Antiangiogenic Effects Of Ktp Laser Activated Gold Nanoparticles In Prevention Of Neovascularization

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Abstract: This study examines the antiangiogenic effect of activated naked gold nanoparticles (AuNPS) in dorsal skinfold window chamber rat model after laser coagulation of dorsal blood vessels, to investigate whether the antiangiogenic effect of AuNPs can inhibit reperfusion and neovascularization of photocoagulated blood vessels in an animal model.This study includes 2 groups, each group contains 10 rats, laser group (control group), and AuNPs group. After laser photocoagulation of dorsal blood vessels through the dorsal window chamber fixed on the dorsum of albino rats, the structure of blood vessels and flow dynamics were documented with color digital photography to evaluate photocoagulation and reperfusion. The laser sessions and injection of AuNPs to the epidermal side of the window were twice weekly for 2 weeks. In the laser group (control group), 18 out of 20 photocoagulated blood vessels reperfused within 4-12 days with reperfusion rate 90%. In AuGNPs group 9 out of 22 photocoagulated blood vessels reperfused within 4-12 days with reperfusion rate 40%. Laser activated naked gold nanoparticles have significant antiangiogenic effect, with minimal side effects detected with local use.

Keywords: angiogenesis, antiangiogenesis, gold nanoparticles, Ktp laser, diode laser, skinfold window chamber.

1. INTRODUCTION:

Solid tumor growth is strongly dependent on angiogenesis. Tumor angiogenesis is a proliferation of blood vessels in the surrounding areas of a cancerous site which plays an important role in cancer growth. [1]
Blood vessels supply tumors with oxygen, nutrients and remove waste products enhancing cancer growth, Thus, blocking the supply of blood to the tumors has been suggested as an approach to fight cancer. [2].
Tumor angiogenesis is the result of a complex interaction between tumor cells, endothelial cells, and other stromal cells, Angiogenesis can be due to:
(a) vascular destabilization mediated by the Angiopoietin/Tie2 axis.
(b) endothelial cell activation strongly dependent on Vascular Endothelial Growth Factor (VEGF) and its receptor VEGFR-2. [3]
There are sub-types of angiogenesis e.g., Sprouting angiogenesis which involves stimulation of endothelial cells to proliferate into the surrounding matrix and form solid sprouts extending to the angiogenic stimulus leading to the formation of an entirely new vessel [4],

Intussusception or splitting angiogenesis which involves division of the lumen of a vessel resulting in formation two vessels [5]

Multiple angiogenetic factors are secreted by blood-deprived (ischemic) cells and these interact with the inner lining (endothelium) of existing blood vessels to cause the budding out of new capillaries [6].

Angiogenesis is controlled by chemical signals in the body. Some of these signals, such as vascular endothelial growth factor (VEGF), which bind to receptors on the surface of normal endothelial cells. When VEGF and other endothelial growth factors bind to their receptors, signals within the endothelial cells are initiated promoting growth and survival of new blood vessels. Other chemical signals, called angiogenesis inhibitors, interfere with new blood vessel formation. [7]

Angiogenesis inhibitors interfere with various steps in blood vessel growth. Some are monoclonal antibodies that specifically recognize and bind to VEGF eg: bevacizumab(Avastin). When VEGF is attached to these drugs, it is unable to activate the VEGF receptor. [8]

Other angiogenesis inhibitors bind to VEGF and/or its receptor as well as to other receptors on the surface of endothelial cells or to other proteins in the signaling pathways, blocking their activities.

Some angiogenesis inhibitors are immunomodulatory drugs agents that stimulate or suppress the immune system that also have antiangiogenic properties eg: Thalidomide [9]. Searching for new drug which can modify angiogenic process to treat human diseases is highly needed.

Despite the availability of anti-angiogenic drugs, the use of these drugs is limited by the lack of therapeutic benefits in specific medical conditions, adverse effect profile, and the potential for the development of drug resistance.

Recent cancer therapies like: surgery, radiation therapy, chemotherapy, immunotherapy and photodynamic therapy (PDT), have several limitations and side effects. [10]

So development of an economically affordable alternative technique for the treatment of cancers that will specifically target the tumor without harming the surrounding healthy tissues is urgently needed. [11]

Naked gold nanoparticles(AuNPs) have received a wide spread interest in molecular imaging, diagnosis and targeted therapy of malignant tumors.

In our study we examine whether that AuNPs, activated by light with the appropriate wavelength, can act as efficient photo thermal agents mediating antiangiogenic therapy. [12]

Photothermal Therapy (PTT) has been modified a lot recently, due to the enhanced photothermal capabilities of the new generations of nanoscale photothermal agents.

Among these nanoscale agents, gold nanoshells and nanorods have optimal properties for translation of near infra-red radiation into heat. smaller gold nanoparticles (AuNPs) are easier to produce, less toxic and show improved photoconversion capability that may profit from the irradiation in the near infra-red region via standard green lasers. [13]

A KTP laser is a solid-state laser that uses a potassium titanyl phosphate (KTP) crystal as its frequency doubling device.

The KTP crystal is implicated by a beam generated by a neodymium: yttrium aluminum garnet (Nd: YAG) laser.

This is directed through the KTP crystal to produce a beam in the green visible spectrum with a wavelength of 532 nm, [14] which can activate AuNPs with small diameters.

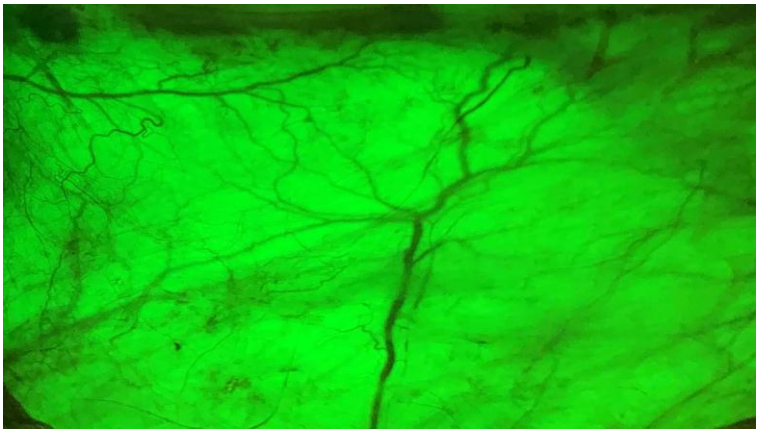

Figure.1 (KTP laser activation of AuNPs)

AuNPs has unique physicochemical properties, including their surface Plasmon resonance (SPR), which depend on the interaction between an electromagnetic wave and free conduction electrons at the AuNPs' surface, causing them to oscillate coherently in resonance with the frequency of visible light, forming a strong electromagnetic Felds.

This phenomenon enhances both the scattering and the absorption of light by the AuNPs and make it suitable for different biomedical applications. [15]

The dorsal skinfold window chamber model is one of the few methods that allows direct examination of in vivo tumor microcirculation. Several modifications to the original design have made it less susceptible to trauma-induced damage, it`s main advantage is that it allows for noninvasive serial observation of tumor microvascular development and tumor blood flow after various treatments. [16]

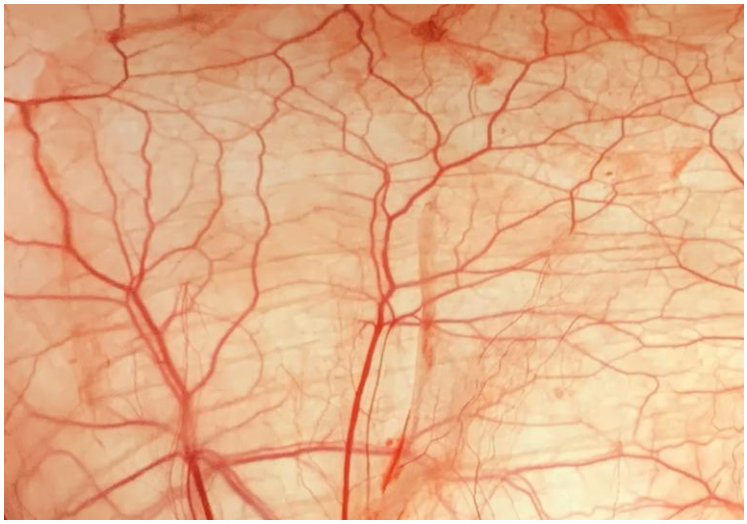

Figure.2(normal dorsal skin vasculature.)

2. MATERIALS AND METHODS:

Animals

All experiments were initiated after a protocol approved by the Animal Care and Use Committee, nuclear materials authority (CU - 1580), Egypt. twenty Adult albino male rats with an initial bodyweight of 100-150 g were assigned to tow groups: laser group (control group), gold nanoparticles group.

All rats anesthetized by intra peritoneal injection of 0.5mg ketamine mixed with xyla-ject solution.

Dorsal Window Chamber Model:
A dorsal window chamber (DWC) was installed on each animal. This model was first described by Algire in 1943, consists of a lengthwise fold of dorsal skin with an implanted clear glass window that permits in vivo visualization and manipulation of the subdermal blood vessels (figure 2).

The window chamber, when properly prepared, provides excellent view in subdermal blood vessels for up to 3-4 weeks.

Briefly, after the animal was anesthetized, the dorsal skin was shaved, epilated, and midline dorsal incision was done, one layer of skin lifted to form a skinfold. A pair of rigid polyvinyl chloride (PVC) window frames were attached to the front and backsides of the dorsal skinfold with sutures.

But tissue reaction occurs within hours after installation causing hazy and unclear images. Another pair of plastic widow frames was used, one layer of skin and subcutis with the panniculus carnosus was completely removed within the circular area of the frame's observation window to expose the subdermal blood vessels in the underlying intact skin, and clear images of dorsal blood vessels was taken.

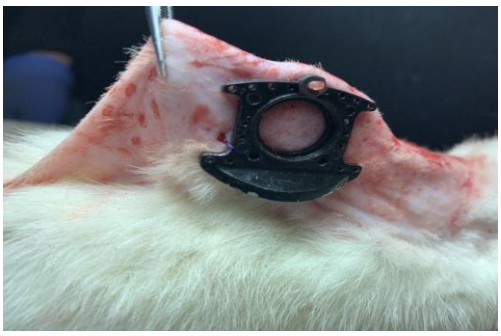

Figure.3 (Plastic window chamber installation)

A thin glass window (12mm in diameter and 0.2mm in thickness) was then inserted into the window frame to protect the sub dermis from dehydration and contamination as shown in figure (3) The window frames were strategically placed on the backs of the animals to enable visualization of a tree-like vascular network for the experiments.
Locaol antibiotic cream was applied daily to the surgical site to prevent wound suppuration.

Laser Irradiation

laser irradiation performed on the window (subdermal) side of the preparation. Blood vessels irradiated with a class 4, diode laser 980 nm (quanta system spa 21058 Solbiate Olona (VA), Italy) which emits a sequence of a variable number of pulses.

Diode laser has a potent thermal perifocal effect in tissues and a shallow penetration depth, and is associated with fewer hazards to deep structures [17].

The number of pulses could be varied from 3 to 8 and the pulse repetition rate could be up to 2 Hz. The duration of an individual pulse is 10 milliseconds and the radiant exposure varies from 20 to 25J/cm^2 with the 2mm spot used in this study.
Laser pulse energies were verified using an energy meter (FL250A-SH with Nova display, Ophir, Logan, UT). Both single and multiple laser pulses were used to irradiate blood vessels.

Gold nanoparticles

Injection of 5 IU GNPs with an average diameter 13±1-2 nm and optical density (OD518 nm =3) were directly injected intradermal twice weekly for 2 weeks.

GNPs prepared by the citrate reduction of tetraachloroauric acid following the method introduced by Turkevich et al and refined by Frens [18-19]. Before the reduction process, all glass wares were cleaned in aqua regia (3 parts HCl, 1 part HNO3), rinsed with deionized water and then dried.

An aqueous solution of tetraachloroauric acid (HAuCl4 · 3H2O, 1 mm) purchased from Sigma- Aldrich, was brought to boiling condition and stirred continuously then a 38.8 mm of trisodium citrate dehydrate solution (Sigma-Aldrich) was added quickly at one time, resulting in a change in solution color from pale yellow to black to deep red. GNPs were diluted in deionized water and the quality and size of the prepared nanoparticles were investigated using UV-visible spectrophotometry (T80+ PG instruments) and transmission electron microscopy (JEM 1230 EM, JEOL).

Rats were extracorporeally exposed to superficial green laser light (Fig 1) with parameters (CW λ 535 nm, power 0.5–1.0 W, laser fluency 200 J/cm2, and duration 15–30 minutes), once every 3 days, for 2 weeks.

After every session plastic cover was loosely attached to the backside of the chamber to prevent sub-dermis from dehydration and contamination.

Assestment of vessels reperfusion:

- Color images by high definition 12 megapixel Sony digital camera are recorded before and after laser irradiation to locate photocoagulated blood vessels in which complete flow stoppage occurred. The blood vessels were followed in the color images to determine if tributaries or reperfusion occurred and to observe neovascularization. Epidermal images were also analyzed to determine if adverse cutaneous effects (e.g., skin irritation, scabbing, or ulceration) occurred.
- In each group of the study some experimentals discontinued the trial due to death or sever adverse effects (data not shown).

3. RESULTS:

1- Laser group (control group):

This group contains 10 rats; a window surgery was performed in order to monitor the stability of this animal model.

It can be noted that the window skin did move downward slightly due to the effects of gravity over the course of 14 days. 6 rats with plastic DWC eroded the outer side of the chamber.

In each window, a 2mm segment of all major branches which normally had a pair of arteriole and venule was irradiated. The stem of the tree-like vascular network in the window was left intact.

Intense photocoagulation of the blood vessels was observed after laser irradiation (Fig. 4) and blood flow in the irradiated blood vessels was absent.

Vasoconstriction and vessel disappearance immediately after laser irradiation were observed. The skin typically became erythematous with the appearance of vascular regeneration and reperfusion around the irradiated sites 3 days after laser exposure (Fig. 4).

On Day 14, the vessels appeared similar to those observed before laser irradiation. The results from all rats in the laser group are summarized in Table (1). In total, 20 vessels were

irradiated and photocoagulated, No blood flow on the same day of irradiation. 18 out of 20 vessels reperfused during a week after irradiation.

Typically, reperfusion and excessive neovascularization occurred between 3 and 14 days after irradiation.

The interval between laser exposure and reperfusion did not appear to be related to the radiant exposure applied (data not shown).

The only adverse effect noted in this study group was that skin necrosis on the top corner of the window chambers occurred in several rats which might have been caused by insufficient blood supply due to vessel coagulation.

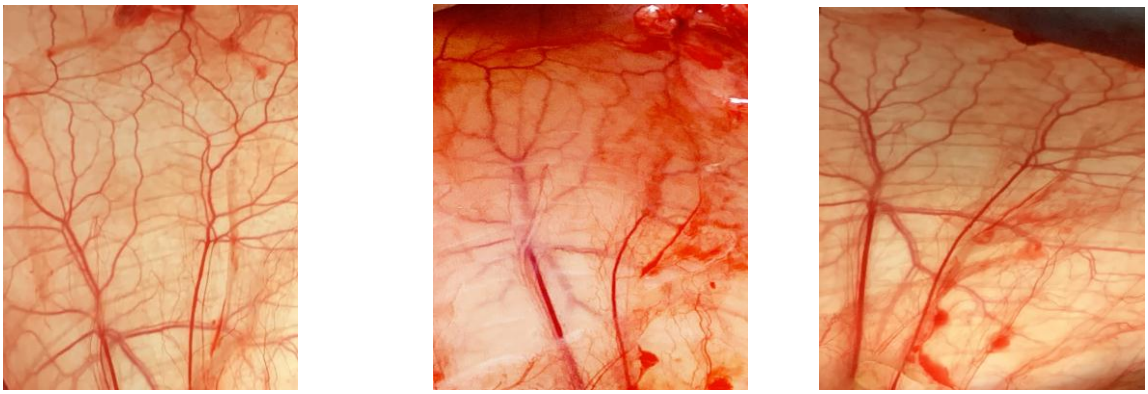

Figure (4):
a. Before laser **b. after laser ablation** **c. day (14)**

TABLE 1. Detailed Results in the Laser Group:

Animal	**coagulated**	**Reperfused**
1	**2**	**2**
2	**2**	**2**
3	**2**	**2**
4	**1**	**1**
5	**2**	**2**
6	**2**	**2**
7	**1**	**1**
8	**1**	**1**
9	**3**	**2**
10	**4**	**3**
Total :	**20**	**18**

2.laser+AuNPs:

This group contains 10 rats, all were received 0,1 ml GNPs with an average diameter 13±1-2 nm, injected intradermal after laser coagulation of dorsal blood vessels on day 0.

Laser irradiation parameters used for this group were identical to those for control group, photocoagulation of the blood vessels was observed after laser irradiation (Fig. 5 a).

Blood flow in the irradiated blood vessels was absent. at Day 3, the window was erythematous and small blood vessels persisted as shown in (Fig 5).

On Day 14, 9 vessels appeared similar to those observed before laser irradiation. The results from all rats in AuNPs group are summarized in Table (2). In total, 22 vessels were irradiated

and photocoagulated, No blood flow on the same day of irradiation. 9 out of 22 vessels reperfused during 2 weeks after irradiation (Fig 5).

TABLE 2. Detailed Results in the AuNPsGroup:

Animal	coagulated	Reperfused
1	2	1
2	2	1
3	2	2
4	1	1
5	2	1
6	2	1
7	2	0
8	2	0
9	3	1
10	4	1
Total :	22	9

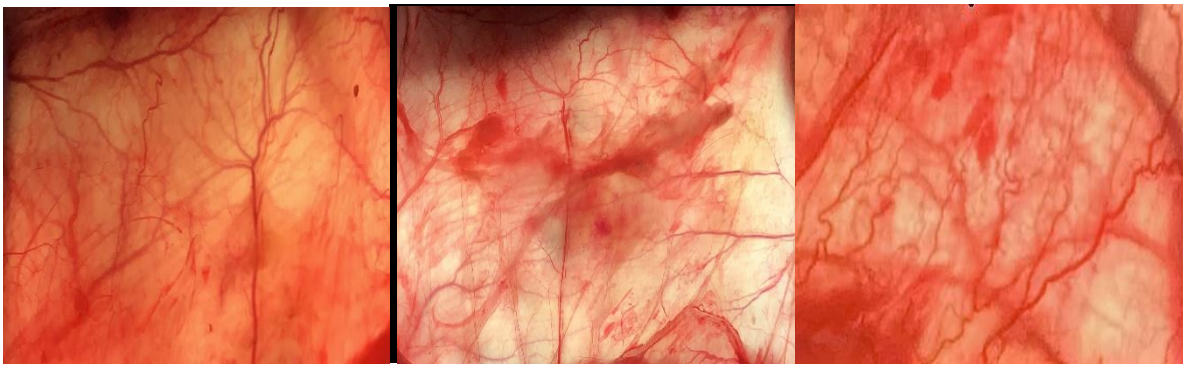

Figure (5): a b c

4. DISCUSSION:

Intravascular thrombus formation has long been regarded as a process that involves an interaction between the blood stream and an altered inner blood vessel surface.
Thrombogenesis as visualized microscopically in the living animal, has been produced experimentally by mechanical, chemical, and electrical injuries to blood vessel, these diverse types of injury have generally been induced through the outer layers of the vessel wall and have resulted in a consistent reaction on the inner vascular surface. This has consisted of an adherence of blood cells, especially platelets, to the endothelium close to the site of injury, the adherence of cells to each other, and the development of a thrombus.
The thrombogenic lesions produced by means of an optically focused laser beam are in many respects comparable other techniques of microvascular injury, offers certain distinct advantages [20].

In addition to its ready availability and ease of handling, it presents the possibility of a high degree of localization and a fine gradation in the severity and size of the induced intravascular lesions.

The induction of intravascular micro thrombosis, embolization, recanalization, and interstitial hemorrhage by means of the laser presents a unique opportunity for the study of these phenomena without the necessity of introducing a foreign material or instrument into the circulation or surrounding tissue [21].

Naked gold nanoparticles (AuNPs) can inhibit the function of pro-angiogenic heparin-binding growth factor, such as VEGF 165, basic fibroblast growth factor (bFGF).

These inorganic anti-angiogenic agents may overcome the unusual toxicities associated with traditional anti-angiogenic agents currently used in the clinics. [22]

AuNPs with a diameter of 14nm are taken up quickly by cells within the first two hours of incubation, gradually slowing the uptake rate until reaching a plateau at 4–7h. [23]

AuNPs are also recognized by their photo thermal capacities, converting electromagnetic radiation into heat due to electron excitation and relaxation, which used for thermal ablation of tumor cells. AuNPs of specific sizes and shapes, including gold Nano rods, Nano shells and Nano rods are capable to convert NIR radiation into heat. NIR lasers (ex, KTP 523nm) are used in AuNP induced PTT due to the optical window in the near-infrared, where hemoglobin, melanin and water absorption is reduced, allowing deeper light penetration into fluids and tissues. [24]

Our results clearly indicate that neovascularization and persistent reperfusion occurred in photocoagulated blood vessels in this animal model treated by laser (Fig. 4 and Table 1).

However, local injection of antiangiogenic materials (AuNPs) inhibits neovascularization and reperfusion of such vessels in the DWC model (Fig.4,5).

Reperfusion can be caused by mechanical and biological mechanisms such as flow restoration in incompletely photocoagulated blood vessels, angiogenesis and neovasculogenesis [25].

Because vessel coagulation was essentially complete in this study, it is hypothesized that angiogenesis/neovasculogenesis is responsible for vessel reperfusion.

Shutdown of major branches in the window induced a severely hypoxic microenvironment which can cause overexpression of hypoxia inducible factor-1 alpha (HIF-1a) which in turn promoted the secretion of angiogenesis-stimulating factors [29] such as platelet-derived growth factor [26] and VEGF [27].

The exact underlying mechanism deserves further study to optimize a combined laser and antiangiogenic drugs in prevention of neovascularization.

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

The discrepancy might be related to the length of the irradiated blood vessel segment (2mm in this study as compared to 7–10mm for PDL). Although further study is needed to determine if a lower reperfusion rate can be achieved when the segment irradiated is longer, it is reasonable to assume that reperfusion would occur and topical antiangiogenic drugs would still be required.

The DWC model is one of the few in vivo animal models which permits serial imaging and application of topical agents.

Although rat skin is thinner and contains some elements (e.g., subdermal muscle) not seen in human Skin, the ultra-structure of the post-capillary venules within rodent skin is comparable to those in humans because the neovascularization is believed to be caused by the progressive ectasia [28].

The major difference between the rat skin and human skin is that there are numerous hair follicles in rat skin. Fur regrowth after depilation may alter the window's microenvironment

because VEGF expression in hair dermal papilla cells (specialized mesenchymal cells in the hair follicle) was previously reported [29].

Also of concern with the DWC model is that the direct contact between sub-dermis and glass window can induce an inflammatory response which may affect the window's microenvironment.

This concern can be alleviated by coating the glass window with a thin layer of biocompatible material (sterile physiological saline).

The advantage of topical application is that it could be delivered to the dermis while avoiding significant systemic drug absorption and associated side effects [30].

70% of rats in Gold nanoparticles group showed loose diarrhea and irritability.

5. CONCLUSION:

Laser activated naked gold nanoparticles have significant antiangiogenic effect, with minimal side effects detected with local use.

Photothermal therapy is a minimally invasive treatment modality in which photon energy is converted to thermal energy sufficient to induce cellular destruction. This modality holds a great promise as a selective hyperthermia in cancer treatment by using AuNPs in combination with laser irradiation of the appropriate wavelength.

Compliance with Ethical Standards

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

-Role of funding source: No funding source, the research is self-funded.

-Ethical Approval: By ethical committee of national institute of laser enhanced Sciences-Cairo University-Egypt-Ref no. :12122017527077.

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