Neovascularization after ischemic injury. Evaluation with ^{99m}Tc-HYNIC-RGD[1]

Neovascularização após lesão isquêmica. Avaliação com ^{99m}Tc-HYNIC-RGD

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ABSTRACT
Purpose: Angiogenesis involves many mediators including integrins, and the tripeptide RGD is a target amino acid recognition sequence for many of them. Hindlimb ischemia is a simple and convenient animal model however standardization of the injection procedures in the devascularized and control limb is lacking, thus rendering difficult the interpretation of results. The aim of this investigations was to evaluate neovascularization in a hindlimb murine model by means of ^{99m}Tc-HYNIC-ß-Ala-RGD. **Methods**: ^{99m}Tc-HYNIC-RGD analog was prepared using coligands. Ischemia was induced in *Wistar* rats by double- ligation of the common femoral artery. Radiolabeled RGD was injected after 2h, as well as 1, 3, 5, 7, 10 and 14 days. Uptake was evaluated by planar imaging and biodistribution studies. **Results**: The highest ratio between ischemia and control was achieved at the 7th day (2.62 ± 0.95), with substantial decrease by the 14th day. For pertechnetate the 7th day ratio was 0.87 ± 0.23. Scintigraphic image confirmed different uptakes. **Conclusion**: ^{99m}Tc-HYNIC-RGD analog concentrated in ischemic tissue by the time of widespread angiogenesis and pertechnetate confirmed reduction in blood flow. In this sense, the protocol can be recommended for ischemic models.
Key words: Technetium. Isotope Labeling. Radionuclide Imaging. Ischemia. Diagnosis. Rats.

RESUMO
Objetivo: A angiogênese em resposta a fenômenos isquêmicos envolve vários mediadores como as integrinas, sendo que o tripeptídeo RGD possui uma seqüência de aminoácidos com reconhecimento para este alvo. O modelo animal de isquemia de pata traseira é simples e conveniente, porém não há uma padronização do procedimento de injeção e controle radioisotópico em membro desvascularizado, dificultando, portanto a interpretação de resultados. O objetivo deste estudo foi avaliar a neovascularização em modelo murino de isquemia de pata traseira através do radiotraçador ^{99m}Tc-HYNIC-ß-Ala-RGD. **Métodos**: O análogo ^{99m}Tc-HYNIC-RGD foi preparado usando coligantes. A isquemia foi induzida em ratos Wistar por dupla-ligação da artéria femoral comum na prega inguinal. Peptídeo RGD radiomarcado foi injetado após 2h, assim como 1, 3, 5, 7, 10 e 14 dias. A captação foi avaliada por imagem planar e estudos de biodistribuição. **Resultados**: A maior diferença de captação entre isquemia e pata controle foi obtida no 7º dia (2,62 ± 0,95), com decréscimo acentuado no 14º dia. Para o pertecnetato a razão no 7º dia foi 0,87 ± 0,23. A imagem cintilográfica confirmou as diferentes captações. **Conclusões**: O análogo ^{99m}Tc-HYNIC-RGD concentrou-se no tecido isquêmico na etapa em que a angiogênese é mais acentuada, e o estudo do pertecnetato confirmou a redução no fluxo sanguíneo. Desta maneira, este protocolo diagnóstico pode ser recomendado para modelos isquêmicos.
Descritores: Tecnécio. Marcação por Isótopo. Cintilografia. Isquemia. Diagnóstico. Ratos.

Introduction

Several partially overlapping definitions apply to the development of blood vessels. Neovascularization is the broad physiological response to ischemia and corresponds to blood flow recovery detected *in vivo*.

Angiogenesis is not only involved in cancer progression but also plays an important role in the improvement and healing of ischemic lesions[1]. It is a complex process involving many components as integrins.

Integrins are a notable class of receptor proteins, from the large family of cell adhesion receptors which are involved in cell-extracellular matrix and cell-cell interactions[2].

They consist of two transmembrane glycoproteins represented by non-covalently associated α and ß-units, which are essential for the healing of ischemic lesions. Accordingly, elevated $\alpha_v\beta_3$ integrin expression has been observed in ischemic tissue of the brain, ophthalmological diseases, and muscles[3].

A majority of integrins, including the $\alpha_v\beta_3$ unit, recognize a conserved amino acid sequence, arginine-glycine-aspartic acid (Arg-Gly-Asp or RGD).

Many of the first generation of RGD peptides were low-binding $\alpha_v\beta_3$ integrins, because the molecule was linear and highly susceptible to chemical degradation. That occured due to reaction of the aspartic acid residue with the peptide backbone. Cyclization of the molecule conferred rigidity to the structure improving binding properties, and may serve as a vehicle to carry radionuclides to the integrin $\alpha_v\beta_3$.

RGD analogs have been radiolabeled with different radioisotopes, including, iodine-123 copper-64, indium-111, fluorine-18, technetium-99m, yttrium-90, bromine-76, with the scope of achieving a radiopharmaceutical targeted for angiogenesis processes in both tumors and regenerative neovascularization[4,5].

Technetium-99m (^{99m}Tc) has the advantage of optimal nuclear properties (6h half-life and monochromatic 140 keV photons), along with favorable logistics, being transportable and easily available from ^{99}Mo/^{99m}Tc generators at low cost. The labeling was performed using the precursors ^{99m}Tc-nitrido and ^{99m}Tc- tricarbonyl[6] as well as BFCA 2-hydrazino-nicotinic acid (HYNIC)[4,7].

The most common approach for designing a target-specific ^{99m}Tc agent has been to attach a chelating group to a bioactive molecule, resulting in a combined ligand that can form a complex with ^{99m}Tc in a reduced oxidation state. This procedure is commonly known as a bifunctional approach, and the specific ligands employed as bifunctional chelating agents (BFCAs).

HYNIC has been reported as a BFCA of great interest due to its high efficiency, fast radiolabeling and high radiolabeling yield[7].

The molecule used in the current study is the integrin $\alpha_v\beta_3$-targeted radiotracer composed of a targeting biomolecule, cyclic RGD peptide (RGDyK), and a radiometal chelate. The lysine residue (K) serves as an ideal building block for further chemical conjugation reactions[8]. For the molecule containing radiotracer, a BFCA (HYNIC) was used to attach the metallic radionuclide (Figure 1).

The animal ischemic hindlimb technique has been often employed in neovascularization[9], and other vascular investigations, even though results are occasionally questioned on account of inadequate standardization of the procedure[10].

The aim of this investigations was the use ^{99m}Tc-HYNIC-ß-Ala-RGD as a diagnostic radiotracer to evaluate neovascularization in the extended period of 14 days, in a hindlimb murine model with double arterial ligation.

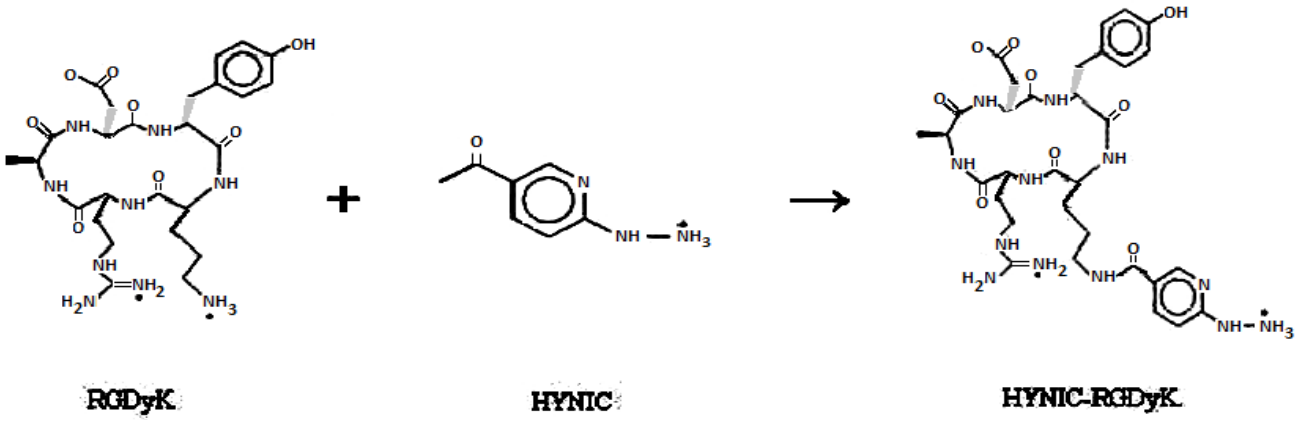


FIGURE 1 – Scheme of HYNIC-RGDyK conjugation.

Methods

Conventional reagents were purchased from Merck, Brazil and Sigma-Aldrich, Brazil, unless otherwise stated, and were used without further purification.

- HYNIC-RGD derivative was synthesized by Biosynthan, Berlin, Germany.

- ^{99}Mo/^{99m}Tc generator: it is routinely manufactured and made available by the Institute of Energetic and Nuclear Research (IPEN-CNEN/Sao Paulo, Brazil).

- Animals for imaging and biodistribution studies: *Wistar* rats were supplied by the animal facility of IPEN-CNEN/Sao Paulo, Brazil

Labeling procedure using EDDA/tricine as exchange products

HYNIC-Peptide labeling procedure was reported before[7]. Briefly, to a sealed reaction vial containing 20 mg Tricine and 5 mg of EDDA it was added 0.5 mL of 0.1M phosphate buffer solution, previously nitrogenated, for the dissolution of the salts. Then 10 µl of a solution 1.32 mM of [c[Arg-Gly-Asp-D-Tyr-(HYNIC)-Lys] plus 5 µL of 8.9 mM $SnCl_2.H_2O$ solution in 0.1N HCl (nitrogen-purged) and 500 µL of $Na^{99m}TcO_4$ was added. The mixture was heated for 15 minutes in water bath at 100°C and cooled to room temperature; the pH of the reaction was 7.

Radiochemical control

Radiochemical analysis of ^{99m}Tc-HYNIC-RGDyK was performed by thin-layer chromatography (TLC) on silica gel strips (ITLC-SG, Gelman Sciences, Ann Arbor, MI) using a two solvent system, namely methylethylketone (MEK) for detection of $^{99m}TcO_4$- and 50% Acetonitrile (ACN) for $^{99m}TcO_2$.

Radiolabeled conjugate was also characterized by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC). This analysis was performed on a Waters 600E system equipped with a Waters 486 tunable absorbance detector, an in-line Packard 150TR flow scintillation analyzer, and a Waters 746 data module. HPLC solvents consisted of H_2O containing 0.1% trifluoroacetic acid (Solvent A) and acetonitrile containing 0.1% trifluoracetic acid (Solvent B). A Symmetry C-18 column (5.0 µm, 100 Å, 4.6 x 250 mm, Waters, Milford, MA) was used with a flow rate of 0.5 ml/min. The HPLC gradient system began with a solvent composition of 95% A and 5% B and followed a linear gradient of 30%A:70%B from 0-25 min, and 30%A:70%B to 5%A:95%B from 25-30 min.

Biological studies

The study was approved by the institutional Animal Welfare Committee, and all procedures were conducted in agreement with the principles of the Brazilian College of Animal Experimentation. The *in vivo* studies were performed in male *Wistar* rats submitted to common femoral artery double occlusion. The study was performed in groups of 6 animals for each time studied.

Surgical technique

The animals were anesthetized (1 mL/Kg IP ketamine and 0.5 ml/Kg IP xylazine) and shaved for surgical intervention. The right femoral vessels were exposed through an inguinal skin incision, and the common femoral artery was carefully separated and double-ligated (Figure 2). The first ligation was done at the common artery proximal to the bifurcation of the deep femoral artery and a second ligation followed on the superficial artery below the bifurcation (Figure 3). After the incision was sutured rats were allowed to recover. By 2h and after 1, 3, 5, 7, 10 and 14 days the radiolabeled peptide was injected.

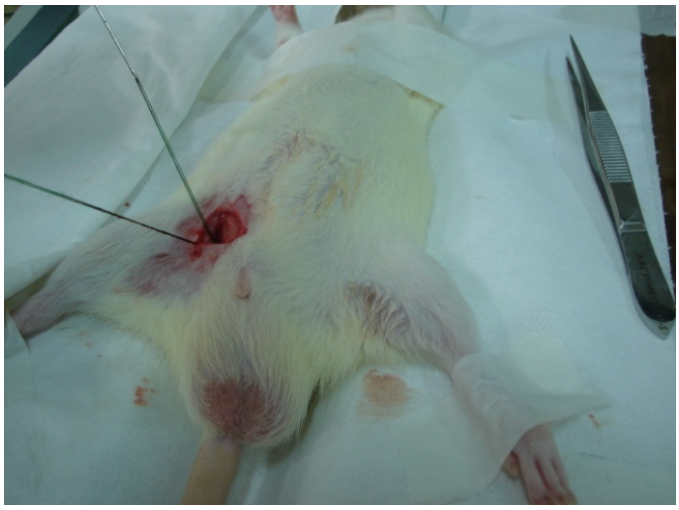


FIGURE 2 – Femoral artery occlusion procedure.

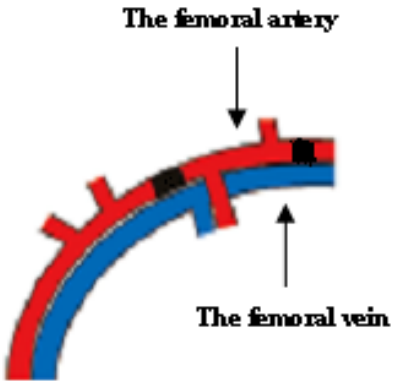


FIGURE 3 – Schematic illustration of femoral artery occlusion[10].

Biodistribution study and imaging evaluation

Each animal was injected with 0.1 mL of ^{99m}Tc-HYNIC-RGD analog via the tail vein. The animals were sacrificed 2 hours post-injection. Evaluation of the uptake by the ischemic limb and normal contralateral limb was done on each occasion. Hindlimb pertechnetate, which has no affinity for regenerating tissue and in the circumstances indicates just blood flow, was also registered for confirmation.

Complete biodistribution assessment was done 7 days after the surgical procedure. The animals were sacrificed by cervical dislocation, and tissues and organs were excised, weighted and radioactivity measured in a gamma counter (Cobra 5002, Packard, USA), using the injected dose as standard for calculation. Results were expressed in percentage of injected dose per gram (%ID/g).

For acquisition of images the rats were anesthetized and horizontally placed under the collimator of a Mediso Imaging System, Budapest, Hungria, employing a LEHR collimator. Images were acquired at 2h post injection using a 256 x 256 x 16 matrix size with a 20% energy window set at 140 keV for a period of 180 seconds.

Ratio between homologous images of the same animal was calculated by means of the Region of Interest (ROI) technique.

Statistical analysis

Discrepancies among the uptakes ratios between of ^{99m}Tc-HYNIC-RGD in ischemic hindlimbs and control side at different times were analyzed by Statgraphics Plus 5.0 (Statistical Graphics Corp., Fairfax, Va., U.S.A.). One-way analysis of variance (ANOVA) followed by post-hoc Tukey test were performed. The adopted significance level (α) was $P<0.05$.

Results

Labeling procedure using EDDA/tricine as exchange products

The conjugate HYNIC-RGDyK was radiolabeled using the exchange labeling technology via tricine and EDDA.

Radiochemical purity of ^{99m}Tc-HYNIC-RGD was 99.45 ± 0.12%. TLC findings were confirmed by HPLC (Figure 4) with a retention time for the product of 12.86 minutes. Only traces (< 0.6 %) of $^{99m}TcO_4^-$ could be detected, with a retention time of 5.33 min. Specific activity was 142.3 MBq/nmol.

Biodistribution studies

Biodistribution of ^{99m}Tc-HYNIC-RGD analog was expressed in %ID/g (Figure 5). Uptake was ordinarily below 1.0 %ID/g. The best values corresponded to kidneys (2.30 ± 0.26 %ID/g), liver, intestine and spleen. Blood uptake (0.07 ± 0.01% ID/mL) showed a good clearance of the radiotracer.

Hindlimb uptake of ^{99m}Tc-HYNIC-RGD can be observed in Table 1. The highest ratio between ischemic hindlimb and control side was achieved at the 7th day (2.62 ± 0.33), with substantial decrease by the 14th day (0.30 ± 0.02). This result was statistically confirmed by one way ANOVA ($p=6.24 \times 10\text{-}24 < 0.05$) and Tukey test.

Pertchnetate uptake in both hindlimbs (control and devascularized), was also documented as a control and the ratio for the 7th day was 0.87 ± 0.23.

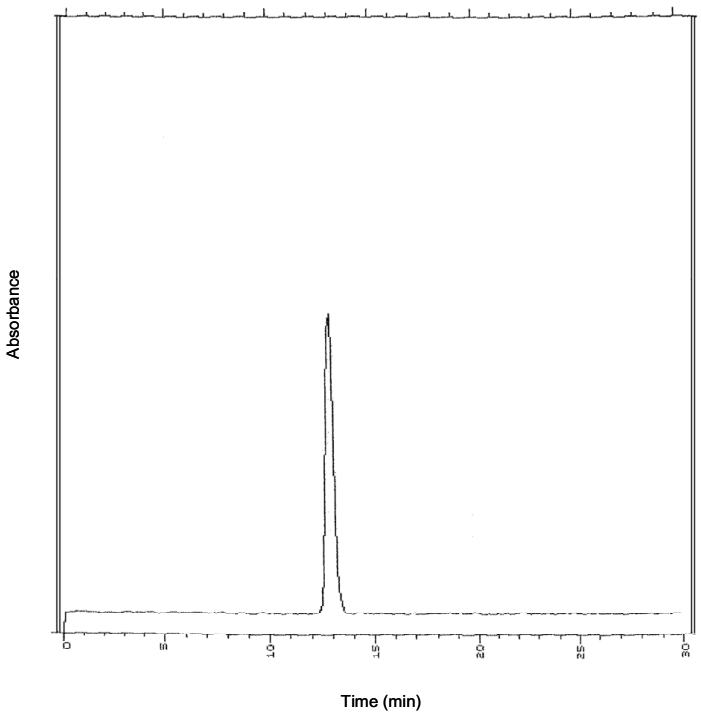


FIGURE 4 – Radio-HPLC elution profile of ^{99m}Tc-HYNIC-RGDyK.

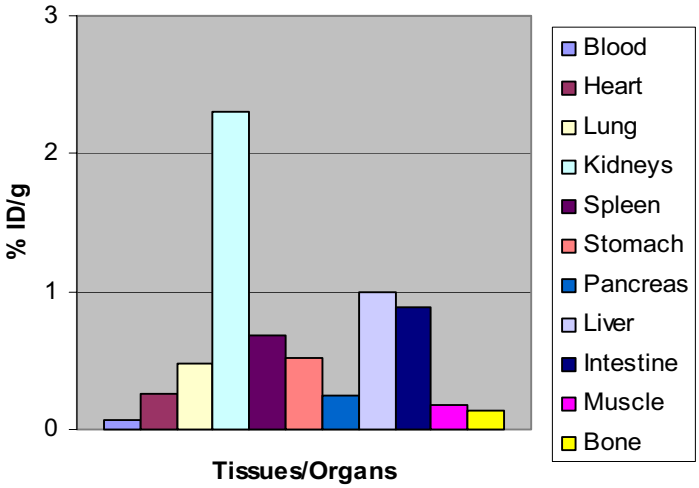
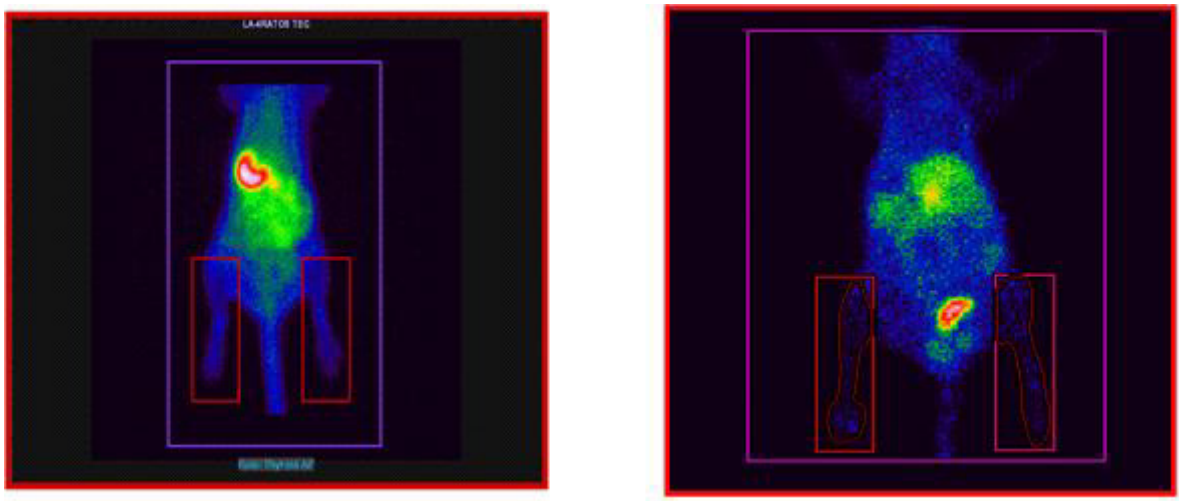


FIGURE 5 – Uptake of ^{99m}Tc-HYNIC-RGD analog in tissues/organs.

TABLE 1 – Uptake of 99mTc-HYNIC-RGD in hindlimbs.

Time after surgical procedure	2 hours	1 day	3 days	5 days	7 days	10 days	14 days
Ischemic Hindlimb (IH)*	0.111±0.015	0.126±0.012	0.169±0.018	0.198±0.008	0.367±0.051	0.083±0.005	0.042±0.004
Control Side* (CS)	0.139±0.004	0.141±0.005	0.142±0.003	0.144±0.003	0.141±0.007	0.139±0.005	0.140±0.006
IH/CS Ratio	0.80±0.12	0.89±0.08	1.19±0.14	1.37±0.04	2.62±0.33	0.59±0.03	0.30±0.02

The images of pertechnetate (A) and ^{99m}Tc-HYNIC-RGD (B) in hindlimbs can be observed in Figure 6. Pertechnetate as a nonspecific marker for new vascularization, didn't show differences between hindlimbs. Ratio of ischemic to normally-perfused hindlimb in the images (B) (2.51), estimated by drawing regions of interest, did not yield much additional information. General values were of the same range as shown by gamma-counter.



(A) **(B)**

FIGURE 6 – (A) Scintigraphic images of $Na^{99m}TcO_4$. **(B)** ^{99m}Tc-HYNIC-RGDyK in ischemic hindlimbs rats on day 7.

Discussion

The major triggers of angiogenesis process can be simplified into three broad categories: mechanical, chemical, and molecular factors[8]. Molecular mediators are not necessarily abundant or present in all phases of the process, but they are specific and can be monitored by radiotracers using imaging or non-imaging techniques.

There are several advantages in using radiolabeled small RGD peptides as radiotracers. They can tolerate harsh conditons for radiolabeling and chemical modification due their small size and rapid blood clearance.

The molecule containing the cyclic sequence RGD usually is designed with more amino acids as fK, fV or yK[4], besides those molecules that compose the multimeric structure to allow the labeling with the radionuclide.

To the sequence RGD it was here added two amino acids, D-Tyrosine and Lysine. Non-natural peptide modifications such as the introduction of D-amino acids, as well as replacement with peptidomimetic structures, tend to grant RGD peptide ligands increased specificity and nanomolar or at least higher affinity.

The radiolabeling of RGD analog using HYNIC as BFCA was easy and quick to perform. Most importantly, it was associated with a high yield and no purification step was needed, so that the radiocompound could be injected right away.

Technetium-99m binds to the hydrazine-moiety forming a ^{99m}Tc-N bond. As HYNIC alone cannot satisfy the coordination requirements of Tc(V) because it occupies one or two coordination sites on the radionuclide, coligands are necessary to complete the coordination sphere of the technetium (V) core.

We had reported before[7] that conjugates prepared by tricine/EDDA exchange labeling exhibit high specific activity and excellent radiochemical stability.

Biodistribution in tissues and organs showed the highest uptake by the kidneys reflecting a renal excretion, followed by liver, intestine and spleen.

Major murine organs, such as liver, spleen and colon showed specific uptake suggesting $\alpha_v\beta_3$ expression in these tissues as announced before by Dijkgraaf *et al.*[2].

The highest uptake ratio of ^{99m}Tc-HYNIC-RGD between ischemic hindlimb and control side was achieved at the 7th day, decreasing in later days probably due to attenuation of the angiogenesis phenomenon.

Lee *et al.*[11] labeled HYNIC-c(RGDyK) with ^{18}F reported that the vascular endothelial growth factor protein expression was maximum on day 7.

Also Hua *et al.*[12] achieved results compatible with ours using ^{99m}Tc-NC100692. Their studies were conducted in mice with NC100692, a product from "Amersham" in which the sequence of amino acids RGD is held in a cycle by a disulphide and a thioether bridge, with a short polyethylene glycol unit. Edwards *et al.*[13] used the same radiotracer, ^{99m}Tc-NC100692, to document the dissociation constant [Kd] for integrin receptors.

For pertechnetate a low ratio was registered because as a passive marker, it does not bind to new blood vessels, confirming just blood flow. The importance of such procedure has not been emphasized in other protocols. The ratio obtained for the 7th day was 0.87 ± 0.23, consistent with reduced perfusion following arterial ligation.

Experimental methods to create hindlimb ischemia are not standardized. Goto *et al.*[10] described a number of methods to create stable hindlimb ischemia in the mouse by occluding the artery, varying from simple ligation, to cutting, or to excision of the artery. The targeted vessel could be the iliac artery, the femoral artery, or the femoral and saphenous trunks. We opted to execute the ligation of the femoral artery in two sites, encompassing

both the common and superficial artery. This modality was easy, convenient and moderately reproducible.

Skjeldal *et al.*[14] reported that, small animals as like mice are endowed with a well-developed innate collateral system, and thus display remarkably high resistance to ischemia. Simple ligation of the femoral artery is known to produce no severe ischemic change.

Within in this context, the animal used in the study (Wistar rats) was not ideal due the well-developed collateral circulation in hindlimb. We achieved only 70% of reproducibility of the model.

The weak image contrast between ischemic and contralateral hindlimb with ^{99m}Tc-HYNIC-RGD is partly due to residual muscle uptake. It does not coincidence with biodistribution results and needs to be improved.

In synthesis, radiotracer assessment demonstrated somewhat less angiogenesis than anticipated. Lee *et al.*[15], employing laser Doppler flowmetry on days 3 and 8 of ischemia, with ^{125}I-c(RGD(I)yV) and then RGD analog labeled with ^{123}I for scintigraphy, were also disappointed by relatively modest image contrast between the hindlimbs.

Conclusions

^{99m}Tc-HYNIC-RGD analog corresponded to expectations, displaying elevated uptake in ischemic tissue by the time of widespread angiogenesis. The animal model showed only 70% of reproducibility. Utilization in other ischemic models can be recommended. The modest image obtained with ^{99m}Tc-HYNIC-RGD does not correspond to a previous result in biodistribution and needs to be improved.

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