Assessment of gait dynamics in rats submitted to limb ischemia[1]

Avaliação da dinâmica da marcha em ratos submetidos à isquemia de membro

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ABSTRACT
PURPOSE: To describe a method for the assessment of gait dynamics in rats submitted to limb ischemia.
METHODS: Twenty-four male Wistar rats (150-160g) were used. Twelve animals were submitted to limb ischemia by ligation of the common left iliac artery (ischemic group: n = 12); and a sham-operated group was used as control (n=12). After a recovery period of 6 weeks, gait dynamics was assessed by counting the complete footprints and the number of hindlimb-floor contacts during a treadmill test for five minutes at a speed of 12 $m.min^{-1}$ and angulation of 15°. The number of contacts of the left hindlimb was divided by the right hindlimb values (LRR) for group comparisons. Ischemic disability was quantified by comparing the area under curve (AUC) created by plotting each contact versus time for each hindlimb. The left hindlimb ischemic disability index (LHDI), which was compared between groups, was defined by the formula: $LHDI = (1- AUC_{left} / AUC_{right}) \times 100$.
RESULTS: Surgery was well tolerated by all animals. Rats did not suffer tissue loss or ulcerations. Complete footprint LRR was 0.3 ± 0.08 for the ischemic group and 1.3 ± 0.9 for controls (p=0.0043). Number of contacts LLR was 0.5 ± 0.2 for the ischemic group and 1.0 ± 0.1 for the control group (p=0.0051). LHDI was 56.83 ± 10.67 for the ischemic group and 2.50 ± 13.10 for the control group (P = 0.031).
CONCLUSION: Assessment of gait dynamics in rats submitted to limb ischemia could be done by footprint analysis and hindlimb contact recording during a treadmill test.
Keywords: Intermittent Claudication. Peripheral Arterial Disease. Reperfusion Injury. Rats.

RESUMO
OBJETIVO: Descrever um método para avaliar a dinâmica da marcha em ratos submetidos à isquemia de membro pélvico.
MÉTODOS: Vinte e quatro ratos Wistar machos (150-160g) foram utilizados neste estudo experimental. Doze animais foram submetidos à isquemia de membro pélvico por meio da ligadura da artéria ilíaca comum esquerda (grupo isquêmico: n=12); e doze animais foram submetidos à cirurgia simulada e usados como controle (grupo controle: n=12). Após seis semanas de recuperação, foi realizada avaliação da dinâmica da marcha por meio da contagem de impressões plantares e da contagem de contatos pata-solo durante teste com esteira durante cinco minutos, velocidade 12 $m.min^{-1}$ e angulação de 15°. Os valores do número de contatos do membro pélvico esquerdo foram divididos pelos do membro pélvico direito (razão esquerda-direita - LRR) para comparação entre os grupos. A quantificação da incapacitação isquêmica foi feita comparando a área sob a curva (AUC) da representação gráfica dos contatos versus tempo para cada membro pélvico. O índice de incapacitação isquêmica do membro pélvico esquerdo (LHDI), que foi comparado entre os grupos, foi definido pela fórmula: LHDI = (1- AUC esquerda / AUC direito) x 100.
RESULTADOS: A cirurgia foi bem tolerada por todos os animais. Nenhum rato apresentou necrose tecidual ou ulceração. A LRR das impressões plantares completas foi 0,3 ± 0,08 no grupo isquêmico e 1,3 ± 0,9 no grupo controle (p=0,0043). A LRR do número de contatos foi 0,5 ± 0,2 no grupo isquêmico e 1,0 ± 0,1 no grupo controle (p=0,0051). O LHDI foi 56,83 ± 10,67 no grupo isquêmico e 2,50 ± 13,10 no grupo controle (p=0,031).
CONCLUSÃO: Avaliação da dinâmica da marcha em ratos submetidos à isquemia de membro pélvico pôde ser feita por meio da contagem de impressões plantares e da contagem de contatos pata-solo durante teste com esteira.
Descritores: Claudicação Intermitente. Doença Arterial Periférica. Traumatismo por Reperfusão. Ratos.

Introduction

Peripheral arterial disease (PAD) affects 4% to 12% of people aged 55 to 70 years and 20% of people over 70 years[1,2]. It is associated with *atherosclerosis obliterans*, manifested clinically by pain during deambulation and, in more advanced cases (critical ischemia), characterized by pain at rest, ischemic ulcer and gangrene which eventually may lead to limb amputation[3-5]. The symptomatic patients require pain relief and prevention of thrombotic complications[6]. Intermittent claudication (IC), a cyclic pain in the legs or buttocks that occurs with exercise and subsides with rest, is the most common debilitating symptom of PAD.

There is good evidence that exercise improves IC in the absence of biochemical or physiological changes[7]. IC patients submitted to physical training frequently show improvement without a correlated elevation in O_2 uptake. This suggests that their gait becomes more efficient, probably due to biomechanical changes[8]. A significant reduction in ground contact area in ischemic limbs has been noted[9].

Recent clinical studies have pointed out a lack of reliable experimental models to allow investigations of the physiopathological basis of PAD, the effects of exercises on it, the use of conservative and interventionist therapies, and its local and systemic effects[10-14]. Rat models of PAD have been obtained using the ligature of the common iliac artery. Hemodynamically, this model is characterized by a pressure fall of 49% and 59% in the flow of the femoral artery, from the third to the ninth week post-ligature despite the development of collateral circulation[15,16].

The functional characteristics after reperfusion of ischemic muscles have been evaluated by measuring the time to muscle exhaustion after electrical stimulation of the motor nerve[17]. Contreras *et al*.[18] studied spatio-temporal sequences of hindlimb movements during gait of ischemic rats. The animals were recorded by means of a single commercial digital video camera located on one side of a transparent acrylic passageway.

Some alterations in the gait patterns in an animal model of hyperalgesia, such as reduction of the contact area of the affected hindlimb when the animals step on the floor, have been previously reported[19], and it is reasonable to assume that this method might be used to assess gait patterns after ischemia.

In the present study we propose a method for the assessment of the gait dynamics of rats suffering from PAD brought on by common left iliac artery ligation.

Methods

Twenty-four male Wistar rats (University of Sao Paulo, Ribeirao Preto, Brazil) weighing 100 to 150g and clinically healthy were used for this study. Animals were maintained in a room with controlled temperature and light and were provided food and water *ad libitum*. Animal care complied with the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC, National Academy Press, 1996). All protocols were approved by the Animal Experimentation Ethics Committee of the University of Sao Paulo, Ribeirao Preto, Brazil.

In order to become habituated to the treadmill protocol, animals were submitted to progressive training for three consecutive days. On the first day, animals were set on the treadmill without movement for 10 minutes. On the second day, they were exercised for 10 minutes at a progressive speed ranging from 5 to 12 $m.min^{-1}$. On the third day, they were exercised at a speed of 12 $m.min^{-1}$ and preoperative gait recordings were obtained. Rats that did not adapt to this protocol were excluded.

The experimental design consisted of two groups. In 12 rats, surgical interruption of the common left iliac artery was performed (ischemic group), and 12 sham-operated rats were used as control.

Anesthesia and surgery

Rats were anesthetized with an intraperitoneal injection of a solution containing 2.5% of tribromoethanol (26 mg/kg, Aldrich Chemical Company – USA). Hair was removed from the abdominal wall with a depilating cream, with care taken to avoid erythema. The common left iliac artery was exposed aseptically through median laparotomy and isolated from the common left iliac vein and nerves, with care taken to avoid damage to vessels or nerves. The artery was ligated with 3-0 cotton suture just distal to the bifurcation of the abdominal aorta.

As a consequence, blood flow to the distal limb becomes completely dependent on the collateral vessels. Control animals underwent sham operations consisting of all surgical procedures except for artery ligation.

Evaluations were performed 6 weeks after surgery. This interval was chosen because the common left iliac artery ligation produces the lowest pressure levels after 6 weeks and no tissue loss or ulcerations were observed[20].

Footprint analysis

Footprint analysis was performed by plunging both hindlimbs into black ink after submitting them to spontaneous exercise in the treadmill for five minutes at a speed of 12 $m.min^{-1}$ and angulation of 15°. Rats were then transferred onto an absorbing paper and walked inside a 10 x 10 x 100 cm tube in order to follow a straight line on a flat surface and at spontaneous gait speed.

The number of complete or incomplete footprints was recorded. The footprint was considered complete if the entire hindlimb surface was printed on the path surface; and incomplete if the posterior area of the hindlimb was not clearly printed. The observer was blinded to both groups. For comparison, the number of complete left footprints was divided by the right ones, originating a left-right ratio (LRR).

Computed treadmill gait analysis

A computed treadmill developed in the Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Brazil, was used for gait analysis. It consisted of a moving belt made of a stainless steel mesh, which transmitted electric impulses (Figure 1). Light electrodes (0.1g) were symmetrically attached to the hindlimbs of each animal with adhesive tape. As animals walked on the stainless steel mesh, contacts between each hindlimb and the moving surface were registered. Animals were placed on the treadmill and exercised spontaneously for five minutes at a speed of 12 $m.min^{-1}$ and angulation of 15°. Time and number of contacts were obtained. Data were analyzed with specially developed software. The gait pattern of the animals was analyzed by plotting each contact versus time for each hindlimb. For comparison, left hindlimb number of contacts (NC) values were divided by the right hindlimb ones, originating a left-right ratio (LRR). Ischemic disability was defined as the deficiency of the ischemic limb to contribute to animal gait. Ischemic disability was quantified by comparing the area under curve (AUC) created by plotting each contact versus time for each hindlimb. The left hindlimb ischemic disability index (LHDI), which was compared between groups, was defined by the formula: $LHDI = (1 - AUC_{left} / AUC_{right}) \times 100$.

FIGURE 1 - Computed treadmill utilized for gait analysis.

Statistics

All variables are reported as mean ± standard deviation. Values for the same group were compared by the Wilcoxon test and Groups were compared by the Mann-Whitney test. $p<0.05$ was considered significant.

Results

Surgery was well tolerated by all animals. Mean weight gain six weeks after surgery was not significantly different. Rats did not suffer tissue loss or ulcerations.

Footprint analysis

The control group showed symmetric hindlimb and plantar footprint morphology. Most of the control animals touched the surface only with the anterior region of both hindlimbs, leading to a prevailing pattern as they walked on the treadmill belt. The mean number of incomplete footprints did not differ significantly between the left (8.33 ± 0.81) and right (8.66 ± 0.81) hindlimbs of the control group. However, in the ischemic group, the number of complete footprints was significantly higher for the right foot (6.66 ± 1.50) than for the left foot (2.16 ± 0.75) (p=0.002) (Figure 2). Mean complete footprint LRR was 0.3 ± 0.08 in the CLI group and 1.3 ± 0.9 in the controls (p=0.0043).

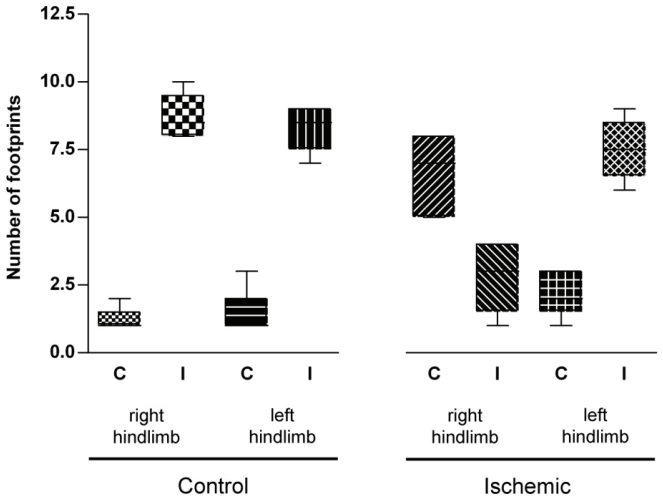

FIGURE 2 - Number of footprints after exercise in the treadmill of rats of Ischemic and Control groups. C- Complete footprints; I – Incomplete footprints. (—) mean; (□) confidence interval; (‡) standard deviation.

Computed treadmill gait analysis

NC values are presented in Figure 3. In the ischemic group, there was a decrease in mean left hindlimb (ischemic) NC (219 ± 84) compared to the right hindlimb (477 ± 52). In the control group, NC did not differ between hindlimbs. There was no significant difference in NC values for the right hindlimb between ischemic rats (454 ± 69) and controls (477 ± 52) (p=0.58). LLR for NC was 0.5 ± 0.2 in the ischemic group and 1.03 ± 0.1 in the control group.

LHDI was 56.83 ± 10.67 in the ischemic group and 2.50 ± 13.10 in the control group (p=0.031) (Figure 4).

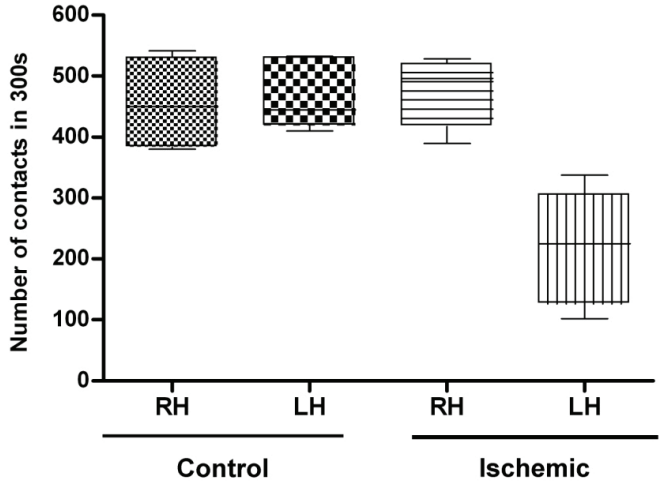

FIGURE 3 - Number of contacts in 300 s of righ hinbdlimb (RH) and left hindlimb (LH) of rats of Ischemic and Control groups. (—) mean; (□) confidence interval; (‡) standard deviation.

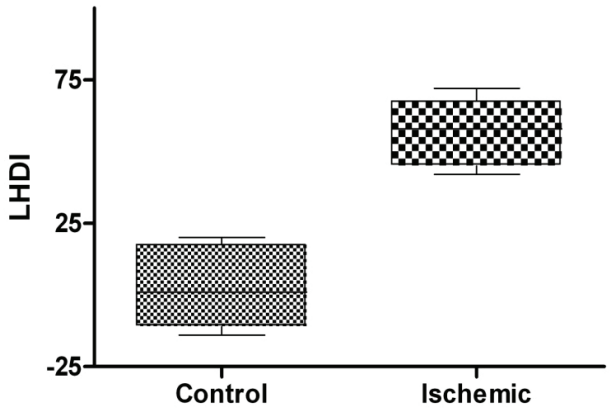

FIGURE 4 - Left hindlimb disability index (LHDI) in rats of Ischemia and Control groups. (—) mean; (□) confidence interval; (‡) standard deviation.

Discussion

The initial purpose of our study was to identify any change in gait patterns due to unilateral ligation of the common iliac artery in rats. Rochester *et al*.[9] defined six weeks as the period of time needed to establish chronic limb ischemia due to femoral artery stenosis. Common left artery ligation was chosen and caused no major tissue loss or pain that would affect food intake by the animals. Such procedure allows comparing the ischemic limb to the normal one during the treadmill test. Similar to a previous study[21], the plantar impression of the ischemic group was therefore asymmetric compared to control animals. Data obtained from plantar impression revealed that the posterior region of the ischemic hindlimb was spared producing incomplete impressions, while the contra-lateral hindlimb produced complete impressions. The sham group showed prevailing incomplete impressions of both hindlimbs. The overload of the contralateral hindlimb was the mechanism used to spare the injured hindlimb. This model cannot be considered analogous to human limb claudication because rats use four limbs, but can be a possible model to test evolving therapies.

The severe ischemia brought on by common iliac artery ligation justifies the lower treadmill speed compared to other studies.

In the present study, control rats showed symmetric hindlimb and plantar footprint morphology. Most of the control animals touched the surface only with the anterior region of both hindlimbs, leading to a prevailing pattern as they walked on the treadmill belt. The number of incomplete footprints in the left and right hindlimbs of the control group was not significantly different.

In contrast, the number of complete footprints of the right foot was significantly higher than that of the left foot in the ischemic group. The gait pattern of the ischemic group was therefore asymmetric compared to control.

The initial goal of the present study was to observe asymmetries of the contact of the ischemic hindlimb against the floor while the animal was walking, as described elsewhere[21]. We found in the present study that most of the plantar surface of the left (ischemic) hindlimb was not used for loading distribution (incomplete footprint). Sham ischemic animals produced incomplete footprints with both hindlimbs. In contrast, the right hindlimb (normal) of the ischemic rats yielded mostly complete footprints, probably as a compensation or protection mechanism for the disability of the ischemic hindlimb. The protective mechanism would be better understood if hyperalgesia (or allodynia) induced by the ischemic process could be demonstrated.

The instantaneous correlation between NC and the instant of contact allowed us to detect precisely the progressive disability triggered by exercise. The LHDI compared the performance of the ischemic hindlimb with the contralateral one, estimating the interaction of the two hindlimbs and defining the utilization rate of the ischemic hindlimb along a predefined path. There was also a difference in LRR for NC. Thus, it was possible to define the total disability of the posterior part caused by unilateral ischemia.

Many clinical studies have tested drug efficacy using the distance for claudication onset and the maximum claudication distance as parameters during a treadmill series test at defined speed and inclination[22-25]. The variation rate was obtained by the difference between pre- and post-treatment values and the initial distance. The LRR is an expression of how many times the rats stepped on the ischemic hindlimb versus the contralateral one. It was demonstrated that NC of the ischemic hindlimb was significantly smaller than that of the contralateral hindlimb. Therefore, the system was sensitive in detecting signs of disability.

We found no significant difference in NC values for the right hindlimb between rats from the ischemic group and control animals. When LHDI was analyzed, the difference between the ischemic and control groups was much larger. As expected, the index for the control animals remained around zero. Some animals belonging to this group display figures below zero, indicating that along the path the left foot is much more active. This difference may represent normal biological variations.

This study is novel in nature and was able to functionally assess ischemia in animals. The model proposed to quantify the gait variables of rats with disability resulting from limb ischemia was objective and sensitive. In future studies, the model may be used to test and compare established or evolving therapies for intermittent claudication.

Conclusion

Gait dynamics in rats submitted to limb ischemia could be assessed by footprint analysis and hindlimb contact recording during a treadmill test.

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