The effect of Antithrombin-III on routine hematological and biochemical parameters in an experimental animal model of skeletal muscle ischemia-reperfusion injury

Karamanos DG,1 Karkos CD,1 Kambaroudis AG,1 Kritsepi M,2 Papadopoulos S,1 Spyridis CT,1 Gerassimidis TS1

15th Department of Surgery, Medical School
2Diagnostic Laboratory, Faculty of Veterinary Medicine
Aristotle University of Thessaloniki, Thessaloniki, Greece

Abstract

Background: Antithrombin III (AT-III) has been shown to attenuate the local and systemic harmful effects of skeletal muscle ischemia-reperfusion (I-R) injury. The aim of the present study was to monitor the fluctuation of routine hematological and biochemical parameters in an experimental animal model of tourniquet-induced skeletal muscle I-R injury and to investigate how these are influenced by the protective administration of AT-III.

Methods: Sixty male Wistar rats were submitted to a 6-hour, tourniquet-induced, complete ischemia of the right hind-limb. Animals were divided into those receiving AT-III (dose, 250 IU/kg) 30 minutes before the reperfusion (group A, n=30) and those receiving placebo (group B, n =30). Another 10 animals were sham-operated (group C). White blood cell (WBC) and platelet (PLT) count, aspartate and alanine aminotransferases (AST and ALT), alkaline phosphatase (ALP), and γ-glutamyl transferase (γ-GT) were estimated in blood samples taken from the inferior vena cava at 3 different time points post-reperfusion (at baseline, at 30 minutes and at 4 hours) and groups A and B were compared using the Mann-Whitney U test.

Results: There were no statistically significant differences between the AT-III and the placebo groups at 0, 30 minutes and 4 hours with regard to the WBC, ALT and γ-GT levels, however, there was a significant decrease of AST levels 4 hours post-reperfusion in the AT-III group compared to the placebo group (p=0.002). An increased PLT count and ALP levels 30 minutes post-reperfusion were also noted in the AT-III group compared to placebo (p<0.001; and p=0.001, respectively).

Conclusions: Of the routine hematological and biochemical parameters tested, AST was found to be significantly suppressed at 4 hours in the AT-III-treated animals, suggesting a possible beneficial effect of AT-III in mouse skeletal muscle I-R injury. The effect of AT-III on PLTs and ALP levels merits further investigation. Hippokratia 2014; 18 (3): 234-239.

Keywords: Experimental study, lower limb, ischemia-reperfusion, skeletal muscle, antithrombin-III

Introduction

Skeletal muscle ischemia is accompanied by a series of biochemical and electrolytic disturbances which are directly associated with the extent and the duration of ischemia. Reperfusion of ischemic tissues may lead to deleterious effects, both locally and systemically, also affecting non-ischemic remote organs, such as lungs, heart, liver or kidneys1,2. The pathogenesis of ischemia-reperfusion (I-R) injury is complex and several factors, including leukocytes, oxygen free radicals, and complement have been implicated.

In recent years, considerable research has been made into preventing or reducing the extent of the skeletal muscle I-R syndrome. Various therapeutic strategies with monoclonal antibodies or anti-oxidant agents, such as N-acetylcysteine, mannitol, katalase, allopurinol and vitamin E have been evaluated. Although such strategies produced promising results in the experimental setting, none proved to be beneficial in daily clinical practice.3-5. Antithrombin III (AT-III) is a serine protease inhibitor, which, in addition to its anticoagulant function, is believed to exhibit an anti-inflammatory activity in sepsis and in I-R. Recent experimental studies suggested that pre-treatment with AT-III may attenuate the deleterious effects of I-R on local and remote organs.6-17. Mimicking the conditions of real-life emergency vascular surgery practice, we aimed, first, to monitor the fluctuation of routine hematological and biochemical parameters in an experimental animal model of tourniquet-induced skeletal muscle I-R injury, and, second, to investigate how these are influenced by the protective administration of AT-III. Such hematological and biochemical blood tests are routinely given in patients presenting with acute limb ischemia and undergoing revascularization procedures and may help to monitor the progress of I-R and guide therapeutic interventions.
Methods

Seventy male Wistar rats weighing between 250 and 300 g were included in this study. The rats were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, kept under a 12-hour light/dark cycle, and permitted ad libitum access to standard laboratory rodent chow and tap water for 2 weeks before the beginning of the experimental procedure. Initially, the rats were anesthetized for a short period. They were placed in a glass bell containing a cotton wisp impregnated with ether (diethyl ether, Panreac Quimica SAU, Barcelona, Spain) and were weighted. Subsequently, ischemia was induced by the application of a polypropylene suture (Prolene 0, Ethicon, Inc., a Johnson & Johnson company, Gargrave, England) on the right hind limb and the absence of flow was checked by Doppler ultrasound (Huntleigh Diagnostics Ltd, South Glamorgan, UK). A previous in-house study, using both Doppler ultrasound and arteriography, verified that the application of a nylon suture produces almost complete limb ischemia. Similarly, previous studies by other investigators had shown that this method reduces blood flow by 98% and leads to acute ischemia.

The routine haematological and biochemical parameters tested on the inferior vena cava blood samples were the following: white blood cell (WBC) count; platelet (PLT) count; aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP); and γ-glutamyl transferase (γ-GT). Hematological examination of WBCs and PLTs (blood sample in vial with EDTA KE/1.3ml) was performed in a Scil Vet ab (animal blood counter) veterinary analyzer. Expression of values was in 10^3 μL. The plasma biochemical examination (blood sample in vial with Li-heparin/1.3ml) was carried out in an open type analyzer (VITALAB, Flexor E, Clinical Chemistry Analyzer, Vital Scientific, NV). Centrifugation in order to obtain plasma was performed in an STAT spin MP multipurpose centrifuge for 3 minutes. Expression of values was in U/L.

Results

The results for every parameter studied in the control (C1, C2), placebo (B1, B2, B3) and AT-III - treated animals (A1, A2, A3) are summarized in Table 1.

Table 1: Median value (interquartile range, IQR) for every parameter studied in the control (C1, C2), placebo (B1, B2, B3) and antithrombin III (AT-III) - treated animals (A1, A2, A3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C1</th>
<th>C2</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (x10^3/μL)</td>
<td>7.7</td>
<td>4.1</td>
<td>11.95</td>
<td>10.1</td>
<td>13.3</td>
<td>8.58</td>
<td>10.05</td>
<td>14.64</td>
</tr>
<tr>
<td>(7.3-7.75)</td>
<td>(3.8-6.1)</td>
<td>(8.6-12.4)</td>
<td>(8.4-12.4)</td>
<td>(11.8-15.8)</td>
<td>(7.7-10.1)</td>
<td>(9.6-11.95)</td>
<td>(13.9-17.2)</td>
<td></td>
</tr>
<tr>
<td>PLT (x10^3/μL)</td>
<td>829</td>
<td>635</td>
<td>609</td>
<td>711.5</td>
<td>748</td>
<td>760</td>
<td>928</td>
<td>781</td>
</tr>
<tr>
<td>(809-892)</td>
<td>(608-715)</td>
<td>(440-661)</td>
<td>(704-717)</td>
<td>(676-766)</td>
<td>(684-797)</td>
<td>(847-945)</td>
<td>(757-842)</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>49</td>
<td>72</td>
<td>320.5</td>
<td>473.5</td>
<td>716</td>
<td>349</td>
<td>375</td>
<td>307.5</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>24</td>
<td>24</td>
<td>92.5</td>
<td>105.5</td>
<td>185</td>
<td>101.5</td>
<td>116.5</td>
<td>232</td>
</tr>
<tr>
<td>(23-26)</td>
<td>(23-26)</td>
<td>(80-113)</td>
<td>(100-130)</td>
<td>(170-222)</td>
<td>(88-123)</td>
<td>(103-128)</td>
<td>(201-280)</td>
<td></td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>568</td>
<td>310</td>
<td>441.5</td>
<td>329</td>
<td>388</td>
<td>421</td>
<td>395</td>
<td>425</td>
</tr>
<tr>
<td>γ-GT (U/L)</td>
<td>2</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>(1-2)</td>
<td>(1-2)</td>
<td>(1-2)</td>
<td>(1-2)</td>
<td>(1-2)</td>
<td>(1-2)</td>
<td>(1-2)</td>
<td>(1-1)</td>
<td>(1-2)</td>
</tr>
</tbody>
</table>

The median WBC counts in the study subgroups are presented in Figure 1a. Both the placebo and the AT-III groups showed a gradual increase of WBC levels as reperfusion progressed, which was statistically significant 4 hours post-reperfusion in the AT-III group, but failed to reach statistical significance in the placebo group (B1 vs B3, \( p=0.45 \); A1 vs A3, \( p=0.001 \)). Compared to the control subgroup C2 (sham operation, no AT-III), this increase was statistically significant in most phases of reperfusion (B1 vs C2, \( p=0.008 \); B2 vs C2, \( p=0.007 \); B3 vs C2, \( p=0.002 \); and A1 vs C2, \( p=0.066 \); A2 vs C2, \( p=0.002 \); A3 vs C2, \( p=0.002 \)). No statistically significant differences were encountered when comparing groups A and B at the different time points of reperfusion (B1 vs A1, \( p=0.07 \); B2 vs A2, \( p=0.677 \); and B3 vs A3, \( p=0.226 \)), and therefore, we could not establish an improvement in WBC levels due to AT-III administration.

**PLTs**

The median of PLT counts in the different study groups are shown in Figure 1b. An increased PLT count was noted 30 minutes post-reperfusion in the AT-III group compared to the C2 control subgroup (A2 vs C2, \( p=0.002 \)) and the placebo group (A2 vs B2, \( p=0.001 \)). The C1 control subgroup which received AT-III had a higher PLT count compared to C2 subgroup (C1 vs C2, \( p=0.009 \)). The platelet count in the AT-III group returned to baseline levels at 4 hours (A2 vs A3, \( p=0.004 \); A1 vs A3, \( p=0.29 \)).

**Transaminases (AST, ALT)**

i) Differences within the same group

AST levels (medians) are graphically presented in figure 1c. These tended to increase significantly following reperfusion in the placebo subgroups, (B1 vs B2, \( p=0.038 \); B2 vs B3, \( p=0.016 \); B1 vs B3, \( p=0.002 \)). In the AT-III group, no statistically significant increase was noted post-reperfusion (A1 vs A2, A2 vs A3 and A1 vs A3, all \( p=NS \)).

Similarly, ALT levels increased significantly post-reperfusion in the placebo group. This increase was statistically significant at 4 hours (B1 vs B3, \( p=0.011 \)). In contrast to AST, ALT increased significantly in the AT-III group, too (A1 vs A3, \( p=0.001 \)).

ii) Differences between placebo and AT-III subgroups at the same time point

AST levels were no statistically different between placebo and AT-III treated animals at 0 and at 30 minutes (B1 vs A1, \( p=0.545 \); and B2 vs A2, \( p=0.096 \)). However, at 4 hours post-reperfusion, AST levels in the AT-III group were significantly lower than in the placebo group (309.4 ± 120.2 U/L vs 714.0 ± 274.6 U/L, \( p=0.002 \)).

With regard to the ALT, no statistically significant differences existed between the placebo and AT-III groups at 0, at 30 minutes and 4 hours.

Both groups which had been subjected to I-R (i.e. A and B) had significantly higher levels of AST compared to the control subgroup C2 which had no I-R and received no AT-III (A1 vs C2, \( p=0.006 \); and B1 vs C2, \( p=0.002 \)). Finally, in subgroups C1 and C2 where no I-R was effected, the transaminases levels were significantly

---

**Figure 1:** Scatter plots presenting median values of white blood cell (WBC) counts (a), platelet (PLT) counts (b), aspartate aminotransferase (AST) levels (c), and alkaline phosphatase (ALP) levels (d) in the antithrombin III (AT-III) and the placebo-treated animals during reperfusion. Parameters not achieving statistical significance are not graphically presented here [i.e. alkaline phosphatase (ALT) and γ-glutamyl transferase (γ-GT)].
lower in the C1 subgroup that received AT-III compared to placebo subgroup C2 (AST: C1 vs C2, p=0.009; and ALT: C1 vs C2, p=0.009).

**ALP**

The median ALP levels are shown in Figure 1d. There were no differences within the AT-III group or the placebo group during the progression of reperfusion. Comparing the AT-III and the placebo subgroups, a statistically significant increase was only found 30 minutes post-reperfusion in the AT-III group, whereas no differences were noted at baseline or at 4 hours (A1 vs B1, p=0.545; A2 vs B2, p=0.001; A3 vs B3, p=0.241). Finally, ALP levels were significantly higher in the C1 compared to the C2 subgroup (C1 vs C2, p=0.009). All AT-III subgroups (i.e. A1, A2, and A3) also had significantly higher levels compared to C2 (A1 vs C2, p=0.007; A2 vs C2, p=0.050; and A3 vs C2, p=0.027).

**γ-GT**

With regard to the γ-GT levels, there were no statistically significant differences between the medians in any subgroups.

**Discussion**

Clinical and experimental studies suggested that administration of AT-III may improve the inflammatory response and disseminated intravascular coagulation caused by sepsis, trauma and endotoxins5. In several experimental models of lung, hepatic and intestinal I-R, administration of AT-III prior to ischemia was shown to exert a protective effect7-16. A similar protective effect of administration of AT-III prior to ischemia was shown on experimental models of lung, hepatic and intestinal I-R, attenuating the harmful effects of skeletal muscle I-R17. Duru et al. published a study which examined the role of AT-III in alleviating the harmful effects of skeletal muscle I-R. This study further investigated factors that could impact on the results. Whether the protective action of AT-III is mediated through reducing the infiltration of tissues by local polymorphonuclear cells8,16,17. The lack of statistical significance with regard to the fluctuation of WBCs during reperfusion may be due to small animal numbers in each subgroup and represent a type II statistical error. Other confounding factors that could impact on the results may be the length of ischemia and of reperfusion, as well as the dose of AT-III. Whether the protective effect against I-R could be enhanced by higher initial and/or repeated doses of AT-III remains to be investigated.
An interesting finding is the statistically significant increase in the PLT count which was found in the AT-III group at 30 minutes post-reperfusion and which returned to baseline at 4 hours. There are no literature data to explain this observation and further studies will be needed to clarify this issue.

The levels of transaminases showed a statistically significant increase in both I-R groups, i.e. both AT-III and placebo groups, compared with the sham-operated group. This observation is in keeping with previous studies\(^2\)–\(^23\). Yassin et al., who also studied the levels of Interleukin-6 and TNF-\(\alpha\), attributed the increased levels to their production from muscle tissue, without being able to exclude with certainty the participation of liver Kupffer cells\(^1\). Comparing AT-III and placebo groups, an improvement (i.e. suppression) in the AST levels was noted at 4 hours post-reperfusion in the AT-III treated animals. This observation may be attributed to the protective action of AT-III. It could be asserted that this kind of protective action predominantly concerns skeletal muscles, since AST is mainly a muscle enzyme. Equally interesting are the results derived from the sham-operated (without I-R) subgroups C1 and C2. The levels of transaminases were significantly lower in AT-III subgroup C1 and it may be that AT-III also protects from the stress of anesthesia and surgery.

A notable observation is the fluctuation of ALP, whose levels increased significantly in C1, A1 and A3 subgroups (which received AT-III) compared to C2 subgroup (no AT-III administration), as well as in A2 subgroup compared to B2. This increase in ALP could be, again, attributed to the AT-III administration, but this does not explain the lack of differences in other instances. Therefore, this observation merits further investigation.

With regard to the \(\gamma\)-GT, this parameter seems to remain relatively unaffected by the I-R phenomenon and the administration of AT-III during the first 4 hours of reperfusion.

Finally, this study has, indeed, some limitations, most of which have already been highlighted. One such limitation is also the lack of backing evidence from cytometry and histological examination of tissues involved. Nevertheless, further evidence in favor of the beneficial role of AT-III in I-R comes from a recent study from our group, which, using an identical experimental protocol, investigated the protective role of AT-III in remote I-R injury, focusing specifically on the lungs and heart. The magnitude of I-R injury was estimated measuring malondialdehyde (MDA) levels, an indirect measure of oxygen free radicals which play a pivotal role in I-R injury\(^2\). Because quantifying oxygen free radicals is difficult due to their reactive nature and short lives, it has been suggested that MDA can be used instead to estimate the extent of oxidant damage to tissues. The study concluded that pre-treatment with AT-III 30 minutes before the reperfusion of an acutely ischemic limb can reduce the oxidant stress to lung and, less so, to myocardial tissue owing to its anti-inflammatory and antioxidant action.

**Conclusion**

Of the routine hematological and biochemical parameters tested, AST was found to be significantly suppressed at 4 hours in the AT-III-treated animals, suggesting a possible beneficial effect of AT-III in mouse skeletal muscle I-R injury. The effect of AT-III on PLTs and ALP levels merits further investigation.

**Conflict of interest**

No funding had been received for this study. There is no conflict of interest.

**References**


