The role of the exogenous supply of adenosine triphosphate in the expression of *Bax* and *Bcl2L1* genes in intestinal ischemia and reperfusion in rats[1]

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Abstract

Purpose: To investigate the role of the exogenous supply of adenosine triphosphate (ATP) in the expression of *Bax* and *Bcl2L1* genes in intestinal ischemia and reperfusion (IR) in rats.
Methods: The study was designed as a randomized controlled trial with a blinded assessment of the outcome. Eighteen adult male Wistar-EPM1 rats were housed under controlled temperature and light conditions (22-23°C, 12 h light/dark cycle). The animals were randomly divided into 3 groups: 1. Sham group (SG): no clamping of the superior mesenteric artery; 2. Ischemia and reperfusion group (IRG): 3. Ischemia and reperfusion plus ATP (IRG + ATP). ATP was injected in the femoral vein before and after ischemia. Afterwards, intestinal segments were appropriately removed and processed for Endothelial Cell Biology Rat RT2 Profiler PCR Array.
Results: ATP promoted the upregulation of *Bcl2L1* gene expression, whereas it did not have significant effects on *Bax* gene expression. In addition, the relation of *Bax/Bcl2L1* gene expression in the IRG group was 1.39, whereas it was 0.43 in the IRG + ATP group. *Bcl2L1* plays a crucial role in protecting against intestinal apoptosis after ischemia and reperfusion. Increased *Bcl2L1* expression can inhibit apoptosis while decreased *Bcl2L1* expression can trigger apoptosis.
Conclusion: Adenosine triphosphate was associated with antiapoptotic effects on the rat intestine ischemia and reperfusion by upregulating of *Bcl2L1* gene expression.
Key words: Mesenteric Ischemia. Adenosine Triphosphate. Oxidative Stress. Gene Expression. Rats.

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■ Introduction

Intestinal ischemia / reperfusion (IR) is a serious event that occurs in several situations such as abdominal aortic aneurysm surgery, small intestine transplantation, liver transplantation, strangulated hernia, hemorrhagic shock, neonatal necrotizing enterocolitis, extracorporeal circulation and because of collapse of systemic circulation, as in hypovolemic and septic shock[1].

Restoration of blood flow to an ischemic organ is essential to prevent irreversible cell damage. However, reperfusion may aggravate ischemic cell damage. The degree of damage associated with ischemia in different tissues depends on its duration, and many of the lesions are developed during the reoxygenation stage due to tissue reperfusion[2-4]. Indeed, the histological changes induced by 3 hours of intestinal ischemia, followed by one hour of reperfusion, are more severe than those induced by 4 hours of intestinal ischemia[5].

The change in energetic metabolism is a crucial feature in these conditions due to interruption of blood supply and oxygen to cells in tissues[6]. Hypoxia or anoxia results in insufficiency of the respiratory chain and adenosine triphosphate (ATP), the main organic energy source, is then generated by anaerobic glycolysis. Once the glycogen reserve is consumed, ATP depletion occurs, and depending on the severity of the IR injury, cell damage can lead to cell death due to necrosis or apoptosis with consequent dysfunction or absence of organic function[7]. Adenosine triphosphate therefore plays a key role in the sequence of deleterious events of ischemia and reperfusion.

The biochemical events responsible for apoptosis depend on a family of caspases proteases, which are synthesized from their inactive precursors (pro-caspases). Once activated, the caspases cleave and thus activate other pro-caspases, resulting in amplification of the proteolytic cascade. This mechanism, besides being destructive and self-propagating, is irreversible[8].

Bcl2 (B-*cell lymphoma 2*) is a family of mammalian genes that encodes several proteins that directly regulate the process of caspase activation. They regulate the permeability of the outer membrane of the mitochondria and may be pro-apoptotic (*Bax, Bad, Bak and Bok*) or anti-apoptotic (*Bcl-2, Bcl-xL, Bcl-w and Bcl2L1*). The relative balance between the pro - and anti- apoptotic proteins, reflecting the formation of homodimers and heterodimers (neutralization), defines the pathway of action on the mechanism of programmed cell death[9].

Thus, the objective of the present study was to investigate whether the exogenous supply of ATP acts significantly on the expression of *Bax* and *Bcl2L1* genes in intestinal dysfunction caused by IR.

■ Methods

The study was designed as a randomized controlled trial with a blinded assessment of the outcome. The Ethics Committee in Research of the Universidade Federal de São Paulo (UNIFESP) (nº 1849010714), approved it according to the recommendations of international legislation on animal protection.

Eighteen adult male Wistar-EPM1 rats weighing 250 to 300g, from the Center for the Development of Experimental Models for Medicine and Biology, were used. The animals were housed under controlled temperature and light conditions with a 12-hour light/dark cycle. The rats had free access to water and standard pellet chow until 6 hours before the surgical procedures. After anesthesia (intramuscular injection of 80 mg/kg of Ketamine and 10 mg/kg of Xylazine), a median laparotomy was performed, exposing the superior mesenteric vessels.

The animals were randomly distributed into 3 groups (6 rats/group):

1. Sham group (SG): no clamping of the superior mesenteric artery;

2. Ischemia and reperfusion group (IRG): clamping of superior mesenteric vessels for 60 minutes followed by 120 minutes of reperfusion; 0.9% saline solution was injected into the femoral vein 5 minutes before ischemia, 5 minutes after reperfusion, and after 55 minutes of reperfusion;

3. Ischemia, reperfusion and ATP group (IRG + ATP): clamping of superior mesenteric vessels for 60 minutes followed by 120 minutes of reperfusion, plus ATP (100 mg/Kg). ATP in the IRG + ATP, or 0.9% saline solution in the SG and IRG, were injected in the femoral vein 5 minutes before ischemia, 5 minutes after reperfusion, and after 55 minutes of reperfusion. Thereafter, intestinal segments (3 cm) were removed at a distance of 20 cm from the duodenum-jejunum flexure, opened longitudinally, gently washed in saline solution, wrapped in aluminum foil, and immediately frozen in liquid nitrogen. The tissue samples were prepared for Endothelial Cell Biology Rat RT2 Profiler PCR Array (SABiosciences, a Qiagen Company, Frederick, Md).

Endothelial cell biology rat RT2 profiler PCR array

Total RNA was extracted from tissues by using TRIzol reagent (Life Technologies, Carlsbad, Calif) and purified by using a RNeasy MiniKit (SABiosciences). Concentration of each total RNA sample was determined by using spectrophotometry, and the quality was assessed with electrophoresis on 2% agarose gels. The first strand of complementary DNA was synthesized by using 1 mg of total RNA and the RT2 First Strand Kit (SABiosciences). Equal amounts of complementary DNA and the Master Mix SYBR Green qPCR Mastermix (SABiosciences) were distributed to each well of the PCR microarray plate containing the pre-dispensed gene-specific primer sets. PCR was performed according to the manufacturer's instructions in 96-well plates to detect the expression of 84 genes related to oxidative stress, 5 housekeeping genes (ACTB, Gapdh, Hsp90ab1, Hprt1, and Gusb) used for normalizing the PCR microarray data, and 1 negative control for genomic DNA contamination. The negative control primer set specifically detects repetitive genomic DNA with a high level of sensitivity.

Three wells of reverse transcription control samples were used to verify the efficiency of the reverse transcription reaction, with a quantitative PCR assay that specifically detects template synthesized from the RNA control of the first-strand synthesis kit. The replicate positive PCR control samples were used to determine the efficiency of the PCR itself. These controls use an artificial DNA sequence and a specific primer set to detect it. The 2 sets of replicate control wells (reverse transcription control samples and positive PCR control samples) also test for inter-well and intraplate consistency. The software (MxPro Equipment Real Time Systems, Stratagene [San Diego, GE Healthcare, Little Chalfont, United Kingdom) calculates the cycle threshold (Ct) values for the 2 genes in the array. Finally, it performs pair-wise comparisons by calculating fold changes in gene expression from the raw Ct data by using the DDCt method. The method used is contained in the spreadsheet for PCR Array Data Analysis version 3.3 (SABiosciences)[10].

Statistical analysis

Gene expression data for each sample were evaluated in triplicate. The Student's t-test was applied to validate the homogeneity of the expression reaction of each gene ($p < 0.05$). The software calculated the variation in the Ct in the study group (IRG) compared

with the Ct of the sham group (SG), and this outcome was expressed as a logarithmic base (2) value according to the formula [2ˆ (ΔΔCt)]. The gene expression results are presented as positive/up-regulation expression (IRG > SG) or negative/down-regulation expression (IRG < SG). The numbers represent how many folds each gene was expressed above negative or below positive. The software established the results 3-fold above up-regulation or 3-fold below down-regulation. The threshold allowed by the algorithm [2ˆ (ΔΔCt)] are biologically relevant[11,12]. The analysis were performed according to the criteria of positive (+) (above the threshold) expression, negative (-) (below the threshold) expression; hyperexpression (three times above the threshold), and hypoexpression (three times below the threshold).

■ Results

Table 1 show the expression profiles of Bax and Bcl2L1 genes related to programmed cell death, which encode, proapoptotic and antiapoptotic proteins, respectively[9]. A significant upregulation ($p < 0.05$) of Bcl2L1 was observed in IRG + ATP, whereas ATP did not have significant effects on *Bax* expression (Table 1). Meanwhile, the relation of *Bax/Bcl2L1* gene expression in the IRG group was 1.39, whereas it was 0.43 in the IRG + ATP group (Table 2).

Table 1 – Expression of *Bax* and *Bcl2L1* genes related to enteric endothelial cell biology from rats in the IRG and IRG+ATP groups.

		Groups		
Bank	**Genes**	**IRG**	**IRG + ATP**	**p**
NM_017059	*Bax*	-1.53	-1.54	0.2064895
NM_031535	*Bcl2L1*	+1.10	+3.58	0.0105467*

IRG, ischemia and reperfusion group; IRG+ATP, ischemia and reperfusion +ATP group.
Significant values of fold up-regulation (+) [$2^{-(\Delta\Delta Ct)}$]. *p<0.05.

Table 2 - Relation between expressions of *Bax/Bcl2L1* genes related to enteric endothelial cell biology from rats in the IRG and IRG+ATP groups.

	Groups	
Relation	IRG	IRG + ATP
Bax/Bcl2L1	1.39	0.43

IRG, ischemia and reperfusion group; IRG+ATP, ischemic and reperfusion +ATP group.

■ Discussion

This study evaluated the modulation effect of Adenosine triphosphate (ATP) in a rat model of intestinal I/R. In our study, we found that ATP promoted the up-regulation of Bcl2L1, which plays a crucial role in protecting against intestinal apoptosis after ischemia and/or reperfusion. A decrease in Bcl2L1 levels triggers a caspase cascade that ultimately lead to apoptosis, whereas increased Bcl2L1 levels can inhibit apoptosis[13]. Conversely, our results illustrate the up-regulation of Bax that is expressed during stress conditions and acts to accelerate programmed cell death, which encodes a protein that is responsible for triggering apoptosis. This protein forms a heterodimer with BCL2 and interacts with mitochondrial membrane, which leads to the release of cytochrome C to the cytoplasm. It also activates caspase 3, which is related to apoptosis[14].

BAX is a protein that is difficult to alter its levels in any study model[13]. However, this study showed that ATP promoted the upregulation of *Bcl2L1* gene expression, which has been shown to favor cell survival in different tissues and organs[15]. Moreover, the relation of *Bax/Bcl2L1* gene expression in the IRG group was 1.39, whereas it was 0.43 in the IRG + ATP group. According to Chueh *et al*.[16], the protective effect of an agent against apoptosis can be assessed by observing the Bax/Bcl2 ratio. The lower its value, the lower is the chance of the cell undergo apoptosis, indicating that the ATP in this study presented a protective effect against apoptosis.

During ischemia and after reperfusion, formation of reactive oxygen species (ROS) occurs, which is responsible for the cascade of deleterious cellular responses that can induce cell death[13-16]. Administration of exogenous ATP has been showing to protect tissues against toxic or ischemic injury and ameliorate the oxidative stress secondary to IR[17]. ATP acts by increasing nitrous oxide (NO) bioavailability, promoting vasodilation of the microvasculature and inhibition of platelet aggregation and adhesion. Operating in the interaction between endothelial surface and leukocytes, NO can mitigate inflammatory activity[18-20].

Moreover, NO has a significant capacity to down-regulate the inflammatory response caused by ischemia to eliminate ROS. It is a key regulator of gene expression, capable of playing either positive or negative roles. NO can be cytotoxic as well as beneficial during IR. It may act both, as a protector of the lesion due to its vasodilator effect, antiplatelet and scavenger of ROS, and as a deleterious agent by interaction with superoxide radicals, forming the radical peroxynitrite[18,19]. The NO produced by endothelial cells would act to modulate IR through its vasodilator activity and counterbalance endothelin activity[18].

Our hypothesis in this experiment was that during intestinal IR, ROS causes cellular changes (particularly cell membrane damage), which results in modifications on gene expression of proteins from the Bcl-2 family, such as Bax (pro-apoptotic) and Bcl2L1 (antiapoptotic). In addition, it is expected that ATP can promote modulating effects on the gene expression that encode these two proteins[21]. Although mixed apoptotic and necrotic cell death occurs, abundant findings implicate the Bcl-2 family as mediators of the hyperoxic cell death response.

The Bcl-2 family proteins act directly on the mitochondrial outer membrane permeabilization, often a critical early step in apoptosis[22]. Regulation of cytochrome C released by both pro-apoptotic and antiapoptotic members of the Bcl-2 family of proteins has been well documented[22]. To determine the function of a protein, extensive genetic and biochemical analyses are frequently necessary, unless the amino acid sequence of the protein resembles another whose function is known[23].

With complete genome sequences and total messenger RNA expression patterns, new strategies have become available. To establish the real meaning of gene expression is challenging. This research, therefore, offers an option that can be followed to determine the association of gene expression as a primary and reliable response in the biochemical pathways of the inflammatory response to intestinal IR.

Analyzing the difference in BcL2 gene expression in this study, we can suggest that ATP would be useful in the treatment of intestinal IR. Nevertheless, other experimental studies must be conducted before clinical application is possible.

■ Conclusion

Exogenous adenosine triphosphate was associated with antiapoptotic effects on

the rat intestine ischemia and reperfusion by upregulating of *Bcl2L1* gene expression.

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