Different patterns of intestinal response to injury after arterial, venous or arteriovenous occlusion in rats

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Abstract

AIM: To investigate the differences in injury patterns caused by arterial, venous or arteriovenous mesenteric occlusion.

METHODS: Male Wistar rats were separated equally into four groups. Occlusion was performed by clamping the superior mesenteric artery (A), the mesenteric vein (V) or both (AV) for 30 min, followed by 60 min of reperfusion. A control group received sham surgery only. Intestinal sections were examined for histological damage and serum tumor necrosis factor-α (TNF-α), endothelin-1 (ET-1), P-selectin, antithrombin III (AT-III) and soluble intracellular adhesion molecule-1 (ICAM-1) concentrations were measured.

RESULTS: All groups showed significant mucosal injury compared to controls. Furthermore, mucosal injury was significantly more severe in the V and AV groups compared to the A group (3.6 ± 0.55, 3.4 ± 0.55 and 2 ± 0.71, respectively, P = 0.01). ICAM-1 was similarly elevated in all groups, with no significant differences between the groups. P-selectin levels were significantly elevated in the V and AV groups but not the A group (1.4 ± 0.5 ng/mL, 2.52 ± 0.9 ng/mL and 0.02 ± 0.01 ng/mL, respectively, P = 0.01) and ET-1 was significantly elevated in the A and V groups but not the AV group (0.32 ± 0.04 pg/mL, 0.36 ± 0.05 pg/mL and 0.29 ± 0.03 pg/mL, respectively, P = 0.01) compared to sham controls. AT-III levels were markedly depleted in the V and AV groups, but not in the A group (29.1 ± 5.2 pg/mL, 31.4 ± 21.8 pg/mL and 55.8 ± 35.6 pg/mL, respectively, P = 0.01), compared to controls. Serum TNF-α was significantly increased in all groups compared to sham controls (1.32 ± 0.87 ng/mL, 1.79 ± 0.20 ng/mL and 4.4 ± 0.69 ng/mL, for groups A, V and AV, respectively, P = 0.01), with higher values in the AV group.

CONCLUSION: Different patterns of response to ischemia/reperfusion are associated with venous, arterial or arteriovenous occlusion. Venous and arteriovenous occlusion was associated with the most severe alterations.

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Key words: Arterial occlusion; Rat; Intestine; Ischemia/reperfusion; Venous occlusion

INTRODUCTION

Acute mesenteric ischemic disease can be either arterial or venous in etiology. Acute arterial ischemia results
from reduced blood flow caused by mesenteric artery thrombosis or embolism, leading to bowel necrosis and high mortality[15]. Mesenteric vein occlusion is usually due to thrombosis, is less common than arterial occlusion and carries a lesser risk of bowel necrosis and therefore reduced mortality[2,16]. Greater knowledge of the differences between these conditions would aid the search for therapeutic interventions, and also expand our understanding of ischemia/reperfusion (I/R) pathophysiology. This information would also be relevant for transplantation and aortic reconstruction procedures, which are invariably related to periods of I/R[17].

The intestine is very susceptible to ischemic injury. Ischemia causes mucosal damage through the production of substances like oxygen-derived free radicals, pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and the complement system[18]. The pattern of injury has been found to be different after experimental arterial or venous occlusion of the mesenteric circulation followed by reperfusion. Generally, the histological injury grade is thought to be greater after venous occlusion, even though energy metabolism is altered to a lesser extent than in arterial occlusion[19]. However, another study found that venous congestion alone could not cause reperfusion injury, while arterial occlusion could do so easily[20]. This issue could be resolved if the differences between the injury response to these two situations could be found.

During I/R, endothelial functions are altered, resulting in microcirculatory dysfunction which modulates mucosal integrity[21]. Endothelial injury and increased vascular permeability also occur[22]. The vascular endothelium regulates vascular tone, leukocyte adhesion and the coagulation cascade, among other factors. Endothelial function serum marker levels such as intracellular adhesion molecule-1 (ICAM-1), endothelin-1 (ET-1) and coagulation factors have also been found to be altered after I/R. One study showed that ET-1 modulates intestinal I/R injury in rats[23], while another showed that ICAM-1 is overexpressed, possibly contributing to injury by promoting leukocyte infiltration[24]. Another adhesion molecule, P-selectin, is also thought to modulate intestinal I/R injury, through complement deposition and leukocyte interaction[25]. Intestinal I/R also causes hemostatic alterations, namely coagulation induction, thrombin generation, and fibrin deposition, which correlate with the extent of injury[26].

In this study we evaluate the possibility that arterial, venous and arteriovenous occlusion, followed by reperfusion, might cause different types of response to I/R injury in the intestine. These differences were investigated by measuring ET-1, antithrombin III (ATIII), soluble ICAM-1 and P-selectin serum concentrations, soluble markers of endothelial function, as well as by measuring TNF-α, a cytokine that plays a central role in I/R injury.

MATERIALS AND METHODS

Animal procedures

Animal procedures were performed in accordance with the proper use and care of laboratory animals, approved by the ethics committee of our institution. Experiments were performed on 20 male Wistar rats weighing 200-250 g. Animals were maintained under standard conditions, such as stable room temperature (24 ± 3°C), a 12 h light/12 h dark cycle, and access to commercial rat pellets and water ad libitum.

Animal model

Briefly, after pentobarbital sodium anesthesia (Anestesal, Pfizer Inc, Mexico) (35 mg/kg, i.p), a midline laparotomy was performed, and the small intestine was externalized and wrapped in humid gauze. Animals were placed under a heating lamp in order to preserve core body temperature at (37°C). The cecum was occluded using a 2-0 nylon suture to prevent collateral blood flow, and ischemia was induced by obstructing either the superior mesenteric artery, the mesenteric vein before its union with the portal system, or both, with microvascular clamps for 30 min (ischemia), and removed to allow 60 min reperfusion. Immediately after clamping the mesenteric vessels, the intestines were returned to the peritoneal cavity where they remained for the entire duration of the procedure. Occlusion was confirmed by loss of pulsation and characteristic red dark coloring of the intestine in the venous group, or pale pink coloring and loss of pulsation in the arterial group.

Rats were divided in four groups (n = 5). The first group received only sham surgery (Sham), where laparotomy was performed but intestines were only manipulated but not made ischemic. Group A was subjected to arterial occlusion and reperfusion as described above. Group V was subjected to venous occlusion and reperfusion as described above. Group AV was subjected to both arterial and venous occlusion and reperfusion as described above.

Morphological examination

Immediately after concluding the reperfusion period, rats were sacrificed by exsanguination from the aorta and tissue samples were obtained and fixed in 10% neutral buffered formalin overnight. Samples were then embedded in paraffin, and 4 μm-thick sections were stained with hematoxylin and eosin (H/E) and examined under a light microscope by a blinded pathologist. The Chiu score of mucosal injury was used to evaluate the degree of histological damage[15]. The scale consists of values from 0 to 5, where 0 is normal mucosa; 1, development of sub epithelial (Gruenhagen’s) spaces; 2, extension of the sub-epithelial space with moderate epithelial lifting from the lamina propria; 3, extensive epithelial lifting with occasional denuded villi tips; 4, denuded villi with exposed lamina propria and dilated capillaries; and 5, disintegration of the lamina propria, hemorrhage, and ulceration.

Inflammatory cell infiltration

Under light microscopy, H/E stained tissue samples were analyzed for leukocyte infiltration by a blinded pathologist. The predominant type was recorded in both mucosa and
muscular layers, and intensity of infiltration was quantified with a numerical scale with values from 0 to 3, where 0, no infiltration; 1, mild infiltration; 2, moderate infiltration and 3, severe infiltration. Two slides and five high power fields per slide were examined and averaged.

**Serum levels of ET-1, AT III, P-selectin, ICAM-1 and TNF-α**

At the end of the reperfusion period 3 mL of blood was obtained from each rat and left to clot for serum acquisition. Serum levels of ET-1 were determined using a rat ET-1 EIA kit (Immuno-Biological Laboratories, Japan). Serum levels of AT III were determined using a rat AT III ELISA kit (GenWay Biotech, USA). Serum levels of soluble ICAM-1 were determined using a quantikine rat sICAM-1 ELISA kit (R & D Systems, USA). Serum levels of P-selectin were determined using a quantikine rat P-selectin ELISA kit (R & D Systems, USA). These were used as markers of endothelial function. Serum concentrations of TNF-α were determined using a rat TNF-α Elisa kit (PeproTech, Mexico).

**Statistical analysis**

SPSS 11.0 statistical software (SPSS Inc. Software, Chicago, Illinois, USA) was used to analyze data using one-way analysis of variance (ANOVA) and with the Tukey-Kramer post-hoc test so as to determine comparisons between the groups, and differences between the groups, respectively. The relationships between the variables studied were determined by the calculation of Pearson correlation coefficients. All values are expressed as mean ± SD and P < 0.05 was considered statistically significant.

**RESULTS**

**Morphological examination**

The sham group showed normal intestinal mucosa (Chiu score 0.2 ± 0.45). All occlusion groups had significantly higher injury scores compared to the sham group (Figure 1, P < 0.01). Groups V and AV had the highest injury scores (3.6 ± 0.55 and 3.4 ± 0.55, respectively), showing severe epithelial lifting and severe capillary congestion (Figure 1), with no difference observed between these two groups. However, both the AV and V groups had significantly higher scores compared to group A (2 ± 0.71, P < 0.01), which showed moderate lifting and very little congestion.

**Inflammatory cell infiltration**

Compared to the sham group (1.2 ± 0.45), groups V and AV (2.6 ± 0.55 and 2.6 ± 0.55) both had a significantly higher leukocyte infiltration intensity (Figure 1, P < 0.05). Group A (2 ± 0.7) was not statistically different from either the V or sham group. The infiltrate type was similar in all groups, and was composed mostly of neutrophils, with few macrophages or lymphocytes observed.

**Serum markers of endothelial function**

The sham group had soluble P-selectin serum concentrations of 0.83 ± 0.47 ng/mL. In group A the levels remained barely detectable, and were not statistically different to the sham group (0.02 ± 0.01 ng/mL). Groups V and AV had significantly higher values compared to the sham group (1.4 ± 0.5 ng/mL and 2.52 ± 0.9 ng/mL, respectively, P < 0.01), with the latter being significantly higher than the former (P < 0.05, Figure 2). AT III serum concentrations were significantly depleted in groups V and AV compared to the sham group (29.1 ± 5.2 pg/mL and 31.4 ± 21.8 pg/mL, respectively, P < 0.01), while in group A they remained within the sham group range (55.8 ± 35.6 pg/mL). However, there was no difference between the V or AV groups (Figure 2). No difference was found between ET-1 levels in the sham and AV groups (0.23 ± 0.01 pg/mL and 0.29 ± 0.03 pg/mL, respectively, Figure 2). Levels in both the A and V groups were significantly elevated compared to the sham group (0.32 ± 0.04 pg/mL and 0.36 ± 0.05 pg/mL, respectively, P < 0.01), with levels in the V group also being significantly higher that in that in the AV group, but not the A group. Finally, all groups had significantly elevated soluble ICAM-1 serum concentrations compared to the sham group (A: 569.2 ± 170.8 pg/mL, V: 529 ± 191.5 pg/mL and AV: 483.9 ± 41.6 pg/mL vs 194 ± 38.9 pg/mL, respectively, P < 0.01), but no differences were found between each of these three groups (Figure 2). In summary, venous and arteriovenous occlusion elevated P-selectin, and depleted AT III, to a greater extent than arterial occlusion only. Venous and arterial occlusion, but not arteriovenous occlusion, elevated ET-1 levels compared to sham groups, with no differences between them. ICAM-1 levels were elevated in all groups compared to the sham group, with no differences between them.

**TNF-α serum concentrations**

The sham group had TNF-α serum concentrations of 0.11 ± 0.14 ng/mL. In groups A, V and AV the levels were significantly increased compared to sham controls (1.32 ± 0.87 ng/mL, 1.79 ± 0.20 ng/mL and 4.4 ± 0.69 ng/mL, respectively, P < 0.01, Figure 2). TNF-α serum concentrations were significantly higher in the AV group compared to both the A and V group, with no differences observed between these two groups.

**Linear correlations between variables**

Linear correlations were observed between the systemic concentrations of endothelial function markers, TNF-α and injury scores. These correlations are shown in Table 1. All the measured variables, except for AT III, showed a significant positive correlation with the histological injury score. AT III showed a significant negative correlation with

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**Table 1 Pearson’s correlation coefficient between the variables studied**

|                  | TNF-α  | ET-1   | P-selectin | ICAM-1  | AT III |
|------------------|--------|--------|------------|---------|--------|
| Chiu Score       | 0.715  | 0.714  | 0.552      | 0.608   | -0.633 |
| TNF-α            | x      | 0.26   | 0.664      | 0.337   | -0.534 |
| ICAM-1           | x      | -0.015 | 0.655      | -0.409  |
| P-selectin       | x      | 0.044  | -0.275     |         |
| AT III           | x      |        |            | -0.532  |

*P < 0.05, †P < 0.01. Data are presented as correlation coefficients (r).
DISCUSSION

Ricci et al.\(^{[15]}\) found greater mortality in rats after intestinal arterial versus arteriovenous occlusion. However, Yano et al.\(^{[16]}\) found that the degree of intestinal damage due to venous occlusion was greater than the damage resulting from both arteriovenous and arterial occlusion, with no difference between the latter two procedures. Greater intestinal hemorrhage has also been observed after venous occlusion compared with arterial occlusion\(^{[6]}\). Other studies confirmed these findings, showing that venous occlusion could induce intestinal injury as early as within 5 min, while arterial occlusion could not even at longer time periods\(^{[17]}\). Some of the molecular bases of these differences have been investigated. Venous congestion has been shown to induce increased levels of inflammatory cytokines, in a similar way to arterial occlusion\(^{[18]}\). However, in one I/R study, intestinal malondialdehyde levels (as an index of free radicals) were found to be higher in venous occlusion compared to arterial occlusion, despite no obvious difference in tissue injury\(^{[19]}\). In other organs, such as the kidney, renal vein occlusion has been found to induce a stronger inflammatory response (TNF-\(\alpha\), free radicals, but not ICAM-1), neutrophilic infiltration, and greater functional damage, compared to arterial occlusion\(^{[20]}\). In our study, I/R due to venous occlusion was shown to cause greater damage to the intestinal mucosa than arterial occlusion alone, in agreement with the previous studies. However, we found no difference between the type or the intensity of injury between venous and arteriovenous occlusion. Arterial occlusion caused limited but significant histological damage and was associated with little leukocyte infiltration. The pro-inflammatory cytokine TNF-\(\alpha\) is one of the main orchestrators of the inflammatory response and a key mediator of injury during I/R\(^{[21]}\). In our study,
occlusion, compared to venous occlusion. We speculate subsequent flow recovery after reperfusion with arterial more pronounced reduction of blood flow and increased outflow. Supporting this idea, Tsunada might only cause reduced inflow perfusion with normal increased intravascular pressure, arterial occlusion alone causes severe congestion and vasodilation as a compensatory mechanism ischemia cause diminished arteriolar resistance and reductions in perfusion pressure during mesenteric explaining this result might be at play. It is known that ET-1 is involved mainly by modulating leukotriene production changes important part in the development of these microvascular substances which induces intense vasoconstriction of cells, and endothelial cell function were strongly correlated to injury intensity.

Intestinal I/R affects the microvasculature, endothelial cells, and endothelial cell function. ET is a vasoactive substance which induces intense vasoconstriction of blood vessels, and its activation is thought to play an important part in the development of these microvascular changes. ET-1 participates in intestinal I/R injury by modulating leukotriene production. Additionally, it has been demonstrated that ET-1 is involved mainly in post-reperfusion induced vasospasm and damage, and ET receptor antagonists are able to protect against mucosal injury. In agreement with previous reports, we found that I/R due to arterial occlusion induces elevations in ET-1 levels, and further, we demonstrated that venous occlusion has a similar outcome, although there was a tendency for higher levels in the venous occlusion group. Moreover, arterial occlusion was unable to alter these values compared to the venous occlusion group. ATIII depleted after intestinal I/R, and that they were restored by protective therapy. Additionally, ATIII was an endogenous anticoagulation molecule produced in lung, liver and endothelium which limits thrombin formation. Thrombin activation and the formation of thrombin-antithrombin complex is increased after intestinal I/R, contributing to cytokine production and inflammatory injury. Furthermore, these alterations would theoretically result in endogenous ATIII depletion. One rodent study found that ATIII levels were indeed depleted after intestinal I/R, and that they were restored by protective therapy. Additionally, ATIII

Figure 2 Serum concentrations of endothelial function markers and TNF-α. A: ET-1 serum concentrations; B: ATIII serum concentrations; C: P-selectin serum concentrations; D: Serum levels of soluble ICAM-1; E: TNF-α serum concentrations. *P < 0.05 vs sham; **P < 0.01 vs sham; ***P < 0.01 vs A; †P < 0.05 vs V; ‡P < 0.01 vs V.
administration can protect the intestinal mucosa against I/R injury. We found that I/R due to both venous and arteriovenous occlusion caused AT III depletion whereas arterial occlusion alone did not. In our study, a negative correlation between AT III levels and injury scores was observed, suggesting that depleted AT III levels are indeed reflective of damage intensity. A possible explanation for our results is that the microvascular congestion and venous stasis caused by the venous occlusion component promotes the coagulation cascade to a greater extent than the arterial component during ischemia and reperfusion. Indeed, temporal and histological differences have been found to exist between thrombus generation in arterial and venous endothelium. While stasis seems to be a more important factor in veins, impaired laminar flow and plaque formation seem to be essential for arterial thrombus generation. Furthermore, studies have shown that decreased levels of AT III after major surgery can predict higher complication and mortality rates. Therefore, the differences in AT III depletion between arterial or venous mesenteric ischemia might be of clinical relevance. The physiopathological consequences of these results warrant further investigation.

ICAM-1 is another adhesion molecule that mediates leukocyte reactivity and adhesion to endothelium. Intestinal I/R is also associated with an upregulation of ICAM-1 and subsequent leukocyte infiltration. Systemic ICAM-1 activity is also upregulated, possibly contributing to multisystem failure and remote organ injury. Serum levels of ICAM-1 have been shown to be markedly elevated after intestinal I/R, and low serum concentrations are associated with beneficial outcomes after the administration of protective agents. The inhibition of ICAM-1 with monoclonal antibodies is able to reduce the intensity of the functional and morphological alterations in the intestine subjected to I/R. In our study, we found that soluble ICAM-1 serum concentrations were elevated by intestinal I/R, in agreement with the previous reports. However, there was no difference between any of the occlusion methods employed, suggesting that soluble ICAM-1 is non-specifically elevated in arterial, venous or arteriovenous I/R.

In conclusion, we showed that a different pattern of response to I/R characterize different forms of mesenteric occlusion methods. Venous occlusion seems to cause more severe tissue damage and changes in P-selectin and AT III depletion compared to arterial occlusion. This suggests that these molecules are sensitive to changes in the intensity of the histological injury and therefore might be partly responsible for some of the pathophysiological differences between venous and arterial occlusion. Arteriovenous occlusion showed greater tissue damage and TNF-α levels compared to arterial occlusion alone, but only differences in P-selectin and ET-1 were found between them. Finally, arterial occlusion was related to the least severe changes compared to controls, and ICAM-1 values were not different between the different I/R groups.

We discussed the possibility of these differences being dependent on local microvascular factors, but since serum concentrations are indicative of the systemic state, the explanation could lie in differences in the systemic consequences of the occlusion methods employed. Studies on the local and remote tissue expression of these molecules would be helpful in clarifying these issues. However, our results confirm that there are differences in the inflammatory responses associated with venous, arterial or arteriovenous occlusion that could partly explain the molecular bases of the clinical differences between these conditions.

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COMMENTS

Background
Intestinal ischemia can occur when blood flow from the mesenteric vasculature is obstructed. Obstructions can be embolic, atherosclerotic or vasospastic in etiology. Mesenteric veins or arteries can be affected. Ischemia and reperfusion also takes place in transplant procedures and states of hypoperfusion (shock). The pathophysiological differences between venous, arterial or total ischemia of the intestine are not fully understood. In this study, the aim was to investigate these differences using a rat model.

Research frontiers
Ischemia/reperfusion is an important topic of research. The molecular mediators responsible for ischemia/reperfusion injury could be modulated to improve transplantation outcomes.

Innovations and breakthroughs
In this study, it is demonstrated that venous, arterial or arteriovenous occlusion followed by reperfusion, cause different patterns of injury and serum elevations of inflammatory markers.

Applications
To understand the pathophysiological roles of ischemia/reperfusion injury could lead to novel treatments, specifically tailored for conditions of venous, arterial or total ischemia.

Peer review
This is a very interesting study which examines changes in rat intestinal histology and inflammatory serum cytokine markers subsequent to arterial, venous or concomitant arterial and venous occlusion and reperfusion. The authors demonstrated the differences in injury patterns caused by arterial, venous or arteriovenous mesenteric occlusion. This study is well-investigated and has a novel finding.

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