Changes in thymus size, cellularity and expression of CD4+ and CD8+ coreceptors induced by *O*-GlcNAcylation, through regulation of AMPK

Alterações no tamanho do timo, celularidade e expressão dos correceptores CD4+ e CD8+ induzidas pela O-GlcNAcilação, através da regulação da AMPK

Running title: *O-GlcNAc alters thymic cellularity*

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doi:
Submetido: 26/12/2024
Aceito: 12/06/2025

ABSTRACT
Introduction: Glycosylation with N-acetyl-glucosamine (*O*-GlcNAc) has been shown to be essential during T cell activation. **Objective:** The present study evaluated the impact of increased levels of *O*-GlcNAcylation on thymic size, cellularity and expression of CD4+ and CD8+ surface markers, through the regulation of adenosine monophosphate-activated protein kinase (AMPK) and caspase-3. **Material and Methods:** Male Wistar rats were treated with glucosamine 300 mg/kg or saline for 21 days. The thymus was collected for analysis of size, cellularity, morphometry and protein quantification by western blotting (*O*-GlcNAc, OGT, CD4+, CD8+, phospho-AMPK, caspase-3). **Results:** Glucosamine treatment increased global levels of *O*-GlcNAc [1.00 vs 2.14 ± 0.19 AU] and OGT [1.00 vs 1.31 ± 0.10 AU] in thymic tissue. *O*-glycosylation increased thymic index (0.10 vs 0.12 ± 0.00 kg/rat) and cellularity (446.90 ± 55.12 vs 891.10 ± 142.30 cells/mL), but was not able to promote morphological changes. The active form of AMPK

was overexpressed (1.00 vs 1.48 ± 0.09 AU) and caspase-3 was reduced (1.0 vs 0.44 ± 0.07 AU). *O*-GlcNAc reduced the expression of CD4+ (1.00 vs 0.68 ± 0.08 AU) and CD8+ (1.00 vs 0.66 ± 0.10 AU) markers. **Conclusion:** *O*-GlcNAcylation induces an increase in thymic size and cellularity by inhibiting the caspase-3 apoptotic pathway through AMPK activation. However, *O*-GlcNAcylation reduces the expression of CD4+ and CD8+ coreceptors, demonstrating that *O*-GlcNAc may represent a novel mechanism for thymocyte modulation.

Keywords: Glycosylation, Thymus, T Lymphocyte, AMPK, Caspase-3.

RESUMO

Introdução: A glicosilação com N-acetil-glucosamina (*O*-GlcNAc) demonstrou ser essencial durante a ativação das células T. **Objetivo:** O presente estudo avaliou o impacto dos níveis aumentados de *O*-GlcNAcilação no tamanho do timo, celularidade e expressão de marcadores de superfície CD4+ e CD8+, por meio da regulação da proteína quinase ativada por monofosfato de adenosina (AMPK) e caspase-3. **Material e Métodos:** Ratos Wistar machos foram tratados com glucosamina 300 mg/kg ou solução salina por 21 dias. O timo foi coletado para análise de tamanho, celularidade, morfometria e quantificação de proteínas por *western blotting* (*O*-GlcNAc, OGT, CD4+, CD8+, fosfo-AMPK, caspase-3). **Resultados:** O tratamento com glucosamina aumentou os níveis globais de *O*-GlcNAc [1,00 vs 2,14 ± 0,19 UA] e OGT [1,00 vs 1,31 ± 0,10 UA] no tecido tímico. *O*-glicosilação aumentou o índice tímico (0,10 vs 0,12 ± 0,00 kg/rato) e a celularidade (446,90 ± 55,12 vs 891,10 ± 142,30 células/mL), mas não foi capaz de promover alterações morfológicas. A forma ativa da AMPK foi superexpressa (1,00 vs 1,48 ± 0,09 UA) e a caspase-3 foi reduzida (1,0 vs 0,44 ± 0,07 UA). *O*-GlcNAc reduziu a expressão dos marcadores CD4+ (1,00 vs 0,68 ± 0,08 UA) e CD8+ (1,00 vs 0,66 ± 0,10 UA). **Conclusão:** *O*-GlcNAcilação induz o aumento no tamanho e celularidade do timo ao inibir a via apoptótica da caspase-3 por meio da ativação de AMPK. No entanto, a *O*-GlcNAcilação reduz a expressão do correceptor CD4+ e CD8+, demonstrando que a *O*-GlcNAc pode representar um novo mecanismo para a modulação de timócitos.

Palavras-chave: Glicosilação, Timo, Linfócito T, AMPK, Caspase-3.

INTRODUCTION

Thymus is a primary lymphoid organ responsible for T cells' development. This bilobed organ is subdivided into two subcapsular regions, cortex and medulla, comprising numerous subtypes of cells including thymic epithelial cells (TECs), dendritic and mesenchymal cells[1,2]. Histologically, the thymic cortex exhibits many developing T cells, delivering mature T cells to thymic medulla. During development, thymocytes undergo events to become mature cluster of differentiation CD4+ or CD8+ single positive cells[3]. CD4+ cells will become helper T cells, and CD8+ cells will become cytotoxic T cells.

Nutritional factors influence cellularity and thymic atrophy, as

lymphocyte development and proliferation require significant energy from glucose. Metabolic changes are observed during thymocyte development to accommodate increasing cellular energy demands through aerobic glycolysis, glutaminolysis, and an increase pentose phosphate pathway flux. All these metabolic pathways produce substrates used in the Hexosamine Biosynthetic Pathway (HBP), which promotes the synthesis of UDP-GlcNAc, the substrate for *O*-linked β-N-acetylglucosamine (*O*-GlcNAc)[4,5].

O-GlcNAc is a post-translational modification (PTM) of proteins that occur in the nuclear and cytosolic compartments of eukaryotic cells. The addition and removal of this monosaccharide is a reversible process catalyzed by two enzymes, *O*-GlcNAc transferase (OGT), which catalyzes the addition of N-acetylglucosamine, and *O*-GlcNAcase (OGA), which catalyzes the hydrolytic removal of *O*-GlcNAc from proteins[6]. Interestingly, studies have demonstrated that *O*-GlcNAc is essential during T-cell activation[7–9].

Similarly to *O*-GlcNAc, 5' adenosine monophosphate-activated protein kinase (AMPK) acts as a cellular energy sensor[10]. Evidence suggests that OGT and AMPK regulate various intracellular nutrient-sensitive processes synergistically, contributing to cellular metabolism and proliferation, protecting cells from metabolic stress[11]. Apoptotic protein inhibition, such as caspase-3, can also be regulated by AMPK. The effect of this kinase during cellular stress may also constitute a potential therapeutic target[12,13].

Considering that the availability and transport of nutrients can play a significant effect on T-cell activation and function; the involvement of regulatory proteins such as AMPK seems to be key to this process[14], and that elevated levels of *O*-GlcNAc can modulate the activity and function of thousands of biological targets[15,16], this study aimed to verify if elevated levels of *O*-GlcNAc can alter thymus size, cellularity, and morphology through regulation of AMPK and caspase-3.

MATERIALS AND METHODS

Animals

Male Wistar rats, 10 to 12 weeks (200-250g) were used in this study. The experimental analysis was performed at the Institute of Biological and Health Sciences of the Federal University of Mato Grosso (ICBS/UFMT). Animals

were housed in cages and exposed to a 12:12 hour light-dark cycle with a standard diet and water *ad libitum*. All procedures were approved by the Animal Use Ethics Committee (CEUA/UFMT), protocol number 23108077041/2020-43, considering the Federal Law number 11.794/08 (Arouca Law), which regulates procedures for the scientific use of animals in research.

Experimental groups

The rats were separated into two groups: control group (n=10) and glucosamine group (n=15). The animals in the glucosamine group were treated with a glucosamine solution (Sigma#G4875, diluted in 0.9% sodium chloride solution), at a dose of 300 mg/kg, intraperitoneally, for 21 days, as described by [17]. The control group received saline solution via the same route and treatment time.

Oral Glucose Tolerance Test (OGTT)

On the 21st day of treatment, blood glucose level was measured, as well as an oral glucose tolerance test was performed. Initially, the animals were fasted for 6 hours, and subsequently, caudal peripheral blood was collected, and glucose was measured with a digital glucometer (Accu Check® Active). Then, a glucose solution (2g/Kg animal; MERCK, D (+)-anhydrous glucose) was intragastrically administered (gavage) to the animals, and blood glucose was measured at 30, 60 and 120 minutes after glucose administration.

Thymic index and cellularity

The gross weight of each rat was recorded on 21st day of treatment. After the end of treatment (21 days), the animals were anesthetized with a mixture of ketamine and xylazine, intraperitoneally. Subsequently, the animals were killed in a CO_2 chamber. A laparotomy was performed to completely remove the thymus, weighed on an analytical balance. The thymic index was calculated using the following equation: organ weight (g)/animal weight(g) x 100[18]. For thymic cellularity, a portion of the thymic tissue was macerated in a solution of 500µl of PBS (phosphate-saline solution). After homogenization, this solution was diluted (1:50). The total number of cells was estimated by counting in a Neubauer chamber, using a mixture of 10µl of the diluted solution and 20µl of Turk's liquid (adapted by[19]).

Morphometry

The thymic tissue was fixed in Methacarn [methanol, chloroform, and acetic acid, 7:2:1; (v:v:v)], for 3 hours with agitation at 4°C, dehydrated in a series of increasing ethanol concentrations, and then embedded in paraffin. After embedding, it was sectioned at a thickness of 4 µm using an automatic microtome (Thermo Fisher HM355S). For morphometric analyses, the sections were stained with hematoxylin and eosin (H&E), and 3 fields of the tissue, belonging to the same slide, were analyzed with a microscope and a 4X objective lens (Nikon Eclipse E-200, Tokyo, Japan), photographed with an attached camera (Fujifilm 18x optical f = 5.0-9.0mm 1:3.1-5.6), and their area quantified using Image-Pro Plus software.

Western Blotting

Thymic tissues were pulverized and homogenized in cold RIPA lysis buffer (cat. #R0278; Sigma) and added with 10% protease inhibitor (cat. #S8820; Sigma). The samples were centrifuged at 12,000×g for 15 min at 4 °C, and the supernatant obtained as total proteins were quantified in duplicate, using the BSA standard curve (2 mg/mL to 0.125 mg/mL) and the Bradford reagent (cat. #B6916; Sigma). Protein extracts were separated by 10% SDS-PAGE gel for electrophoresis run at 4ºC. Subsequently, the proteins were transferred to a nitrocellulose membrane (cat#GE10600004, Sigma, USA) by the sandwich technique, through electrophoresis. After the transfer, nonspecific sites were blocked by submitting the membranes to shaking with 5% TBS-T/BSA for 1 h. The membranes were incubated with the primary antibodies described: *O*-GlcNAc [cat#9875S; Cell Signaling (1:500); mouse]; OGT [cat#2408s; Cell Signaling (1:1000); rabbit]; CD4+ [cat#237722; Abcam (1:1000); rabbit]; CD8+ [cat#PA5-79011; Thermo Fisher (1:1000); rabbit]; phospho-AMPK Thr172 [cat#2535T; Cell Signaling (1:1000); rabbit]; Caspase-3 [cat#9665s; Cell Signaling (1:1000); rabbit]; and β-actin [cat#SAB5500001; Sigma Aldrich (1:200); rabbit] overnight at 4 ºC under constant agitation. Membranes were subsequently incubated with secondary antibodies for 1 h at 24 ºC. The detection was performed by chemiluminescence (cat#32,209, Thermo Fischer, USA), detected in photographic equipment (ImageQuant LAS 4000). The bands were evaluated using the Uni Scan gel 6.1 software and membrane normalization was performed by β-actin expression.

Data Analysis

The results were expressed as mean ± standard error of the mean (SEM), for each animal (n) per group. Statistical analysis was performed using the Prisma program (GraphPad Prism 5.0, Graph Pad Software Incorporated, CA). The statistical significance of the data was determined by *Student t test*. Protein expression analyses were performed by the column test, using a one-sample *Student t test* with statistical value = 1. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Glucosamine treatment (300mg/kg), over 21 days, increased the expression of the enzyme OGT, responsible for catalyzing the addition of *N*-acetylglucosamine to the target proteins [1.00 vs 1.31 ± 0.10 AU (**Figure 1A**)]. Consequently, *O*-GlcNAcylation in thymic proteins was increased compared to the control group [1.00 vs 2.14 ± 0.19 AU (**Figure 1B**)].

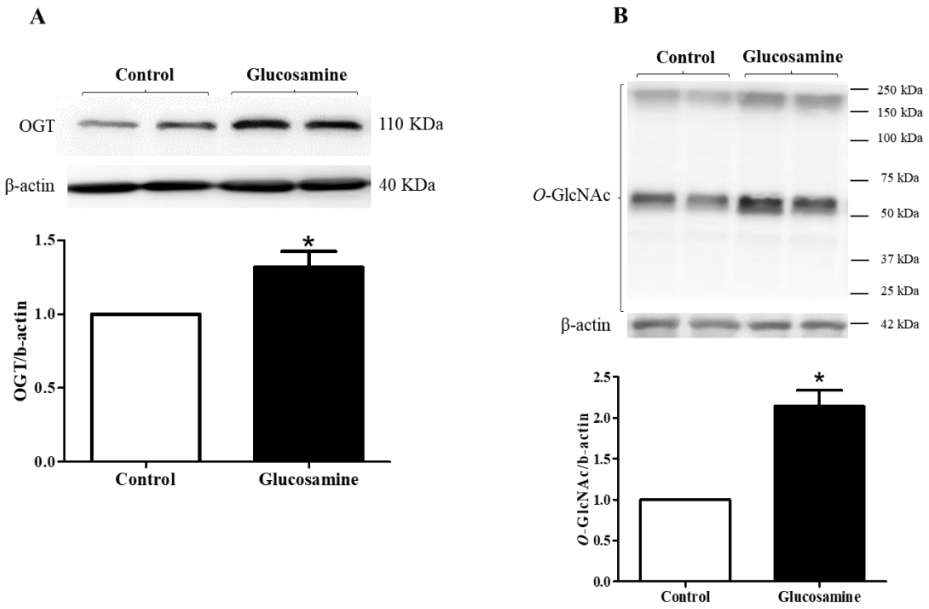
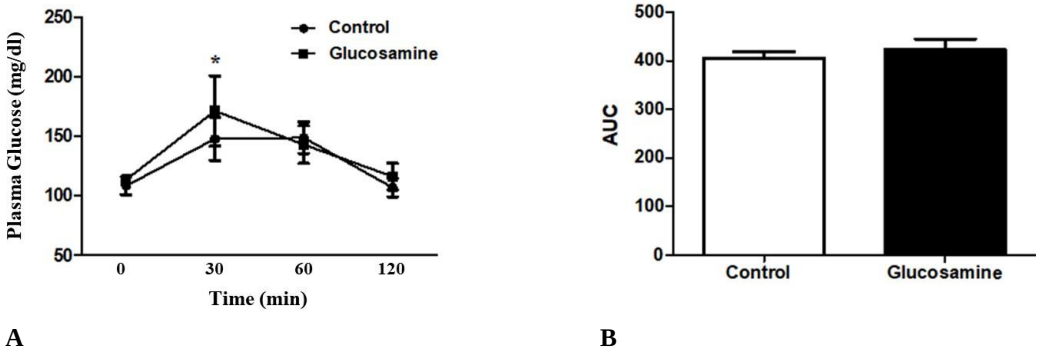


Figure 1 - Glucosamine treatment increases thymic expression of OGT and *O*-GlcNAc in rats. Treatment with glucosamine was able to stimulate OGT expression **(A)** and consequently increase *O*-GlcNAcylation **(B)** in thymic tissue. Top, representative picture of Western blot membrane with the respective molecular weights of the proteins evaluated. Bottom, the quantitative graph is showing the mean ± SEM of OGT expression or *O*-GlcNAc-modified proteins (n = 10). The values of protein expression were evaluated individually and corrected by β-actin expression. The statistical comparison was performed using the Student's t test. *$p < 0.05$ vs. respective control group.

The OGTT, which was used to determine whether this *in vivo* treatment had any effect on fasting glucose levels, showed that glucosamine treatment did

not alter this parameter. During the OGTT, the glucosamine-treated group exhibited a glycemic peak at the 30-minute mark compared to the control group. However, the augmented glucose level did not persist, and after 60 and 120 minutes the blood glucose returned to normal levels **(Figure 2A)**. No alteration in the area under the curve analysis was observed between treatments **(Figure 2B)**.



A **B**

Figure 2 - Glucosamine treatment did not alter the Oral Glucose Tolerance Test (OGTT). Oral glucose tolerance test **(A)** and area under curve **(B)** in glucosamine-treated rats their respective controls. Representative graphs show the mean ± SEM for each group (n = 7). The statistical comparison was performed using the Student's t test, comparing the groups at the same time-point. *p < 0.05 vs. respective control group.

Our next aim was to investigate if augmented *O*-GlcNAcylation leads to changes in thymus size, cellularity, and morphology. In the morphometric analysis, no changes were observed in the total [(mm^2) 9.9 ± 0.5 control vs. 9.2 ± 0.6 glucosamine, **(Figure 3A)**], cortex [(mm^2) 8.2 ± 0.4 control vs. 7.4 ± 0.6 glucosamine, **(Figure 3B)**] or medulla [(mm^2) 1.7 ± 0.1 control vs. 1.8 ± 0.1 glucosamine, **(Figure 3C)**] area of the thymus, after glucosamine treatment. However, augmented thymic index (0.10 *vs* 0.12 ± 0.00 kg/rat **(Figure 3D)**] and hyperplasia [446.90 ± 55.12 *vs* 891.10 ± 142.30 cells/ml **(Figure 3E)**] were observed after glucosamine treatment, compared to the control group.

The effect of glucosamine treatment was also evaluated on important regulators of metabolic pathways and energy homeostasis. The expression of phosphorylated AMPK was increased after glucosamine treatment [1.00 *vs* 1.48 ± 0.09 AU (**Figure 4)**]. Since the inhibition of the pro-apoptotic process can be regulated by AMPK, the expression of caspase-3 was also evaluated.

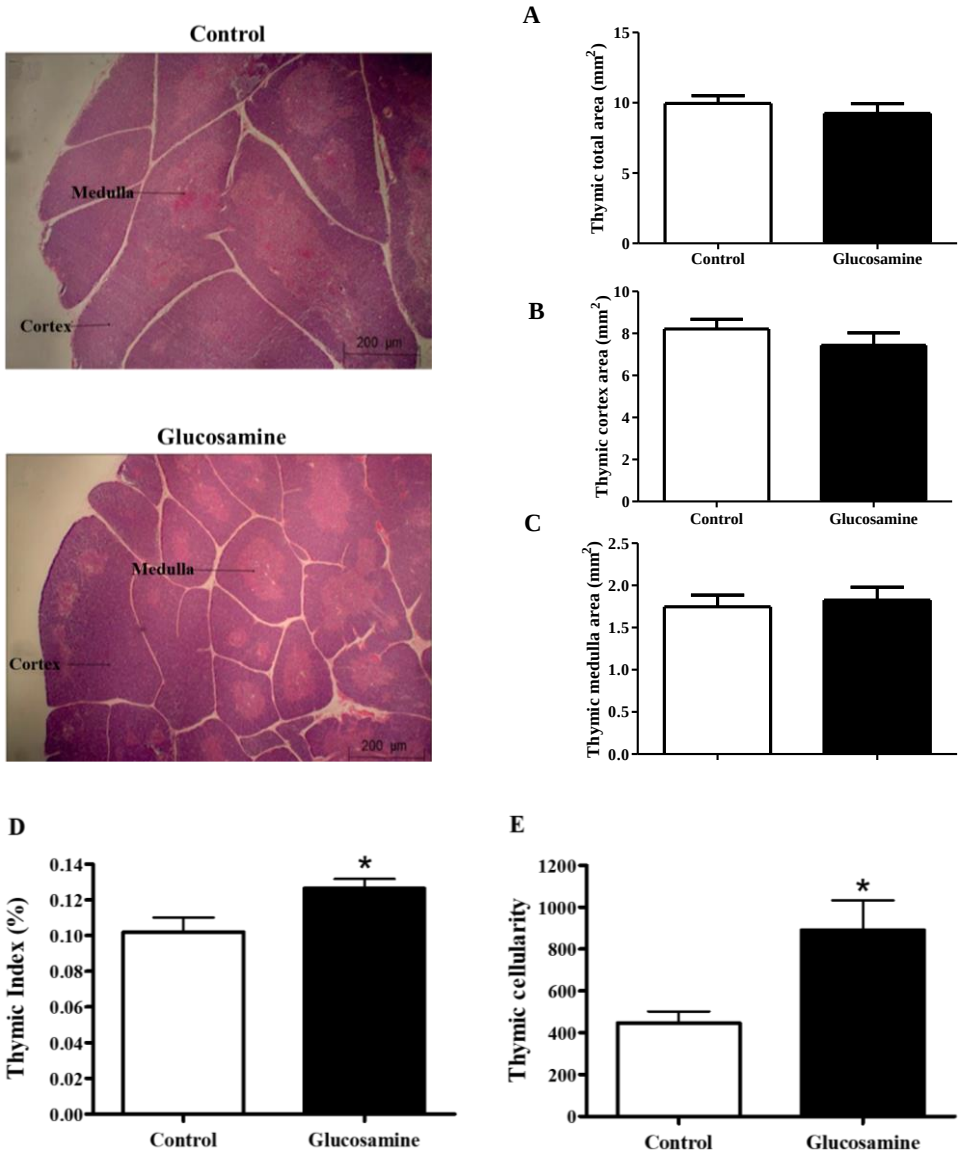


Figure 3 - Effects of glucosamine-treated on thymus morphology (A-C), size (D) and cellularity (E). In the morphometric analysis, no changes were observed in the total **(A)**, cortex **(B)** and medulla **(C)** area of the thymus, after 21 days of treatment with glucosamine. Increased amounts *O*-GlcNAc-proteins lead to cause an increase in thymic index **(D)**, and cellularity **(E)**, compared to the control group. The quantitative graph is showing the mean ± SEM for each group (n = 7). The statistical comparison was performed using the Student's t test. *p < 0.05 vs. respective control group.

Interestingly, the expression of caspase-3, a pro-apoptotic factor, was reduced in glucosamine-treated animals [(1.0 *vs* 0.44 ± 0.07 AU (**Figure 4)**], compared to the control group.

To determine the influence of increased levels of *O*-GlcNAcylation on the expression of T cell coreceptors, we observed the expression of CD4+ and CD8+ cell surface markers. We observed that increased levels of O-

GlcNAcylation in thymic tissue promoted a reduction in the expression of CD4+ (1.00 vs 0.68 ± 0.08 AU) and CD8+ (1.00 vs 0.66 ± 0.10 AU) molecules **(Figure 5)**.

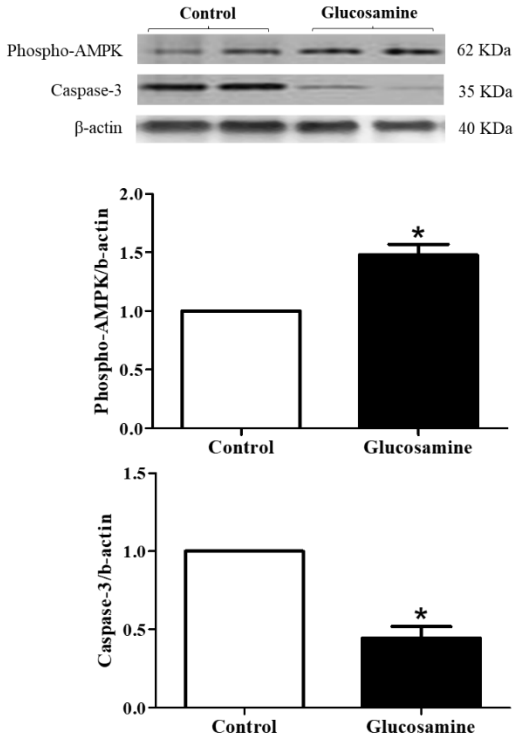


Figure 4 - Glucosamine treatment increases expression of phosphorylated AMPK, but reduce expression of caspase-3 in thymic tissue from rats. Top, representative western blot image of phosphor-AMPK and caspase-3 (n = 10). Bottom, quantitative graph shows the mean ± SEM of phosphor-AMPK and caspase-3 expression in thymus (n = 10). The values of protein expression were evaluated individually and corrected by β-actin expression. Statistical comparison was performed using the Student's t test. *p < 0.05 vs. respective control group.

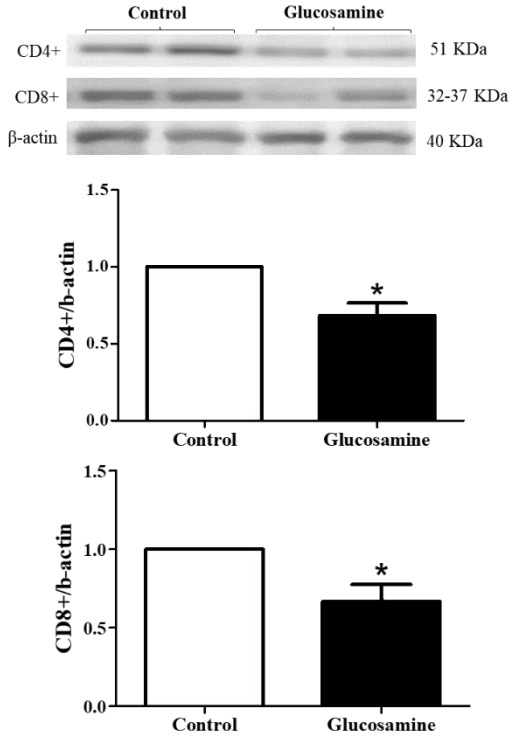


Figure 5 - Glucosamine treatment decreases expression of the co-receptor molecules CD4+ and CD8+ in thymic tissue from rats. Top, representative western blot image of CD4+ and CD8+ (n = 10). Bottom, quantitative graph shows the mean ± SEM of CD4+ and CD8+ expression in thymus (n = 10). The values of protein expression were evaluated individually and corrected by β-actin expression. Statistical comparison was performed using the Student's t test. *p < 0.05 vs. respective control group.

DISCUSSION

The effects of elevated levels of *O*-GlcNAc have already been evaluated in various organs, such as the heart, lungs, placenta, and kidneys[17,20–22]. However, this is the first study to elucidate the effects of this post-translational modification (PTM) on the thymus, a primary lymphoid organ essential to adaptive immunity. The findings of this study provide evidence that *O*-GlcNAcylation can increase the size and evoke thymus hyperplasia, at least in part, by inhibiting the caspase-3

apoptotic pathway through the activation of AMPK. On the other hand, it appears that the expression of CD4+ and CD8+ coreceptors does not accompany this increase in the thymic cellularity.

Glucosamine is a monosaccharide capable of increasing global levels of *O*-GlcNAc by directly entering the hexosamine biosynthesis pathway[5]. Initially, glucosamine treatment was effective in stimulating OGT expression in the thymus and, consequently, promoting an increase in global thymic protein *O*-GlcNAcylation. However, it is estimated that 2-5% of the total cellular glucose is channeled into the hexosamine biosynthesis pathway[23]. Therefore, OGTT was performed to rule out that glucosamine treatment was impacting the glycemic levels. It is important to stress that the glucosamine treatment did not significantly alter OGTT. Therefore, the increased *O*-GlcNAcylation observed in the thymus is directly associated with the greater availability of glucosamine, rather than glucose.

In this study, it was observed that glucosamine treatment increased the thymic index compared to the control group, suggesting that the *O*-GlcNAcylation promotes a change in thymus size. Rytter et al. (2017) observed a correlation between thymus size and the nutritional status of children, showing that malnutrition was directly related to reduced thymus size[24]. These findings strengthen the hypothesis that thymus size can be used as a marker of malnutrition-induced mmunodeficiency, although it is still unknown to what extent thymus size reflects immune competence[25,26].

Interestingly, hyperplasia was also confirmed upon augmented *O*-Glycosylation. However, despite an increase in both the thymic index and thymic cellularity, morphological analysis of the tissue revealed that the thymic structure is preserved, with no statistical difference in the cortex and medulla areas between the groups. Considering the changes observed so far, we can speculate that longer glucosamine treatment might trigger alterations in thymic structure since the role of *O*-GlcNAc is also related to the exposure time (acute or chronic), as evidenced in cardiac and vascular tissue[11,16,27,28].

Glycoside mapping studies were performed, via direct glycopeptide measurement, on resting and activating primary human T cells, 227 proteins were identified as targets for *O*-GlcNAc[29]. Considering these findings and the evidence that AMPK is a

nutritional sensor that regulates the plasticity process in the metabolic reprogramming of T cells[11], we decided to evaluate the effects of increased *O*-GlcNAc on the protein expression of this protein in the thymus. It was observed that the active form of AMPK is overexpressed in the glucosamine-treated group.

Bullen et al. (2014) demonstrated that AMPK is a target of *O*-glycosylation in an in vitro study using different cell lines[11]. Rolf et al. (2013) also demonstrated that AMPK is indispensable for the survival and function of effector T cells, and its genetic deletion induces lymphocyte death during activation[30]. Besides promoting T lymphocyte survival, AMPK activation also enhances their antitumor function, particularly CD8+ T lymphocytes and regulatory T cells, CD4+ CD25+ Tregs[31,32].

Advances in molecular technology have identified the potential of therapeutic enzymes in the treatment of diseases related to apoptosis, such as cancer, heart failure and neurodegenerative diseases. Among all therapeutic enzymes, caspase-3 is one of the most significant[33]. Increased *O*-GlcNAc decreased the expression of caspase-3 in this study. Altogether, caspase-3 may be inhibited due to AMPK overexpression, mediated by high levels of *O*-GlcNAc. Other studies have also shown that AMPK activation protected endothelial cells against cell death induced by hyperglycemia or high-fat content, suggesting a negative regulation of caspase-3 activity via AMPK activation[34,35].

Glucosamine treatment also interfere with the expression of T cell coreceptors, reducing the expression of CD4+ and CD8+ surface markers. Considering that a T cell expresses numerous CD4+ or CD8+ molecules on its surface and that the intensity of cell activation signals is also related to the number of interactions between these molecules and the peptide-MHC II and I complexes, respectively[36], we can speculate that lower expression of these coreceptors in the thymus may subsequently interfere with the activation capacity of T cells in the periphery, impairing or altering immune responses associated with the participation of these molecules[37].

On the other hand, silencing the OGT gene in mice was used as a model to evaluate the importance of *O*-GlcNAc in thymocyte development. The knockout animals had a ≥70% reduction in the number of these cells, with a consequent reduction of mature T cells in the periphery, indicating immune failure[38]. Similarly, OGT ablation in regulatory

CD4+ T cells (Tregs) induce an aggressive autoimmune syndrome in mice, resulting in from Treg lineage instability and deficiency. Conversely, pharmacological induction of *O*-GlcNAc increases the suppressive activity of human Tregs[8,9].

All these findings indicate that this PTM appears to be a dynamic modulator of cellular signaling pathways that play a significant role in T cells[8]. However, a paradox also arises regarding the benefits and/or harms this modification could cause in immune response. In our study, elevated levels of *O*-GlcNAc were able to increase thymus size and cellularity but decreased the expression of CD4+ and CD8+ coreceptors, which are involved in the activation of mature T cells[36].

It is worth mentioning that this paradoxical effect of *O*-GlcNAc has also been observed in other target tissues[16,39]. In the cardiovascular system, for example, Lima et al. (2012) described that the positive and/or negative role of *O*-GlcNAc in the vasculature could be associated with exposure time, cellular metabolic state, and the target proteins of this modification in different cell/tissue/organ types[16]. Therefore, further studies should be encouraged for a better understanding of how this PTM can modulate the innate and adaptive immunity system in physiological and pathophysiological conditions.

CONCLUSION

In this study, we observed for the first time that *O*-GlcNAc appears to exert a modulatory effect on thymic cells, regulating thymic cellularity and T cell coreceptor expression, inhibiting caspase-3-dependent apoptosis, likely via AMPK kinase activation. Further studies will be necessary to better characterize the involvement of *O*-GlcNAc in the thymic microenvironment. However, the findings of this work provide evidence that *O*-GlcNAc may represent a novel mechanism for the modulation of thymocytes, which could aid in the future understanding of immunological alterations in situations where levels of this PTM are elevated, such as in diabetes, hypertension, and cancer.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

FUNDING INFORMATION

This work was supported by Fundação de Amparo à Pesquisa do Estado de Mato Grosso (FAPEMAT-PRO.001786/2023 to VVL and FAPEMAT-PRO.000112/2023 to FRG), Conselho Nacional de Desenvolvimento Científico e Tecnológico [(CNPq) grant number 308899/2022-9 (to F.R.G.)], and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) in Brazil.

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