and epithelial cells, and located in the cytoplasm. In sham-operated group, the expression of M30 CytoDEATH-positive cells (i.e. amount of cellular apoptosis) was low, and the percentage of M30 CytoDEATH-positive cells was (1.35 ± 0.27)% (Fig.4A).
In 30-min ischemia alone group (reperfusion 0 h), the M30 CytoDEA-TH-positive cells were scattered in cytoplasm, and the percentage achieved to (2.58 ± 0.85)% (Fig.4B, E). At the onset of reperfusion, the rate of apoptotic positive cells rapidly increased, and the percentage of the M30 CytoDEA-TH-positive cells achieved the maximum at 1 h after reperfusion, increasing 7.34-fold compared with that in sham-operated group (P<0.05) (Fig.4C, E). Following prolonged reperfusion, the M30 CytoDEATH-positive cells decreased, and the percentage of M30 CytoDEATH-positive cells reached the bottom at 24-hour reperfusion [(0.45 ± 0.13)%, P<0.05 compared with that in sham-operated group] (Fig.4D, E). There was no significant difference between the sham-operated group and 72-hour reperfusion group. These results suggest that the apoptotic positive cells increased rapidly after reperfusion, and the increased course was transient and induced mainly by reperfusion.

2.5 The quantitative changes of proliferative cells in gastric mucosa at different reperfusion time after ischemia by immunohistochemical assay

As shown in Fig.5, the expression of PCNA-positive cells (i.e. proliferative positive cells) was predominantly in the neck region of the gastric glands. In sham-operated group, the expression of PCNA-positive cells was abundant, the percentage was (8.47 ± 1.34)% (Fig.5A, E). In the GI-R groups, 30-min ischemia alone (reperfusion 0 h), the percentage of PCNA-positive cells were (5.78 ± 1.02)%(Fig.5B, E); the quantities of the PCNA-positive cells decreased rapidly after reperfusion, the percentage of PCNA-positive cells reached the lowest level at 1-hour reperfusion (0.41 fold vs sham-operated group, P<0.05) (Fig.5C, E). Following prolonged reperfusion, the PCNA-positive cells increased gradually, and reached the peak at 24-hour reperfusion (2.66 folds vs sham-operated group, P<0.05) (Fig.5D, E). There was no significant difference between 72-hour reperfusion group and sham-operated group. These results suggest that the percentage of proliferative positive cells decreased rapidly at the initial stages of reperfusion, and then increased gradually, reaching the maximum at 24 h after reperfusion. The result is dramatically opposite to...
the percentage of apoptotic positive cells at the same time after reperfusion.

3 DISCUSSION

The GI-R of organs as a complex phenomenon is frequently encountered in biology and medicine, and has interested more and more researchers since the 80s of last century. Voluminous literatures in this respect represent that the reperfusion injuries of important organs, such as heart, lung, brain and kidney, except stomach, have been extensively explored theoretically and clinically. The available information concerning the acute gastric mucosal injury induced by ischemia-reperfusion was limited[11,16,19,20].

In the present study, our results clearly indicated that the gastric mucosal injury induced by GI-R was mainly the result of reperfusion, because gastric ischemia alone only induced the mild gastric mucosal injury, and the gastric mucosal severe lesions occurred in the initial stages of reperfusion and reached the peak at 1 h after reperfusion. Whereafter, the gastric mucosal started repair, and the recovery was almost completed in three days. These facts indicated that gastric mucosa had an amazing self-repair ability, which was possible through promoting the amount of proliferative cells and inhibiting gastric mucosal cellular...
apoptosis.

On the other hand, in recent years although reports on the gastric mucosal cell injury and repair induced by GI-R were raised, little information is available concerning gastric mucosal cellular apoptosis and proliferation induced by GI-R, and related reports were also contradictory and unsystematic. In the study we systematically investigated the time-course changes in the gastric mucosal cellular apoptosis and proliferation induced by GI-R using histological and immunohistochemical staining methods, the cellular mechanism of gastric mucosal injury and repair were elucidated.

Previous related studies and our results demonstrated that the apoptotic cells and proliferative cells were coexisted in the gastric mucosa. The cellular proliferation and apoptosis maintained a dynamic balance under normal conditions. The apoptotic cells were localized in the gastric mucosal surface layer; the proliferative cells were predominantly distributed in the vicinity of gastric glandular neck region. Our results further showed that the major place apoptosis and proliferation occurred after GI-R were consistent with that in sham-operated group.

Our experiments indicated that the changed courses of gastric mucosal cellular apoptosis was consistent with and that of proliferation were opposite to the gastric mucosal injury induced by GI-R at identical times. These results suggest that the gastric mucosal injury at the initial stages of reperfusion were caused by rapid apoptotic aggravation and inhibitive proliferation of gastric mucosal cells. Following the prolongation of reperfusion time, the gastric mucosal cellular apoptosis gradually decreased and proliferation increased, therefore, the gastric mucosal injury was repaired. Thus the molecular mechanism of gastric mucosal injury and repair was clarified.

In the present study, we have not involved the detailed cellular and molecular mechanisms of gastric mucosal cellular apoptosis and proliferation induced by GI-R, because the regulative factors were very complex in gastric mucosal cellular injury and repair after GI-R. In our previous study, we have reported that the PVN was involved in the regulation of GI-R and was one of the special CNS area attenuating the GI-R injury and related to decrease of MDA content, gastric acidity and pepsin activity, while gastric juice volume, total acid output and gastric barrier mucus seemed not to be active in the mechanisms.

In addition, a variety of bioactive compounds and cytokines, such as platelet-activating factor, ICAM-1, TNF-α, IL-1, IL-6, EGF, TGF-α, participate in regulative effects of gastric mucosal cellular apoptosis and proliferation. Recently, we have extraordinarily attended to the effect of gastric mucosal blood flow after reperfusion on gastric mucosal injury induced by GI-R, and already observed that the adequate decrease of gastric mucosal blood flow after reperfusion could inhibit gastric mucosal cellular apoptosis and accelerate cellular proliferation in our other experiments (data not shown). Presently, our investigation will further focus on the molecular mechanisms and signaling pathway of GI-R, and correlative paper will be reported in the future.

REFERENCES


