Journal of Veterinary Science & Technology

Vale, et al., J Veterinar Sci Technol 2016, 7:1
DOI: 10.4172/2157-7579.1000291

Research Article Open Access

The Correlation of Glycogen Metabolism in Rabbit Myocardial Ischemia

Daniela Fantini Vale[1], Renato Martins da Silva[2], Raquel Ribeiro de Aguiar[1], Guilherme Alexandre Monteiro[1], Fernanda Antunes[1], Renato Abdala Karam Kalil[3], Carlos Jorge Logullo de Olveira[2] and André Lacerda de Abreu Oliveira[1,*]

[1]Laboratório de Clínica e Cirurgia Animal (CCTA/UENF), and Unidade de Experimentação Animal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, RJ, Brazil

[2]Laboratório de Química e Função Proteínas e Peptídeos-CBB and Unidade de Experimentação Animal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, RJ, Brazil

[3]Instituto de Cardiologia do Rio Grande do Sul, Fundação Universitária de Cardiologia. Av: Princesa Isabel, 395 Porto Alegre-RS, Brazil

[*]Corresponding author: André Lacerda de Abreu Oliveira, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Unidade de Experimentação Animal-RJ, Avenida Alberto Lamego, 2000, Horto, CEP 28013-602, Campos dos Goytacazes, RJ, Brazil, Tel: +55 (22) 2748-6084; E-mail: lacerdavet@uol.com.br

Rec date: Dec 16, 2015; **Acc date:** Jan 18, 2016; **Pub date:** Jan 20, 2016

Abstract

Ischemia is responsible for several heart injuries, leading to functional disorders and higher mortality in animals. This process is a condition of blood circulatory arrest, leading to hypoxia and an anaerobic glycolysis. In this case, glycogen is fundamental to maintain energy homeostasis, through glycogen synthase kinase 3 (GSK3) regulations. This enzyme is usually involved in cardio protection, as well as several other biological processes. To study glycogen synthase kinase 3β (GSK3β), analyzing the involvement of this enzyme on cardiac system protection to understand its role in energetic metabolism during ischemia and reperfusion. Using the inflow occlusion (IO) application, the circulatory blood to the heart was blocked in adult New Zealand white rabbits. Parameters such hemogasometry as lactate levels were evaluated during the transoperatory period, using CG4+test strips (i-STAT® *System*). GSK3β transcription and activity analysis was performed by real time qRT-PCR and western blotting respectively, and glycogen quantification was determined enzymatically.GSK3β transcription increased during ischemia, followed by a decrease in glycogen content, suggesting that the consumption of this substrate during ischemia is mediated by GSK3β. Lactate level is highest in ischemia, and the pH value decreased during the same period. The results suggest the importance of GSK3β in the heart metabolic adaptations after ischemia and reperfusion injuries, sustaining glucose anaerobic metabolism through glycogen reserves modulation. The results show that the transcription of GSK3β correlated with cardiac metabolic adaptations after ischemia and reperfusion injuries, sustaining glucose anaerobic metabolism.

Keywords: Glycogen synthase kinase 3; Gene expression; Ischemia; Myocardium; Rabbits

Abbreviations:

GSK3β: Glycogen Synthase Kinase 3 Beta; IO: Inflow Occlusion; GS: Glycogen Synthase; ATP: Adenosine Triphosphate; PaO_2: Partial Pressure of Oxygen in Arterial Blood; TCO_2: Total of Carbonic Dioxide; SO_2: Oxygen Saturation in Arterial Blood; HCO_3-: Bicarbonate; PCO_2: Partial Pressure of Carbon dioxide in Arterial Blood; BE: Base Excess

Background

Myocardial ischemia is caused by a disruption between blood flow and metabolic requirements. In this event, the oxygenation and metabolic substrates delivered to the myocardium are not sufficient to meet its energetic requirements. Therefore, myocytes convert from aerobic to anaerobic metabolism, as a consequence of reduced oxygen availability. Additionally, lactate acidosis is observed accompanied by buildup of high lactate levels [1,2].

Such damage leads to a specific physiologic consequence, like activation of cardioprotective proteins, to ensure the cardiac tissue integrity [3]. This cardioprotection against ischemia involves the activation of kinases, anti-apoptotic proteins, antioxidant enzymes, calcium regulators, and membrane permeability defects [4,5]. Recently, it was observed that the pharmacologic inhibition of a particular enzyme, glycogen synthase kinase 3β (GSK3β), reduced infarct size and improved postischemic function [6]. Based on RNA interference, other studies have demonstrated the importance of a protection-signaling pathway operating via GSK3β in cardiac myocytes [7]. One function classically associated with GSK3 is the regulation of glycogen reserves, phosphorylating glycogen synthase (GS), responsible for the synthesis of this carbohydrate reserve [8,9]. Elevated glycogen levels in heart have been shown to have cardioprotective effects against ischemic injury [10]. Schaefer and Ramasamy observed a decrease in glycogen content after ischemia in isolated rat heart, but the authors did not investigate glycogen regulator enzymes [11].

Glycogen is the main storage form of glucose in animal cells and is used as energy source in these organisms. In mammals, most glycogen is found in the liver, contributing to bloodstream glucose homeostasis. Skeletal muscle, heart muscle and other tissues utilize the remaining glycogen [12]. These carbohydrate reserves may be mobilized to obtain energy readily, even under anaerobic conditions, ensuring a proper functioning in tissues under hypoxia. Despite of the importance of glycogen, it homeostasis involves a coordinated regulation of the rate of glycogen synthesis and the rate of glycogen breakdown. These two processes are fine regulated by GSK3 [8].

GSK3β is a serine/threonine kinase, localized in different intracellular compartments such as nucleus, mitochondria, and cytosol [13]. This enzyme phosphorylates and inhibits glycogen synthase, decreasing glycogen levels [8,14]. Previous studies gave evidence to support GSK3β as a negative regulator of the hypertrophic response in

cardiomyocytes culture [15,16], and suggest additional roles of GSK3β in the heart. Therefore, numerous cardioprotective agents converge to GSK3β, suggesting that this enzyme is a therapeutic target to minimize the consequences of heart ischemia [6,17]. However, these studies focused on enzyme inhibition, and did not consider the molecular regulation of GSK3β. In this study, we demonstrate the transcription levels of GSK3β after ischemic injury and reperfusion. Additionally, we show the metabolic changes associated to this damage, aiming to better understand its protective effect on the myocardium.

Methods

Experimental design

The procedures were performed in the Unidade de Experimentação Animal (UEA) of the State Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). Were used 18 rabbits (Oryctolagus cuniculus), Leporidae family, Albino type, male, weighing between 2.5 and 3.5 kilograms (kg), weighed on a precision scale with a maximum capacity of 15 kg and a minimum of 100 g (Ramuza® DCR-15 bat). The animals were provided by the rabbit sector of the Universidade Federal de Viçosa (UFV), Minas Gerais. These animals passed for a quarantine period, kept in the same room at 22°C to 27°C, controlled lighting and noise, allocated in individual cages lined with wood shavings, where they received balanced feed for rabbits (Presence® animal feed) and water *ad libitum*.

Anesthetic protocol

The animals were pretreated with acepromazine maleate (0.3 $mg.kg^{-1}$) (Acepran® 1%, Univet S.A., São Paulo-Brazil), associated with pethidine hydrochloride (10 $mg.kg^{-1}$) (Dolosal ®, Cristália, São Paulo-Brazil) intramuscularly. Twenty minutes after premedication, animals were prepared for catheterization of the marginal vein and central auricular artery. Subsequently the ventral neck was prepared for tracheal intubation (tracheostomy). During the anesthetic and surgical procedures, animals received an infusion of Ringer's solution containing lactate (7 ml/kg/h). Anesthesia was induced intravenously with propofol (10 $mg.kg^{-1}$) (Propovan® Cristália, Itapira-SP, Brazil). Anesthetic maintenance was performed with isoflurane (1.5%) (Isoforine®, Cristália, São Paulo, Brazil) in 100% oxygen in a semi-open circuit. Oxygen was provided to animals using a manual ventilator, except during the cardiac circulatory arrest period.

Cardiac circulatory arrest or Inflow occlusion

The rabbits were randomly distributed in three groups of six animals: (1) the control group, (2) the ischemia group, and (3) the reperfusion group. All groups underwent thoracotomy. The cardiac circulatory arrest (inflow occlusion) was performed in both the ischemia and reperfusion groups.

The rabbits were placed in the left lateral decubitus position for the right unilateral thoracotomy in the fourth intercostal space. The pleural cavity was opened, and the cranial and caudal cava vein and the azygous vein were dissected in 200-millimeter (mm) segments (Figure 1A). Before applying the vascular clamps, Satinsky closure was performed by hyperventilation for 30s in order to empty the cardiac chambers (Figure 1B).

Subsequently, the total circulatory arrest was established for a period of 5 min. Then, left ventricle biopsy (100 mg of tissue) was performed and the veins were released at the end of the occlusion period, inversely (Figure 1C). However, the azygos vein remained connected (Figure 1A). The same procedure described for the ischemia period was performed in the reperfusion group. Subsequently, left ventricle biopsy was performed in the ischemia group during occlusion for 5 minutes. However, in the reperfusion group the left ventricle biopsy was performed after 5 minutes of reperfusion. The control group did not undergo occlusion, and was subjected to left ventricle biopsy after 5 minutes of thoracotomy.

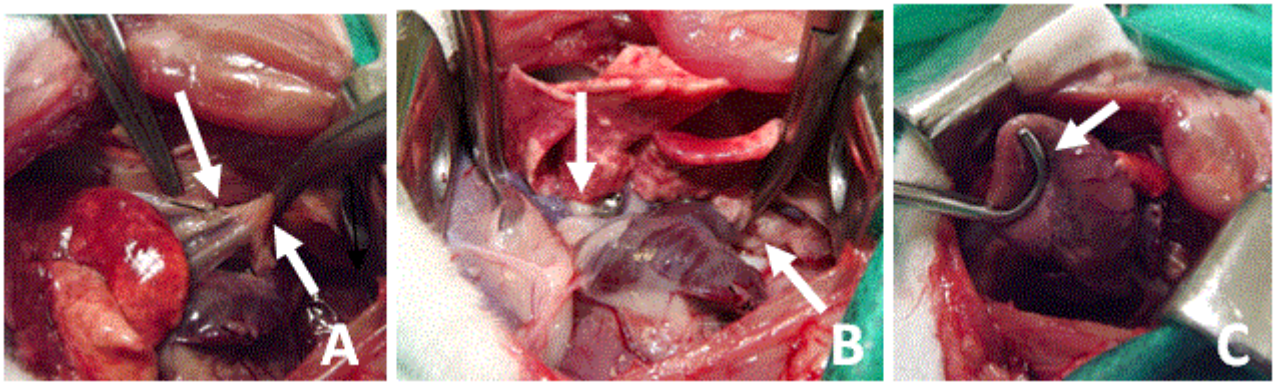

Figure 1: Application of inflow occlusion technique. (A) Azygos vein ligation and occlusion of the cranial vena cava, (B) Occlusion of cranial and caudal vena cava with vascular clamps, at the moment of inflow occlusion and (C) clamping of the left ventricle to perform cardiac biopsy.

The procedures were performed in the Animal Experimentation Unit (UEA) Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) in Campos dos Goytacazes, RJ, Brazil. All experiments were performed in accordance with the standards and ethics of animal experimentation, established by Brazilian federal law no. 11794/08 after approval of the Ethics Committee of Animal Use (CEUA) Universidade Estadual do Norte Fluminense Darcy Ribeiro, under protocol number: 171/2012.

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Molecular analysis

The cardiac tissue (50 mg) from the left ventricle was placed in RNA later™ (Quiagen) and stored at -20°C for RNA extraction according to the Trizol protocol (Invitrogen Life Technologies, Inc., Carsbad, CA, USA). cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Gene expression analysis was performed by quantitative RT-PCR (StepOne PlusTM Real Time PCR-Applied). GSK3 specific primers were designed from rabbit (*O. cuniculus*) sequences available in GenBank (NCBI - XP. 002716707.1). To validate amino acid sequences, a multiple sequence alignment was performed using sequences deposited in GenBank from rabbit GSK3β (XP_002716707.1) (*O. cuniculus*), human (NP_001139628.1) (*H. sapiens*), dog (XP_535751.2) (*C. Falimiaris*) and mouse (XP_006522488.1) (*M. musculus*), which show the conserved kinase motifs (list motifs) and the amino acids serine 9 and tyrosine 216 (Figure 2). The primers used to amplify the targets are listed in Table 1.

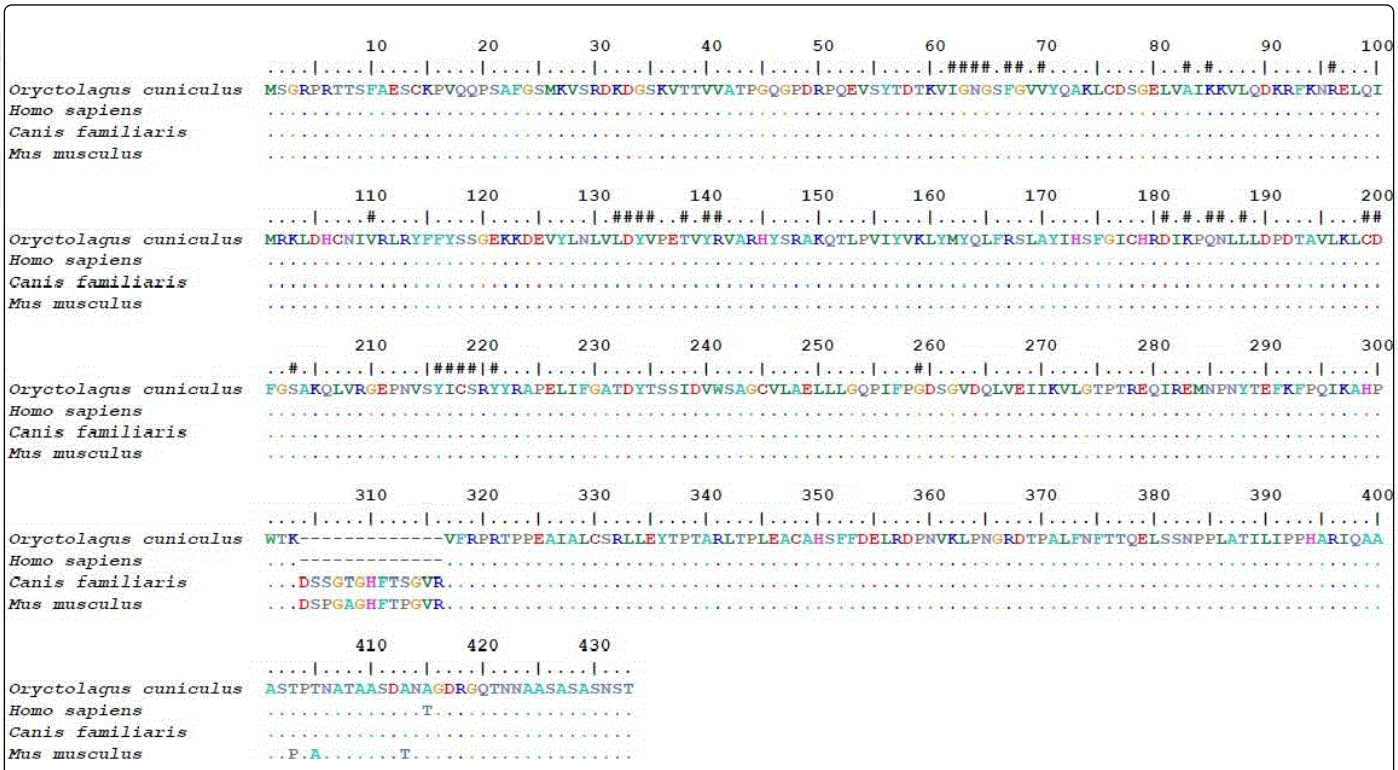

Figure 2: Multiple sequence alignment of glycogen synthase kinase 3 beta amino acid sequences. The symbol "#" stands for amino acid residues that are part of the active site. Access Code: *O. cuniculus* (XP_002716707.1), *H. sapiens* (NP_001139628.1), *C. familiaris* (XP_535751.2), and *M. musculus* (XP_006522488.1).

Gene	Abbreviation	Primers	Fragment Length (bp)
Cyclophylin A	Cyclo	5′AAGAAGATCACCATTGCCAAC	78
		3′GAGCTAGAGGAATGGTCAGGTG	
Glycogen Synthase Kinase 3β	GSK3β	5′GAAAAGGTGATTCGGGAAGA	141
		3′ AAGGATGGCAGCAAGGTAAC	

Table 1: Gene sequences for Cyclophylin A and GSK3β.

The relative expression was determined using the Relative Expression Software Tool-REST [18]. The Cyclofilin A gene was utilized as an internal reference gene to normalize the reactions [19].

Protein extraction and Western blot analysis

To extract GSK3β protein cardiac tissue (30 mg), the left ventricle was macerated in nitrogen and extracted using 500 µL of lysis buffer (20 mM Tris-HCl pH 7.5, 10% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium pyrophosphate, 2.3 mM ammonium

molybdate, 10 mM sodium fluoride, 0.02 mM pepstatin A and Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA). Protein samples were quantified using the Bradford protein assay [20].

Relative GSK3β expression levels were determined using routine western blot analysis. Samples were resolved on a 10% SDS-PAGE denaturing gel using 30 μg of total protein and transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). To confirm protein transfer the nitrocellulose membrane was stained in Ponceau S solution (1%) for 1 min. After staining the membrane was destained with distilled water and placed in blocking solution (3% skim milk in 1X TBS: 50 mM Tris, 150 mM NaCl, pH 7.5 and 2% Tween-20) overnight. Subsequently after blocking, the membrane was exposed to primary antibody (1:10000) to p-GSK3β (Santa Cruz, Dallas, Texas, U.S.A) in blocking solution for 2 h at 4°C with gently shaking. The membrane was washed with 1X TBS four times in intervals of 5 mins (20 min in total) and exposed secondary antibody (1:2000) (goat anti-rabbit IgG HRP conjugated) (Sigma Aldrich, St. Louis, MO, USA) in blocking solution for 1 h at room temperature with gentle shaking (Table 2).

Antibody	Origin	Weight Molecular	Dilution
GSK3βtotal	Sigma (G7914)	47 kDa	1:10000
p-GSK3β (Ser9)	Santa Cruz (SC-11757-R)	47 kDa	1:10000
Anti Rabbits IgG (Whole molecule) peroxidase antibody produced goat	Sigma	47kDa	1.4305556

Table 2: Primary and secondary antibodies list used for analysis of protein expression by Western blotting.

To visualize GSK3β, the membrane was washed with 1X TBS four times at 5-min intervals (20 min in total) and exposed to 3,3'-diaminobenzidine (DAB) and hydrogen peroxide solution (800 μL 1 M Tris-HCl pH 7.5; 2400 μL 0.1 M imidazole; 40 mg DAB and 40 μL of hydrogen peroxide to 8.8 mM). The membranes were dried on an A4 sheet paper and photographed together with the gels. The experiment was performed in triplicate for each group evaluated and repeated twice. The images were scanned and the band intensities were determined by ImageJ software (National Institutes of Health Cambria, USA) for quantitative pixel analysis.

Glycogen quantification

Glycogen is the largest carbohydrates reserve in mammals, and under a condition of ischemia, these reserves can be mobilized to sustain cardiac metabolism. Cardiac tissue (20 mg) from left ventricle was homogenized in 1 mL extraction buffer containing 200 mM sodium acetate pH 4.8 and centrifuged at 10,000×g for 10 min. The supernatant was incubated with 1 unit α amyloglucosidase (Sigma-Aldrich Chemicals) in acetate buffer for 4 h at 40°C. Liberated glucose was detected with a commercial kit for glucose dosage (Glucox®, Doles) at A510. Endogenous glucose was subtracted from control conditions (without the addition of α-amyloglucosidase). Glycogen content was determined using a standard curve as described elsewhere [21], and normalized by total protein content. Results are presented as a mean and standard deviation from three independent experiments.

Statistical analysis

To verify the data normality of the variables was performed the Shapiro-Wilk test. For non-parametric data (TCO_2, PO_2 and SO_2) the Kruskal-Wallis was performed, followed by Dunn's Multiple Comparison post-test. The analysis of variance (ANOVA) and Tukey's test were performed for other hemogasometric variables, as well as for lactate and glycogen levels, heart rate, and transcriptional analysis. The significance level (α) was 5% ($P<0.05$). The statistical tests were calculated by the Graph Pad Prism® Software version 5.0 (Graph Pad, USA).

Results

The decrease of pH and consequently increase of lactate describe a lactate acidosis

In order to identify the hemogasometric variations during 5 min of ischemia and reperfusion, we analyzed the pH, plasma bicarbonate, and blood gases in control rabbits or after inflow occlusion application. PH averages were statistically significantly lower in the ischemia groups, compared to other treatments (Figure 3A). After reperfusion pH values remained low (Figure 3A). In the ischemia group, CO_2 blood pressure increased significantly, as a result of not ventilating the animals during IO. This effect also persisted in the reperfusion group, even 5 minutes after ventilation and recirculation were resumed (Table 3). To investigate the flow of anaerobic glycolysis under ischemia and reperfusion conditions, the amount of lactate is determined. Lactate levels were statistically significantly higher in the ischemic group, when compared to the control, and remained high in the reperfusion group (Figure 3B).

Glycogen synthase kinase 3 beta amino acid sequences alignment

Specific primers were designed outside the conserved region using the Primer 3 software version 4.0 [22] (Figure 4A), and the deduced amino acid sequence was also analyzed (Figure 4B). A Multiple sequence alignment of glycogen synthase kinase 3 beta amino acid sequences was performed to demonstrate the higher identity of this enzyme between four organisms (*Oryctolagus cuniculus*, *Homo sapiens*, *Canis familiaris*, *Mus musculus*) and evidencing the amino acid residues that are part of the active site (Figure 2). The amino acid sequences were highly conserved, showing 96 to 99.7% identities and

confirming that the GSK3 sequence is from rabbit (Figure 2). The primers used to amplify the targets are listed in Table 1.

Variables Hemogasometry		Treatments		
		Control	Ischemia	Reperfusion
pH	Mean	7.27	7.12***	7.12***
	SD	± 0.04	± 0.05	± 0.06
HCO3 (mmol L-1)	Mean	24.97	24.87	24.35
	SD	± 5.18	± 5.70	± 3.22
PaCO2 (mmHg)	Mean	52.81	76.47*	73.75
	SD	± 8.47	± 25.87	± 12.23
BE (mmol L-1)	Mean	-1.57	-4.42	-6.85
	SD	± 5.71	± 5.31	± 4.56
TCO2 (mmol L-1)	Median	26.0	27.0	28.0
	IQR	2.0	8.0	2.5
PaO2 (mmol L-1)	Median	66.0	35.0	97.50
	IQR	225.5	25.4	194.5
SO2 (%)	Median	99.0	84.0	86.0
	IQR	15.0	45.0	18.0

Table 3: Hemogasometric variables observed in ischemia, reperfusion, and control groups. The mean and standard deviation of hemogasometric pH, HCO_3-, $PaCO_2$, BE variables; and interquartile range of hemogasometric TCO_2, PaO_2 and SO_2 variables observed in rabbits subjected to occlusion for the three treatment groups. ***Different from the control group (P<0.05).

Glycogen content decreases in response to high GSK3β transcription levels

Transcription analysis of GSK3 was performed in order to determine the gene response to ischemia and reperfusion conditions in accordance with real-time PCR strategy (Table 1 and Figure 5). Figure 5A shows a graphical representation of the PCR strategy. During ischemia we observed that GSK3β transcriptional level increased by approximately 50 times. After reperfusion, the transcript levels were equivalent to the values observed for the control conditions (Figure 5B). This enzyme is associated with glycogen synthesis regulations by GS inhibition. A reduction of glycogen levels after ischemia induction was observed, however, during reperfusion the glycogen amount remained unaltered, compared to the control group (Figure 5C). The glycogen rate does not follow the same proportion of GSK3β transcription analysis.

A higher amount, approximately 50%, of phosphorylated GSK3 (p-GSK3β) was observed in the ischemia group compared to reperfusion group (Figure 5D) in accordance of western blotting strategy (Table 2).

The comparison of the ratio of p-GSK3β to GSK3β between control to ischemia and control to reperfusion groups revealed no differences in the former, but a reduction in the latter (Figure 5D).

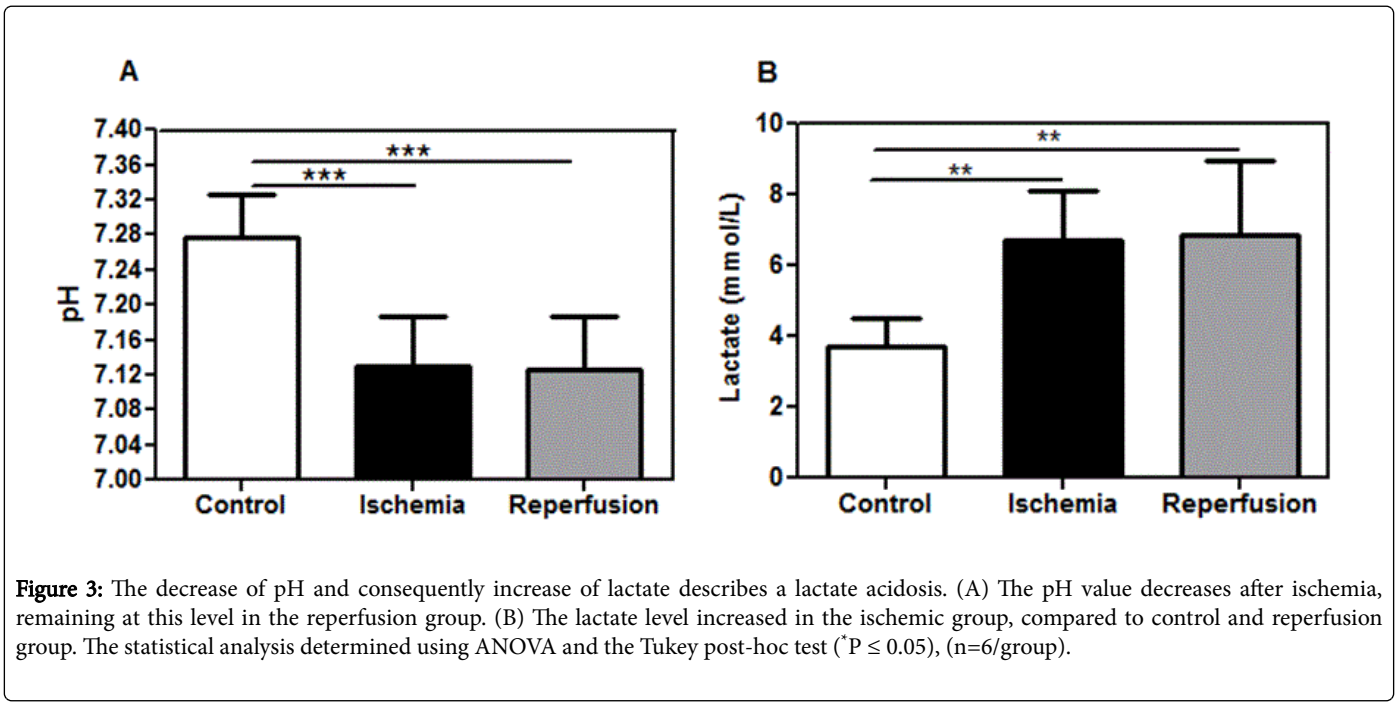

Figure 3: The decrease of pH and consequently increase of lactate describes a lactate acidosis. (A) The pH value decreases after ischemia, remaining at this level in the reperfusion group. (B) The lactate level increased in the ischemic group, compared to control and reperfusion group. The statistical analysis determined using ANOVA and the Tukey post-hoc test ($^{*}P \leq 0.05$), (n=6/group).

A

GGAGGAAGGAAG**GAAAAGGTGATTCGGGAAGA**GAGTGATCATGTCAGGGCGGCCCAGAACCACCTCCTTTGCGGAGAGCTGCAA
GCCAGTGCAGCAGCCTTCAGCTTTTGGCAGCATGAAAGTTAGCAGAGAC**AAGGATGGCAGCAAGGTAAC**TACAGTGGTGGCAACT
CCTGGGCAGGGTCCAGACAGGCCACAAGAAGTCAGTTATACAGACACTAAAGTGATTGGAAATGGGTCATTTGGTGTAGTATATCAA
GCAAAACTTTGTGATTCAGGAGAATTGGTTGCCATCAAGAAAGTATTACAGGACAAGAGATTTAAGAACCGAGAGCTCCAGATTATG
AGAAAGCTAGATCATTGTAACATAGTCCGTTTGCGTTATTTCTTCTACTCGAGTGGTGAGAAGAAAGATGAGGTCTATCTTAATCTGG
TGCTGGACTATGTTCCGGAAACAGTATACAGAGTTGCCAGACACTATAGTCGAGCCAAACAGACACTCCCTGTCATCTATGTCAAGT
TGTATATGTATCAGCTGTTTCGAAGTTTAGCCTATATCCATTCCTTTGGAATCTGCCATCGGGATATTAAACCACAGAACCTCTTGTT
GGATCCTGATACAGCTGTTTTAAAACTCTGTGACTTTGGAAGTGCAAAGCAGCTGGTCCGAGGAGAACCCAATGTTTCGTATATCTG
TTCTCGGTACTATAGGGCACCAGAGTTGATCTTTGGAGCCACTGATTATACCTCCAGTATAGATGTATGGTCTGCAGGCTGTGTATT
GGCTGAGCTGTTGCTAGGACAGCCAATATTTCCAGGAGACAGTGGTGTGGATCAGTTGGTGGAAATAATCAAGGTCCTGGGAACAC
CAACAAGGGAGCAAATTAGAGAAATGAACCCAAATTACACAGAATTCAAATTTCCTCAAATTAAGGCACATCCTTGGACTAAGGTCTT
CCGACCCCGAACTCCACCAGAGGCAATTGCACTGTGTAGCCGTCTGCTGGAGTACACACCAACTGCCCGACTGACACCACTGGAA
GCTTGTGCACATTCATTTTTTGATGAATTACGGGACCCAAATGTCAAACTACCAAATGGGCGAGACACACCTGCACTCTTCAACTTCA
CCACTCAAGAACTGTCAAGTAATCCACCTTTGGCTACCATCCTTATTCCTCCTCATGCTAGGATTCAAGCAGCTGCTTCAACCCCTAC
AAATGCCACAGCAGCCTCAGATGCTAATGCTGGAGACCGTGGACAGACCAATAATGCCGCTTCTGCATCAGCTTCCAACTCCACCT
GAACAGTCGCAAGCAGCCAGCTGCACAGGAAGAACCACCAGTTACTTGAGTGTCACTCAGCAACACTGGTCACGTTTGGAAAGAAA
ATT

B

MSGRPRTTSFAESCKPVQQPSAFGSMKVSRDKDGSKVTTVVATPGQGPDRPQEVSYTDTKVIGNGSFGVVYQAKLCDSGELVAIKKVLQ
DKRFKNRELQIMRKLDHCNIVRLRYFFYSSGEKKDEVYLNLVLDYVPETVYRVARHYSRAKQTLPVIYVKLYMYQLFRSLAYIHSFGICHRD
IKPQNLLLDPDTAVLKLCDFGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSIDVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLG
TPTREQIREMNPNYTEFKFPQIKAHPWTKVFRPRTPPEAIALCSRLLEYTPTARLTPLEACAHSFFDELRDPNVKLPNGRDTPALFNFTTQE
LSSNPPLATILIPPHARIQAAASTPTNATAASDANAGDRGQTNNAASASASNST

Figure 4: Design of GSK3β specific primers. (A) Specific primers were designed outside the conserved region, (B) The deduced amino acid sequence was also analyzed. The bold sequence represents the forward and reverse primers, and the kinase domain protein is represented by the underline regions.

Discussion

The aim of this work was to evaluate the GSK3β transcription response to ischemia and reperfusion and to determine its role on the overall metabolism after these heart injuries. GSK3β is a kinase that is described as an important factor involved in many different genes and metabolic control, such as ischemia. GSK3β signaling in ischemia and cardioprotection has been studied quite extensively [17,23,24], although the GSK3β transcriptional response remains unclear at this point. All these changes during ischemia lead to a specific physiological response that, in turn, triggers the activation of cardioprotective proteins, ensuring the integrity of cardiac tissue [3].

GSK3β participates in a variety of physiological and pathophysiological processes, including the cardiac system protection [17,25]. However, the majority of these studies have relied upon overexpression approaches or nonselective inhibitors [26]. GSK3β transcripts level increased significantly during ischemia (Figure 5B); however, no apparent differences were observed in the phosphorylation levels of protein, when compared to the control under the same conditions (Figure 5D). As discussed previously, GSK3β regulates GS negatively by phosphorylation, blocking glycogen synthesis (GS) [8].

Although this regulation occurs at enzymatic level, not transcriptionally, such genic response was observed after ischemia, suggesting that this injury activates a GSK3β signaling genic program. During the ischemic period, the cardiac tissue undergoes glucose and oxygen privation, followed by a decrease in the oxidative phosphorylation rate and ATP formation, although the heart energy demands remain as high as before the injury [27]. In this context of hypoxia and impaired mitochondria metabolism, cardiac myocytes may mobilize glycogen to maintain energy homeostasis, therefore sustaining anaerobic glycolysis. Such increased GSK3β transcription observed in ischemic rabbits could reflect the need for GS inhibition, ensuring the appropriate glycogen mobilization. Glycogen content was evaluated in our treatments, and was shown to decrease in ischemic animals, compared to the control (Figure 5C), suggesting an essential role of the GSK3β and glycogen reserves to maintain the heart metabolism integrity at the critical ischemic condition.

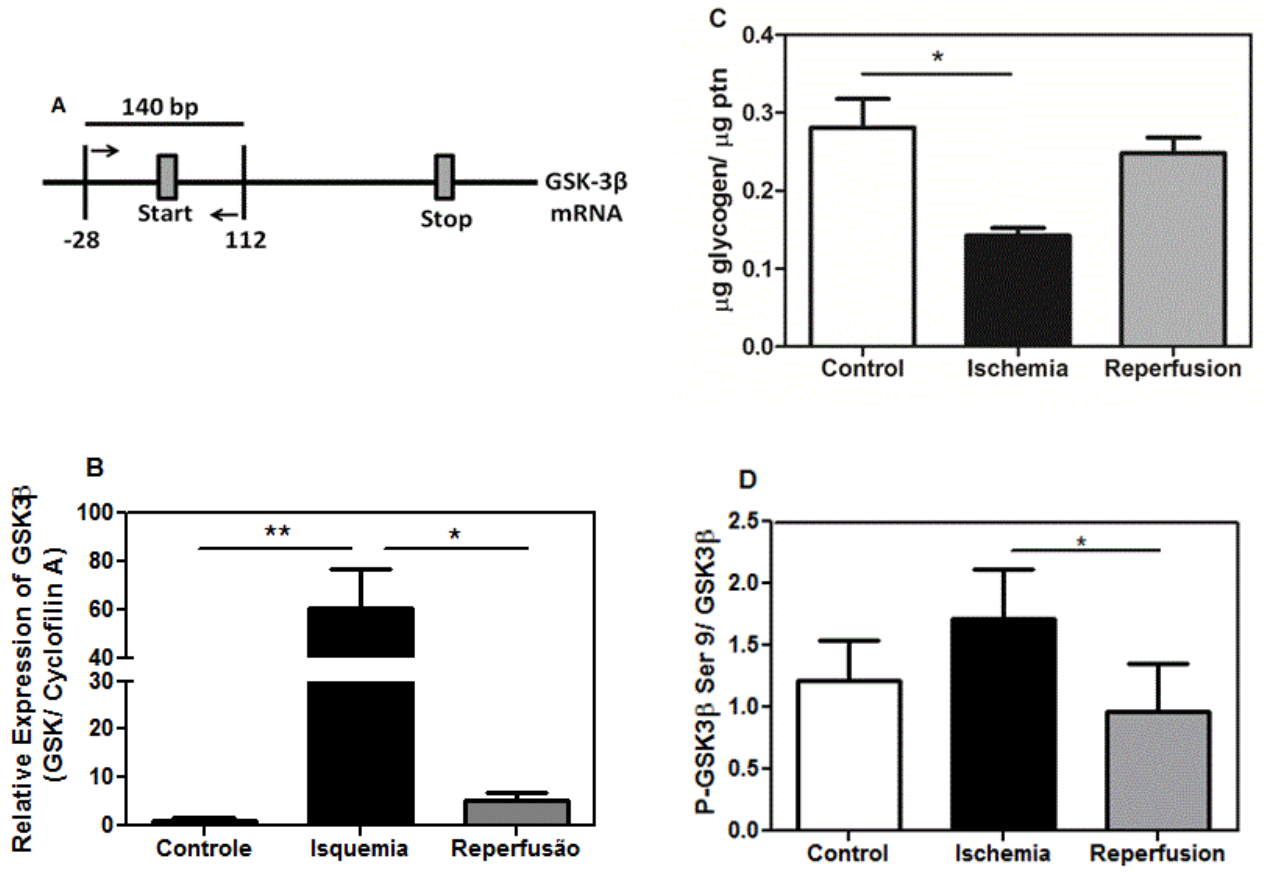

Figure 5: Glycogen content decreases in response to high GSK3β transcription levels. (A) The top diagram shows a representation of the PCR strategy, Gray box=start and stop codons, vertical lines=primers to amplify 140-bp amplicon. (B) The bottom diagram shows the GSK3β transcription levels in ischemia, reperfusion, and control groups. (C) Glycogen content during ischemia. (D) The p-GSK3β/GSK3β relation is higher in the ischemia condition compared to reperfusion. The statistical analysis determined using ANOVA and the Tukey post-hoc test ($^{*}P \leq 0.05$), (n=6/group).

The metabolic changes during ischemia are comparable between mammals, and one of the major effects is the decrease in the heart oxygen concentration [28]. We observed a reduction in partial oxygen pressure in rabbit arterial blood after ischemic injury (Table 3). With IO, the circulatory blood to the heart was blocked, limiting oxygen and glucose supplies into the rabbit heart. During ischemia, glycogen is the main carbohydrate source for glucose catabolism (Figure 5C), a process that is mediated by GSK3β (Figure 5B). One of the consequences of hypoxia in the heart is the increase in glycolysis rate, to sustain the tissue high-energy demand [29] (Opie 1990). However, in absence of oxygen, metabolism changes from aerobic to anaerobic glycolysis, which generates lactate as end product.

The lactate level increased during ischemia in the rabbit blood (Figure 3B). Furthermore, lactate concentration did not change after reperfusion, compared to the ischemic group, suggesting that 5 minutes of reperfusion is not sufficient to reestablish the normal lactate level observed in the control rabbits (Figure 3B). We also observed an apparent decline in pH during ischemia and reperfusion, compared to the control (Figure 3A), although no statistical difference was found between ischemic and reperfusion groups. Anaerobic glycolysis associated with glycogen depletion and lactate accumulation is well documented, but GSK3 genic regulation during this injury is unclear.

Reperfusion is characterized by the recovery of blood circulation, with the restoration of oxygen and nutrient levels. After reperfusion, GSK3β transcription level decreases to similar levels as observed in the control group (Figure 5B). The same happens with protein phosphorylate level (Figure 5D), suggesting enzyme activation after reperfusion. The return of circulatory blood, oxygen, and nutrients lead to aerobic glycolysis and oxidative mitochondrial metabolism. In contrast, glycogen content returns to a similar level as seen in the control, and to the GSK3β transcript levels (Figure 5B and 5C). Otherwise, the amount of lactate remains unaltered after reperfusion, since recycling to pyruvate usually takes place hours after blood circulatory reestablishment. Although reperfusion and ischemia occur in a short period of time (5 minutes), GSK3β transcription was altered and, consequently, glycogen content and related metabolites also changed, revealing that a refined GSK3β genic signaling program is activated during ischemia and reperfusion conditions. Other authors propose that this enzyme has pro-apoptotic effect involving the regulation of metabolism and signaling proteins, transcription factors,

and gene expression [13]. The increased GSK3β transcription observed in ischemic rabbit heart (Figure 5B) may activate the apoptosis cascade, in order to avoid necrosis injuries in the heart due to metabolic effects. However, the involvement of this kinase in the apoptotic events during ischemia needs to be further investigated.

Conclusion

The results of this study show that the transcription of GSK3β correlates with heart metabolic adaptations after ischemia and reperfusion injuries. Probably it was sustained by the glucose anaerobic metabolism through glycogen reserves modulation. GSK3β has emerged over the years as a core component of energy metabolism, cell growth and development, and of cardioprotection, which contributes to further understanding its role in mammals during ischemia and reperfusion.

Future studies will have to include the inhibition assays as well as the study of other components associated with this enzyme as upstream and downstream agents of GSK3β signaling in order to comprehend the cause and effect of GSK3β signaling during ischemia and reperfusion.

Acknowledgements

The authors are grateful to the Brazilian agencies Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento Pessoal de nível Superior (CAPES) for supporting this project.

Disclosure of Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Piacentini L, Karliner JS (1999) Altered gene expression during hypoxia and reoxygenation of the heart. Pharmacol Ther 83: 21-37.
2. Luft FC (2001) Lactic acidosis update for critical care clinicians. J Am Soc Nephrol 12 Suppl 17: S15-19.
3. Rubin CS, Rosen OM (1975) Protein phosphorylation. Annu Rev Biochem 44: 831-887.
4. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94: 1621-1628.
5. Halestrap AP, Clarke SJ, Khaliulin I (2007) The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta 1767: 1007-1031.
6. Tong H, Imahashi K, Steenbergen C, Murphy E (2002) Phosphorylation of glycogen synthase kinase-3ß during preconditioning through a phosphatidylinositol-3-kinase-dependent pathway is cardioprotective. Circ Res 90: 377-379.
7. Trivedi CM, Luo Y, Yin Z, Zhang M, Zhu W, et al. (2007) Hdac2 regulates the cardiac hypertrophic response by modulating Gsk3 beta activity. Nat Med 13: 324-331.
8. Embi N, Rylatt DB, Cohen P (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Em J Biochem 107: 519-527.
9. Jope RS, Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 29: 95-102.
10. Vigoda A, Mamedova LK, Shneyvays V, Katz A, Shainberg A (2003) Glycogen metabolism in rat heart muscle cultures after hypoxia. Mol Cell Biochem 254: 311-318.
11. Schaefer S, Ramasamy R (1997) Glycogen utilization and ischemic injury in the isolated rat heart. Cardiovasc Res 35: 90-98.
12. Berg JM, Tymoczko JL, Stryer L (2002) Biochemistry. WH Freeman (Ed) New York.
13. Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65: 391-426.
14. Plyte SE, Hughes K, Nikolakaki E, Pulverer BJ, Woodgett JR (1992) Glycogen synthase kinase-3: functions in oncogenesis and development. Biochim Biophys Acta 1114: 147-162.
15. Haq S, Choukroun G, Kang ZB, Ranu H, Matsui T, et al. (2000) Glycogen synthase kinase-3beta is a negative regulator of cardiomyocyte hypertrophy. J Cell Biol 151: 117-130.
16. Morisco C, Zebrowski D, Condorelli G, Tsichlis P, Vatner SF (2000) The Akt-glycogen synthase kinase 3beta pathway regulates transcription of atrial natriuretic factor induced by beta-adrenergic receptor stimulation in cardiac myocytes. J Biophys Chem 275: 14466-14475.
17. Miura T, Nishihara M, Miki T (2009) Drug development targeting the glycogen synthase kinase-3beta (GSK-3beta)-mediated signal transduction pathway: role of GSK-3beta in myocardial protection against ischemia/reperfusion injury. J Pharmacol Sci 109: 162-167.
18. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.
19. Blaauw E, Lorenzen-Schmidt I, Babiker FA, Munts C, Prinzen FW (2013) Stretch-Induced Upregulation of Connective Tissue Growth Factor in Rabbit Cardiomyocytes. J Cardiovasc Transl Res 6: 861-869.
20. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
21. Vital W, Rezende GL, Abreu L, Moraes J, Lemos FJA (2010) Germ band retraction as a landmark in glucose metabolism during Aedes aegypti embryogenesis. BMC Dev Biol 10: 25.
22. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, et al. (2012) Primer3--new capabilities and interfaces. Nucleic Acids Res 40: e115.
23. Gao HK, Yin Z, Zhou N, Feng XY, Gao F, et al. (2008) Glycogen synthase kinase 3 inhibition protects the heart from acute ischemia-reperfusion injury via inhibition of inflammation and apoptosis. J Cardiovasc Pharmacol 52: 286-292.
24. Miura T, Tanno M, Sato T (2010) Mitochondrial kinase signalling pathways in myocardial protection from ischaemia/reperfusion-induced necrosis. Cardiovasc Res 88: 7-15.
25. Medina M, Wandosell F (2011) Deconstructing GSK-3: The Fine Regulation of Its Activity. Int J Alzheimers Dis 2011: 479249.
26. Sugden PH, Fuller SJ, Weiss SC, Clerk A (2008) Glycogen synthase kinase 3 (GSK3) in the heart: a point of integration in hypertrophic signalling and a therapeutic target. A critical analysis. Br J Pharmacol 1: 137-153.
27. Taegtmeyer H, Roberts AF, Raine AE (1985) Energy metabolism in reperfused heart muscle: metabolic correlates to return of function. J Am Coll Cardiol 6: 864-870.
28. Kwasnicka KL, Stopiglia AJ, Freitas RR, Fantoni DT (2000) Avaliação hemogasométrica durante a parada circulatória total -Inflow Occlusion- aplicada por diferentes períodos de tempo em cães sadios. Braz. J Vet An Sci 37: 234-242.
29. Opie LH (1990) Myocardial ischemia--metabolic pathways and implications of increased glycolysis. Cardiovasc Drugs Ther 4 Suppl 4: 777-790.