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LOWER LIMB PERFUSION SCINTIGRAPHY WITH 99mTc-MIBI
SCINTIGRAPHY AND DETERMINATION OF ENDOTHELIN IN DIABETIC
AND NONDIABETIC PATIENTS

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Abstract
Background / Aim. Peripheral artery disease (PAD) is a common macrovascular complication in patients with diabetes mellitus (DM) as a result of impairment of homeostatic mechanisms of the endothelium, thus initiating the process of atherosclerosis. The imbalance between endothelium-derived vasodilators and vasoconstrictors plays an important role in the pathogenesis of diabetic microangiopathy, as well as in other vascular complications in diabetes. Perfusion scintigraphy using $^{99m}$Tc-MIBI (methoxyisobutilisonitrile) can be very useful method for evaluation of the lower limbs muscle perfusion. 1) To compare the results of dynamic and static studies of lower limbs tissue muscle perfusion scintigraphy with $^{99m}$Tc-MIBI (one-day rest-stress protocol) in patients with and without diabetes mellitus and to determine the perfusion reserve for diagnostic evaluation of peripheral artery disease (PAD) in patients with DM type 2; 2) To assess the correlation of endothelin levels as a vasoconstrictor agent between the two groups (with and without diabetes). Methods. Prospective study was performed in 90 patients, divided in two groups according to the presence of diabetes – patients with DM type 2 (DP) 60/90 (67%), and patients without DM (NDP) 30/90 (33%). Lower limbs tissue muscle perfusion scintigraphy with $^{99m}$Tc-MIBI including two studies (“rest” and “stress”). Results. In diabetic patients group significantly lower pick of radioactivity was detected in comparison with non diabetic group, in both phases (rest and stress), for both calves. Lower counts from the static phase were registered in the region of both calves. Lower inter-extremity indexes as well as perfusion reserve were found in diabetic group. Concentration of ET-1 between groups showed significant difference and higher concentration in diabetic patients. Conclusion. This one-day protocol (rest-stress with $^{99m}$Tc-MIBI) of perfusion scintigraphy of lower limbs is considered a useful procedure in PAD assessment, especially the asymptomatic form.

Key words: perfusion reserve, diabetes, microcirculation, endothelin, lower limbs.

Introduction

Peripheral artery disease (PAD) is a common macrovascular complication in patients with diabetes mellitus (DM) as a result of impairment of homeostatic mechanisms of the endothelium, thus initiating the process of atherosclerosis. The normal, healthy endothelium regulates vascular tone and structure and exerts anticoagulant, antiplatelet, and fibrinolytic properties. The maintenance of vascular tone is accomplished by the release of numerous dilator and constrictor substances. A major vasodilative substance released by the endothelium is nitric oxide (NO), originally identified as endothelium-derived relaxing factor (EDRF). The endothelium also produces vasoconstrictor substances, such as endothelin (the most potent endogenous vasoconstrictor identified to date) and angiotensin II. Angiotensin II not only acts as a vasoconstrictor but is also pro-oxidant and stimulates production of endothelin. Endothelin and angiotensin II promote proliferation of smooth muscle cells and thereby contribute to the formation of atherosclerotic plaque. Activated macrophages and vascular smooth muscle cells, characteristic cellular components of atherosclerotic plaque, produce large amounts of endothelin.

The imbalance between endothelium-derived vasodilators and vasoconstrictors initiates a number of events/processes that promote or exacerbate atherosclerosis; these include increased endothelial permeability, platelet aggregation, leukocyte adhesion, and
generation of cytokines. Decreased production or activity of NO, manifested as impaired vasodilation, and increased production of endothelin, may be one of the earliest signs of atherosclerosis. All these processes play an important role in the pathogenesis of diabetic microangiopathy, as well as in other vascular complications in diabetes. Development of endothelial dysfunction involves several biological mediators including increased expression of endothelin (ET)-1 and altered expression of ET receptors. Increased endothelial ET-1 expression enhances lipid biosynthesis and accelerates the progression of atherosclerosis.

There are a number of diagnostic procedures that, according to the accepted protocols for this vasculopathy, are successively involved in different levels of diagnosis. Despite good anatomic information for the large arteries provided by computed angiography, it is insufficient for the small vessels perfusion. Perfusion scintigraphy using ⁹⁹mTc-MIBI can be very useful for evaluation of the lower limbs muscle perfusion. After intravenous application, ⁹⁹mTc-MIBI is rapidly cleared from the circulation and preferentially is accumulated in muscular tissues (including heart) proportionally to regional blood flow. These characteristics of ⁹⁹mTc-MIBI make it very suitable for examining regional blood flow, visualization with gamma camera, as well as getting quantitative parameters for regional blood flow changes, including quantitative assessment of tissue perfusion in basal conditions ("rest" study) and after workload ("stress" study).

**Aim**

1) To compare the results of dynamic and static studies of lower limbs tissue muscle perfusion scintigraphy with ⁹⁹mTc-MIBI (one-day rest-stress protocol,) in patients with and without diabetes mellitus and to determine the perfusion reserve for diagnostic evaluation of peripheral artery disease (PAD) in patients with DM type 2;

2) To assess the correlation of endothelin levels as a vasoconstrictor agent between the two groups (with and without diabetes).

**Methods**

Lower limbs tissue-muscle perfusion scintigraphy of (TMPS) was performed, through this one-day rest-stress protocol with ⁹⁹mTc-MIBI. The study has been approved by the Ethics Committee and all subjects signed double informed consent form. This was a prospective study performed in 90 patients, divided in two groups according to the presence of diabetes – patients with DM type 2 (DP) 60/90 (67%), and patients without DM (NDP) 30/90 (33%). In the non-diabetic group, 10 (33.33%) patients had hypertension – HTA, 8 (26.67%) were obese, 7 (23.33%) had hyperlipidemia - HLP and 6 (20%) were smokers. Analyzing the symptoms 18 (60%) had calf pain, 11 (36.67%) complained of numbness, and 7 (23.33%) had cold lower extremities. In DP 44 (73.33%) had HTA, 26 (43.33%) HLP, 20 (33.33%) were smokers, and 50% were obese. 48 (80%) patients had calf pain, 34 (56.67%) had numbness and 24 (40%) complained of cold legs.

**⁹⁹mTc-MIBI scintigraphy**

Lower limbs tissue muscle perfusion scintigraphy with ⁹⁹mTc-MIBI is a non-invasive, functional method that evaluates tissue perfusion in resting condition ("rest" study) and after workload ("stress" study), as visually, as well as through several quantitative parameters.
Tissue muscle perfusion studies were done with planar technique, with two-headed gamma camera (DHV MEDISO Nucline SPIRIT), low energy high resolution collimator (LEHR). Before the initiation of the rest study the patient was positioned in resting mode for 20-30 minutes (separate isolated room was used to avoid external influence and the patients were instructed to remain in a horizontal position during this period of resting mode). Rest study was started with a dynamic phase of tissue-muscle vascularization of both calves after i.v application of 300 MBq of $^{99m}$Tc-MIBI, (the rest study time interval was 7 minutes, consisted of 28 frames, with time interval 15s per frame) (Fig.1), followed with a whole body scan (WBS) for tissue perfusion of the whole body in posterior position (PA), matrix size 512x1024x16, speed 15cm/min. Stress study was carried out afterwards and the patient was instructed to perform 30 flexion/extensions of both feet, followed by i.v application of 600 MBq $^{99m}$Tc-MIBI, when the dynamic phase was started with the same acquisition protocol as in the rest study (Fig. 2). After application of the radiopharmaceutical, the patient performed another 30 flexion/extension of the feet. WBS was performed afterwards (with the same acquisition as in the rest study) (Fig. 3).

With quantitative analyses of the dynamic phase, radioactive curves were constructed in a time manner (time activity curve – TAC) above the region of interest (Fig. 2), positioned above both calves and these parameters were investigated:
* $T_{\text{maximum}}$ (Tmax): time of maximal uptake of the tracer in each calf and impulses collected in Tmax
* $\text{Radioactivity in 1\text{-}st\text{ minute in calves}}$: (radioactivity above calf in 1\text{-}st minute) x 100 / maximal radioactivity above calf
With quantitative analyses of WBS, with registered impulses in the ROI, positioned over calves and the whole body these indexes were evaluated:
* $\text{Radioactivity in calves}$: accumulated impulses in both calves in both studies, after drawing symmetrical ROI (Fig. 3)
* $\text{Intra-extremity index}$: (for both studies) left calf/left ankle (LC/LA) and right calf/right ankle(RC/RA).
* $\text{Index calf/whole body}$: (for both studies) left calf/whole body and right calf/whole body
* $\text{Perfusion reserve (PR %)}$ for both calves: as a percent of grow of tissue blood flow in stress study, in comparison with rest study, calculated with the formula.

\[
\text{PR (\%)} = \frac{(\text{radioactivity in calf in stress} - \text{radioactivity in calf in rest})}{\text{radioactivity in calf in rest}} \times 100\%
\]

$\text{Endothelin-1 measurements}$

For the determination of endothelin-1 in our study we used a commercial RIA by the manufacturer Phoenix Pharmaceuticals, Inc. After blood withdrawal, the samples were centrifuged and the serum was stored in a refrigerator at -20°C until analyses performed simultaneously for all samples. ET-1 measurements were taken by a competitive radioimmunoassay. The method is based on a competitive reaction of the analyte (endothelin-1 in the test sample) and the radiolabelled endothelin ($^{125}$I-endothelin) in the kit, for the limited amount of antibody-specific antibodies in each of the test tubes. According to the competitive conditions, there is an inverse correlation of the bound radioactivity in the formed immune complex and the concentration of the analyte (endothelin-1). The
procedure for the determination of endothelin-1 was carried out in accordance with the conditions and protocol prescribed by manufacturer.

Results

In diabetic group patients had significantly lower pick of radioactivity detected in the dynamic phase in comparison with control group, in both studies (rest and stress), for both calves (Table 1). The number of impulses in the 1st minute for both calves was also significantly lower in DP in both studies as well (Table 2.).

The accumulated counts in the region of both calves was insignificantly lower in DP compared to NDP in the rest study and significantly lower in the stress study (p=0.03; p=0.018 consequently). The counts accumulated in the rest study were for LC 16967.78±3520.9 in DP vs. 17726.83±3285.3 in NDP, while for RC 17228.07±4287.5 in DP vs. 17772.87±3242.2 in NDP. In the stress study total counts for the LC were 75546.95±15864.5 in DP, 84098.9±19954.7 in NDP and in the RC 75059.9±14851.9 in DP, 83972.8±19489.8 in NDP.

The mediana for intra-extremity index of left and right calf was lower for diabetic patients, without significance, (Table 3). Non-significant index of calf/whole body was registered in both studies for both calves (Table 4).

Perfusion reserve (PR) of calves (LC, RC) was calculated with the formula “(ROI stress-ROI rest) × 100% / ROI rest”. The results showed insignificantly lower PR of LC in DP compared to NDP (40.25 ± 14.7 vs. 44.77 ± 10.3 in ND, p=0.32) and significant difference in the PR of RC was registered in the diabetic patients (40.02 ± 11.2 vs 44.53±10.5 in NDP, p=0.045).

Concentration of ET-1 between groups showed significant difference and higher concentration in diabetic patients (Table 5).

Discussion

Diabetes mellitus is a chronic disease caused by impaired insulin secretion or insulin resistance. Peripheral arterial disease in diabetes is a consequence of an atherogenic process in the lower limb arteries accelerated by multifactorial pathophysiologic mechanisms underlying DM. This process is accompanied also with atherotrombosis in vasculature of other organs including coronary and cerebrovascular system. Having in mind all complications arising from this pathological condition it is of great clinical significance to recognize the early abnormalities in the peripheral circulation. The precise assessment of the prevalence of PAD in diabetic patients is aggravated by the high prevalence of asymptomatic forms, peripheral neuropathy, and the absence/impared function of pain perception, as well as the present limitation of screening methods for its diagnosis. Therefore, in the resolution of asymptomatic and subclinical forms of PAD in these patients, both preventive and diagnostic and curative medical procedures should always be included.

For this purpose in nuclear medicine 99mTc-labelled perfusion tracers are used to provide better image quality as well as quantitative processing of the scans. Radiopharmaceutical that was used in our study, 99mTc-MIBI, is a lipophilic cationic component that injected into animals is distributed into the tissues proportionally to blood flow and is retained in the mitochondria. Given the negative plasma membrane potential
and even more negative mitochondrial membrane potential, both potentials contribute to a strong driving force for $^{99m}$Tc-MIBI accumulation and sequestration in the mitochondrial matrix. Studies showing that $^{99m}$Tc-MIBI cultured myocardial cells accumulate 1000 times more in mitochondria than in the cytosol, have contributed to its wide application in the field of nuclear cardiology.$^{8}$ Biodistribution and kinetics of the $^{99m}$Tc-detected components allowed combining myocardial perfusion with perfusion of the lower limbs.

The results from our study clearly pointed to abnormal microvascular perfusion in the affected regions of lower limbs, while the quantification of the tested parameters indicated the extent of perfusion insufficiency. Lower number of accumulated counts was detected in both calves for both phases in the diabetic patients. In rest phase of the left calf total count number was $75546.95 \pm 15864.5$ in DP, and $84098.9 \pm 19954.7$ in ND. And for the right calf the total count number was $75059.9 \pm 14851.9$, and $83972.8 \pm 19489.8$ consequently for both groups. Still significant decrease of the counts was registered in the stress phase only, due to reactive hyperemia. This is a state when under resting conditions, the limb used all possible resources for blood supply and self-protection from ischemic consequences, such as collateral circulation and, vasodilator response under the action of stimuli that are excreted in response to hypoxia or steel phenomenon, so under loading conditions it is unable to raise the blood flow to a higher level in order to provide an appropriate metabolic response to the effort.

Perfusion of the lower extremities was also performed in the study of Taillefer in 35 patients using method of post-occlusive reactive hyperemia and resting state. Regions of interest over both thighs and calves were drawn in PA position of imaging, and afterwards inter- and intra-extremity index were calculated. Paradoxically larger uptake showed muscle blood supply from significantly stenosed blood vessels, which resulted in false positive and false negative results.$^{9}$

In 2001 Cosson et al., investigated by thallium-201 scanning, circulation in the muscles of the lower limb in diabetic patients without clinical peripheral vascular disease but with a high cardiovascular risk profile and suggested that scanning of the lower limbs coupled with myocardial scintigraphy is a convenient method of investigating peripheral muscle circulation. They found muscle perfusion defects in 42% of the patients, mainly in the calves.$^{10}$

Significantly lower PR of diabetic patients (without peripheral artery disease) versus control group (without DM) $70.2 \pm 10.7\%$ и $98.6 \pm 9.4\%$ were registered in 2004 by Lin and co-workers. They used method of 60 plantar and dorsal flexions of the right foot and calculated the perfusion reserve by the formula $PR = (ROI \text{ right foot} – \text{left foot}) / ROI \text{ (right foot)} \times 100\%$.  

Lower extremity ischemic disease assessed by thallium-201 was also used by Cizmic in evaluation of diabetic angiopathy. Their results of lower extremities perfusion scintigraphy showed reliable indice of muscle microcirculatory perfusion, with statistically significant correlation between the Doppler hemodynamic indices and thallium-201 perfusion scintigraphy.$^{12}$

Younes, in 2017, performed 30-40 dorso-plantar flexions and extensions of the right foot in sitting position and afterwards ROI were drawn over both calves. Using the formula $PR = \text{Stress (right foot)} – \text{Rest (left foot)} / \text{ROI (left foot)} \times 100\%$ significantly lower PR was detected in patients with peripheral artery disease versus control group $28.4 \pm 20.3\%$ vs. $65.0 \pm 11.4\%$, $P < 0.001$.  


Perfusion muscle scintigraphy of the lower limbs can help in the algorithm for starting using more invasive diagnostic methods such as angiography. In 2007, Soyer published a case of a patient with intermittent claudication in one leg, a preserved circulation evaluated by the Doppler technique, a striking reduction in perfusion in stress phase recorded with $^{99m}$Tc-MIBI muscular scintigraphy and the detection of multiple stenosis with peripheral arterial angiography. Additionally, through the visual analysis of the scans it is possible to locate regions with impaired microvascular circulation, which would contribute to the appropriate therapeutic modalities.

In our case report of diabetic patient in 2016 we performed TMPS and confirmed diabetic angiopathy in both calves, with a borderline value for perfusion reserve of the left calf 57%, and a lower perfusion reserve of the right calf 42% (reference values 50-80%).

Tan and co-workers used two-day protocol of $^{99m}$Tc-MIBI tissue-muscle perfusion scintigraphy in patients with Behcet disease, using pharmacologic stress plus adding 30 plantar flexions and extensions of the feet. PR calculated with the formula $\text{PR} \% = ((\text{ROI stress} - \text{ROI rest})/ \text{ROI rest}) \times 100\%$. They got significantly lower PR in the control group - 3.34±8.7%, vs. 8.6±8.5%.

The detection of PR with the method of TMPS was used in patients with rheumatoid arthritis, as a screening tool in the evaluation of the atherosclerotic process by Amin in 2012. Higher PR were noticed in the control group vs. patients with RA (48.3 ± 27.2% vs. 30.7 ± 22.6%, P=0.015).

The concentrations of ET-1 showed significant higher mean values in the group of diabetic patients versus the control group, which is consistent with the pathogenetic mechanisms for the involvement of ET-1 in the onset of microangiopathy. In that context, in several studies it was found that vascular endothelial dysfunction may precede type 2 diabetes, implying that elevated levels of ET-1 can be part in the development of the metabolic syndrome, mainly through reduction of insulin sensitivity. Considering the conducted studies, it was found that ET-1 increase the production of reactive oxygen species (mainly superoxide anions) and thus contributes to the endothelial activation and consecutive endothelial dysfunction in vascular endothelial cells as the main place of ET-1 production. Also, increased circulating levels of ET-1 may promote the initiation and progression of atherosclerosis by inhibiting endogenous NO production in VSMCs, through its inhibitory effect endothelial nitric oxide synthase (eNOS), and additionally contributes to the development of microcirculatory disorders.

Conclusion

This one-day protocol (rest-stress with $^{99m}$Tc-MIBI) of perfusion scintigraphy of lower limbs is considered a useful procedure in PAD assessment, especially the asymptomatic form. The investigation of the functional haemodynamic parameters are important for relevant guidance, treatment and risk stratification of patients with PAD.
References

Table 1. Number of impulses accumulated at the peak of radioactivity for both calves

<table>
<thead>
<tr>
<th>Peak of radioactivity</th>
<th>group</th>
<th>REST</th>
<th>STRESS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean±SD</td>
<td>mean±SD</td>
<td></td>
</tr>
<tr>
<td>Tmax RC</td>
<td>DP</td>
<td>2158.75 ± 410.6</td>
<td>7223.62 ± 1383.4</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>2427.40 ± 278.8</td>
<td>8019.47 ± 946.3</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.0018**</td>
<td>0.0057**</td>
</tr>
<tr>
<td>Tmax LC</td>
<td>DP</td>
<td>2234.75 ± 423.7</td>
<td>7240.07 ± 1673.8</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>2445.43 ± 384.1</td>
<td>7995.53 ± 1098.3</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.024*</td>
<td>0.028*</td>
</tr>
</tbody>
</table>

p(Student t-test for independent samples) *p<0.05  **p<0.01
RC = right calf, LC=left calf, DP=diabetic patiens, NDP = non diabetic patients

Table 2.

Number of counts accumulated in the 1st minute of dynamic phase

<table>
<thead>
<tr>
<th>T1min</th>
<th>group</th>
<th>REST</th>
<th>STRESS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean±SD</td>
<td>mean±SD</td>
<td></td>
</tr>
<tr>
<td>RC</td>
<td>DP</td>
<td>1949.32 ± 404.9</td>
<td>6752.88 ± 1248.6</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>2230.87 ± 284.4</td>
<td>7671.73 ± 978.1</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.001**</td>
<td>0.00068**</td>
</tr>
<tr>
<td>LC</td>
<td>DP</td>
<td>2048.45 ± 435.1</td>
<td>6924.87 ± 1314.9</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>2248.6 ± 442.1</td>
<td>7646.87 ± 1080.5</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.044*</td>
<td>0.011*</td>
</tr>
</tbody>
</table>

p(Student t-test for independent samples) *p < 0.05  **p < 0.01
RC = right calf, LC=left calf, DP=diabetic patiens, NDP = non diabetic patients

Table 3.

Intra-extremity index for both calves in both studies (rest and stress)

<table>
<thead>
<tr>
<th>variable</th>
<th>group</th>
<th>REST</th>
<th>STRESS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean±SD</td>
<td>median</td>
<td>mean±SD</td>
</tr>
<tr>
<td>LC/LA</td>
<td>DP</td>
<td>82.17 ± 23.72</td>
<td>74.47</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>82.79 ± 23.31</td>
<td>88.09</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.7 ns</td>
<td>0.2 ns</td>
</tr>
<tr>
<td>RC/RA</td>
<td>DP</td>
<td>84.48 ± 29.09</td>
<td>79.86</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>81.65 ± 19.08</td>
<td>83.23</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.86 ns</td>
<td>0.43 ns</td>
</tr>
</tbody>
</table>

p(Mann-Whitney test) **p < 0.01
LC/LA = left calf/left ankle, RC/RA=right calf/right ankle, DP=diabetic patiens, NDP = non diabetic patients
Table 4.

Index calf/whole body

<table>
<thead>
<tr>
<th>variable</th>
<th>group</th>
<th>REST mean ± SD</th>
<th>STRESS mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC/WB</td>
<td>DM</td>
<td>1.98±0.4</td>
<td>3±0.5</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>1.78±0.3</td>
<td>3.05±0.6</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.23</td>
<td>0.69 ns</td>
</tr>
<tr>
<td>RC/WB</td>
<td>DM</td>
<td>2±0.4</td>
<td>2.98±0.5</td>
</tr>
<tr>
<td></td>
<td>NDP</td>
<td>1.8±0.3</td>
<td>3.05±0.6</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.24</td>
<td>0.55 ns</td>
</tr>
</tbody>
</table>

p(Student t-test for independent samples) **p < 0.01
LC/WB = left calf/whole body, RC/WB=right calf/whole body,
DP=diabetic patients, NDP = non diabetic patients

Table 5.

Endotelin concentration

<table>
<thead>
<tr>
<th>variable</th>
<th>DP</th>
<th>NDP</th>
<th>p-level</th>
</tr>
</thead>
<tbody>
<tr>
<td>endothelin</td>
<td>mean±SD</td>
<td>mean±SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>105.22±8.8</td>
<td>98.58±8.6</td>
<td>p=0.042*</td>
</tr>
</tbody>
</table>

p (Student t-test for independent samples) *p < 0.05
Fig. 1– Dynamic phase of both calves in rest study.
Fig. 2 – Dynamic phase of both calves in stress study.
Fig. 3 – Whole body scan in stress study.