

**INDUCED ANGIOGENESIS WITH INTRAMEDULLARY DIRECT CURRENT: AN  
EXPERIMENTAL RESEARCH**

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**Running Title: Induction of Angiogenesis with current**

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**ABSTRACT**

The purpose of this study was to evaluate angiogenesis after using intramedullary direct electrical current in rabbit tibia. Thirty-two New Zealand rabbits were divided into four groups: 1 - false electrode, 2 - hole group, 3 - control, and 4 - intramedullary electrical stimulation. Half the rabbits in each group were evaluated angiographically, pathologically, and scintigraphically on day 7, and the rest were evaluated on day 21. Results proved that electrical stimulation was not capable of induction of angiogenesis in the subjects killed on day 7 and day 21. Furthermore, we found some fibrotic changes secondary to electrical stimulation on day 7 ( $p: 0.04$ ) and day 21 ( $p: 0.01$ ). However, an increase in new capillary vessels occurred in the false electrode group ( $p: 0.02$ ). We found no useful effect of electrical stimulation in our study, a finding that is possibly due to our use of a method previously undocumented in the literature. We believe that this study can be the new baseline for further studies into the stimulation or inhibition of angiogenesis using intramedullary wire with or without electrical stimulation.

**Key Words:** angiogenesis, direct current, medullary canal, Alar's transparency

## **INTRODUCTION**

Ischemic vascular diseases of the limbs are still one of the most important problems of human kind in spite of developed technologies in the current age. Unfortunately, ischemic vascular diseases may cause limb amputations due to limited therapeutic options unless patients have distal macroarteries suitable for bypass grafting operations (14). To prevent amputation, new investigations have been directed to stimulate angiogenesis.

According to the literature, the observation of microvascular growth, “angiogenesis,” was first made in 1853 by Meyer (21). Since then, several angiogenic factors, such as vascular growth factor, and gene therapies have been used to improve angiogenesis (7,9,13,14,21). However, growth factors or supplementary gene therapies require complex and expensive treatments. We believe that induction of natural angiogenic behavior of the organism is more useful and more applicable. The purpose of this study was to evaluate whether intramedullary electrical stimulation can stimulate angiogenesis in muscles due to the release of some angiogenic factor in the medullary canal in an animal model.

## MATERIALS AND METHODS

The Ethics Committee of Animals Care of Inonu University approved the study. Thirty-two New Zealand rabbits obtained from a government institution were used. The animals were divided into four groups:

1 - False electrode group (n=8): The animals were anesthetized intramuscularly 35 mg/kg Ketalar (Ketamin ®) and 3 mg/kg Xylazin (Rompun®). All surgical procedures were performed under aseptic conditions. A hole was opened percutaneously using 1-mm Kirschner wire at the proximal and distal metaphysis of the tibia towards the medullar canal. A rhodium-coated, 26-gauge, stainless-steel wire electrode with a 10-mm uninsulated tip was placed down the medullar canal for a distance of 10 mm through the hole. The electrodes were fixed with silk sutures on the skin and not attached to a power pack. After the intervention, the electrode positions were checked using radiography.

2 - Hole group (n=8): Two holes were drilled at the proximal and distal metaphysis of the tibia.

3 -Control group (n=8): These animals served as controls and received no intervention.

4 - Electricity group (n=8): Electrodes were placed within the tibia, with the proximal electrode as the cathode and the distal as the anode, as described above. Prior to beginning this study, we tested intramedullary resistances of four rabbits' tibiae as 10 kΩ. According to ohm rules, to obtain 0.1 μA direct current within the medullary canal, voltage from the power pack should be 10 mV and 10 μA. Based on these data, electrical current was determined as 10 mV and 10 μA, repetitive square wave pulse with an interval of 0.5 second, and a 0.5 second stimulus width at 60 stimulus/minute frequency. The medullary canal was stimulated continuously up to the time the animals were killed, and the electric currency within the entire circuit was checked daily.

In all groups, the contralateral tibiae also served as controls.

Each of these four groups was divided into two subgroups. Half of the subjects in each group were evaluated on day 7 of the experiment. The rest of them were evaluated on day 21. Evaluations consisted of angiographic and scintigraphic studies that were carried out when the subjects were alive. Pathologic measurements were performed after euthanasia via air injection into the pericardium.

***Angiography Procedure:*** All angiographies were performed under anesthesia. To detect both limbs at the same time, radio-opaque materials were injected with a 22-gauge intravenous cannula from the abdominal aorta through the retroperitoneal surgical aortic approach.

***Scintigraphic Procedure:*** After angiography, the scintigraphic material (4 mCi [148 MBq] in 2 cc volume) was administered via the same route as above. <sup>99m</sup>Tc Technetium human albumin macroaggregates (<sup>99m</sup>Tc-MAA) (Pulmocis, CIS Bio Int. France) were used as the radioactive material. According to the manufacturer's instructions, the particle numbers per vial ranged between 2 and 4 million, and at least 80% ranged in size between 30 and 50  $\mu\text{m}$ . No <sup>99m</sup>Tc-MAA particles were larger than 100  $\mu\text{m}$  or less than 10  $\mu\text{m}$ . After 20 minutes, animals were euthanized. Next, the imaging was performed using a dual-head gamma camera with a low-energy high resolution parallel hole collimator (ADAC vertex plus digital gamma camera, ADAC Vertex V60 digital gamma camera, Milpitas, CA, USA). Anterior static planar images of the hind limbs (3 min and 256x256 image matrix) were taken.

***Pathological Procedures:*** After scintigraphy, the crural muscles of the two limbs (the legs operated upon and the legs without surgery) were removed, and muscle specimens were fixed with 10% formaldehyde for fixation. After 24 hours of fixation, 3-mm samples in full-thickness were obtained from three different levels of the muscles: the first level was at 1 cm from the proximal end of muscles, the second level was 2 cm, and third level was 3 cm (Fig. 1). Each sample was 5  $\mu\text{m}$  in thickness and stained with hematoxylin-eosin.

**Angiographic Measurements:** All branches of the crural arteries in each subject were counted. To obtain the most correct account, a transparency was designed (Alat's transparency) (Fig 2). To correctly count the whole arteries observed in the angiography, the Alat's transparency was placed onto the angiography paper. The arteries in each region were counted in order, and the sum of the results of each region equaled the number of visible arteries in the leg.

**Scintigraphic Measurements:** Rectangular regions of interest (ROI) were drawn on the images. Scintigraphic results were evaluated by two experienced observers.

**Pathological Measurements:** The number of capillaries, which are capable of containing only 1-1.5 erythrocyte in their lumen (7-10  $\mu\text{m}$ ), were counted from three of the most vascularized areas after detecting all specimens under 400X magnification (Light Microscope, Olympus CH30 Japan). The arithmetic mean value of those three results equaled the number of the first level. This method was repeated for the second and third levels. This result was the general number of the capillary arteries of a leg. In addition, necrosis, fibrotic changes, and granulations were evaluated pathologically. Pathological procedures were carried out by the same pathologist.

The radiologist, a specialist in nuclear medicine, and the pathologist were blinded to the codes or interventions of any subjects.

**Statistical Calculations:** The accumulated results were analyzed with Wilcoxon signed rank tests, Kruskal Wallis, Mann Whitney U, and Pearson tests on an SPSS V 10.0 computer program, and a p value of  $<0.05$  was accepted as statistically significant.

## RESULTS

The results of this study proved that electrical stimulation was not capable of inducing angiogenesis in the subjects euthanized on day 7. On day 21, we determined an inhibition effect instead of stimulation by electricity; results are summarized in Figure 3. A statistically significant difference was found when the results of the false electrode group were compared with the electricity group ( $p:0.02$ ), and when the hole group and the electricity group were compared ( $p:0.02$ )

The mean values of the numbers of the capillaries in the granulations in each group that was euthanized on day 7 are shown (Fig 4). The biggest values were obtained in the electrical stimulation group, and there was a significant statistical difference between the electricity group and the control group ( $p:0.01$ ). No statistically significant differences were obtained among the groups on day 21.

When fibrotic changes were compared, we found another important finding (Fig 5). It is possible to see the side effects of electrical stimulation. The biggest change was due to electrical stimulation, and this had a statistical meaning ( $p:0.04$ ) when we compared it with the control group. There was an increase in fibrotic changes in the false electrode group; no significant statistical relation was detected between this group and others. The mean values of the numbers of fibrotic changes in each group sacrificed on day 21 are shown (Fig 5). There was a statistically significant difference between the control group and the electricity group. ( $p:0.01$ ).

We found no statistical difference in the angiographic results and in the scintigraphic results when we compared the groups with each other in the subjects killed on day 7 and day 21.

To show the effects of each procedure, the results of both the operated-upon legs and the untouched legs of each group were compared. We found no statistically significant results in these comparisons on day 7 or on day 21.

## DISCUSSION

Based on the information obtained from this study, electrical stimulation had no superior features for inducing angiogenesis in rabbit leg. Also, use of electricity showed more fibrotic changes in the muscle. When we compared the fibrotic effects of the electrical stimulation with the control group, there was a statistically significant difference ( $p:0.01$ ). The side effects of electricity applied 21 days were more dangerous than its side effects in 7 days because the  $p$  value was 0.04 on day 7 and was 0.01 on day 21. Whereas, the number of capillary vessels were increased using the false electrode without significant harmful changes.

The research on stimulation of angiogenesis includes gene therapies, supplements of angiogenic factors, or inductions of native angiogenic capacity (3,7,13,19,22). Gene therapies are expensive and require a qualified team. Recombinant angiogenic growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), salvaged ischemic areas of myocardium and hindlimb in animal models (9,17). However, the clinical application requires large amounts of these recombinant proteins and is not feasible at this time (13).

Electrical stimulation to induce angiogenesis has been indicated as a new idea in the search for novel approaches to treat ischemia (15,17,19). The mechanism of angiogenesis has been described by indirect effect of increased expression of VEGF in muscle cells. Kano et al (13) reported that when cultured skeletal muscle cells were electrically stimulated, there was a significant increase in blood flow within the muscle and significantly increased VEGF protein in the stimulated muscle after a five-day stimulation. A similar result was obtained by Zaho et al. with a constant direct current electrical field (22). In the present study, we hypothesized that electrical current stimulates precursor cells with a direct or indirect cellular mechanism in the medullary canal because this part of bone contains many living cells, a capillary network,

a blood supply, and some precursor cells. However, unexpectedly, the intramedullary direct current led to inhibition of angiogenesis with unknown reason.

The electrical form chosen may be important in stimulating angiogenesis. Electrical pulse has been used to produce muscle contraction (1,2,6,11,12,16). It was hypothesized that hypoxia caused by muscle contraction was relevant to the increase of capillary density of skeletal muscles (12). Electromagnetic fields have also been used to stimulate cells in culture (5,8,18). Despite successful results with previous studies, we do not prefer this technique because there is no intramedullary application. An intramedullary direct current described by Brighton and Hunt was used in the present study (4). They applied constant direct current for stimulate osteogenesis in rabbit tibia using an intramedullary stainless-steel electrode, and they observed the hemopoietic cells were gradually replaced by polymorphic cells, which was a main purpose for the current study. However, a negative effect was observed. Despite observed inhibition of angiogenesis, we did not conclude that any intramedullary application of direct current has an inhibition effect on angiogenesis.

The magnitude of direct current can play a role in the induction of angiogenesis; however, the mechanism of how electrical stimulation induces the expression of VEGF is unknown. We applied the minimum direct current that could be passed through the entire medullary canal from cathode to anode, which were 10mV and 10  $\mu$ A. If we compare these results with Bringthon and Hunt's study (3), the amount of direct current used in the present study was one-tenth that used in the other study. Consequently, we concluded that the inhibition effect of intramedullary DC could not be related to high voltage of electricity.

Duration of electrical stimulation can also be an important factor affecting angiogenesis. In previous studies, it started on the day after electrode implantation and continued for from 2 hours to 5 days (11,12,13,22). Stimulation time in the current study could be considered longer than other studies. However, for evaluation of capillary new vessels using

angiographic, scintigraphic, and pathologic measurements instead of detection of increased amounts of VEGF protein, we applied electrical stimulation for seven to 21 days. If we criticize the extended duration of electrical stimulation, this might be a reason that inhibition of angiogenesis in the electricity group had a different result from previous studies in the literature.

Placement of intramedullary false electrodes and creation of a hole may stimulate angiogenesis with direct mechanical effects and trauma. Some studies support this idea that electrical currents are insufficient to stimulate osteogenesis without the mechanical and chemical effects of an electrode (4,10,19). This can explain our results that stimulation of angiogenesis occurred in the hole and false electrode groups. However, inhibition of angiogenesis is not clear with this hypothesis.

When we compared the pathological results with the angiographic and scintigraphic results, we found no relationship. This discordance may be explained because we counted only newly visible capillaries with a luminal diameter of  $<10\ \mu\text{m}$  in the pathological measurements. According to Takeshita S et al., angiography cannot provide images of arteries measuring  $<200\ \mu\text{m}$  in diameter. The authors developed synchrotron radiation microangiography, which appears to be a powerful means of assessing the development of small collateral arteries and may help to provide a basis for understanding the collateral circulation (20). This can explain why pathological findings are not parallel with angiographic results or scintigraphic results in the current study. Furthermore, our study showed angiographically and scintigraphically that there were no new vessels bigger than  $200\ \mu\text{m}$  at the end of 21 days with intramedullary stimulation.

A limitation of the current study is that the inhibition of angiogenesis could have showed whether or not this effect was due to inhibition of some chemical factors such as VEGF or

FGF. However, when we organized this study, our purpose was more to evaluate end organ response than to discover mechanism or pathway.

In conclusion, we proposed that intramedullary electric stimulation would be valuable to induce angiogenesis in the ischemic limb. But, results showed that this technique led to inhibition of angiogenesis with harmful effects such as fibrotic changes. Also, the application of a false electrode into the medullary canal was found effective in increasing angiogenesis with fewer side effects and may be a good solution to stimulate angiogenesis, rather than use of intramedullary electrical stimulation. As a result, we couldn't find any useful effect of electrical stimulation in our study, a finding that is due possibly to our chosen method, which was different than those cited previously in the literature. However, we believe that this study can be the new baseline for further studies of the stimulation or inhibition of angiogenesis using intramedullary wire with or without electrical stimulation.

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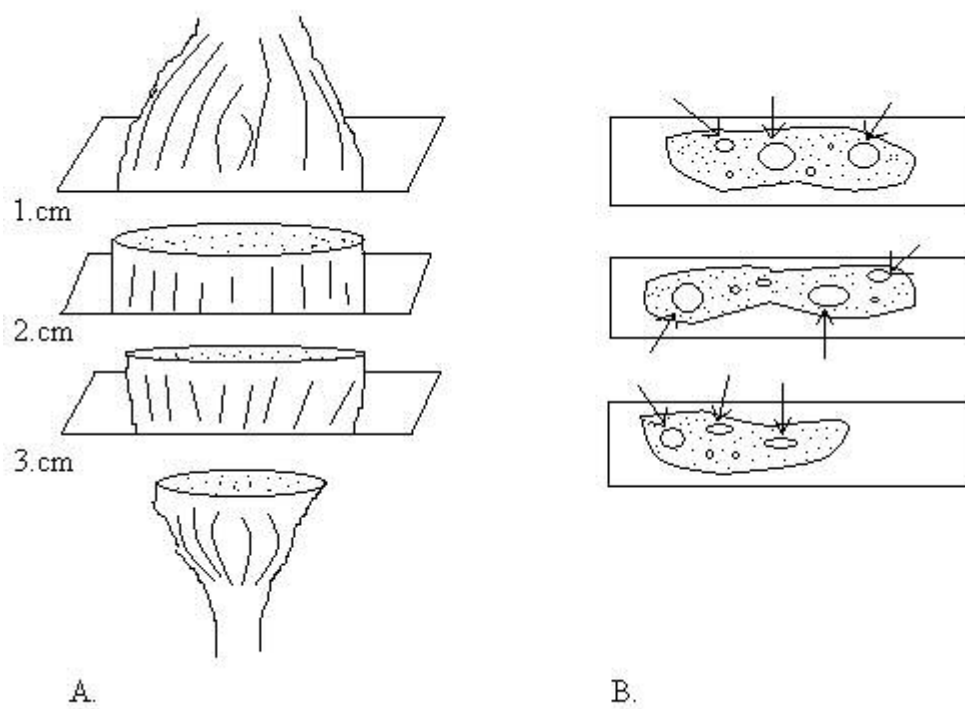
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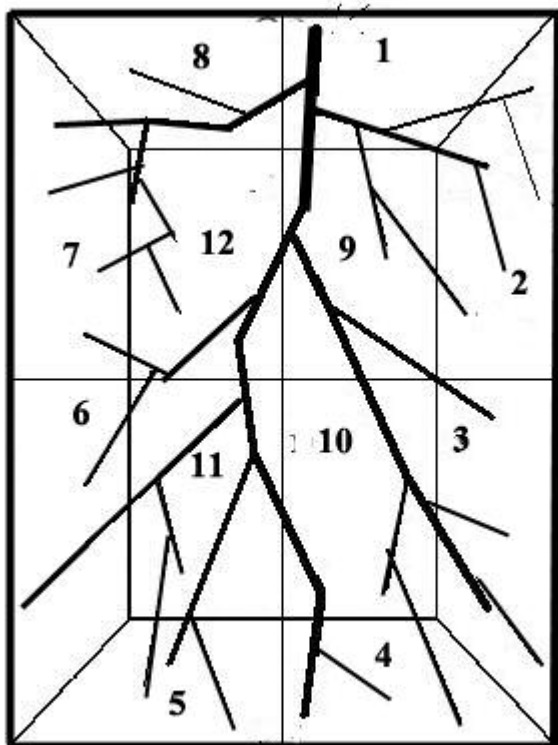
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**LEGENDS**

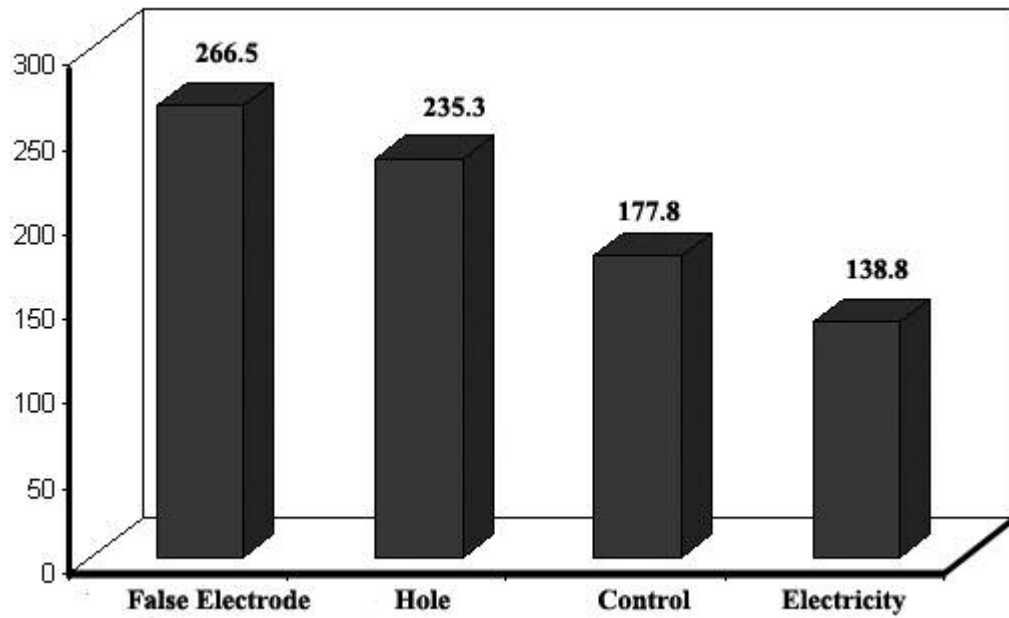
Figure 1: Drawing shows preparing of pathologic specimens



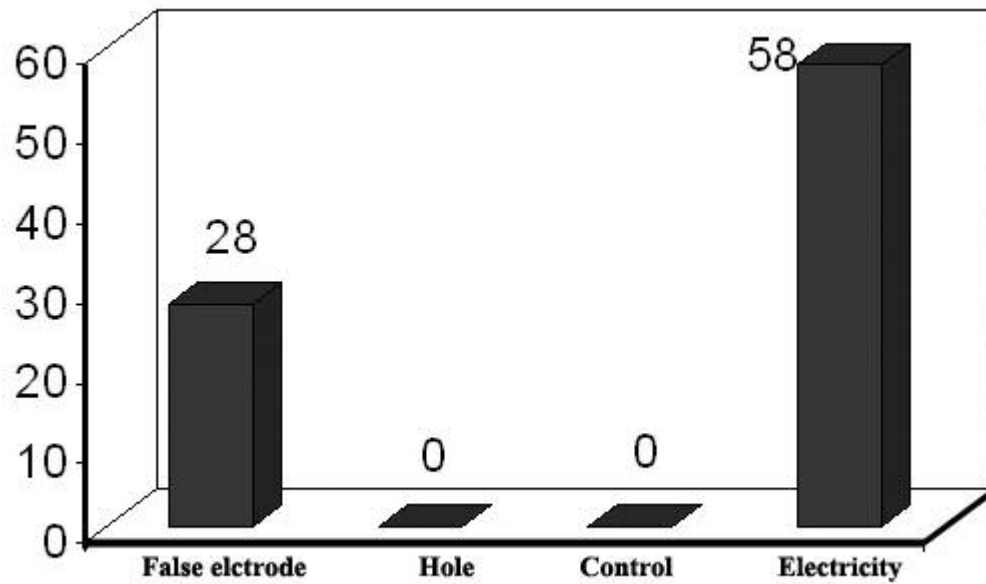
**Figure 2:**The usage of Alat's transparent. The angiography film covered with the Alat's transparent. Then, in order, it's started to count. To prevent the repetitions, counting is started from the outer areas. A vessel counted in the outer is not counted in the inner. For example, the result of the area 1 is 1, area 2 is 4 in this schematic view.



**Figure 3:**Pathological findings in 21<sup>st</sup> day. The numbers of the capillaries in the interfascicular areas. (According to mean levels)



**Figure 4:** The mean values of the numbers of the capillaries in the granulations in each group sacrificed in 7<sup>th</sup> day.



**Figure 5:** The mean values of the numbers of the fibrotic changes in each group sacrificed in 21<sup>st</sup> day.

