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Induction of Ovulation using Low-Level Laser Biostimulation

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Abstract

AIM: Low-level laser therapy (LLLT) has been used in biostimulation of rat ovaries using a fiber optic laser introduced directly through the vagina to deliver laser doses, which is a new modality of laser induction of ovulation, compared with conventional medical induction of ovulation using clomiphene citrate and with non-interventional controls.

MATERIALS AND METHODS: This was a prospective experimental study carried out from January 2014 to February 2016 in a university-based photobiology laboratory. Seventy-two female Wistar Albino rats were used in this study, divided into three groups: 17 rats used as a control group, 19 rats received clomifene citrate (Clomid group), and 36 rats exposed to diode laser 660 nm wavelength (laser group). Histopathological assessment of rat ovaries was done in the three study groups.

RESULTS: Higher number of follicles was found in the laser group compared to controls, the difference was significant in the primordial follicles. Non-significant increase in the number of primordial follicles in the laser group was found compared to the Clomid group. Clomid group showed a non-significant increase in primary, secondary, and antral follicles.

CONCLUSION: This study shows that ovarian laser biostimulation is a new encouraging method for induction of ovulation.

Introduction

Human ovaries are divided into three regions. The hilum which gives attachment to mesovarium, through which pass blood vessels, lymphatics, and nerves. The medulla, which consists of connective tissue stroma and is surrounded by the cortex [1].

The ovarian cortex consists of ovarian follicles in different stages of development and stroma in between them. It can be hard to distinguish between cortex and medulla, but follicles are usually not found in the medulla [2].

Each ovary is located a long side the lateral wall of the uterus in a region called ovarian fossa. The fossa usually lays beneath the external iliac artery and in front of the ureter and the internal iliac artery.

Usually, ovulation occurs in one of the two ovaries (at random) releasing a fertilizable egg each menstrual cycle [3].

Although rat ovary is described as having a cortex and medulla, the division between them is not distinct. Generally, the medullary region has more prominent blood vessels and connective tissue stroma with pale-staining polygonal interstitial cells, while the cortex has more graafian follicles and corpora lutea [4]. The ovaries of cycling rats contain follicles in different stages of development and three or four sets – ages of corpora lutea are present at the same time [5]. The ovaries are located at the distal end of the uterine horns near the kidneys. The oviducts connect the ovaries to each horn of the uterus. The ovaries produce the ova, also called the egg, and certain hormones [6].

Several variances exist between menstrual and estrous cycles, in humans, the reproductive cycle, called the menstrual cycle, last approximately 28 days, in rodents this cycle, called the estrous cycle, lasts approximately 4–5 days. Rats display, most of the time, regular cycles; they are easy to manipulate, and the cycle is not disturbed easily even with the routine stress in the animal facility [7]. Another variance is that animals that have estrous cycles reabsorb the endometrium if conception does not occur during that cycle. Animals that have menstrual cycles shed the endometrium through menstruation instead [8].

The estrus cycle is divided to proestrus, estrus, metestrus (or diestrus I), and diestrus (or diestrus II) phases [9]. Vaginal smear cytology is used to determine phases of the estrus cycle [10]. The classification of

each phase is based on the amount of three types of cells observed in the vaginal smear: epithelial cells, cornified cells, and leukocytes [11].

Low-level laser irradiation of cells at certain wavelengths can activate some of the native components resulting in alteration of specific biochemical reactions, as well as cell metabolism. This alteration forms the basis for low-power laser effects [12].

Cellular targets are mitochondria with the effect of increased adenosine triphosphate (ATP) production, modulation of reactive oxygen species, and initiation of cellular signaling [13].

Low-power lasers do not have a thermal effect on tissue. Photons may influence the proliferation of cells [14].

The final enzyme in the production of ATP by mitochondria is cytochrome-C-oxidase which appears to accept energy (photoacceptor) from low-level laser lights, making it a possible candidate for mediating the properties of laser therapy [15].

The effects of low-level laser therapy (LLLT) appear to be limited to specified wavelengths of laser [16]. The typical wavelength is in the range of 600–1000 nm (red to near infrared) [17]. Administering LLLT below the dose range does not appear to be effective [18]. The depth of penetration of laser light depends on the light's wavelength, mode of the laser, power density, technical design of the apparatus, and the treatment technique used [19].

Clomifene citrate was described as most important medications needed in a basic health system [20]. It has become the most widely prescribed drug for ovulation induction to reverse anovulation or oligoovulation [21].

Clomifene binds to the E2 receptors in the hypothalamus to create a state of hypoestrogenicity, thereby causing an enhanced gonadotropin-releasing hormone release followed by an increased secretion of gonadotropins which induces ovulation [22].

Material and Methods

Eighty-two adult female Wistar Albino rats (Rattus norvegicus) were used in this study since they are the most commonly used experimental animals and easily available, selected at the average fertile period at 10–15 weeks old and 180–220 gm body weight. Rats were obtained from animal's house of faculty of veterinary medicine – Cairo University.

All animal experiments were conducting according to national and international guidelines for laboratory animals. They were allowed to grow at the same environmental conditions of good ventilation, adequate stable diet, temperature, and humidity, also with same housing and management. The animals were housed in standard cages, five per cage, in a controlled temperature room, with free access to food and water.

The ovaries of two sacrificed female rats were subjected to diode laser 660 nm wavelength using an apparatus laser vex inc.-DPSSL II with contact control (Figure 1). The laser beam was delivered using a fiber optic that was introduced directly through the vagina. To estimate the accurate dose reaching the ovaries, a very sensitive power meter (Coherent-LaserCheck = Model 1098293) was placed behind the ovaries to measure the amount of power delivered to the ovaries through the vaginal vault.

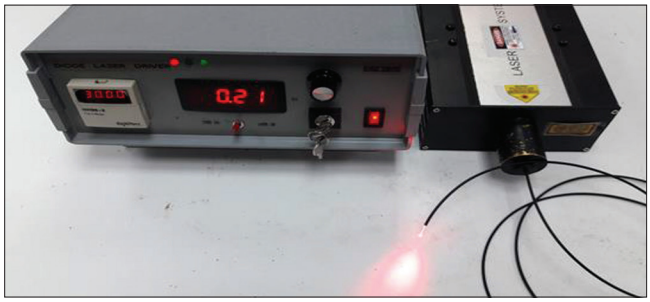

Figure 1: Diode laser 660 nm wavelength (laser vex inc.-DPSSL II)

Many measurements were done until we obtained the recommended power, in which at a current of 210 m MA, the power at the tip of the fiber optic was 9.06 mW and those reaching the ovaries was 5.03 mW, and this was the targeted power [23], [24] (Figure 2).

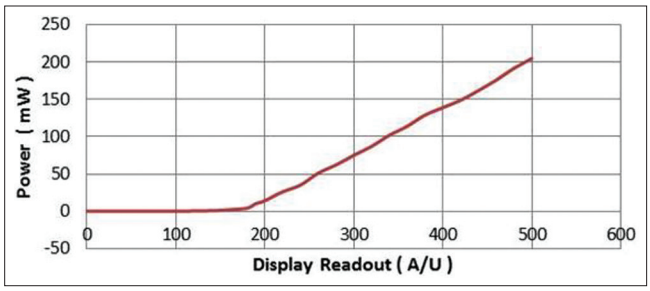

Figure 2: Diode laser 660 nm – fiber out, curve (power mw and current mAMP)

Determination of the phase of estrous cycle was performed through a collection of vaginal secretions with a plastic pipette filled with 0.5–1 mL of normal saline (NaCl 0.9%). Vaginal fluid was placed on glass slides. One drop was collected with a clean tip from each rat. This fresh drop was examined stained by methylene blue solution under a light microscope, using objective lenses, × 10 and × 40.

Vaginal smears was then done every morning daily for at least three consecutive successive regular cycles, to gain experience in handling of rats and to be familiar with the technique and morphological features of each phase of estrous cycle and to determine the diestrus phase of the estrous cycle, in which ovulation induction – either by clomifene citrate or by laser- – should be conducted during this phase [25].

Only cyclic rats were used in this study, whereas rats with irregular cycles (Blocked) were excluded from the study.

Estrous synchronization was then performed, i.e., the stage of estrous was determined by vaginal swab, so that animals in the same estrous stage were allocated to the same group.

A pilot study was conducted using two rats administered clomifene citrate, at a dose of 20ug/day for 2 consecutive days, starting on the diestrus phase and then euthanized on the 4th day of the cycle. Serum samples for estradiol (E2) and progesterone (P) were collected and both ovaries were taken for histopathology. Another pilot study was tried using three rats exposed to laser biostimulation at a dose of 150 mJ/cm^2 (5 mW/Cmc^2 × 30 s) for two consecutive days. Starting in the diestrus phase and then euthanized on the 4th day of the cycle, serum samples for E2 and P were collected and both ovaries were taken for histopathology. A 3rd pilot study was done by exposing three rats to laser biostimulation at a dose of 150 mJ/cm^2, for 3 consecutive days (a cumulative dose of 450 mJ/cm^2) [23] [24], results also recorded.

Following these pilot studies (eight rats were used in the pilot studies), the original study started using a total number of 74 adult female rats. Two died (one from the laser group and one from the clomifene group and were excluded from the study). The remaining 72 rats were divided into three groups.

- Group I (Control Group): Consists of 17 rats used as a control group
- Group II (Clomid Group): Consists of 19 rats received clomiphene citrate 20 micrograms per rat daily orally for 2 consecutive days, starting on the diestrus phase of the estrous cycle [25]
- Group III (Laser Group): Consists of 36 rats, in which their ovaries were exposed to diode laser 660 nm wavelength (laser vex inc.-DPSSL II) with contact control (fiber optic introduced through the vagina to reach the vault), using power density of 5 mW/cm^2, for 30 s (total dose of 150 mJ/cm^2), for 3 consecutive days (a cumulative dose of 450 mJ/cm^2). Starting in the diestrus phase of the estrous cycle [23], [24].

At the end of the study, at metestrus phase (4th day of starting induction), the phase in which serum estradiol or progesterone begins to increase [26]. All animals then received general anesthesia in the form of intramuscular injection of mixture of ketamine (50–75 mg/kg) and xylazine (10 mg/kg), this gives duration of 20–30 min deep anesthesia and sleeping time for 120–240 min [27].

Tissue collection and processing

Laparotomy was then done to obtain ovaries for histopathological assessment. The ovaries of the studied rats were surgically removed, rinsed in a cold saline, immersed in 10% buffered neutral formalin solution, embedded in paraffin wax, sectioned at 4–5u, and finally stained with hematoxylin and eosin [28], and periodic acid–Schiff stain procedure [29].

In our study, we used two-dimensional analysis for serial ovarian sections embedded in paraffin. The number of follicles counted in this way is referred to as the raw count Q2 (without using any correction factors) – an abbreviation of German “Querschnitt” – for the profiles related to histological cross-sections, introduced by [30].

Microscopic examination of stained ovarian sections in the three study groups was done for primordial, 1ry, 2ry and antral follicles.

Statistical analysis

All statistical calculations were done using computer programs, Microsoft Excel Office version 10 (Microsoft Corporation, NY, USA), and SPSS (Statistical Package for the Social Science; SPSS inc., Chicago, IL, USA) for IBM version 22, statistical programs.

Data were statistically described regarding range, mean, standard deviation (± SD), and frequencies (number of cases). Comparison between the three study groups was made by one-way analysis of variance (ANOVA) test, and then analysis between every two groups was done by independent samples *t*-test. A probability value ($P < 0.05$) was considered statistically significant.

Results

This study was conducted on 72 rats, divided into three groups: 17 rats control, 19 rats clomifene, and 36 rats laser group.

Table 1: Descriptive analysis of number of follicles at different follicular stages in the three study groups

Groups	n	Mean	SD	SE	Minimum	Maximum	*F*	p
Primordial								
Clomid	17	2.0000	1.41421	0.34300	0.00	5.00	5.910	0.005
Control	16	1.0625	0.92871	0.23218	0.00	3.00		
Laser	27	2.5926	1.62337	0.31242	0.00	7.00		
Total	60	2.0167	1.52373	0.19671	0.00	7.00		
Primary								
Clomid	17	2.0588	1.39062	0.33727	0.00	5.00	2.282	0.111
Control	16	1.1875	0.91059	0.22765	0.00	3.00		
Laser	27	1.7037	1.17063	0.22529	0.00	4.00		
Total	60	1.6667	1.20263	0.15526	0.00	5.00		
Secondary								
Clomid	17	2.2353	2.07754	0.50388	0.00	7.00	1.573	0.216
Control	16	1.3125	0.87321	0.21830	0.00	4.00		
Laser	27	1.7778	1.33973	0.25783	0.00	5.00		
Total	60	1.7833	1.50808	0.194690	0.00	7.00		
Antral								
Clomid	17	3.5882	3.14362	0.76244	0.00	10.00	3.205	0.048
Control	16	1.7500	1.00000	0.25000	0.00	4.00		
Laser	27	2.5185	1.74026	0.33491	0.00	6.00		
Total	60	2.6167	2.17919	0.28133	0.00	10.00		

There was statistically significant difference in primordial follicles (p = 0.005), as well as antral follicles (p = 0.048) in the three study groups. *F*: ANOVA test, p: Significance. ANOVA: Analysis of variance, SE: Standard error, SD: Standard deviation.

After exclusion of extremes, one rat was excluded from the control group, two were excluded from the Clomid group, and nine from laser group.

The study showed that there was statistically significant difference in the number of primordial follicles (p = 0.005), as well as the number of antral follicles (p = 0.048) in the three study groups as shown in (Table 1). The Clomid group had significantly higher number of primordial, primary, and antral follicles compared to the control group (p < 0.05) (Table 2).

Table 2: Comparison between number of follicles at different follicular stages in the Clomid and control groups

Groups	n	Mean	SD	SEM	*t*	p
Primordial						
Clomid	17	2.0000	1.41421	0.34300	2.236	0.033
Control	16	1.0625	0.92871	0.23218		
Primary						
Clomid	17	2.0588	1.39062	0.33727	2.115	0.043
Control	16	1.1875	0.91059	0.22765		
Secondary						
Clomid	17	2.2353	2.07754	0.50388	1.644	0.110
Control	16	1.3125	0.87321	0.21830		
Antral						
Clomid	17	3.5882	3.14362	0.76244	2.233	0.033
Control	16	1.7500	1.00000	0.25000		

Clomid group have significantly higher number of primordial, primary and antral follicles compared to the control group (p < 0.05). *t*: Independent samples test, p: Significance. SD: Standard deviation, SEM: Standard error of mean.

Significantly higher number of primordial follicles was found in the laser stimulated group compared to controls (p= 0.001) (Table 3). There was no statistically significant difference in the number of different follicular types between Clomid and laser groups (p > 0.05) (Table 4). There was statistically significantly higher number of antral follicles compared to other types of follicles (primordial follicles, primary follicles, and secondary follicles) was found in the Clomid group (p = 0.037, p = 0.021, and p = 0.005, respectively).

Table 3: Comparison between number of follicles at different follicular stages in the laser and control groups

Groups	n	Mean	SD	SEM	*t*	p
Primordial						
Laser	27	2.5926	1.62337	0.31242	−3.441	0.001
Control	16	1.0625	0.92871	0.23218		
Primary						
Laser	27	1.7037	1.17063	0.22529	−1.511	0.138
Control	16	1.1875	0.91059	0.22765		
Secondary						
Laser	27	1.7778	1.33973	0.25783	−1.239	0.222
Control	16	1.3125	0.87321	0.21830		
Antral						
Laser	27	2.5185	1.74026	0.33491	−1.611	0115
Control	16	1.7500	1.00000	0.25000		

Significantly higher number of primordial follicles was found in the laser stimulated group compared to controls. *t*: Independent samples test, p: Significance. SD: Standard deviation, SEM: Standard error of mean.

Data were summarized in (Table 5). The study showed there was significantly higher number of antral follicles compared to primordial follicles in the control group (p = 0.029) (Table 6).

Table 4: Comparison between number of follicles at different follicular stages in the Clomid and laser groups

Groups	n	Mean	SD	SEM	*t*	p
Primordial						
Clomid	17	2.0000	1.41421	0.34300	−1.237	0.223
Laser	27	2.5926	1.62337	0.31242		
Primary						
Clomid	17	2.0588	1.39062	0.33727	0.911	0.367
Laser	27	1.7037	1.17063	0.22529		
Secondary						
Clomid	17	2.2353	2.07754	0.50388	0.890	0.378
Laser	27	1.7778	1.33973	0.25783		
Antral						
Clomid	17	3.5882	3.14362	0.76244	1.455	0.153
Laser	27	2.5185	1.74026	0.33491		

No statistically significant difference in the number of different follicular types between Clomid and laser groups (p > 0.05). *t*: Independent samples test, p: Significance. SD: Standard deviation, SEM: Standard error of mean.

Table 5: Pairwise comparisons between different type of follicles in the Clomid group

Factor (I)	Factor (J)	Mean difference (I−J)	SE	p	95% CI for difference[a]	
					Lower bound	Upper bound
1	2	−0.059	0.201	0.773	−0.484	0.366
	3	−0.235	0.458	0.614	−1.206	0.736
	4	−1.588*	0.697	0.037	−3.066	−0.111
2	1	0.059	0.201	0.773	−0.366	0.484
	3	−0.176	0.356	0.627	−0.931	0.578
	4	−1.529*	0.595	0.021	−2.790	−0.268
3	1	0.235	0.458	0.614	−0.736	1.206
	2	0.176	0.356	0.627	−0.578	0.931
	4	−1.353*	0.420	0.005	−2.242	−0.463
4	1	1.588*	0.697	0.037	0.111	3.066
	2	1.529*	0.595	0.021	0.268	2.790
	3	1.353*	0.420	0.005	0.463	2.242

Factor (I): Group number, Factor (J): Other groups, 1: Primordial follicles, 2: Primary follicles, 3: Secondary follicles, 4: Antral follicles. Significantly higher number of antral follicles compared to primordial follicles was found in the Clomid group (p = 0.037), Significantly higher number of antral follicles compared to 1ry follicles was found in the Clomid group (p = 0.021), significantly higher number of antral follicles compared to 2ry follicles was found in the Clomid group (p = 0.005). CI: Confidence interval, SE: Standard error. *in mean difference column=Significance, [a]in CI column=non significance

Table 6: Pairwise comparisons between different type of follicles in the control group

Factor (I)	Factor (J)	Mean difference (I−J)	SE	p	95% CI for difference[a]	
					Lower bound	Upper bound
1	2	−0.125	0.125	0.333	−0.391	0.141
	3	−0.250	0.250	0.333	−0.783	0.283
	4	−0.688*	0.285	0.029	−1.294	−0.081
2	1	0.125	0.125	0.333	−0.141	0.391
	3	−0.125	0.256	0.633	−0.671	0.421
	4	−0.563	0.316	0.095	−1.236	0.111
3	1	0.250	0.250	0.333	−0.283	0.783
	2	0.125	0.256	0.633	−0.421	0.671
	4	−0.438	0.241	0.089	−0.951	0.076
4	1	0.688*	0.285	0.029	0.081	1.294
	2	0.563	0.316	0.095	−0.111	1.236
	3	0.438	0.241	0.089	−0.076	0.951

Factor (I): Group number, Factor (J): Other groups, 1: Primordial follicles, 2: Primary follicles, 3: Secondary follicles, 4: Antral follicles. Significantly higher number of antral follicles compared to primordial follicles was found in the control group (p = 0.029). CI: Confidence interval, SE: Standard error, *in mean difference column=Significance, [a]in CI column = non significance.

There was statistically significantly higher number of primordial follicles compared to primary and secondary follicles in the laser group (p = 0.001 and p = 0.017, respectively). In addition, there was statistically significantly higher number of antral follicles compared to primary and secondary follicles in the laser group (p = 0.012 and p = 0.007, respectively). Data were summarized in (Table 7).

Table 7: Pairwise Comparisons between different type of follicles in the laser group

Factor (I)	Factor (J)	Mean difference (I−J)	SE	p	95% CI for difference[a]	
					Lower bound	Upper bound
1	2	0.889*	0.247	0.001	0.382	1.396
	3	0.815*	0.320	0.017	0.156	1.473
	4	0.074	0.427	0.864	−0.803	0.951
2	1	−0.889*	0.247	0.001	−1.396	−0.382
	3	−0.074	0.232	0.752	−0.551	0.403
	4	−0.815*	0.302	0.012	−1.436	−0.194
3	1	−0.815*	0.320	0.017	−1.473	−0.156
	2	0.074	0.232	0.752	−0.403	0.551
	4	−0.741*	0.254	0.007	−1.262	−0.219
4	1	−0.074	0.427	0.864	−0.951	0.803
	2	0.815*	0.302	0.012	0.194	1.436
	3	0.741*	0.254	0.007	0.219	1.262

Factor (I): Group number, factor (J): Other groups, 1: Primordial follicles, 2: Primary follicles, 3: Secondary follicles, 4: Antral follicles. CI: Confidence interval, SE: Standard error. *in mean difference column=Significance, [a]in CI column = non significance

Discussion

Low-power laser irradiation of cells at certain wavelengths can activate some of the native components

resulting in alteration of specific biochemical reactions, as well as cell metabolism. This alteration forms the basis for low-power laser effects [31].

The mitochondria are sensitive to irradiation with monochromatic visible and near-infrared light. It increases ATP synthesis and consumption of oxygen [32], as well as RNA and protein synthesis in the mitochondria [33].

This new modality of low-level laser laminated veneer lumber biostimulation was compared with the conventional medical induction of ovulation using clomifene citrate, and with non-interventional controls. The purpose of this study is to assess and compare the number of different types of follicles in the three studied groups, exactly at the same phase of the estrous cycle.

In our study, we used diode laser 660 nm wavelength, since its apparatus forms are small, portable easily modulated. Furthermore, the depth of penetration of diode laser is suitable to reach the human ovary. Using a fiber optic, that was introduced directly through the vagina to deliver laser to the ovaries through the vaginal vault, reduces the number of animal loss (only one rat died in laser group), i.e., 2.7% fatality.

Changes in the primordial, primary, secondary, and graffian follicles were studied in the present study showing statistically significant difference in primordial follicles ($p = 0.005$), as well as antral follicles ($p = 0.048$) in the three study groups.

In our study, Clomid group have significantly higher number of primordial, primary and antral follicles compared to the control group ($p < 0.05$). Furthermore, non-significant rise in 2ry follicles was found in the Clomid group.

Naseri, *et al*. showed non-significant rise in the number of all follicular types in Clomid group compared to controls [34].

In our study, a higher number of all follicles were found in the laser group compared to controls, but the difference was significant in primordial follicles ($p < 0.05$).

Naseri, *et al*., showed that number of primordial follicles increased significantly in red laser (RL) and near-infrared laser (NIRL) groups, compared with control group (This increase was equal in the RL and NIRL groups). The results of 1ry and 2ry follicles were similar to primordial follicles analysis; the only difference was that the growth ratios in RL and NIRL show non-significant increase in the NIRL group. Antral follicles show significant rise in the RL and NIRL groups, compared with the control group, also the growth ratios in RL and NIRL show significant rise in the NIRL group [34].

In our study, no statistically significant difference in the number of different follicular types between laser and Clomid groups ($p > 0.05$). Being non-significant increase of 1ry, 2ry, and antral follicles in the Clomid group, whereas primordial follicles showed non-significant rise in the laser group.

Naseri, *et al*. showed that the number of primordial follicles increased significantly in the RL and NIRL groups, compared with the Clomid group (This increase was equal in the RL and NIRL groups). The results of 1ry and 2ry follicles were similar to primordial follicles analysis; the only difference was that the growth ratios of 1ry follicles in RL and NIRL show non-significant increase in the NIRL group, which was significant in 2ry follicles. Antral follicles shows non-significant rise in the RL group compared to the Clomid group, but the difference was significant in the NIRL group [34].

In the study conducted by Sherein and Hossam Eldein, who compared the effect of low-power laser biostimulation using diode laser 650 nm with clomifene citrate in induction of ovulation on a polycystic ovary syndrome rats, most ovaries of the laser group contain less follicular cysts and multiple luteal cysts compared to the Clomid treated group, indicating ovulation in most animals of this group. As appeared in this study, laser is more effective with less complication compared with Clomid [24].

Conclusion

This study shows that ovarian laser biostimulation is a new encouraging method for induction of ovulation alone or at least complementarily with other methods. This had been proven histopathologically by higher number of follicles that was found in the laser group compared to controls and the difference was significant in the primordial follicles.

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