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Radiolabeling of porcine, murine growth hormone and a potential antagonist G118R-mGH for biodistribution study

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Abstract: This study aimed to radiolabel porcine growth hormone (pGH), murine growth hormone (mGH), and its antagonist (G118R-mGH) with the iodine radioisotopes ^{131}I and ^{123}I, to investigate the biodistribution and brain transport of these hormones. Radiolabeling was performed using the Chloramine T method, a protein iodination technique. The radiolabeled products were characterized by physicochemical techniques such as SDS-PAGE, size-exclusion high-performance liquid chromatography (HPLC-SE), and ascending paper chromatography, with measurements performed using single-photon emission computed tomography (SPECT). These measurements demonstrated high radiochemical purity, exceeding 95%, and preservation of molecular size. *In vitro* stability assays indicated that the radiotracers maintained their integrity for at least 24 hours. *In vivo* biodistribution studies in mice revealed distinct tissue distribution patterns for mGH and G118R-mGH, suggesting different uptake and metabolism mechanisms. The use of ^{123}I allowed for SPECT-CT imaging studies, which proved important for assessing the ability of mGH and G118R-mGH to cross the blood-brain barrier and distribute in specific regions of the brain. The results obtained in this work highlight the versatility of the radiolabeling protocol employed and its potential for investigating complex biological processes and application in research.

Keywords: Growth Hormone 1, Radiolabeling 2, Iodine 3, SPECT-CT 4.

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Radiomarcação do hormônio de crescimento porcino, murino e um potencial antagonista G118R-mGH para estudo de biodistribuição

Resumo: Este estudo teve como objetivo radiomarcar o hormônio do crescimento porcino (pGH), murino (mGH) e seu antagonista (G118R-mGH) com os radioisótopos de iodo ^{131}I e ^{123}I, visando investigar a biodistribuição e o transporte cerebral desses hormônios. A radiomarcação foi realizada utilizando o método de Cloramina T, uma técnica utilizada para iodinação de proteínas. Os produtos radiomarcados foram caracterizados por técnicas físico-químicas como SDS-PAGE, cromatografia líquida de alta eficiência por exclusão molecular (HPLC-SE) e cromatografia em papel ascendente medida através da tomografia computadorizada por emissão de fóton único (SPECT), demonstrando alta pureza radioquímica, superior a 95% e preservação do tamanho molecular. Ensaios *in vitro* de estabilidade indicaram que os radiomarcadores mantiveram sua integridade por pelo menos um período de 24 horas. Estudos de biodistribuição in vivo em camundongos revelaram padrões de distribuição tissular distintos para o mGH e o G118R-mGH, sugerindo diferentes mecanismos de captação e metabolização. A utilização de ^{123}I permitiu a realização de estudos de imagem por SPECT-CT, que se mostraram importantes para avaliar a capacidade do mGH e do G118R-mGH atravessarem a barreira hematoencefálica e se distribuirem em regiões específicas do cérebro. Os resultados obtidos neste trabalho evidenciam a versatilidade do protocolo de radiomarcação empregado e o seu potencial para a investigação de processos biológicos complexos e aplicação na pesquisa.

Palavras-chave: Hormônio de Crescimento 1, Radiomarcação 2, Iodo 3, SPECT-CT 4.

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1. INTRODUCTION

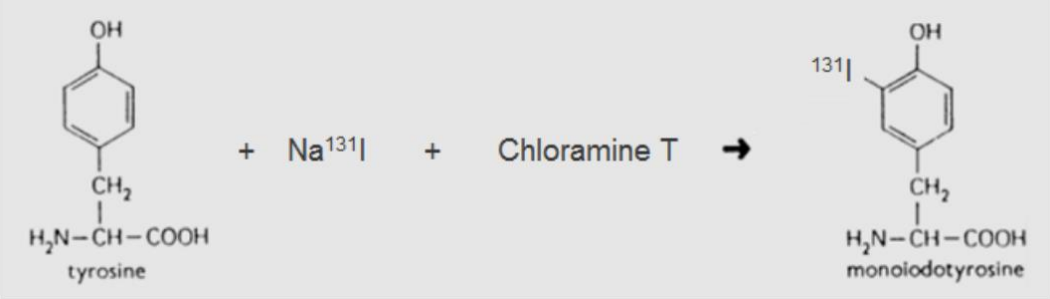
Growth hormone (GH) is secreted by the anterior pituitary and is involved in regulating several important physiological functions, such as cell proliferation, tissue growth, protein synthesis, carbohydrate and lipid metabolism, among others. Traditionally, the main target tissues of GH are the liver, skeletal muscle, bones, and adipose tissue, which are responsible for the primary biological effects of this hormone [1]. However, recent evidence has indicated that the central nervous system (CNS) is also a critical target tissue for many of GH's biological functions, including metabolism regulation, control of various cognitive functions, behavioral modulation, and neurotrophic action [2].

Radiolabeling has been a very valuable tool for discoveries regarding GH's action in the CNS and has contributed to the body of evidence that has led to speculation about this hormone's ability to cross the blood-brain barrier (BBB) [3]. According to the current dogma, the molecular mass of 22 kDa of GH is considered too large to cross the BBB. Restrictive factors include the presence of tight junctions between endothelial cells, the capillaries that make up the BBB, and the underlying continuous basal membrane, which substantially reduce the paracellular permeation of large proteins [4].

The technique of GH radiolabeling with ^{125}I, developed in 1963, involves incorporating radioactive iodine into the protein molecule through electrolytic substitution and the iododeprotonation of aromatic rings of tyrosine residues. GH possesses eight such residues [5].

Chloramine T (CLT) is a sodium salt of N-chlorotoluene sulfonamide and is widely used as an oxidizing agent in the radiolabeling reaction to convert sodium iodide into an electrophilic form, thereby releasing diatomic iodine. On the other hand, sodium metabisulfite ($Na_2S_2O_5$) is added to the reaction to stop it, reduce excess CLT, and transform I_2 into I^- in the molecule (Figure 1) [6].

Figura 1: Illustration of the radioiodination process with sodium iodide ($Na^{131}I$) and the introduction of ^{131}I atoms into the tyrosine residue at the aromatic hydroxyl ring, resulting in an electrolytic exchange through oxidation by CLT.



Source: [7].

The mechanism by which this hormone reaches the central nervous system (CNS) from the peripheral circulation remains obscure, but appears to occur independently of the presence of the growth hormone receptor (GHR). Thus, drugs such as GHR antagonists may cross the blood-brain barrier (BBB) and represent a therapeutic strategy to reverse pathological conditions dependent on the GH-GHR interaction within the CNS.

The development of the growth hormone receptor antagonist (GHA), with a glycine codon mutation (at amino acid 120) replaced by arginine (R), enables site 1 to bind to the growth hormone receptor (GHR) but prevents functional binding at site 2, avoiding intracellular signaling. This led to the creation of a competitive human growth hormone inhibitor (G120R-hGH) and the development of the drug Pegvisomant, used in the treatment of patients with acromegaly and in studies aiming to understand GH activity [8].

However, this remains a poorly explored field lacking research, as previous *in vivo* studies have shown that the hGH antagonist (G120R-hGH) was ineffective in rodents [8] [9] [10]; in other words, it could not antagonize the growth-promoting action of GH and exhibited an agonistic effect in rodents, potentially binding to the prolactin receptor (PRLR) [9]. This underscores the importance of producing and working with homologous hormone and antagonist models.

The primary objective of this work is to perform radiolabeling of GH and its variants with ^{123}I and ^{131}I, to characterize and validate the radiolabeled products obtained, and to conduct preliminary *in vivo* studies using heterologous models (murine growth hormone) to enable future research aimed at deepening our understanding of GH physiology.

2. MATERIALS AND METHODS

Radiolabeling with ^{131}I or ^{123}I: The pGH, mGH and G118R-mGH were labeled with ^{123}I for imaging studies and with ^{131}I for biodistribution studies. A protocol based on the Chloramine T (CLT) method was developed. The labeling method was standardized as follows: 50 μg of GH in 50 μL of phosphate-buffered saline (PBS, pH 7.4) and 37 MBq of $Na^{131}I$ or $Na^{123}I$. To initiate the oxidation process, 50 μg of CLT was added, followed by incubation under orbital shaking for 45 minutes. To terminate the reaction, 100 μg of sodium metabisulfite ($Na_2S_2O_5$) was added.

Radiochemical Purity Evaluation: The radiochemical purity of the labeled product was assessed using ascending paper chromatography, employing iTLC Aluminum Sheets strips as the support and 85% methanol as the mobile phase. Following chromatographic development, the radioactivity was measured using single-photon emission computed tomography (SPECT). The retention factor (Rf) of the labeled product was considered 0, and that of free iodine was 1.0.

***In vitro* labeling stability study:** To evaluate the labeling stability of ^{131}I-mGH and ^{131}I-G118R-mGH, four labeling reactions were performed for each protein (totaling eight samples), and each was diluted in 500 μL of fetal bovine serum. At 1, 3, 6, and 24 hours post-labeling, 15 μL of each sample was used to analyze the radiochemical purity.

Impacts of Chloramine T on GH: To analyze whether the oxidation reaction of CLT formed aggregates and/or dimeric forms in GH, reactions were performed under

conditions similar to labeling: 50 μg of GH in 50 μL of phosphate-buffered saline (PBS, pH 7.4), 50 μg of CLT was added, and incubation was performed under orbital shaking for 45 minutes, followed by termination of the reaction with 100 μg of sodium metabisulfite ($Na_2S_2O_5$), but without the use of the iodine radioisotope. Subsequently, the samples were analyzed by size-exclusion high-performance liquid chromatography (SEC-HPLC), with absorbance measured at a wavelength of 220 nm, and by SDS-PAGE under non-reducing and reducing conditions.

SDS-PAGE Analysis: Samples of the hormones, before and after reaction with Chloramine T, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels used were prepared with a concentration of 15% polyacrylamide in the separating gel and 3% in the stacking gel. The analyses were performed under both reducing and non-reducing conditions. For protein visualization, the gels were stained with Coomassie Brilliant Blue G-250 or silver nitrate [11].

Size Exclusion Chromatography (SEC-HPLC): Size-exclusion chromatography analyses were performed using a high-performance liquid chromatography system equipped with a TSKgel G2000 SW column (60 cm x 7.5 mm, 10 μm particle size, 125 Å pore size) coupled with a TSKgel SW guard column (7.5 cm x 7.5 mm). The mobile phase consisted of a 0.025 M ammonium bicarbonate solution (pH 7.0, adjusted with 2% phosphoric acid). The chromatographic separation was performed isocratically at a flow rate of 1.0 mL/min. Detection of the analytes was carried out using an ultraviolet (UV) detector at a wavelength of 220 nm. The chromatographic conditions used followed the protocol described by Menezes et al. [12].

***In vivo* studies:** In this study, we used C57BL/6J mice, obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed in the Nanci do Nascimento Animal Facility at IPEN under a 12h/12h light/dark cycle, at a controlled temperature of 22

± -1 °C, and with *ad libitum* rodent feed. The project was approved by the IPEN Animal Ethics Committee, protocol number 20/22.

Biodistribution assay in C57BL/6J mice: Saline solution with ^{131}I-mGH or ^{131}I-G118R-mGH or ^{131}I-pGH was injected intraperitoneally in C57BL/6J mice, with an activity of 0.12 MBq and 0.16 μg of hormone per mouse. Four animals were used, two for each protein. After 45 minutes the animals were euthanized. Blood samples were collected from the orbital plexus (75 μL) and the following organs were dissected: liver, heart, lungs, spleen, muscle (gastrocnemius), bone (femur), stomach, intestines, pancreas, kidneys, bladder, and brain. These organs were then weighed and the radioactive activity was measured using a gamma ray counter.

For analysis of graphs and statistics, the software GraphPadPrism (version 8.0.2) was used, employing two way ANOVA. Data were calculated as the percentage of injected dose per gram of tissue (%ID/g). The tissue/blood ratio was obtained by dividing tissue activity (%ID/g) by blood activity (%ID/g).

SPECT-CT Imaging Assay: A saline solution containing ^{123}I-mGH or ^{123}I-G118R-mGH was injected intraperitoneally into C57BL/6J mice at an activity of 37 MBq and 50 μg of hormone per mouse. Euthanasia was performed 5 minutes after injection, and only the heads were placed in the scanning chamber. Images were acquired after 1 hour. The passage of the hormone through the blood-brain barrier and its localization in brain anatomical regions were evaluated by single-photon emission computed tomography-computed tomography (SPECT-CT), analyzed using a microPET (Albira).

3. RESULTS AND DISCUSSIONS

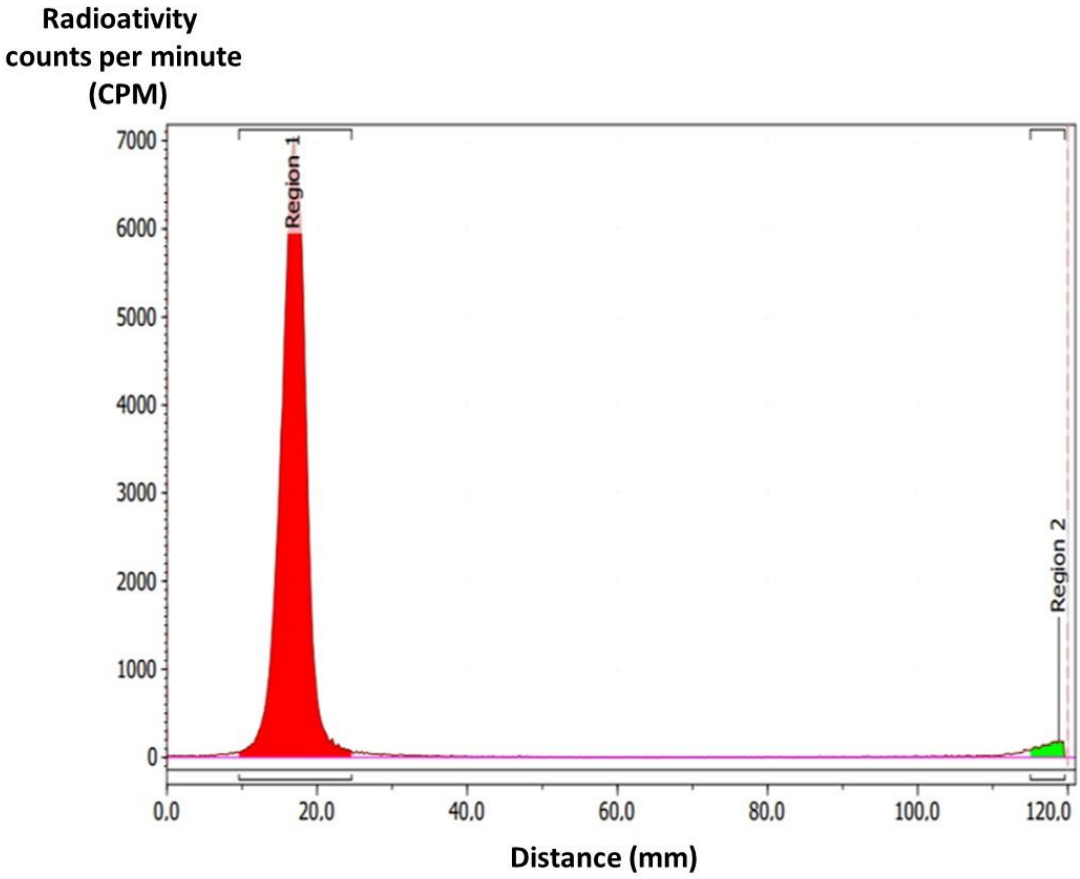
The efficiency of GH radioiodination, i.e., the amount of iodine available in the solution that is transferred to the molecules, can vary depending on factors such as the amount of material available in the solution and the time allowed for the reagents to act. Another crucial factor to consider in this procedure is the impact on the protein during the reaction, which can affect its physical integrity, immunological activity, and biological function [13].

In the development of the GH radiolabeling method using CLT, Greenwood & Hunter (1963) achieved an efficiency of up to 76%, using 5 μg of hGH, 14-28 MBq of ^{131}I, 100 μg of CLT, and quickly adding twice the amount of $Na_2S_2O_5$ after a brief homogenization [14]. In this case, the ^{131}I-labeled hGH was calculated to contain up to 0.5–1 iodine atom per molecule. They observed a progressive loss of immunological activity of the hormone starting from 18 MBq/μg.

In another study by Biscayart et al. (1989) on the preparation of ^{125}I-labeled hGH under two labeling conditions, it was observed that adding ^{125}I with an activity of 37 MBq to 5 μg of hGH, with the addition of 0.22 μg of CLT and a reaction time of 5 minutes, resulted in iodine incorporation into the protein of no more than 20%. However, increasing the hormone concentration to 20 μg and CLT to 0.88 μg yielded an 80% labeling efficiency [15].

This demonstrates that with a longer reaction time, it is possible to reduce the concentration of CLT in a reaction with a higher amount of hormone and achieve a high labeling yield. In our study, we used a standardized protocol from our research group with 50 μg of protein, 37 MBq of ^{131}I, 50 μg of CLT, and a reaction time of 45 minutes. This approach resulted in high radiochemical purity with a labeling yield greater than 95%, as shown in Figure 2.

Figure 2 – Paper chromatography analysis to assess the radiochemical purity of murine growth hormone (mGH) labeled with ^{131}I (red) and free ^{131}I (green). Source: Author's data.



The stability of radiolabeled products is a critical factor, particularly considering the type of study to be conducted. In the context of this work, where the labeled hormones would be used immediately in *in vivo* studies and considering the short half-life of the ^{123}I radioisotope (13.2 h), we limited our stability studies to a period of up to 24 hours. Table 1 presents the results of the assessment of the radiochemical purity of ^{131}I-mGH and ^{131}I-G118R-mGH over this period. The data demonstrate that, for at least 24 hours, the radiolabeled products maintained their stability.

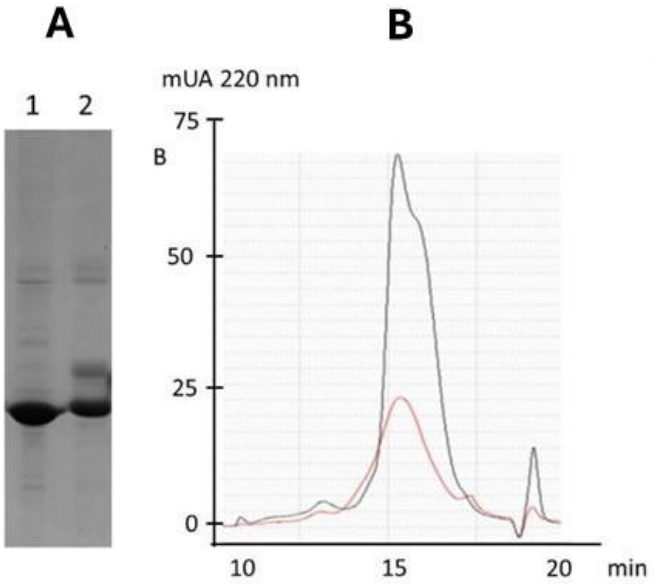
Table 1: *In vitro* stability of ^{131}I-labeled mGH and G118R-mGH. Determination of radioactivity of the tagged hormone and free iodine (^{131}I) at different incubation times (1, 3, 6, and 24 hours).

Study of the stability of iodine-131 labeled												
			Tagged	^{131}I		Tagged	^{131}I		Tagged	^{131}I		Tagged	^{131}I
mGH	1	1 h	3,30 µCi	0,76 µCi	3 h	3,35 µCi	0,81 µCi	6 h	3,4 µCi	0,7 µCi	24 h	3,30 µCi	0,8 µCi
	2		3,20 µCi	0,7 µCi		3,25 µCi	0,7 µCi		3,25 µCi	0,7 µCi		3,2 µCi	0,62 µCi
	3		2,75 µCi	0,55 µCi		2,8 µCi	0,6 µCi		2,8 µCi	0,6 µCi		2,8 µCi	0,7 µCi
	4		3,0 µCi	0,5 µCi		3,1 µCi	0,6 µCi		3,1 µCi	0,6 µCi		3,04 µCi	0,53 µCi
G118R-mGH	1	1 h	5,0 µCi	0,7 µCi	3 h	5,05 µCi	0,7 µCi	6 h	5,1 µCi	0,63 µCi	24 h	4,98 µCi	0,7 µCi
	2		2,0 µCi	0,5 µCi		2,3 µCi	0,5 µCi		2,77 µCi	0,43 µCi		2,07 µCi	0,55 µCi
	3		4,5 µCi	0,68 µCi		4,87 µCi	0,71 µCi		4,51 µCi	0,7 µCi		4,7 µCi	0,86 µCi
	4		3,5 µCi	0,5 µCi		3,8 µCi	0,68 µCi		3,33 µCi	0,68 µCi		3,64 µCi	0,6 µCi

Source: Author's data.

We also evaluated the impact of the oxidation reaction with Chloramine T on the integrity of the GH molecule. To this end, we performed reactions under conditions similar to those used in radioactive labeling, including the addition of sodium metabisulfite ($Na_2S_2O_5$) to quench the reaction, but without the addition of the iodine radioisotope. Samples, both before and after the oxidation reaction, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions and by size-exclusion high-performance liquid chromatography (HPLC-SE) (Figure 3).

Figure 3: Evaluation of the impact of Chloramine T. A. SDS-PAGE analysis: 1. mGH; 2 mGH after reaction with CLT; B. HPLC-SE chromatograms: mGH untreated (black) and treated with CLT (red).



Source: Author's data.

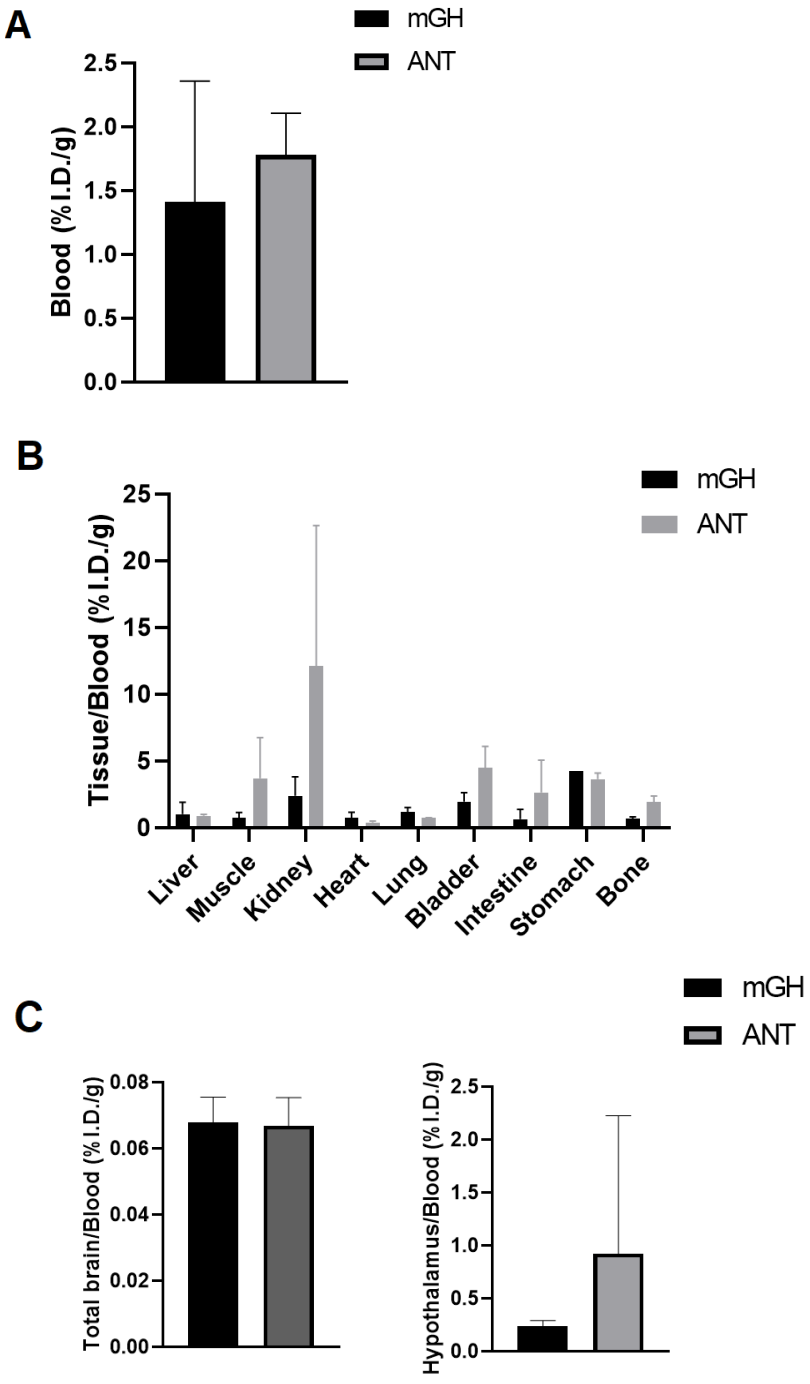
Although we observed a slight increase in dimer formation (estimated at less than 5% of the total hormone), the analyses did not indicate significant alterations in the overall quality of the GH molecule

Studies show that the half-life of GH in plasma is short, with rapid clearance when it is alone ($t_{1/2}$ = 11-20 minutes) [16]. This information is crucial for understanding GH availability in the blood and the time required for its internalization into tissues via receptor binding. In our study, as shown in Figure 4A, the percentage of injected dose per microliter of blood after 45 minutes was similar between ^{131}I-mGH and ^{131}I-G118R-mGH, indicating that both proteins remained available in the circulation of mice over time.

The acute tissue response to recombinant GH in mice shows differential sensitivities across tissues, with the greatest maximal response observed in the gastrocnemius muscle compared to other regions such as the heart, liver, white adipose tissue, and kidney [17]. Our results regarding the availability of ^{131}I-mGH and ^{131}I-G118R-mGH showed variation depending on the tissue collected. However, when comparing the dose of the radiolabeled compounds in the tissues (Figure 4B), we did not observe a statistically significant difference.

This suggests that the potential antagonist G118R-mGH may remain available in the body's tissues over time to counteract the effects of circulating mGH. In the brain, we confirmed previous studies on GH's ability to permeate from the circulation to the brain independently of receptor binding (Figure 4C). The ability of G118R-mGH to remain available in the brain for an extended period provides therapeutic potential for studying the effects of GH antagonism in the murine brain.

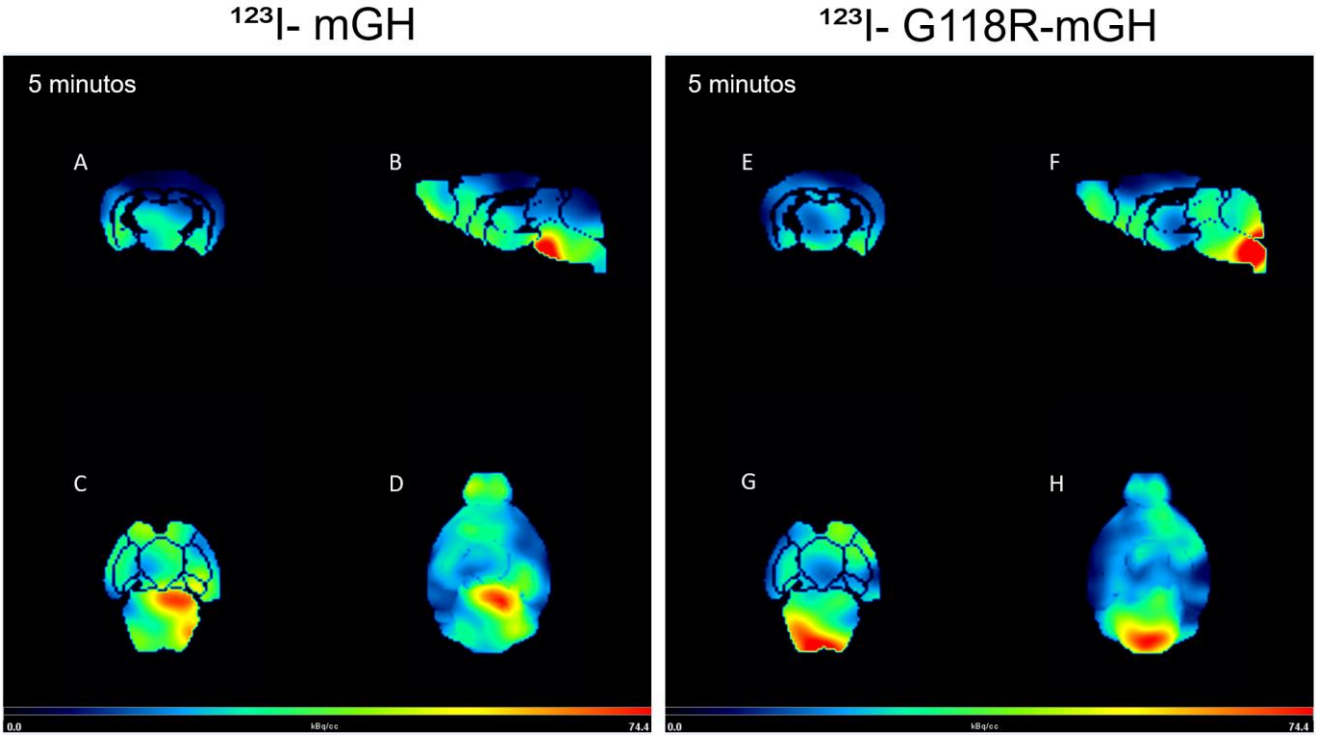
Figure 4 - Biodistribution assay in mice injected with ^{131}I-mGH (black) and ^{131}I-G118R-mGH (gray), with an activity of 0.12 MBq (1 million counts per minute - CPM) and 0.16 µg of each hormone, euthanized 45 minutes after intraperitoneal injection. Injected dose tissue/blood ratio per gram of tissue (%ID/g). (A) Percentage of the injected dose per µL of blood; (B) Tissue-to-blood ratio of the injected dose per gram of tissue; (C) Tissue/blood ratio of the injected dose per gram of tissue for the total brain and the hypothalamus separately.



Source: Author's data.

A SPECT-CT imaging study was conducted using the radioisotope ^{123}I (half-life of 13.2 hours, energy of 159 keV) to investigate the cerebral biodistribution of the radiolabeled hormones ^{123}I-mGH and ^{123}I-G118R-mGH in C57BL/6J mice. Images were acquired with a focus on brain regions [18]. This study aimed to assess the ability of these hormones to cross the blood-brain barrier (BBB) and their specific distribution in different brain regions. The main results are presented in Figure 5.

Figure 5: Single-photon emission computed tomography (SPECT-CT) images of mouse brains euthanized 5 minutes after injection of ^{123}I-mGH or ^{123}I-G118R-mGH. On the left are the images of radiolabeled ^{123}I-mGH: (A) coronal section; (B) sagittal section; (C) transverse section; (D) whole brain viewed from above. On the right are the images of radiolabeled ^{123}I-G118R-mGH, (E) coronal section; (F) sagittal section; (G) transverse section; (H) whole brain viewed from above.



Source: Author's data.

4. CONCLUSIONS

In this study, we demonstrated the efficacy of a radiolabeling methodology for obtaining porcine growth hormone (pGH), murine growth hormone (mGH), and its

antagonist (G118R-mGH) labeled with the radioisotopes ^{123}I and ^{131}I. The high radiochemical purity of the obtained radiopharmaceuticals allowed for *in vivo* biodistribution studies, revealing the ability of these hormones to cross the blood-brain barrier and distribute to different brain regions.

The results obtained open up new perspectives for the development of research and diagnostic tools, enabling more in-depth studies on the mechanisms of action of GH and its antagonists in the central nervous system. Additionally, the developed methodology can be applied to the labeling of other proteins of interest, expanding the possibilities of investigation in various areas of biomedicine.

The ability to track the biodistribution of these hormones in real time, using imaging techniques such as SPECT-CT, offers a powerful tool for research and development of new therapies. The results obtained in this work highlight the versatility of the radiolabeling protocol employed and its potential for investigating complex biological processes and application in research.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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