Effect of Triiodothyronine Administration on the Kidney During Haemorrhagic Shock and Resuscitation

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Abstract

Objective: Apoptosis, measured via caspase activity, can be used to assess renal tissue damage in haemorrhagic shock. We investigated whether Triiodothyronine could attenuate apoptosis and protect against haemorrhagic shock-induced renal injury.

Methods: Haemorrhagic shock was induced in swine until the mean arterial pressure (MAP) was 35–40 mmHg for 40 minutes. Animals were randomly assigned to a control group (n=5), Group-F (Fluid resuscitation, n=6), and Group-T3 (Fluid plus Triiodothyronine, n=6). The swine were resuscitated for 1 hour aiming to MAP restoration (±10% from baseline) and were followed up for another 360 minutes. Haemodynamic parameters, fluids, acid-base status, plasma urea nitrogen, creatinine levels and caspase activity in the kidney were measured.

Results: Haemodynamic parameters did not differ significantly amongst the three groups. Group-T3 required less normal saline (Group-T3: 1083±204 mL versus F: 2500±547 mL, p=0.001) and hydroxyethyl starch (Group-T3: 558±102 mL versus F: 916±204 mL, p=0.004) during resuscitation. Additionally, Group-T3 swine experienced less acidosis following haemorrhage/resuscitation with a pH of 7.39 versus a pH of 7.26 in Group-F (p=0.004) at 360 minutes. Haemodynamic parameters, fluids, acid-base status, plasma urea nitrogen, creatinine levels and caspase activity in the kidney were measured.

Conclusion: Administration of Triiodothyronine in a swine model of haemorrhagic shock seems to interfere with renal cell apoptosis. The exact mechanism needs to be further investigated in future research.

Keywords: Apoptosis, haemorrhagic shock, kidney, resuscitation, triiodothyronine

Introduction

Acute kidney injury (AKI) is a common consequence of traumatic haemorrhagic shock and a strong predictor of multi-organ failure and increased mortality (1). Fluid resuscitation, which is one of the cornerstones in the management of hypovolaemia, can also negatively affect the kidney through ischaemia/reperfusion injury manifested by the activation of the oxidative stress and apoptotic pathways (2). Apparently, AKI related to ischaemia/reperfusion may contribute to acute and chronic renal failure (2, 3). Moreover, the damage initiated at the kidney level can trigger a systemic inflammatory response which affects other organs such as the brain, heart, and liver and negatively aggravates the treatment outcome (4). Thus, resuscitation strategies that mitigate renal damage would be invaluable in decreasing the overall morbidity and mortality of trauma patients.
During haemorrhagic shock and subsequent resuscitation, apoptosis is induced in various tissues, including the kidney. The magnitude of caspase activity has been used to identify the degree of apoptosis in different studies (5).

It is also well-established that thyroid function is altered in critical illnesses, including life-threatening haemorrhage (6). In addition, resuscitation may have a further negative impact on the hypophyseal thyroid axis (6). In recent studies, the administration of triiodothyronine (T3) has been proven to contribute to haemodynamic stability and more importantly, it decreased the level of apoptotic cell death in beta pancreatic cell cultures and in cardiac cells following infarction (7-9).

The aim of the present study was to evaluate whether T3 administration had a protective effect against haemorrhagic shock-induced renal injury by means of alleviating the degree of apoptosis in the organ. In this setting, T3 could attenuate morbidity in haemorrhagic shock and resuscitation through its beneficial impact on renal tissue. To our knowledge, this is the first study to address the effect of thyroid hormone on the kidney during haemorrhagic shock and resuscitation.

**Methods**

The present research was conducted ethically and responsibly, and was in full compliance with all the relevant codes of experimentation and legislation. All the applicable international guidelines for the care and use of animals were followed. The experimental study protocol was formally approved by the scientific committee of Aretaieion Hospital, University of Athens, Greece (Σ-27, 17/02/09) and the Research Committee of the Prefecture of Eastern Attica, Greece.

**Animal Preparation and Anaesthesia**

A total of 17 male adult swine having an average weight of 28 kg were used for the study. The animals were moved to the research centre one week before the experiment. They were housed in a temperature-controlled environment under a 12-hour light and dark cycle. Free access to water and food was provided. The animals were made to fast 12 hours before the experiment, except for water ad libitum. Premedication was administered 1 hour before the induction of anaesthesia, which comprised intramuscular midazolam 0.5 mg kg$^{-1}$, ketamine 15 mg kg$^{-1}$ and atropine 0.045 mg kg$^{-1}$.

Anaesthesia was induced with propofol 3 mg kg$^{-1}$ (Propofol, Fresenius Kabi, Austria) and fentanyl (Fentanyl, Janssen-Cillag, Belgium) via an auricular vein cannulated with an 18-gauge venous catheter. Following endotracheal intubation, cis-atracurium 0.5 mg kg$^{-1}$ i.v (Nimbex, GlaxoSmithKline, Italy) was administered and the animals were placed on volume-controlled mechanical ventilation (Taema Clazy S 2000 Antony Cedex, France). The ventilation settings were adjusted as follows: tidal volume of 10±2 mL kg$^{-1}$ and respiratory rate of 12–15 breaths per minute were maintained to keep the end-tidal CO$_2$ level at 35±5 mmHg. Anaesthesia was maintained with propofol, fentanyl and cis-atracurium at standard infusion doses.

The animals were placed in the supine position and a three-lumen catheter was inserted into the right internal jugular vein for blood sampling and continuous central venous pressure (CVP) monitoring. After surgical exposure, the femoral artery was cannulated with a 20-gauge catheter for continuous arterial blood pressure monitoring (every 5 minutes in 1$^{st}$ hour and every 15 minutes after that). The temperature was monitored with a rectal probe and the swine were kept warm by using a heating pad. A suprapubic catheter was also inserted.

**Experimental Design and Haemorrhagic Shock**

The swine were allocated randomly into three groups: Group-C (Control, n=5), Group-F (Fluid resuscitated, n=6), and Group-T3 (Fluid plus T3-resuscitated, n=6). Following induction and preparation, a midline laparotomy was performed aseptically. Group-C underwent cannulation, suprapubic catheter placement, midline laparotomy and closing of the abdomen. After 6 hours, the animals were euthanised. Group-F and T3 were subjected to haemorrhagic shock established through a left lobe hepatic resection, which was allowed to bleed. This was initiated after a period of observation of 10 minutes and lasted for 30 minutes, i.e., until time point 40'. Bleeding was allowed to a mean arterial pressure (MAP) 35±5 mmHg.

At the end of bleeding period at 40 minutes, the abdomen was reopened, haemostasis was carried out, the total amount of blood was measured and resuscitation was performed for 60 minutes. The blood loss was estimated by removing blood
and clots from abdomen by suction and by weighing the used swabs. During the resuscitation period, the animals received 0.9% NaCl up to 35 mL kg\(^{-1}\) plus hydroxyethyl starch 130/0.4 (Voluven, Fresenius Kabi, Canada) up to 18 mL kg\(^{-1}\) (N/S to Voluven ratio 2:1) with end-point the normalisation of MAP to baseline values ±10%. If swine were still not stable after the first infusion, a second infusion up to the same amounts was administered. After resuscitation, the animals received maintenance fluids; dextrose 5% (D\(_5\)W) plus 30 mL of NaCl 15% in every 1000 mL of D\(_5\)W according to individual MAP values. The T3-group received the same amount of fluids plus T3.

The experiment ended 6 hours after the beginning of shock and animals were euthanised by administering thiopental 2g and vecuronium 30 mg. Aseptic technique was maintained throughout the experiment and no antibiotics were used.

**T3 Administration, Blood and Kidney Tissue Sampling**

In the beginning of haemorrhagic shock establishment, T3 (3’5’3’-T3, Sigma chemicals, St Louis MO USA) was administered as a bolus of 0.8 mcg kg\(^{-1}\), followed by an infusion of 0.1 mcg kg\(^{-1}\) minute\(^{-1}\) until the end of the experiment.

Blood samples from the internal jugular vein were obtained at time points 0’, 40’ and 360’. Arterial blood gases were collected at the same time points and central venous oxygen saturation (ScvO\(_2\)) was also measured. Blood samples were collected in polypropylene tubes, centrifuged at 4000 g for 20 minutes at 4\(^\circ\)C and serum samples were then separated. These were subsequently stored at −80\(^\circ\)C until processed. We measured biochemical indices of kidney function, namely urea and creatinine levels. We also measured the levels of lactate dehydrogenase (LDH), alkaline phosphatase and amylase.

![Figure 1. a-d. Differences in mean arterial pressure (MAP) at time points 0’ (1A), 10’ (1B), 40’ (1C) and 360’ (1D) between groups](image-url)
Kidney tissue samples were shred at 9500 rpm (for 10 s at 4°C) by a homogeniser (WiseTis, HG-15A; Korea) and supernatants were removed. Renal tissue was also stored at −80°C until analysis via a colorimetric assay kit (R&D Systems, Minneapolis, MN). Specifically, the active caspase-3 was assessed in renal tissue homogenates as a reliable biomarker of apoptosis. The colorimetric assay was used for the quantitative determination of caspase-3 activity. This method is based on a microtiter plate reader (at 400 nm or 405 nm) or spectrophotometric detection and quantification of chromophore p-nitroaniline (pNA), which is formed by cleavage from the labelled substrate DEVD-pNA. Comparison of pNA absorbance between apoptotic and uninduced control sample helps to assess the activity. The manufacturer’s instructions were followed for all the procedures (handling of tissue specimens/cytosolic extracts, use of reaction buffers and DEVD-pNA substrate, sample absorbance reading) and safety issues (10). The results were expressed as pg min⁻¹ g⁻¹.

**Statistical analysis**

Statistical analysis was carried out by the use of Statistical Package for the Social Sciences for Mac. Data are reported as mean values±standard deviation for normally distributed values, while median values are presented for non-normal distributions. Normality was assessed through the Shapiro-Wilk test according to the size of our dataset. Histograms were also assessed for the distribution of values. For comparisons between different groups at corresponding time points, the one-way analysis of variance test was applied followed by post hoc analysis using the Bonferroni correction. A p-value of <0.05 was considered statistically significant.

**Results**

No differences were found in body weight, baseline hemodynamic parameters and baseline biochemistry results. Temperature was maintained in all groups during the experiment.

**Table 1. Heart rate changes in the three groups at 0 minutes, 40 minutes and 360 minutes**

|       | Control (C) | Fluids (F) | Fluids and T3 (T3) | p (C/F) | p (C/T3) | p (F/T3) |
|-------|-------------|------------|--------------------|---------|----------|---------|
| HR 0  | 102±25      | 103±14     | 101±11             | 1.000   | 1.000    | 1.000   |
| HR 10 | 107±25      | 132±34     | 128±37             | 0.655   | 0.895    | 0.100   |
| HR 40 | 107±31      | 103±35     | 128±60             | 1.000   | 1.000    | 1.000   |
| HR 360| 115±52      | 148±55     | 112±38             | 0.611   | 1.000    | 0.433   |

Values are expressed as mean±standard deviation (beats/min). A p-value of <0.05 was considered statistically significant.

**Table 2. Arterial blood gases and central venous oxygen saturation (ScvO₂) in the three groups at time points 0 minutes, 40 minutes and 360 minutes**

|       | Control (C) | Fluids (F) | Fluids and T3 (T3) | p (C/F) | p (C/T3) | p (F/T3) |
|-------|-------------|------------|--------------------|---------|----------|---------|
| pH 0  | 7.54±0.12   | 7.53±0.1   | 7.53±0.1           | 1.000   | 1.000    | 1.000   |
| pH 40 | 7.51±0.1    | 7.42±0.1   | 7.49±0.1           | 0.674   | 1.000    | 1.000   |
| pH 360| 7.48±0      | 7.26±0.1   | 7.39±0.1           | 0.004   | 0.443    | 0.062   |
| pO₂ 0 (mmHg)| 231±60     | 252±69     | 269±45             | 1.000   | 0.934    | 0.100   |
| pO₂ 40| 223±68      | 223±68     | 258±59             | 1.000   | 1.000    | 1.000   |
| pO₂ 360| 215±64     | 180±45     | 250±57             | 0.939   | 0.926    | 0.134   |
| pCO₂ 0 (mmHg)| 33±9       | 32±7       | 39±7               | 1.000   | 0.823    | 0.385   |
| pCO₂ 40| 36±9        | 29±5       | 33±10              | 0.452   | 1.000    | 0.100   |
| pCO₂ 360| 38±6       | 40±10      | 40±9               | 0.116   | 1.000    | 0.223   |
| BE 0  | 5.2±2.9     | 3.9±3.8    | 6.1±1.6            | 1.000   | 1.000    | 0.619   |
| BE 40 | 5.7±2.8     | -4.2±5.7   | 1.7±3.9            | 0.007   | 0.457    | 0.114   |
| BE 360| 4.2±3.4     | -5.1±5.8   | -0.7±1.0           | 0.005   | 0.186    | 0.220   |
| HCO₃ 0 (mmol L⁻¹)| 30±4     | 26±3       | 29±3               | 0.238   | 1.000    | 0.473   |
| HCO₃ 40| 29±3        | 19±5       | 24±2               | 0.001   | 0.203    | 0.051   |
| HCO₃ 360| 28±4       | 22±5       | 24±2               | 0.086   | 0.307    | 0.100   |
| ScvO₂ 0 (%)| 78±12   | 88±7       | 87±6               | 0.187   | 0.249    | 1.000   |
| ScvO₂ 40| 81±4        | 56±15      | 58±14              | 0.031   | 0.049    | 1.000   |
| ScvO₂ 360| 80±12      | 69±12      | 78±7               | 0.356   | 1.000    | 0.509   |

Values are expressed as mean±standard deviation. A p-value of <0.05 was considered statistically significant. BE: base excess
The MAP values were similar in all groups at the starting point of 0'. Haemorrhagic shock was successfully established as MAP dropped significantly in the F and T3-groups at 10' and 40' minutes (Figure 1). Restoration of intravascular volume was also satisfactory; as expected, no significant differences were noted regarding MAP at the end of the experiment. The heart rate at time point 360' was 115±52 beats min⁻¹ in the C-group, 148±36 beats min⁻¹ in the F-group, and 111±36 beats min⁻¹ in the T3-group, without statistical significance (F versus T3-groups: p=0.43) (Table 1). Nevertheless, the clinical significance of the above HR difference between the F-group and T3-group should be considered. Blood loss did not differ between the two groups (F: 708 mL versus T3: 700 mL, p=0.93). The volumes of fluids administered differed between groups: the T3-group needed less normal saline (T3: 1083±204 mL versus F: 2500±547 mL, p=0.001) and hydroxyethyl starch (T3: 558±102 mL versus F: 916±204 mL, p=0.004) to maintain similar haemodynamic values during the resuscitation period. However, this difference was somewhat blunted over time as the amount of maintenance fluids (D₅W plus NaCl) required by the F-group was slightly less during the observation period and until the end of the protocol, i.e. from time point 100 minutes to 360 minutes (C: 1000±0 mL, F: 1000±0 mL, T3: 1666±516 mL, F versus T3: p=0.01). Reflecting the smaller amount of total fluids given in the T3-group, the CVP at the end of the protocol was higher in the F-group, but without statistical significance (F:5.6 mmHg versus T3:2.1 mmHg, p=0.27).

Regarding the blood gas analysis, the administration of thyroid hormone was associated with less acidosis following haemorrhagic shock and resuscitation; pH at 360 minutes was 7.26 for the F-group and 7.39 for the T3-group (p=0.004) (Table 2). The metabolic element of the acidosis was suggested by the drop of bicarbonate and the higher base deficit of the C-group as compared to the other two groups. ScvO₂ was higher in the T3-group at the end of the experimental period, but without statistical significance (Table 2).

Biochemistry results are presented in Table 2. Urea was maintained within normal limits in all groups. Conversely, creatinine was significantly elevated after 6 hours in the F-group as compared to the T3-group (p=0.019). Alkaline phosphatase...
tase (ALP) and amylase did not present notable variability between groups, while LDH was significantly higher in the C-group only at 40 minutes (Table 3).

There was a difference in apoptosis amongst the groups by means of caspase-3 activity level, but this was not statistically significant (Figure 2). In kidney homogenates, the mean caspase-3 activity was $84\pm93$ pmol min$^{-1}$ g$^{-1}$ in the C-group. After HS/R, the mean renal caspase-3 level increased in the T3-group, while it was reduced in the F-group ($132\pm174$ and $32\pm18$ pmol min$^{-1}$ g$^{-1}$ respectively, $p=0.245$). Administration of T3 increased the caspase-3 activity in comparison to the fluid resuscitated F-group. The difference was high in absolute values, but statistical significance was not reached. All animals survived the 8-hour period of observation.

**Discussion**

Despite advances in treatment, mortality due to haemorrhagic shock remains high amongst animals. Even amongst survivors, multi-organ failure due to ischaemia/reperfusion injury following resuscitation from hypovolaemic shock is a constant threat. The kidney is amongst the organs that is most seriously affected and at the cellular level, apoptosis is a common expression of an organ's injury (11, 12). Apoptosis is a complex biological process that is fundamental for cell homeostasis and disease (13). Within the kidney, apoptosis is associated with tubular cell death resulting from ischaemia/reperfusion, and several molecules are involved in its origin. Two main pathways have been described in the apoptotic cell death process: (1) an extrinsic pathway, mediated by cell membrane receptors, which generally belong to the tumour necrosis factor family, and (2) an intrinsic pathway that is initiated by the mitochondria. One of the molecules involved in the latter is caspase-3.

Cleavage of caspase-3 indicating increased apoptotic signaling has been identified and used in many different experimental settings, such as chronic kidney disease, sepsis and haemorrhagic shock (14-16). Caspase-3 is a protein encoded by the CASP3 gene (17). The sequential activation of different caspases leads to its eventual activation. The release of cytochrome c by the mitochondria is involved in this process. Otherwise, caspase-3 exists as an inactive pro-enzyme which undergoes proteolysis, dividing the molecule in two subgroups that subsequently unite and mould together into the active form of caspase-3. This active molecule is responsible for the fragmentation of DNA during the process of cellular apoptosis (13).

Decreased levels of T3 and T4 have been documented in critical illnesses, including experimental haemorrhagic shock and more importantly, the degree of thyroid hormone deficiency is linked to survival rates (18, 19). Diminished levels of circulating thyroxine are associated with reduced myocardial energy, haemodynamic instability, and increased need for inotropic support (6). Postoperative i.v. T3 administration after coronary artery bypass surgery has been proven to increase cardiac index without affecting the mortality (20). In life-threatening bleeding, T3 administration has been found to increase survival in animals and contribute to haemodynamic stability (9, 21). In addition, trauma patients with lower T4 levels during admission require a more aggressive crystalloid resuscitation and more units of transfused red blood cells in the first 24 hours (6). Altogether, cardiac function, haemodynamic stability and survival are ameliorated by the administration of T3 in haemorrhagic shock.

The negative consequences of AKI are numerous; the patients are at risk for developing subsequent chronic kidney injury (4), while other organs such as the heart, brain, lung, and liver may also be negatively affected (4). In fact, renal ischaemia/reperfusion injury has been documented to trigger apoptosis in other tissues, such as the lung (3). Regarding survival in trauma, hospital mortality increases nearly four-fold in patients with AKI (22), therefore, protecting the kidney in conditions of hypovolaemia is of vital importance.

In our study, the addition of T3 was associated with a trend towards higher levels of caspase-3 activity in the kidney. The exact mechanism through which the administration of T3 increased programmed cell death in the renal tissue in our study cannot be fully explained. Apoptosis and necrosis share some common pathways in the death signalling pathway (23). It has been demonstrated that hypoxia caused by inhibiting the mitochondrial respiratory chain can trigger cellular necrosis, while other stimuli may lead the same cells to apoptotic cell death (23). In the initial phases of AKI, multiple immediate early genes that participate in the repair and regenerative process are up-regulated (24). Apoptotic genes are also up-regulated and help in the repair process of attenuating injury after IR (4). Considering this, we can speculate that the T3-resuscitated swine had a superior cellular oxygen reserve than the fluid-only resuscitated animals, which enabled them to follow the apoptotic pathway. In fact, the absence of intracellular ATP blocks apoptosis could have happened in the kidneys of the Group-F swine (23). It is also known that haemorrhagic shock depletes the levels of ATP in several organs, including the kidney (25). Prolonged ATP depletion leads to the necrotic pathway instead of apoptotic cell death after renal ischaemia, while repletion of ATP levels restores the ability of cells to undergo apoptosis (26, 27).

The haemodynamic stability offered by T3, along with better acid-base homeostasis, may have also contributed to improved tissue oxygenation and consequently, the increased
caspase-3-related apoptotic activity in the kidney. Fluid overload could also lead to AKI via the renal compartment syndrome and renal venous congestion, which indicates that achieving haemodynamic stability with less fluid administration could potentially have a beneficial effect on renal tissue protection (28). Endothelial injury and glycocalyx breakdown, which occur in trauma and haemorrhage, are also implicated in this phenomenon, thus aggravating tissue oedema. Similarly, CVP values higher than 12 mmHg are also associated with AKI due to renal venous congestion (28). On the contrary, other substances, such as the antineoplastic agent ukrain, have been found to protect against ischaemia/reperfusion injury by reducing the apoptotic activity within the renal cells (2). The related discrepancy in our results may be explained by the timing of sampling or the oxygen delivery associated with T3, as discussed above.

To date, no specific treatment for AKI exists, and supportive measures such as haemodynamic stability, which will ensure adequate renal blood flow and glomerular filtration rate, are the main target in its management. Due to the difficulty in measuring the indices of renal perfusion, surrogate markers are often used in clinical practice and these include, arterial blood pressure, lactate levels, ScvO2, and serum creatinine levels (28). In this context, the T3 administration achieved to maintain all the above parameters within normal limits compared to fluid replacement resuscitation alone.

The kidneys were not affected clinically to a large extent at the end of the experiment in any of the groups, as documented by the plasma urea nitrogen and creatinine levels. However, it can take hours for kidney injury to become apparent (3). It is possible that injury may have occurred at the cellular level, even though common tests failed to identify it, as serum urea nitrogen and creatinine do not invariably measure subclinical kidney injury (29, 30). Similar to our study, although creatinine levels can be the same in rat haemorrhagic shock treated with or without N-acetylcysteine, underlying damage is actually different when measured through caspase-3 levels (30). Thus, the altered level of activity of caspase-3 as mentioned above is a proof of the organ’s underlying damage. A longer protocol is probably necessary to evaluate the effects of T3 on renal function, as this is estimated by biochemical markers in the plasma. Moreover, we could not ignore that, albeit within normal limits, creatinine was much higher in the F-group compared to the T3-group.

A limitation of our study was that the pathway through which T3 reduces apoptosis in renal cells was not fully elucidated. Other parameters of inflammation, apoptosis and necrosis, which would have provided more information, were not measured. The activation of caspase-3 by itself is a sign of an increase in cell death, but cannot be considered a proof of the same (12). Several caspase-like enzymes have been identified and in certain circumstances, the presence or activation of caspase-3 is not indispensable for apoptotic cell death (12). The p-value was not low enough to support significance, which was another limitation that was believed to be related to the small number of animals included in each group. Even though the number of swine was rather small, the minimisation of animal variation and the strictly controlled experimental setting and performance improved the reliability of our results. Further, the huge between-group difference regarding the absolute values presented an important finding that could not be ignored.

Conclusion

Under the present study design, we demonstrated that T3 exerts a significant effect on renal tissue during haemorrhage and resuscitation, modulated through caspase-3 activation and cellular apoptosis. We found that the administration of T3 to a swine model of liver trauma-induced haemorrhagic shock improved renal cell histology and haemodynamic stability. The exact mechanism behind this finding needs to be further investigated in future large-scale studies.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of the University of Athens [X-27, 17/02/09] and from the Animal Research Committee of the Prefecture of Eastern Attica (493, 09/03/10).

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