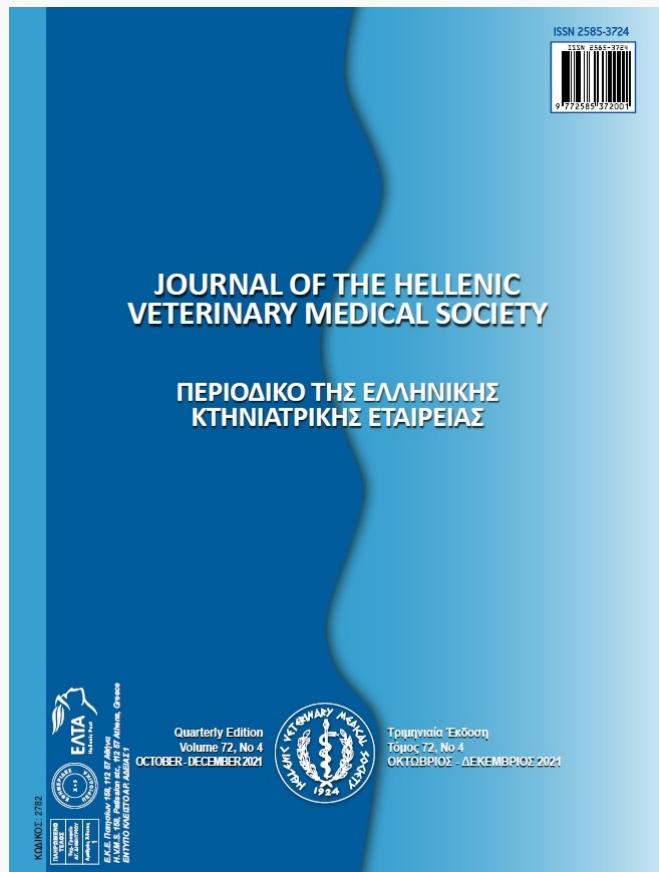


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A report on less-severe, long-duration persistent hind-limb ischemia surgical rabbit model

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ABSTRACT: Development of a persistent hind limb ischemia rabbit model without excessive adverse symptoms and compromised animal welfare.

New Zealand White (n=18, male and female) rabbits of 3-4 months of age and 3.0 ± 0.1 Kg body weight were used. The surgical technique for ischemia avoided the ligation of vessels above the inguinal ligament and included only the ligation and complete excision of the common and superficial femoral arteries along with all their branches up to popliteal and saphenous arteries. Study duration was 84 days.

All animals completed the study period uneventfully. The activity of the animals remained unaffected throughout the study except for the first post-operative day. Adverse symptoms of other models such as loss of limb due to necrosis, loss of nails and necrosis of skin were not observed while successful ischemia was confirmed. There was a significant decrease ($P=0.0381$) in ischemic right limb circumference. Terminal angiography by abdominal aortic cannulation in the animals demonstrated negligible amount of angiogenesis at the distal ischemic thigh in comparison to the control limb ($P=0.001$).

This study reports successful development of a refined chronic hindlimb ischemia rabbit model.

Keywords: Hind Limb Ischemia, Rabbit Model, Welfare, Thigh muscle atrophy, Angiography

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INTRODUCTION

In hindlimb ischemia, the obliterated blood supply to the legs results in intermittent ischemic claudication pain, rest pain, ulceration, and gangrene in human patients. In a normal healing process, spontaneous angiogenesis and revascularization ensure adequate reperfusion of blood to tissues in cases of ischemia in younger individuals and in cases of less severity in the aged. However, the occurrence of revascularization is obviously nonexistent in adult humans with severely aggravated ischemic condition. Unlike human patients, animals have the potential to spontaneously recover from ischemia (Dragneva et al., 2013) since they show an extraordinary capability to form collaterals and they regain normal arterial perfusion within a short period of time. Therefore, the symptoms as well as recovery at the cellular level differs between animal models and humans. This fact makes it difficult to produce animal models of chronic persistent ischemia.

For studies of hindlimb ischemia, researchers tried to develop animal models with ischemia in various animal species (Klausner et al., 1988; Belkin et al., 1989; Belkin et al., 1990; Blebea et al., 1990; Freischlag & Hanna 1991; Sternbergh III & Adelman 1992; Sternbergh III et al., 1994; Blaisdell, 2002) with highly varying results. Mice, rabbits, and pigs are preferred for peripheral arterial ischemia studies owing to a large extend of baseline collateral vasculature and in their closer resemblance to human vascular anatomy while rats, cats, rabbits, and dogs are used due to their relative simplicity to produce models (Waters et al., 2004). Ischemia produced by the simple ligation of arteries is inadequate for a treatment study as this results in only a partial ischemia (Seifert et al., 1985; Hendricks et al., 1990) for a short period. To overcome this, experimental animal models were created by inducing ischemia in the hindlimb by ligation or excision of the iliac artery (Skjeldal et al., 1991), femoral artery (Kalka et al., 2000; Milia et al., 2002) or both the femoral and saphenous arteries (Kanno et al., 1999; Byun et al., 2001). Occlusion of both the femoral artery and vein by strangling the thigh with a tourniquet to produce hindlimb ischemia was also reported in some studies (Wiersema et al., 2000; Messina et al., 2002). Aortic ligation produced models of high severity and the contralateral limb could not be used as the control (Weiss, 1974) since it affects both the hindlimbs.

Most of these above demonstrated models cause

highly damaging side effects with mortality and acute lesions rendering a model of high-severity and clinical signs. Hence, most of the studies that use ligation of femoral arteries and its branches enumerate clinical symptoms as part of observations. Influx arterial ligation and excision at the level above inguinal ligament renders the animals with skin necrosis of hind limb (Varnavas et al., 2010), blackened toenails and limping (Baffour et al., 1992), nonfunctional hind limb (Pu et al., 1994) or limb necrosis (Kyriakides et al., 2001). Reports of mortality are also made by various groups owing to uncontrollable damage and complications resulting by the generation of models of high-severity (Waters et al., 2004).

There exists a necessity to report models of mild to moderate severity and to deliver chronic hind-limb ischemia to study proof of concepts in preclinical research. The model is required to be of high survival rate to aid research and at the same time with milder symptoms to benefit its welfare. In this study, we report the development of a persistent ischemic model and its confirmation in New Zealand White rabbits without any notable clinical symptoms with a 100% survival rate. This safe, simple and effective technique was postulated to withdraw unnecessary animal suffering to create a rabbit model of hind limb ischemia with mild severity to suit pre-clinical studies to evaluate angiogenesis.

MATERIALS AND METHODS

Animals

Eighteen New Zealand white rabbits of either sex (nine males and nine females) with an age of 5-6 months and weight of 3.0 ± 0.1 kg were selected randomly from the colony. Resource equation method was used to find the sample size for a non-blocked study design in which the animals underwent a single treatment. Pre-clinical studies that aim to scrutinize potential therapies are widely encouraged to see the results in both males and females to extrapolate and rule out any sex-influence in interpretation of the results. Hence this study also used both the sexes of animals. Each individual animal formed an experimental unit in this study. Blinding of groups was not applicable since there was only one group involved and the investigators were scoring the clinical signs of all animals in the study. The animals were bred and housed in the Division of Laboratory Animal Science (DLAS) of Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Bio Medical

Technology Wing (BMT Wing), Thiruvananthapuram, Kerala, India. Health monitoring was carried out in accordance with the FELASA guidelines (Nicklas et al., 2010) and the animals were free from any diseases. Ethical clearance was obtained from the competent authority (Institutional Animal Ethics Committee).

Housing Conditions

The animals were housed individually by following the floor space recommendations proposed in "Guide for the Care and use of Laboratory Animals" (NRC, 2010) which is in unison with the recommendations of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Guidelines, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India. The animals were fed with *ad libitum* standard rabbit pelleted feed (Amrut Laboratory Animal Feeds, India) and U.V sterilized drinking water. Treats were provided daily with carrots, sprouted Bengal gram, fresh chopped green grass and cabbages; all washed and cleaned with potassium permanganate solution (1 in 1000). A 12/12 h automated lighting schedule was provided with an intensity not exceeding 325 Lux at 1-meter height from the floor. The temperature of $22\pm2^{\circ}\text{C}$ and relative humidity of 30-70% with 12-15 air exchanges per hour were provided in the experimental animal rooms and the supplied fresh air was HEPA filtered. Cage changes were done in every alternate day. Faecal and urine trays were removed daily and replaced with a cleaned one. All the experiments were carried out between 9.00am and 5.00pm.

Surgical technique

The animals were anesthetized with an intramuscular injection of xylazine (5 mg/kg) and ketamine (50 mg/kg). Pre-emptive analgesia and antibiotics were provided an hour before the surgery using a single dose each of meloxicam 0.5 mg/kg subcutaneously and cephtriaxone 15 mg/kg intramuscularly respectively. Under aseptic precautions, the entire right limb was shaven cleanly and swabbed with povidone iodine solution, and draped with sterile window drapes. The right femoral artery was exposed through a longitudinal skin incision in the medial thigh that extended from the inguinal ligament to the knee. At first, the common femoral artery was dissected along its total length and blunt dissection enabled the separation of artery from vein and nerve. This was followed by dissection of all of its branches distal to the inferior epi-

gastric artery (deep femoral, lateral circumflex, and superficial epigastric arteries). Then, the dissection of the popliteal and saphenous arteries was done distally and all arteries were ligated. Finally, the ligated arteries with all branches were resected and removed (Fig 1). After muscle apposition with 3-0 Vicryl suture, the skin was closed with 3-0 braided silk. The animals were left to recover in a quiet area and upon complete recovery, the animals were left back to their respective home cages. All the animals received cephtriaxone 15 mg/kg intramuscularly once daily for 5 days. Meloxicam injection was given once daily at 0.5 mg/kg subcutaneously for 5 post-operative days and povidone iodine ointment was applied on the wound site until the skin sutures were removed on the 12^h -14th post-operative day.

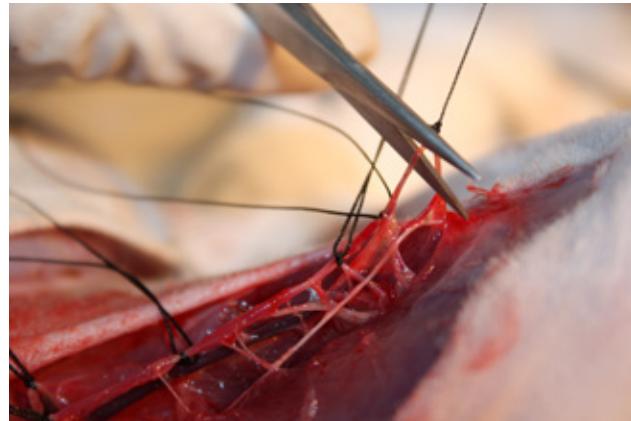


Fig. 1: Ligation and complete excision of the common and superficial femoral arteries along with all its branches up to popliteal and saphenous arteries

Clinical Assessments

All the assessments and procedures were performed by well experienced and trained personnel. Both the hindlimbs of rabbits were clinically evaluated daily until the end of the study. The state of surgical wounds, limping, gait and changes if any in movement patterns, changes of hair and skin, nails, and toes for 6 weeks after the operation were assessed using an objective score-sheet. Thigh circumference on day of surgery of both limbs was obtained and it was compared with the same limb post-operatively to assess muscular atrophy.

Home cage activity assessment

The animals after the intervention for the induction of ischemia were housed individually. Using a camera, (D-Link Surveillance system) five-minute videos were recorded and time of activity during this period was calculated. The observation was conduct-

ed one day before the day of surgery to obtain the baseline value, and then on 1st, 10th and 42nd and 84th post-operative days. Comparison was done with each animal's baseline value with respect to these post-operative days. Activity was defined as time spent on active grooming, drinking and eating, walking and rearing in the cage. Rest comprised of the time of sitting idle in the cage without exhibiting any of the activities enlisted above.

Angiographic analysis

Angiography was done on the 84th post-operative day. The animals were premedicated with xylazine at the rate of 5 mg/kg, intramuscularly and ketamine at the rate of 50 mg/kg, intramuscularly to induce the anesthesia. When the animals were under deep surgical anesthesia, under aseptic conditions laparotomy was performed with a mid-ventral incision towards the lower part of the abdomen and the abdominal aorta was exposed below the level of kidneys. Heparinisation was done with Heparin (Nuparin®, Troikaa, Gujarat, India) at the rate of 1.0 mg/kg, intravenously ten minutes prior to catheterization of abdominal aorta to prevent thromboembolism during the vascular procedure. After looping the aorta with umbilical tape, arterial cannulation was done using 20G intravenous cannula with injection valve (Mediflon®, Eastern Medikit Ltd., India) and the cannula was secured *in situ* with 3-0 braided silk sutures. The aorta was ligated anteriorly to prevent the retrograde flow of the dye while angiography was performed. The animal was positioned dorsoventrally to obtain angiography of both the hind limbs simultaneously. Patency and blood flow to thigh region and shank regions were assessed using 7 ml of Iohexol (Non-Ionic dye) "Omnipaque-350" (GE Healthcare, Ireland). For each rabbit, angiography was performed using Fluoroscopic "C" arm (Powermobil, Germany). An angiogram of four seconds duration was recorded at the level of the medial thigh and arterial count was done to compare the right and left thighs. The number of contrast-opacified arteries crossing over circles and the total number of circles encompassing the medial thigh area were counted in a single blind fashion. An angiographic score was calculated for each film.

Statistical analysis

The statistical tests were performed using GraphPad Prism 8.4.3 (686) (GraphPad Software, San Diego, CA, USA). All data are expressed as the Mean \pm SD. Normality of data was tested using D'Agostino

& Pearson test and normal data was analyzed with repeated measures-one-way ANOVA whereas a non-parametric test, Friedman's test was used to analyze the non-normal data. Comparisons between pre-surgical and post-surgical right hindlimb and left hindlimb thigh circumferences were performed using repeated measures One-way ANOVA, with baseline value as control for respective limbs. For comparing the activity scores on days 1, 10, 42 and 84, the baseline value of activity of each animal was used as the control and a repeated measures ANOVA was done to find out differences in activity levels. Sidek's multiple comparison test was used to assess post-hoc pairwise differences when repeated measures one-way ANOVA was employed whereas Dunn's multiple comparison test was used for Friedman's test. For comparing the angiographic score, each animal's right limb was compared against its left limb using a student's *t*-test. A value of $P<0.05$ was considered to be statistically significant in the study.

RESULTS

Clinical Assessments

All the animals completed the study period uneventfully and are included in the analysis. After surgery, all the animals regained righting reflexes in 15 ± 3 minutes after skin suture and recovered completely from anesthesia in 180 ± 32 minutes after skin closure. Complete wound healing was observed in 12 ± 2 days after surgery. All the animals showed general weakness of the right hind limb on the first postoperative day. No animals exhibited any visible clinical signs indicative of toe or skin necrosis owing to ischemia. No changes of hair and skin, nails, and toes could be observed during the entire study period.

Thigh Circumference Measurements

Pre-operative and post-operative thigh muscle circumference measurements of left limb (Fig. 2a) showed significant increase on days 10, 42 and 84 with respect to the measurements obtained as baseline showing an increase in size of growth attributable to the non-operated and normally well perfused limb ($P<0.0001$, $F(17, 51) = 1.477$). A significant decrease of the ischemic right thigh circumference was noticed on days 10, 42 and 84 (Fig 2b) with respect to the baseline circumference ($P=0.0381$, $F(17, 51) = 1.915$). The trends on left and right limbs with respect to the baseline thigh circumference of animals are given as Fig. 2c.

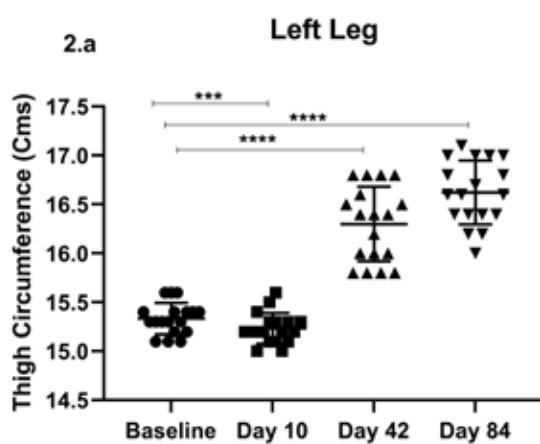


Fig 2a: Left thigh circumference (cm) over time. *** = $P < 0.001$, **** = $P < 0.0001$

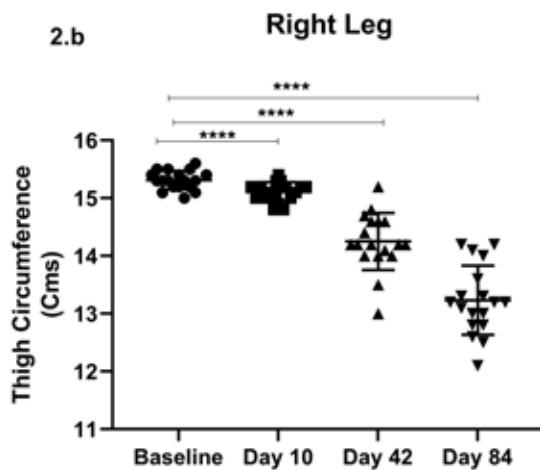


Fig 2b: Right leg circumference (cm) over time. **** = $P < 0.0001$.

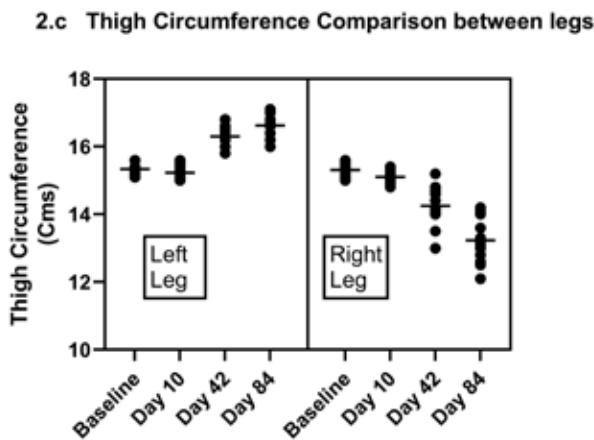


Fig 2c: The graph depicting the loss of thigh circumference of the perfusion deprived right leg with respect to the contra-lateral left leg showing increase in circumference during the entire period of the study

Home cage activity assessment

Significant decrease in activity was noticed on

day 1 post-operatively in activity of the animals ($P < 0.0001$, Friedman Statistic = 34.71) and no differences could be observed at any of the other time points (Fig 3).

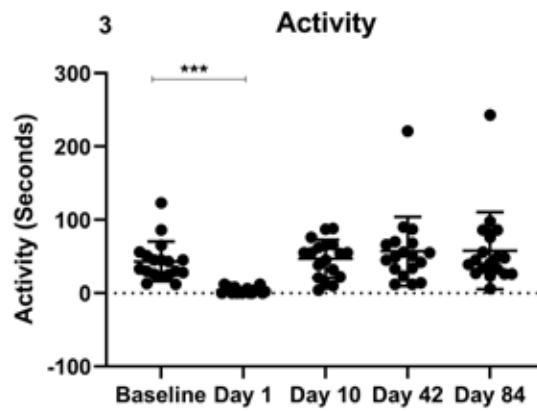


Fig 3: Reduced light phase-home cage activity levels on immediate post-operative day. The animals showed difference in repeated measures ANOVA in comparison to the control (baseline) value on the first post-operative day. *** = $P < 0.001$

Angiographic analysis

The number of patent blood vessel counted differed between the right and the left limbs with consistently higher number of vessels in the left limb ($P = 0.001$, $t = 21.31$, $df = 34$) (Fig 4). Hence this terminal angiography suggests a successful animal model which is consistent and persistent in the perfusion loss of the ischemic hind limb (Fig. 5). This method is a simpler one neither exposing the animal to procedures like cardiac puncture and radioactive substances exposure for the assessment and confirmation of animal model development nor requires repeated general anesthesia within the study period.

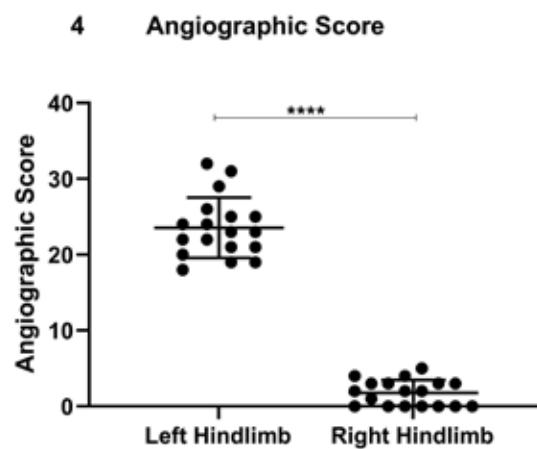


Fig 4: Angiographic score (patent-arterial count) on day-84 showing a significant loss of supply arteries in the ischemic right limb in animals. **** = $P < 0.0001$



Fig 5: Angiograph on day-84 with contrast material showing loss of patent vasculature in the right limb in comparison with the intact left limb. Patent artery of the left leg is indicated with arrow head

DISCUSSION

Many animal species such as dogs exhibit widely established hind limb collateral pathways (Sunder-Plassmann et al., 1984) necessitating complicated procedures like identifying as many as 14 branches of iliac artery and their subsequent ligation to develop the animal ischemia model. There exist at least three collateral routes that take care of the supply when the femoral artery is surgically obstructed in species such as rats making it a difficult surgical model as well (Seifert et al., 1985). Specieswise differences of femoral arterial anatomy is very well documented and in comparison to murine hindlimb, rabbit femoral anatomy is different (Kochi et al., 2013). Limited baseline arterial network in hind legs and lesser collateral network make species such as mice, rabbits and pigs excellent animal models to study hindlimb ischemia to closely simulate human vascular anatomy (Waters et al., 2004). Of these favorable species, rabbits are widely used because of the optimum size of the animal unlike mice and pigs. Apart from ligation and excision of arteries, multiple techniques had been tried in the past decades in animal models like occlusion using pharmacologic and chemical agents resulting in high mortality of experimental animals (Longland, 1953). External tourniquet application was also a widely practiced technique in a wide range of species such as mice, rats, rabbits, cats, dogs and in non-human primates (Barie & Mullins, 1988). However, muscular necrosis occurring at around 6 hours post-isch-

emia and loss of the entire limb renders the technique less adoptable. Further, tourniquet application never results in chronic ischemia even though it produces acute ischemia making the animal model useful only to limited applications. To overcome these shortfalls, an animal model of surgical ligation followed by excision of major supply artery closer to the aorta at the level of the common iliac artery and the ablation of potential sources of collateral supply to produce profound chronic effects rather than performing simple distal occlusion of common femoral artery is demonstrated previously in rabbits (Gao et al., 2020; Waters et al., 2004). This technique documents a persistent model preventing short-bridging collateral vessel formation by ligating above the level of inguinal ligament. Clinically noticeable ischemia with limping of the affected limb on the first post-operative day and after the 10th post-operative day with marked atrophy has been reported (Pu et al., 1994). Superficial tissue necrosis and non-functional hindlimb and mortalities have been reported previously with this technique (Pu et al., 1994) and various groups had reported highly adverse clinical symptoms in rabbits ranging from dysfunctional leg and necrosis of skin and loss of nails (Waters et al., 2004).

For proof-of-concept studies, molecular and local evidence of persistent ischemia is the targeted outcome rather than adverse effects that affects animal's well-being. To achieve this, we planned to spare the influx arteries at the level of aorta and above inguinal ligament and distally ligate and resect local arteries to develop an animal model with effective loss of tissue vascularity. It was postulated that this approach could minimize or avoid the severity of symptoms.

The present work documents a method that could maintain hind limb ischemia successfully up to 12 weeks (84 days) with a modified technique by avoiding influx arterial ligation and excision but at the same time equally persistent in duration when compared to previous reports (Pu et al., 1994; Hong et al., 2001). However, in contrast to several previously reported studies, it was noticed that there existed no readily observable clinical signs in the animals except for a chronic reduction in the thigh circumference, hence resulting in an effective but less severe disease model. This model avoided common iliac artery ligation, which required a laparotomy and is less effective in producing ischemia.

Rabbit models have the ability of forming collaterals and regain the normal arterial perfusion with-

in a short period of time in three to seven days after occlusion of arteries (van Bemmelen et al., 2007). Dissection of the complete femoral artery and its side-branches produced deep distal ischemia in this study. When compared to the established methods (Waters et al., 2004), the present study involved more refined techniques by not involving radioactive chemicals, redundant anesthesia to the animals within the study period and procedures like cardiac puncture under general anesthesia. These techniques if repeated within the study period could affect the percentage of survival and there are possibilities that the increased stress levels can adversely affect the efficacy of treatment adopted in studies of angiogenesis.

The right leg exhibited ischemic atrophy and progressively lower thigh circumference measurements. Interestingly, a significant increase in left thigh circumference was also noticed which is due to compensatory hypertrophy since the left limb was more used to bear weight predominantly after surgery. The home cage activity reduction observed on the first post-operative day may be attributable more to the surgical pain rather than to an effect of arterial perfusion of the operated leg since this phenomenon could not be observed on other post-operative days. Since analgesia was administered daily, the adequacy of coverage of meloxicam needs to be probed into in rabbits in similar surgeries. Multimodal analgesia shall be tried to bring in better welfare in similar procedures. This work demonstrates that less tedious confirmatory measures can be adopted to replace repeated invasive techniques that are stressful to the animal model. Animal activity as a measure of welfare is often overlooked by scientists. This parameter can be of great utility since it is totally non-invasive. It could be

useful in analyzing the difference between groups in terms of limb functionality and as a measure of general physical well-being. The terminal angiographic scoring which is a non-recovery procedure avoids the exposure of the animal to invasive diagnostic procedures or radioactive substances for the confirmation of model development and also avoids repeated general anesthesia within the study period.

CONCLUSION

Less severe animal models that are equally effective and validated are a necessity to evaluate proof of concept in preclinical studies involving hind-limb ischemia. Report on loss of hind limb blood supply and establishment of atrophy with no obvious adverse clinical signs by a modified surgical approach in a group of experimental rabbits is presented. This technique avoids laparotomy and minimizes the chances of animal mortality and at the same time ensures the effectiveness in providing an animal model useful to study hind limb ischemia.

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CONFLICT OF INTEREST

None declared.

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