Original Article

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A Comparison Between the Effect of Low-Level Laser Therapy and Clomiphene on Rats' Ovarian Tissue Blood Vessels Under In Vivo Conditions

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Abstract

Background: Studying the prevalence of infertility as a common disease and its treatment have recently become an important issue. These treatments include drugs and surgery; and lasers have also been used complementarily. However, they affect the ovarian blood vessels number. Accordingly, the present study was conducted to investigate the photo-bio stimulation effect on near-infrared and red laser as of low-level lasers on the blood vessels in ovarian tissue, compared with the clomiphene's effect.

Methods: Twenty-eight female rats were randomly divided into four groups: Control (CT), Clomiphene Drug (D), Red Laser (RL) and Near-Infrared Laser (NIRL). Afterwards, the laser groups received irradiation (5 J/cm^2 dose) and the rats in group D received clomiphene ($1\mu g/kg$). After the experiment, the animals were anesthetized and their ovaries were removed. Henceforth, the laser's effect was investigated and compared with that of clomiphene.

Results: The number of the blood vessels increased in NIRL (71.96%) and RL (67.070%) groups, compared with CT. It was also increased in NIRL (59.81%) and RL (14%) compared to D group. In addition, it increased in NIRL (53.27%) compared with RL. This increase was significant in the NIRL group indicating that NIRL increases ovarian activity to produce blood vessels that can be certainly used in future studies for finding a cure for ovarian negligence to produce more blood vessels and treat diseases caused by it.

Conclusion: The results convinced us to introduce laser as a new method to increase the ovarian activation by increasing the blood vessels in a future study. Although the mechanism of low-level lasers action is not well known, it has been proved to be more effective than clomiphene.

Keywords: Animals, Clomiphene, Female, Infertility, Lasers, Ovary, Prevalence

Introduction

The development of new ovarian blood vessels from the existing ones (angiogenesis) is a complex event (1). Angiogenesis in ovaries is a physiological function (2,3). Also, the ovarian function depends on the complex vascular system due to their follicles receiving nutrition, oxygen and effective hormones (4,5). Moreover, the inhibition of angiogenesis caused the attenuation of follicular growth and hence, disruption of ovulation (5,6). Any disorder in folliculogenesis cycle leads to ovarian tissue damage as Polycystic Ovary Syndrome (PCOS) does (7,8). PCOS is one of the most common causes of an ovulatory infertility which was first described by Stein-Leventhal in 1934 (9,10). Various methods are used to treat PCOS; *e.g.*, clomiphene citrate, insulin sensitizer as metformin, weight reduction, gonadotropin therapy, and lasers (11,12). Nevertheless, there is no exact treatment for PCOS and there is an unmet need for such a treatment (9,13).

Clomiphene as first-line option in women suffering PCOS, is a standard drug for ovulation induction and still considered (14-16). And angiogenesis is critical for follicular growth, and ovulation in the ovary. Angiogenic factor dysregulation may contribute to ovulatory dysfunction and subfertility, which are commonly observed in women with PCOS (8). During the menstrual cycle, clomiphene increased the number of blood vessels in the ovary bearing the dominant ovarian follicle (17). Also, clomiphene as a competitive inhibitor of estrogen is given early in the menstrual cycle which increases the concentrations of circulating Follicle-Stimulating Hormone (FSH) leading to the development of a number of follicles. Unfortunately, approximately 15–25% of PCOS patients fail to respond to clomiphene treatment (18). Therefore, it is very important to find certain treatments, without drugs such as clomiphene (13).

Laser therapy is a new method of treatment that dates back to the 1960s. In this method, light is backscattered or a fraction of the light is back-reflected by a remote target and then is allowed to re-enter the laser cavity. Through this process, both the amplitude and frequency of the lasing field can be modulated (19-21). In 1978, this phenomenon was experimentally demonstrated by Donati who called it Self-Mixing Interferometry (SMI) or Optical Feedback Interferometry (OFI), which is known as the diode laser. Diode lasers have been applied in various fields of science, especially medicine. Although Low-Level Laser therapy (LLLT) is studied widely in gynecology to treat different diseases, in most cases, LLLT is used as a complementary therapy (19,21-23). A previously published study showed that LLLT could increase the growth of cells by increasing the blood vessels in tissues (24). Hence, the present study was aimed at investigating the effects of LLLT on the ovarian tissue as a complementary treatment for increasing the ovarian function to produce oocytes before oocyte formation. For this purpose, we used LLLT optical properties under in vitro conditions.

Materials and Methods

Preparation of the animals

Firstly, 28 sexually mature female Wistar rats within the weight range of 150-300 g were supplied from animal house of Tabriz University of Medical Science (TUMS), Medical Physics Department in Iran. The number of animal samples in the study was designed based on the previous related studies (25,26). According to these studies that used 5 to 8 animals per each group in the study, we used 6 to 10 rats per group in our study to prevent any bias.

After checking their generation health, the rats were placed in the same phase of the menstrual cycle by the estrous test. Then, their skins were shaved and the shaved areas were washed by betadine and the exact location of the laser radiation on their skin was marked by the marker. The animals were divided randomly into four groups with an equal member of members (n=7); Control (CT), Clomiphene drug (D), Red Laser (RL), and Near-Infrared Laser (NIRL). Rats were painted for each group and labeled from one to seven. In the next step, the rats were kept in new cages for one week in the ambient conditions with free food and water at 25-27°C in the laboratory to adapt to the new conditions. After a week, the rats were weighed to ensure they remain in the range. Their behavior in the cage was examined during the experiment. Also, the rats were kept in private cages during the experiment to receive interventions or cleaning (three times a week).

In this basic study, a repeated measurement study on rats was performed while they were respected as

animals and were not subjected to any harassment. Thus, no physical damage was inflicted. During the experiment, they were in the best condition in terms of feed, ventilation and cage hygiene. During the intervention, the rats entered the rat restrainer designed for this test without any stress. Finally, after the intervention, the rats were anesthetized with ketamine/xylazine and sacrificed. The process was done without any damages to the rats. Also, Ethics and Research Committee of TUMS (code number: TBZMED.REC.1394.238) approved the protocol of this study.

The process of intervention

There were seven rats in each group. Group D received 1 *μg/kg* of clomiphene in 1 *ml* water solution (27). Clomiphene can stimulate folliculogenesis cycle and increase sex hormone activation and consequently increase fertility (15) and RL intervention group received red spectrum laser, 33±2 power output, and 142.2 *seconds* of irradiation. On the other hand, NIRL group received near-infrared spectrum laser with 810 *nm* wavelength, 212±2 power output, and 28 *seconds* of irradiation. The laser beam area was 0.79 cm^2 with 10 *mm* diameter of nuzzle for both probes, and power densities were 41.77 mw/cm^2 for red laser and 268.35 mw/cm^2 for NIR laser beams. Continuous pulse laser mode has been found to be optimal for increasing the activity of proteins, ATP synthesis, and cell growth (28). Laser intervention was carried out based on a previously published protocol about laser implementation (22). It is worth mentioning that CT group as the control remained without any intervention.

This intervention was utilized to stimulate the ovarian tissue of rats. Irradiation occurred twice on the first two days in the menstrual cycle for 8 weeks (each 6 days, for 49 days). The rats in RL and NIRL groups were irradiated by the same nozzle on the skin by two diode lasers (MUSTANG 2000 +, Moscow, Russia) which transferred the same dosage during the experiment in each estrous cycle. Due to the same stress condition for CT and D groups, they have gone under the same nozzle without any irradiation. The laser probes were placed in direct contact with the skin, which was marked by a marker on a perpendicular angle (Figure 1). However, the percentage of transferred dose and irradiation time was already determined by a primary experiment. Henceforth, the same dose of laser was transferred to ovary for two groups. Moreover, water was used for impedance coupling between their skin and lasers. The average dose reaching the target was 5 J/cm^2 that was found to be optimal for increasing the activity of proteins, ATP synthesis, and cell growth (25). In this study, we introduced the irradiated time for two groups, red and infrared by calculating average dose to ensure how much the average dose has been reached the target. Transcranial Low-Level Laser Therapy (LLLT) treatment was done 16 times in 8 weeks. Also, the marked skin area thickness was measured to calculate skin attenuation coefficient by Lambert-Beer equation (28) (Table 1, Figure 2).

$I = I_0 e^{-\mu x}$

$\mu x = ln\ I_0/I$

Treatment process

All conditions were similar for all the rats, except for the treatments. After passing the experiment period and the overnight food deprivation, the animals were weighted again to have the same weight range. They were anesthetized with an intraperitoneal injection of ketamine/xylazine (60/10 *mg/kg*). The ovary was immediately removed and rinsed in a cold saline and weighed. The ovarian tissue was divided into two parts; one part was frozen in liquid nitrogen and stored at -80°C refrigerator until biochemical assay and the other part was immersed in 10% formalin for histopathological evaluations. For histopathological studies, some parts of the ovarian tissue were isolated and placed in a 10% formalin solution, dehydrated in ethanol, cleared in xylene and embedded in paraffin. After tissue processing steps, several serial sections of the ovary (5 *μm* thicknesses) were prepared and stained with Hematoxylin and Eosin (H&E) for microscopic observations and studies (Figure 3). The thickness of media tunica was measured using Motic Images version 2.0 and light microscope. The stained sections were also evaluated by a blind histologist (26,28).

The samples were evaluated and the results were analyzed using SPSS software, version 19. The significance of the data was determined and table and chart were drawn, accordingly.

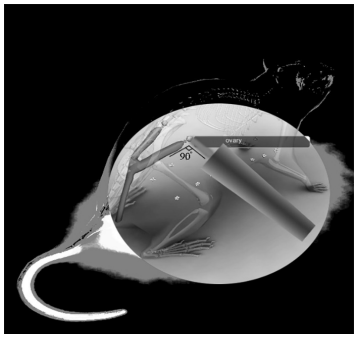

Figure 1. Laser irradiation.
It showed the laser's position during the experiment.

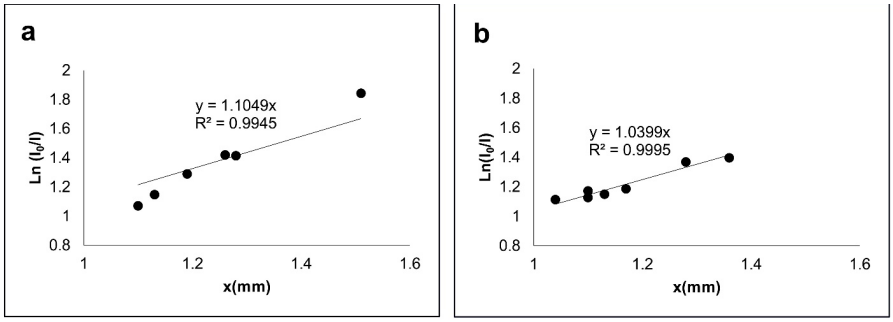

Figure 2. The skin attenuation coefficient.
It was calculated by Lambert-Beer equation (a) for the red laser in 630 *nm* wavelength and (b) infrared laser in 810 *nm* wavelength.

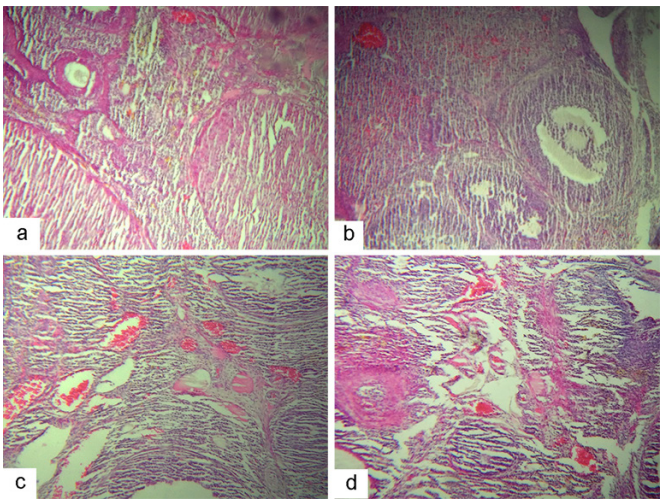

Figure 3. Sections of ovary.
There are four parts in this picture. (a) The control picture with less than blood vessels. (b) D group. There is an increase in the number of blood vessels compared to CT. (c) RL group which shows an increase in growth number of blood vessels more the D and CT group. (d) The changes of NIRL samples which have the most increase in the number of blood vessels.

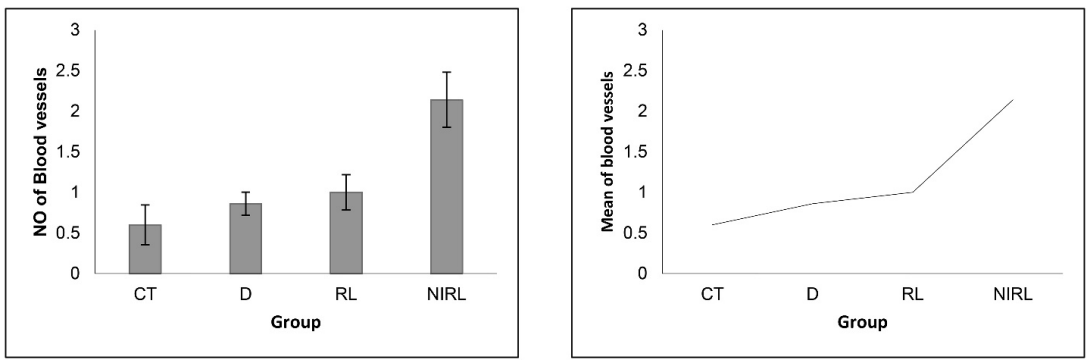

Figure 4. The number of blood vessels
An increase in the number of blood vessels in D, RL, and NIRL groups compared to CT. The maximum increase is for NIRL group and the lowest increase is in D group.

Table 1. The skin thickness

Wave length (*nm*)	I_0 (*w/cm²*)	I (*w/cm²*)	Ln I_0 /I	x±0.03 (*mm*)	*μ* (1/*mm*)
630	32.3	7.8	1.4209	1.26	1.12770
	32.7	11.2	1.0715	1.1	0.97410
	31.6	8.7	1.2898	1.19	1.08387
	34.2	8.3	1.416	1.28	1.10625
	33.5	5.3	1.8438	1.51	1.22106
	32.1	10.2	1.1465	1.13	1.01460
		Mean of *μ* = 1.0880.152±			
810	202	50	1.3962	1.36	1.026618
	216	67	1.1706	1.1	1.064182
	188	61	1.1256	1.1	1.023273
	216	55	1.3679	1.28	1.068672
	219	67	1.1844	1.17	1.012308
	195	64	1.1141	1.04	1.07125
	218	69	1.1504	1.13	1.018053
		Mean of *μ*= 1.0370.138±			

The marked skin area thickness was measured in two 630 nm and 810 nm wavelength to calculate skin attenuation coefficient.
CT control group without any injection
D group with clomiphene drug injection
RL group with red laser injection in 630 nm wavelength
NIRL group with near-infrared laser injection in 810 nm wavelength
*p< 0.05

Table 2. The number of blood vessels

Group	Groups	Mean difference	Standard error	Significance
CT	D	-0.257	0.373	0.900
	RL	-0.400	0.373	0.709
	NIRL	-1.543*	0.373	0.002
D	CT	0.257	0.373	0.900
	RL	-0.143	0.340	0.974
	NIRL	-1.286*	0.340	0.005
RL	CT	0.400	0.373	0.709
	D	0.143	0.340	0.974
	NIRL	-1.143*	0.340	0.014
NIRL	CT	1.543*	0.373	0.002
	D	1.286*	0.340	0.005
	RL	1.143*	0.340	0.014

It increased with injection in D, RL and NIRL groups compared with CT. This increase is significant for NIRL group compared to the other groups.
CT control group without any injection
D group with clomiphene drug injection
RL group with red laser injection in 630 nm wavelength
NIRL group with near-infrared laser injection in 810 nm wavelength
*p< 0.05

Table 3. Mean of blood vessels

Parameters	CT	D	RL	NIRL
Blood vessels	0.60±0.2460	0.86±0.143	1.00±0.218	2.14±0.340***, ****, *****

* Abbreviations: C: control, D: clomiphene drug injection, RL: Red Laser injection, NIRL: Near Infrared Laser injection.

** Data are presented as Mean ± SD

***p< 0.05 as compared with control

Laser beam transmission measurement

Ovary and skin samples were taken and the data were analyzed by a statistical program. Also, attenuation coefficient was calculated by Lambert-Beer law. By using that, laser absorption has been carried out to a percentage reaching potential that ovaries were calculated. Each data was obtained by three repetitions. They were compared with the standard information. Skin thickness was measured by a standard caliper (Pittsburgh 6-inch digital caliper with 0.03 mm precision), (28).

Statistical analysis

The number of blood vessels were statistically analyzed by the IBM SPSS statistics 19 software. Once, Tukey's HSD test and another time, analysis of variance (ANOVA Post Hoc) was used. All data were reported as mean ± SD. Significance level was set at $p< 0.05$ (28).

Results

After the experiment period, 25 of 28 tested rats, (89.30%) were sacrificed and their ovarian tissues were removed. The results of intervention effects on rats were investigated and compared with the results of laser and clomiphene drug effects on blood vessels in ovarian tissue. For this purpose, we measured the histology changes in a number of capillaries in the ovaries generated in the estrous cycle in each group by stained E&S and then compared them.

Histological analysis

After the experiments, the animals' blood vessels in ovarian tissue were counted in each group. At first, the results of lasers in two 630 *nm* and 810 *nm* wavelengths were compared which showed that the number of blood vessels in NIRL and RL groups increased, compared with CT group (Table 2). The increase ratio in NIRL group was 2.14 times more than RL. The increase was significant in NIRL group, compared with CT (71.96%) and RL groups (53.27%). It was also significant in RL as compared with NIRL group (Figure 4).

Second, the results of laser in RL group with 630 *nm* wavelength were compared with those in D group. It showed that the number of ovarian blood vessels increased in both groups, compared to CT group, but this increase was rather observed in RL group (14%). The increase ratio in RL group was 1.163 times more than D group (Figure 4). No significant increase was observed in each group in this section (Table 2).

Third, the results of NIRL were compared with 810 *nm* wavelength and D groups. There was an increase in the number of ovarian blood vessels in both groups, compared with CT (Figure 4), which was rather observed in NIRL group (2.49 times more than D). While this result indicated that there was a significant increase in NIRL group, compared with CT and D (59.81%), it was not significant in D group, compared with CT group (Table 2).

Finally, we compared the results of all groups together. There was an increase in the blood vessels in each group with intervention. But this increase was the most in NIRL group, compared with others. Such an increase is significant for NIRL group, compared to CT, and significant for D and NIRL, too. Compared with RL group, there was a significant relation merely between RL and NRL groups. However, it was not significant for RL, compared to both CT and D groups (Table 2). Moreover, there was a significant relationship between NIRL and three other groups but not between CT and RL groups. There was not a significant relationship between CT and D groups, too (Figure 4). Therefore, the increase in the number of blood vessels in

the samples of separate groups can be written as: CT < D < RL < NIRL (Table 3).

In our last project, we investigated the effect of low-level-lasers on the folliculogenesis cycle in rats' ovarian tissue and compared it with the effect of clomiphene drug. Lasers were utilized with two 630 and 810 *nm* wavelength, too. The results demonstrated an increase in the number of ovarian follicles in the folliculogenesis cycle. An appropriate change was also observed in the level of effective hormones on the folliculogenesis cycle, which was most effective using 810 *nm* laser intervention.

Also, in the statistical measurement, the results were significant only for laser at near-infrared laser with 810 *nm* wavelength compared with the other intervention groups. Thus, the laser with 810 *nm* wavelength stimulated the rats' ovarian tissue, developing the folliculogenesis cycle to produce mature oocyte.

Conclusion

This basic study was conducted based on the rats' ovarian activation to produce oocyte with fertilizing ability in the estrous cycle. Nevertheless, the current study showed a significant increase ratio in the production of ovarian blood vessels following the use of low-level lasers in near-infrared (NIR) spectrum which was greater than the use of clomiphene. Meanwhile the laser in Red (R) spectrum increased the ovarian blood vessels, but not significantly. Accordingly, previous studies indicated that the increase in blood vessels in the ovarian tissue could also increase the ovulation (21,25). More ovarian blood vessels which increase the nutrition, oxygen and effective hormones for producing oocytes, result in increasing ovarians' tissue follicular developmental activity in the ovary and its activation is boosted (23). It was also shown that angiogenesis increased with the ovarian follicle's development (29) since the ovarian follicles contain and produce angiogenesis factors (26,30). In our previous study, we showed that diode lasers in two 630 *nm* and 810 *nm* increased the ovarian activation to produce oocytes significantly. It appears that increasing the folliculogenesis cycle growth produced different follicles. Also, lasers significantly affected the level of hormones which in return influenced the follicles' growth. FSH as the effective hormone on folliculogenesis, stimulated the vessels' growth. Despite the increased number of blood vessels, the folliculogenesis cycle grew to produce oocytes (28,31). Therefore, angiogenesis plays an important role in the sequence of events leading to development of ovarian follicular cycle to produce more oocytes during the folliculogenesis cycle (32,33). This result is of importance due to the indication of increasing the ovarian function for producing oocyte showing the increase in ovarian function to produce oocytes. This process occurs to stimulate the blood vessels by LLLT in two wavelengths. Hence, increasing the angiogenesis increases the ovarian function by increasing the production of oocytes.

Discussion

In 2012, Abramovich *et al* investigated the factors affecting the blood vessels and it was shown that increasing the number of blood vessels was associated with an increase in the activity of ovarian tissue in producing various follicles and mature oocytes which indicated an increase in angiogenesis in the ovary tissue. It can be stated that stimulating the ovarian tissue with 810 *nm* laser can cause more increased ovarian blood vessels compared with clomiphene drug, which indicates an increase in ovarian tissue activity to produce mature oocytes (34).

In 2018, the number of ovarian tissue blood vessels in PCOS patients was examined and showed a decrease in the number of blood vessels, which led to a decrease in the level of effective hormones, oxygen and adequate nutrients in the folliculogenesis cycle and as a result, ovarian cysts. Therefore, increasing the number of blood vessels indicated an increase in the follicular production activity of the ovarian tissue (7). Also, in this study, it was shown that the 810 *nm* laser can increase angiogenesis more efficiently; thus, we can maintain that near-infrared laser with 810 *nm* wavelength which can increase the ovarian function as compared with clomiphene drug.

Therefore, according to the results in this study and compared with the previous studies showed an increase in the number of follicles and changes in the level of effective hormones' in the folliculogenesis cycle compared with clomiphene drug (28), it can be stated that stimulating the ovarian tissue with 810

nm wavelength laser increases the ovarian tissue follicular production activity and eventually mature oocytes.

In 2013, low-level lasers treatment and their function were evaluated. The results showed that low-level lasers can increase the activity of irradiated tissues by stimulating cells producing more ATPs; although its functional mechanism was not well understood. This study was investigated on the nervous tissue and it was noted in other tissues over the years as low-level lasers' treatment was used to stimulate the ovarian tissue. Finally, it was shown that these lasers can increase the activity of ovarian tissue, one of the symptoms of which is an increase in ovarian blood vessels (25).

In 1989, effective hormonal drugs for PCOS patients were investigated, and this study indicated that clomiphene could be more effective on ovarian tissue activity in mature oocyte production than other drugs. Although after several uses of clomiphene, ovarian tissue may resist to drug stimulation and would not respond to treatment, it is still the first and most widely used drug effective on ovulation. Compared with our study, these results showed that low-level lasers' treatment can be more effective than clomiphene treating ovarian hypoxia by stimulating ovarian tissue (27). In 2006, laser was noted as a complementary treatment with drugs and ultrasound, and as a dependent treatment, it could increase the effectiveness of ovulation in ovarian tissue in women with PCOS but it was not investigated autonomously, which we noted in our study. The results represented that low-level lasers treatment can increase ovarian function independently (21).

The results can be used for future researches and solve such problems caused by ovarian negligence such as PCOS (35). This syndrome caused by ovarian dysfunction to produce mature oocytes is a reason for infertility. Therefore, PCOS occurs by ovarian slumber (22) and finding a certain and convenient treatment method is important. Thus, clomiphene can be utilized as a common drug to treat infertility caused by PCOS, and certainly lasers are appropriate as complementary approaches (36). Laser can also be employed in surgery treatment for infertility caused by PCOS (37). Although the mechanism of low-level lasers action is not well known (22), it has been proved to be more effective than clomiphene. Hence, the results can be employed for future studies to treat PCOS without using any drug.

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