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Thalidomide is better than Rapamycin in prevention of neovascularization after laser photocoagulation in dorsal window chamber rat model

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Abstract:

Objectives: -This study examines the effect of two different antiangiogenic drugs (rapamycin and thalidomide) in prevention of neovascularization when locally used, after laser coagulation through dorsal skinfold window chamber rat model.

- Determine what is the most effective antiangiogenic drug when used locally and its potential side effects.

Methodology:

The study includes 3 groups, each group contains 10 rats Thalidomide, Rapamycin and control group (laser group). Blood vessels in the dorsal window chambers implanted on dorsal skin of albino rats were irradiated by Diode laser pulses until coagulation occurs. Blood flow and vessels structure were documented with high definition camera to evaluate photocoagulation and reperfusion. Treatment groups received the treatment drug which was injected to the widow's medial side, every 3 days' intervals for 2 weeks after laser irradiation.

Results:

Control group, 26 out of 28 photocoagulated blood vessels reperfused within 5-12 days with reperfusion rate about 93%, in Rapamycin group the reperfusion rate was about 46%. We also found that the reperfusion rate in Thalidomide group was about 8% which was the lowest reperfusion rate in all groups.

Conclusions:

Thalidomide group reperfusion rate was about 8% which was the lowest reperfusion rate in all groups. Thalidomide is more effective than Rapamycin, and it`s antiangiogenic effect lasts for weeks with no side effects detected with local use.

Keywords: Angiogenesis-Antiangiogenic Drugs-Diode laser-Sirolimus-Thalidomide-Skinfold window chamber

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Introduction:

Angiogenesis is defined as formation of new blood vessels and capillary beds from normally existed vessels, angiogenesis processes involve endothelial cells differentiation and migration, which line the inner walls of blood vessels. [1]

Angiogenesis plays an important role in embryonic development, wound repair, subside of inflammation, and onset of neoplasia, Dysregulation of angiogenesis is the main cause of different pathologic conditions. [2]

Angiogenesis is necessary for growing tissues, with its increasing metabolic needs, so it provides an adequate blood supply and proper waste drainage [3].

Types of angiogenesis (1). Sprouting angiogenesis: it involves stimulation of endothelial cells to proliferate into the surrounding matrix and form solid sprouts extending to the angiogenic stimulus forming a new vessel [4]

(2). splitting angiogenesis or Intussusception: it is the division of the lumen of an existing vessel to form two vessels [5-6]

Some angiogenesis inhibitors are immunomodulatory drugs which activate or inhibit the immune system beside its antiangiogenic properties. [7-8]

Antiangiogenic treatment of tumors is a promising therapeutic regimen. [9-10].

The dorsal skinfold window chamber**:** presented in 1943 by Algire, consists of a longitudinal fold of dorsal skin with a fixed clear glass window that allows in vivo visualization and manipulation of the subdermal blood vessels(fig.1). Dorsal window chamber (DWC) is one of the few models that allows for direct examination of in vivo vasculature and microcirculation. Multiple modulations to the original design have made it less susceptible damage. [15]

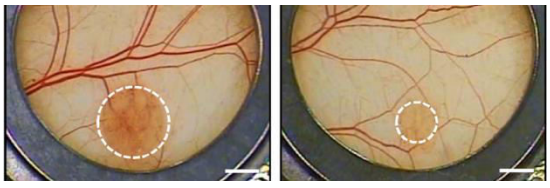

Fig.1(dorsal skinfold window chamber (15))

Thalidomide was prescribed as an antiemetic and sedative in 1950s but it was quickly prohibited when it`s teratogenic effects discovered. Recent studies show that thalidomide has strong antiangiogenic effect, which may be related to its teratogenic effects. [11]

Thalidomide has immunomodulatory, ant inflammatory, and antiangiogenic effects, but the exact mechanisms of action are not fully understood.
Thalidomide was one of the first angiogenesis inhibitors used in multiple myeloma and showed clinical efficacy [12].

Rapamycin(Sirolimus)which is a macrolide antibiotic, is used recently as an immunosuppressive drug to inhibit organ transplantation rejection. It blocks the mammalian target of rapamycin (mTOR) [13], which known to be an important controller of multiple mitogenic signaling pathways, it controls the cell growth in different physiological and pathological conditions.

Recent studies showed that immunosuppressive doses of rapamycin inhibit the development of tumors and tumor metastases, this effect of rapamycin suggested to be based on its anti-angiogenic properties, as it reduce vascular endothelial growth factor (VEGF) production and decrease vascular endothelial cell stimulation. [14]

Methodology:

All experiments done after a protocol approval by the: Animal Care and Use Committee, medical department of Nuclear materials authority (cu- 5818). Thirty adult albino male rats with an initial body weight of 100-150 grams divided into three groups each group has 10 rats as follows: Control group, Rapamycin group, Thalidomide group.

Dorsal Window Chamber Model:

A dorsal window chamber (DWC) fixed on each animal, when properly prepared, it provides clear view to the subdermal blood vessels for up to 4 weeks. after the animal was anesthetized by intra peritoneal injection of 0.5mg ketamine mixed with xyla-ject solution (manufactured by ADWIYA pharmaceutical Egypt), the dorsal skin shaved, epilated, and midline dorsal incision was done, one layer of skin lifted to form a skinfold and the chamber fixed by plastic screws and 3-0 polypropylene sutures. The window chamber purchased online from APG company USA.

Laser Irradiation

Laser irradiation was done on the subdermal side of the window. Blood vessels irradiated with a class 4, diode laser 980 nm (quanta system spa 21058 Solbiate Olona (VA), Italy) which produce a sequence of a variable number of pulses [16]. The pulses number could be varied from 3 to 8 and the repetition rate could be up to 2 Hz.

The duration of an individual pulse is 20 milliseconds and the radiant exposure varies from 150 to 250J/cm^2 with the 2mm spot used in this study.

Rapamycin

Rapaimmune (Sirolimus) 1 mg (Wyeth pharmaceutical, Pfizer) used in this study. It was dissolved in sterile filtered dimethyl sulfoxide solution (DMSO) to have a concentration (1mg/ml). Immediately after vessel coagulation by laser irradiation on Day zero, 2.5 international units (IU) equivalent to rapamycin doses (1.5mg/kg/day) given through local injection to the epidermal side of the window; once every 3 days, for 2 weeks.

Thalidomide:

Thalidomide 100 mg (nostrum impex division of M.P.C pharma private Limited India) dissolved in Dimethyl sulfoxide (DMSO) to have concentration (0,5mg/ml), 5 IU local injection to the epidermal side of the window was done, once every 3 days, for 2 weeks, immediately after laser irradiation on day 0. [17]

After every session loose plastic cover was attached to the inner side of the chamber to prevent sub-dermis from dehydration and contamination. Daily application of local antibiotic cream to the surgical site was done to prevent wound suppuration.

Assessment of vessel reperfusion

Color images by 24 megapixel Sony camera used to capture high definition images through the window chamber with special connector.

The vessels imaged before laser irradiation to identify photocoagulated blood vessels in which complete flow stoppage occurred after laser photocoagulation (day 0) and on days 7,14 to assess the efficacy of each drug used and its duration of action.

Data analysis

The number of perfused vessel are given as mean ± SEM from all groups. For statistical analysis of differences between the control group and antiangiogenic treatments groups, a F-test (ANOVA test) was used.

Results:

1-Control group:

Laser Group contains 10 rats, in this group the window chamber was installed then laser photocoagulation done to rat vessels in dorsal skin fold in order to monitor the normal

regeneration process of new vessels or recanalization of coagulated vessels without effect of any angiogenitic inhibitor, it was noted that the window chamber moved downward slightly due to the effect of gravity over the days of the experiment.

In each window, after identification of major branches of the dorsal vessel which had a pair of arteriole and venule, they were lased with a series of laser pulses to a 2mm segment of the branches until photocoagulation was observed.

The stem of the vascular plexus in the window was left intact, and the flow dynamics in the majority of the irradiated blood vessels was absent.

Day 0: before laser coagulation (fig. 2 a), then laser coagulation of the major dorsal vessels (fig. 2 b).

Day 3-7: The window and the skin over showed erythematous reaction during the follow up days, vascular reperfusion and regeneration around the irradiated sites was noted (Fig. 2 b,c).

Day 14: Vessels appearances were similar to those before laser irradiation (fig.2 d).

Totally, 30 vessels were irradiated and 28 photocoagulated, No blood flow on Day 1 after irradiation, After 14 days26 out of 28 vessels reperfused.

Typically, reperfusion and excessive neovascularization occurred between 3 and 14 days after irradiation. The results from all rats' blood vessels in laser group are summarized in diagram (1).

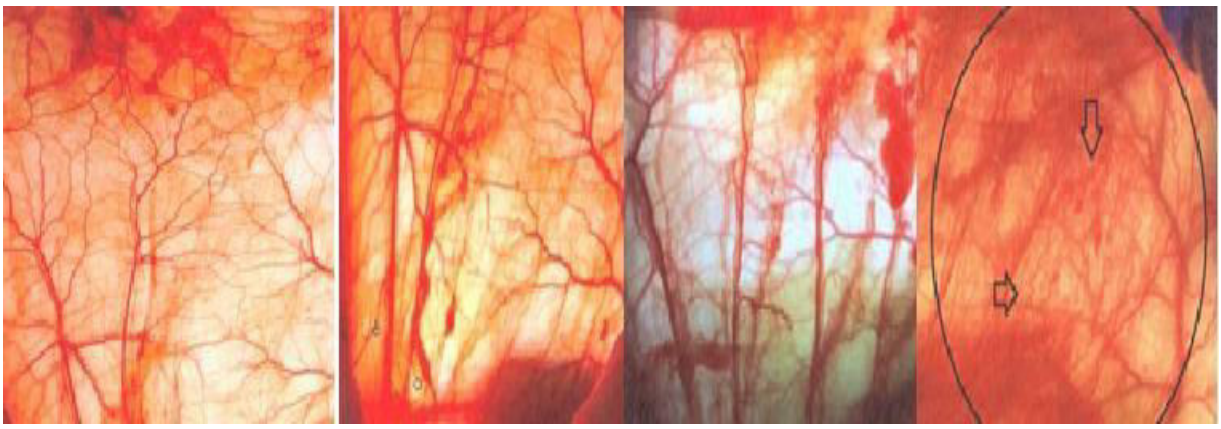

Figure.2:

a. Before laser b. after laser ablation c. day (7) d. day (14)

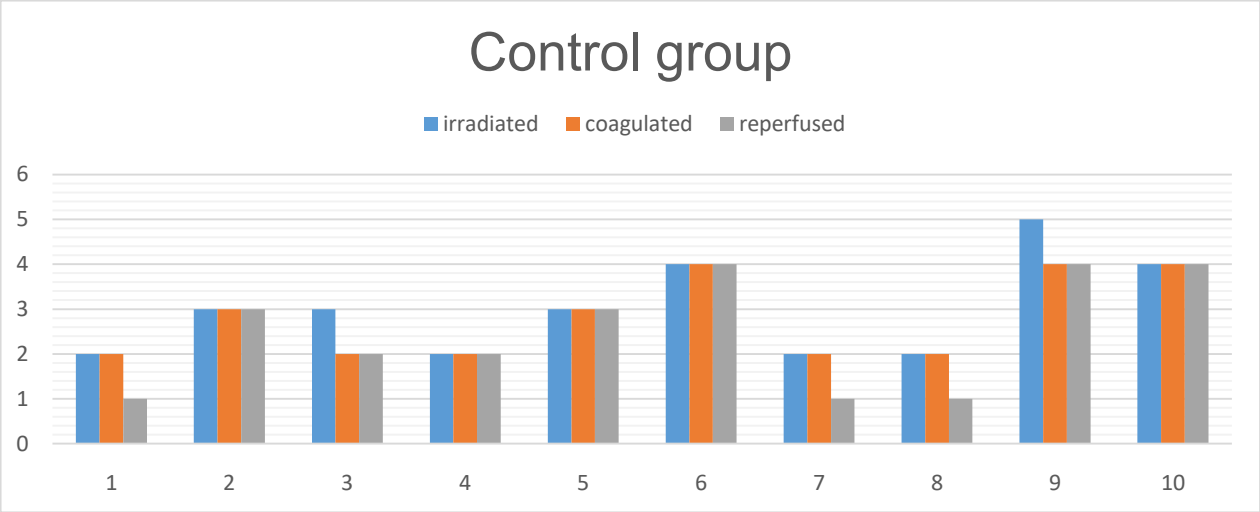

Diagram.1: Results of Control group

2. Thalidomide group:

This group contains 10 rats, all were received 0,05ml (5IU) thalidomide 50mg injected intra dermal after laser irradiation of 30 dorsal blood vessels in day 0, the parameters of laser irradiation used for this group were similar to those for the control group.

Day 0: laser coagulation of 26 vessels as shown in fig. (3 a) then thalidomide injection was done.

Day 3-7: significant reduction in cutaneous blood vessels and no reperfusion occurred in all vessels fig. (3 b)

Day 14: no reperfusion occurred and minimal neovascularization are observed in 2 major vessels fig. (3 c)

It was noted that skin necrosis on the corners of the window chambers occurred in several rats which might be due to insufficient blood supply after vessels coagulation.

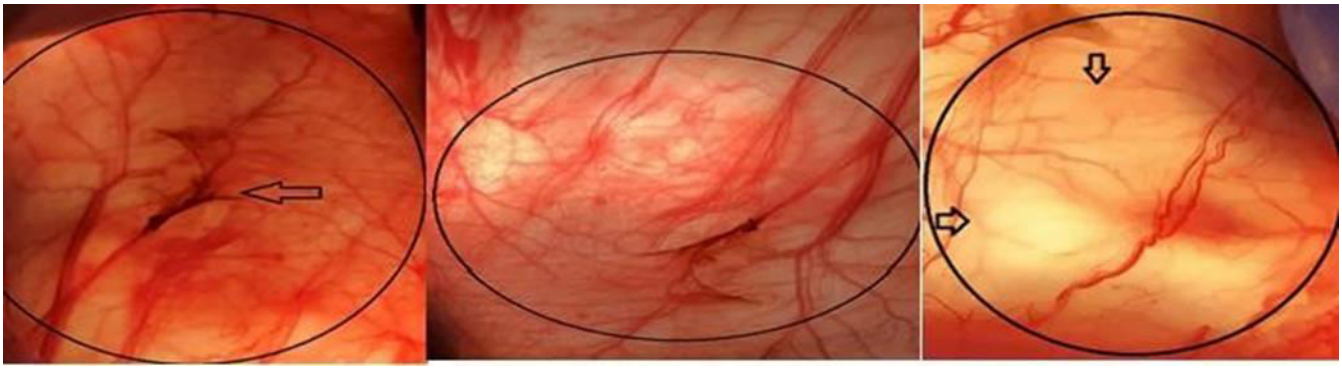

Figure.3: a b c

3.Rapamycin group:

This group contains 10 rats, first all were received 0,1 ml (10 IU) rapamycin (Sirolimus) 1mg injected intradermal after laser irradiation on day 0, but no significant effect in post irradiated days (3,7,10), so we increased the dose to 0.2ml.

Day 0: laser parameters used for this group were similar to those for the control group,30 blood vessels irradiated, 26 photocoagulated blood vessels were observed (Fig. 4 a), then 0,2 ml rapamycin 1 mg injection was done.

Blood flow in the coagulated vessels was absent immediately and 1 day after laser irradiation in most rats.

Day 3-7: the window showed erythema and small vessels persisted as shown in (Fig. 4 b). But those vessels therefore regressed and no reperfusion was observed in several coagulated vessels.

Day 14:12 major vessels reperfused (Fig. 4c). fur grew was diminished as a common side effect of rapamycin.

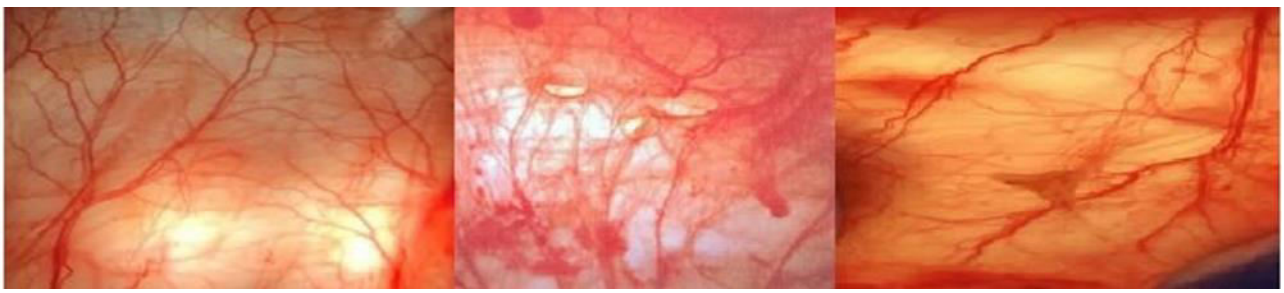

Figure.4: **a** **b** **c**

Table 1: Results of Control, Rapamycin and Thalidomide:

Group	Coagulated	Reperfused	Reperfusion %
1.Control	28	26	93%
2.Rapamycin	26	12	46%
3.Thalidomide	26	2	8%

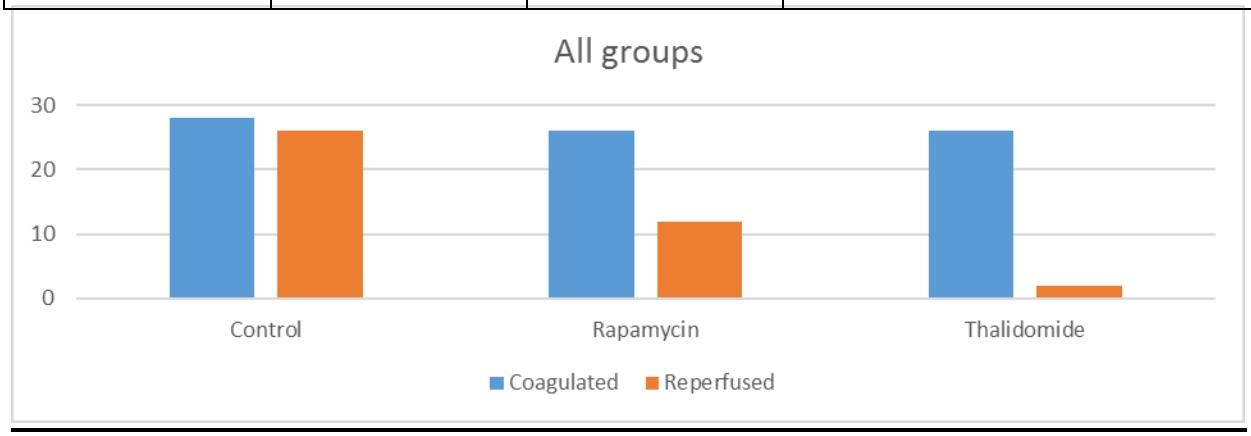

Diagram. 2: All groups results

Results of data analysis:

ANOVA test was used to detect the most significant antiangiogenic drug.

F-statistic value = 0.53306

P-value = 0.61222

Thalidomide group has significant antiangiogenic effect in comparison to other groups.

Discussion:

Based on mechanism of action, the angiogenesis inhibitors divided into:

1-Drugs inhibit the endothelial cells growth:

e.g. Endostatin and Combretastatin A4: cause endothelial cells apoptosis.

Thalidomide is a powerful inhibitor of endothelial cell growth.

2-Drugs block the angiogenesis signaling:

e.g. anti-VEGF antibodies (Avastin which has a food and drug agency "FDA" approval for colorectal cancer), Interferon-alpha (inhibits the production of basic fibroblast growth factor "bFGF" and VEGF) [18].

3-Drugs block the extracellular matrix breakdown:

Inhibitors of matrix metalloproteinases enzyme "MMPs" e.g: (Sunitinib,Sorafenib,rapamycin) [18].

Antiangiogenic drugs are essential lines of treatments to several diseases like: Vascular malformation (port wine stain), Diabetic retinopathy, Multiple myeloma, Solid tumors and Metastasis.

Tumors angiogenesis: angiogenesis is one of the most important steps in the hematogenous metastasis as it provides the routes for the tumor cells to migrate from the primary tumor site and allows their seeding into distant organs. Tumor angiogenesis is generation of a network of blood vessels within the cancerous site. This process can occur by :1- Release of signaling molecules by the tumor cells; these molecules activate the surrounding tissue to promote growth of new blood vessels which stimulates vascular endothelial cells to divide rapidly.

2-Generation of new vasculature by vasculogenesis. The tumor cells trans-differentiate in endothelial-like cells and create structures from inside of the tumor tapping into a nearby blood vessel [33].

Since angiogenesis is critical for tumor growth and metastasis, anti-angiogenic treatment is a highly promising therapeutic regimen. Thus, there has been a lot of researches aimed towards the discovery of angiogenesis inhibitors. More than forty anti-angiogenic drugs are being tested in clinical trials all over the world. [4]

Thalidomide and its analogues (Linalidomied, pomalidomide) were synthesized several decades ago as a drugs against respiratory infections and were administered to pregnant women for relief of morning sickness. The drugs were withdrawn from the market when its teratogenic effects discovered. Researchers in Japan showed that the developmental defects caused by thalidomide stem from the drug's ability to bind to the protein cereblon (CRBN). The findings provide the first step for developing new thalidomide analogs and derivatives with reduced risk of birth defects. [30] Thalidomide suppresses cell proliferation and angiogenesis and control the invasion and metastasis of tumors in pre-clinical studies.

With its immunomodulatory and anti-inflammatory, properties, thalidomide is used in the treatment of many forms of cancer and autoimmune conditions by modulating the signaling pathways via the mediation of vascular endothelial growth factor, phosphoinositide-kinase/protein kinase B and nuclear factor kappa B, and mammalian target of rapamycin, which integrates these signaling systems. [19]

Thalidomide researches showed that: thalidomide is used to treat or prevent certain skin conditions related to Hansen's disease which known as leprosy (erythema nodosum leprosum). Thalidomide is also used to treat a certain type of cancer (multiple myeloma). It works in Hansen's disease by reducing swelling and redness (inflammation). It also reduces the formation of blood vessels that feed tumors. It is effective as an adjuvant therapy in newly diagnosed cancer patients with small residual tumor, because thalidomide inhibits neovascularization of microscopic residual tumor cells and prevents the development of macroscopic, angiogenesis dependent tumor masses. [20]

Thalidomide is already listed as a treatment of multiple myeloma. Angiogenic cytokines such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor are expressed by myeloma cells and appear to play a role in the increased angiogenesis seen in myeloma. In addition, VEGF may serve as a paracrine growth factor for myeloma cells. Based on the increased angiogenesis observed in myeloma, thalidomide has been studied as antiangiogenic therapy. Although its mechanism of action in myeloma is still unclear, thalidomide appears to be active in 25–30% of patients with refractory myeloma. Major toxicities include constipation, sedation, skin rash, fatigue, and peripheral neuropathy. Studies are ongoing to determine its role as initial treatment for myeloma. [31]

Thalidomide is well sustained with systemic use, the most common side effects of thalidomide systemic use are: constipation, peripheral neuropathy and sedation. (after treatment with thalidomide for more than a year). [21]

Clinical studies on local use of thalidomide demonstrated that low-dose thalidomide was safe and effective for the therapy of epistaxis in Hereditary Hemorrhagic Telangiectasia (HHT) patients. However, following two months of oral administration, important adverse effects have been registered obliging the discontinuation of the therapy. The local administration into the nose of the drug is a way to maintain the positive result obtained, eliminating adverse effects. The nasal powders will enable thalidomide maintenance therapy as a complement of systemic oral administration. This result contributes to a safer re-positioning of a very active drug. [32]

Rapamycin is used as an immunosuppressant and angiogenesis inhibitor drug, we examined whether immunosuppressive doses of rapamycin can inhibit neovascularization.

Rapamycin diminish endothelial cell splitting or sprouting by its effect on the expression of proliferating cell nuclear antigen (PCNA), apoptotic cell death and vascular endothelial growth factor (VEGF). [22]

Another studies showed that fur grew much slower as compared to other shaved and depilated skin when topical rapamycin was applied to animal models. [23]

The dorsal skin fold window chamber is one of the few in vivo animal models which allows serial imaging and application of topical drugs.

Although rat skin is thinner and contains different structures (e.g., subdermal muscle) from human skin, the ultra-structure of the post-capillary vessels within rodent skin is similar to those in humans because the neovascularization is believed to be caused by the progressive ectasia [24].

The obvious difference between the human skin and rodent skin is that there are multiple hair follicles in rodent skin. Fur growth after depilation may change the window's microenvironment [25].

Direct contact between the glass window and sub-dermis can cause an inflammatory response which may change the window's microenvironment.

This concern can be avoided by coating the glass window with a thin layer of bio-compatible material (sterile physiological saline).

The preference of local application of drugs is that it could be delivered to the dermis while avoiding systemic drug absorption and associated side effects [26].

Our results strongly indicate that neovascularization and reperfusion occurred in the majority of the photocoagulated blood vessels in laser group (control group) (Fig. 2 and diagram 1).

The nearly 100% reperfusion rate (diagram 1) in laser group suggests a lower therapeutic efficacy of laser only modality.

The discrepancy might be related to the length of the irradiated blood vessel segment (2mm). Although other studies are needed to determine if a lower reperfusion rate can be achieved when longer segment is irradiated.

Reperfusion could be caused by biological mechanisms or mechanical mechanisms such as: blood flow return in partially photocoagulated blood vessels, angiogenesis and neovasculogenesis.

Because vessel coagulation was essentially complete in this study, we assumed that angiogenesis (neovasculogenesis) is responsible for vessel reperfusion and local injection of antiangiogenic drugs inhibits neovascularization and reperfusion of such vessels (Fig.2,3,4).

Majority of studies demonstrate the ability of thalidomide to inhibit basic fibroblast growth factor (bFGF)-induced angiogenesis.

Antitumor effects of thalidomide may not due to its antiangiogenic effect only but due to other mechanisms, such as the immunomodulatory effect of thalidomide. [27]

Recently, Thalidomide shown to increase the anti-tumor effect of carboplatin in breast cancer experimental, but the mechanisms involved in this therapeutic regimen are still in need to be studied. [28]

Future studies on thalidomide and other cytotoxic drugs will be required to determine that serum bFGF could be a useful biologic marker to the antiangiogenic effect of these drugs.

In case of rapamycin, inhibition of the mTOR–HIF-1a–VEGF pathway is expected to play an important role in preventing vessels reperfusion after laser irradiation and the antiangiogenic effect of rapamycin is also due to a direct antiproliferative response on VEGF-stimulated endothelial cells by inhibition of the PI3K–p70S6 kinase pathway [29].

However, in our study newly formed blood vessels in rapamycin-treated animals were regularly perfused and showed normal vessel morphology, so we conclude that rapamycin impairs the vascularization by inhibition of VEGF-mediated angiogenesis, but production and release of different angiogenic growth factors causes reperfusion, which may explain our results that rapamycin treatment was not sufficient to prevent angiogenesis significantly.

Two different rapamycin formulae were tested in this study. The results indicate that Dosing of rapamycin is important to achieve an optimal antiangiogenic effect.

-Side effects: no significant side effects detected.

Rapamycin group: Fur grew normally.

Thalidomide group: No constipation or sedation observed.

Conclusion:

Finding a tumor-selective, nontoxic cancer therapy based on the simple idea of starving the tumor of its blood supply by inhibition of angiogenesis, has led to a significant amount of interest in recent researches.

Thalidomide group had the lowest reperfusion rate 8%. Thalidomide is the most effective antiangiogenic drug and its antiangiogenic effect remains for weeks with minimal side effects detected with local use.

Compliance with Ethical Standards:

-Conflict of interest statement: I declare no competing interests.

-Ethical Approval: By ethical committee of national institute of laser enhanced Sciences-Cairo University-Egypt-Ref.no.:1212201752707

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