Open Access Maced J Med Sci **electronic publication ahead of print,**
published on June 25, 2018 as https://doi.org/10.3889/oamjms.2018.216

ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences.
https://doi.org/10.3889/oamjms.2018.216
eISSN: 1857-9655
Basic Science

Assessment of Laser Biostimulation in Induction of Ovulation

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Citation: El-Nourya MA, El-Begawyb MB, Morsyc ME, Al Sherbineyd EM. Assessment of Laser Biostimulation in Induction of Ovulation. Open Access Maced J Med Sci. https://doi.org/10.3889/oamjms.2018.216

Keywords: Laser; Biostimulation; Induction; Ovulation

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Received: 27-Feb-2018; **Revised:** 09-Jun-2018; **Accepted:** 10-Jun-2018; **Online first:** 25-Jun-2018

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: This study aimed to evaluate a new modality of low power laser biostimulation in rat ovaries, in comparison with the conventional medical therapy by clomiphene citrate that depends on up-regulation of the hypothalamic-pituitary-ovarian axis to induce ovulation.

STUDY DESIGN: A Prospective experimental study carried out from January 2014 to February 2016.

SETTING: University-based photobiology laboratory.

MATERIALS AND METHODS: Seventy-two (72) Female-Wistar Albino rats were used in this study, divided into three groups: 17 rats used as a control group, 19 rats received clomiphene citrate (Clomid group), 36 rats exposed to diode laser 660 nm wavelength (laser group).

RESULTS: Biochemical assessment of serum Estradiol and serum Progesterone was done in the three study groups. Serum Estradiol & Progesterone levels were statistically significantly higher in clomiphene and laser treated groups than non-interventional controls, while no significant difference between clomiphene and laser groups as regard of both hormones.

CONCLUSION: This study shows that ovarian laser biostimulation is a new encouraging method for induction of ovulation, at least in animals. This had been proven biochemically by a significant increase in serum Estradiol and serum Progesterone.

Introduction

In humans as well as animals including rats, the reproductive function of the ovaries is controlled through complex feedback mechanisms, involving releasing factors from the hypothalamus and FSH and LH from pituitary gland. The resulting estrogen and progesterone produced by the ovarian follicles and corpora lutea, respectively, are the basis for the oestrous and menstrual cycles and also for normal reproductive function [1]. Many differences exist between menstrual and oestrous cycles, in humans, the reproductive cycle, called the menstrual cycle, last approximately 28 days, in rodents this cycle, called the oestrous 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 [2]. Another difference is that animals that have oestrous cycles reabsorb the endometrium if conception does not occur during that cycle. Animals that have menstrual cycles shed the endometrium through menstruation instead [3].

The estrus cycle is characterised as proestrus, estrus, metestrus (or diestrus I) and diestrus (or diestrus II) [4]. Vaginal smear cytology is used for the determination of the estrus cycle phases [5]. The characterisation of each phase is based on the proportion of three types of cells observed in the vaginal smear: epithelial cells, cornified cells and leukocytes [6].

Clomiphene citrate (CC) is a non-steroidal selective estrogen receptor modulator (SERM) of the triphenylethylene group that has become the most

widely prescribed drug for ovulation induction to reverse anovulation or oligo-ovulation [7]. It was described on the 19th WHO Model List of Essential Medicines [8], the most important medications needed in a basic health system.

Clomiphene binds to the E2 receptors in the hypothalamus (interfering with recycling of receptors) to create a state of hypo-estrogenicity, thereby causing an enhanced gonadotropin-releasing hormone (GnRH) release followed by an increased secretion of gonadotropins which induces ovulation [9].

Ovulation is known to occur in 70% of cases, while pregnancy occurs only in about 25-30% of the cases. The low pregnancy rate is due to the antiestrogenic effects of CC on the cervix, which would make it difficult to sperm penetration and on the endometrial growth which would, therefore, be unreceptive to the embryo [10].

Some studies have suggested that clomiphene citrate if used for more than a year, may increase the risk of ovarian cancer [11].

This, however, is disputed, and some feel there is no significant increase in risk [12]. It is not recommended by the manufacturer to use clomiphene for more than 6 cycles [13].

The clinical effect of light can be classified as direct and indirect, depending on whether the light causes an effect to occur within the irradiated tissue or whether a nervous or neuroendocrine signal is generated in the irradiated area and causes a systemic effect in another part of the body [14].

Low power lasers do not have a thermal effect on tissue. Photons may influence the proliferation of cells [15].

Low-level laser therapy (LLLT) is a form of laser medicine used in physical therapy and veterinary treatment that uses low-level lasers or light-emitting diodes to alter cellular function [16].

Photobiology works on the principle that, when the light hits certain molecules called chromophores, the photon energy causes electrons to be excited and jump from low-energy orbits to higher energy orbits. In nature, this stored energy can be used by the system to perform various cellular tasks, such as photosynthesis [17].

Cellular targets are mitochondria with the effect of increased adenosine triphosphate production, modulation of reactive oxygen species, and initiation of cellular signalling [18].

The final enzyme in the production of ATP by mitochondria, cytochrome-C-oxidase does appear to accept energy (photoacceptor) from laser-level lights, making it a possible candidate for mediating the properties of laser therapy [19].

The effects of LLLT appear to be limited to specified wavelengths of laser [20]. The typical wavelength is in the rage of 600-1000 nm (red to near infrared) [21].

Administering LLLT below the dose range does not appear to be effective [22]

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 [23].

Material and Methods

All Institutional and National Guidelines for the care and use of experimental animals were followed.

Eighty-two adult female-Wistar Albino rats (Rattus norvergicus) 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.

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. The laser beam was delivered using a fibre-optic that was introduced directly through the vagina.

To estimate the accurate dose reaching the ovaries, a very sensitive power meter (Coherent-Laser Check = Model 1098293) was placed behind the ovaries to measure the amount of power delivered to the ovaries through the vaginal vault.

Many measurements were done until we obtained the recommended power, in which at a current of 210 m AMP, the power at the tip of the fibre-optic was 9.06 mW and those reaching the ovaries was 5.03 mW, and this was the targeted power [24] [25] (Figure 1).

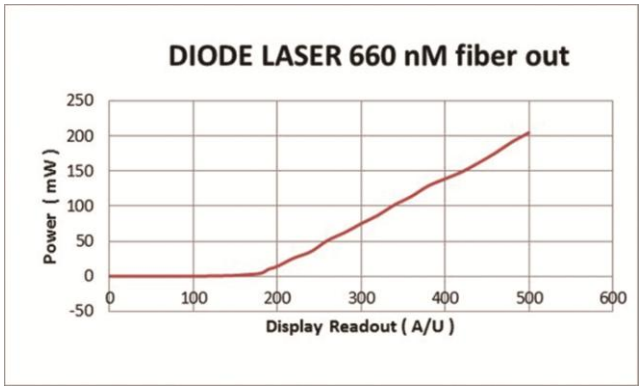

Figure 1: Diode laser 660 nm-fibre out, curve (power mW& current mAMP)

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 clomiphene citrate or by laser-should be conducted during this phase [26]. Only cyclic rats were used in this study, whereas rats with irregular cycles (Blocked) were excluded.

Oestrous synchronisation was then performed, i.e. the stage of oestrous was determined by vaginal swab microscopically in each animal so that animals in the same oestrous stage were allocated to the same group.

Determination of the phase of oestrous 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%) by inserting the tip into the vagina, but not deeply. Vaginal fluid was placed on glass slides. One drop was collected with a clean tip from each rat. This fresh drop was examined (either unstained or stained by methylene blue stain) under a light microscope, without the use of the condenser lens, with 10 and 40 x objective lenses.

A pilot study was conducted using two rats administered clomiphene citrate, at a dose of 20ug/day for 2 consecutive days, starting on the diestrus phase and then sacrificed on the 4th day of the cycle. Serum samples for E_2 and P were collected. Another pilot study was tried using three rats exposed to laser biostimulation at a dose of 150 mj/Cm^2 (5 MW/Cm^2 x 30 sec) for two consecutive days. Starting in the diestrus phase and then sacrificed on the 4th day of the cycle, serum samples for E_2 and P were collected. A 3rd pilot study was done By exposing three rats to laser biostimulation at a dose of 150 mj/Cm^2, for three consecutive days (a cumulative dose of 450 mj/Cm^2) [24] [25], results also recorded.

Blood samples were collected by puncture of the ophthalmic venous plexus for E_2 and P hormonal assay.

Following these pilot studies (Eight rats were used in the pilot studies), the original study was started in January 2014 and continues to February 2016, using a total number of 74 adult female rats. Two died (one from the laser group and one from the clomiphene 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. Nothing was done for this 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 oestrous cycle [26].

- *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 seconds (total dose of 150 mj/Cm^2), for three consecutive days (a cumulative dose of 450 mj/Cm^2). Starting in the diestrus phase of the oestrous cycle [24] [25].

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 [27], blood samples were collected by puncture of the ophthalmic venous plexus & serum were stored at -20ºC until assayed.

The serum Estradiol and Progesterone assay were done by fully automated access system for immunoassay analysis using Electro-Chemi-Luminescence (ECL) detection system.

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. 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.

A probability value (P value) less than 0.05 was considered statistically significant.

Results

This study was conducted on 72 rats, divided into three groups: 17 rats control, 19 rats' clomiphene & 36 rats' laser group (Table 1).

Table 1: Comparison between the three study groups as regard serum Estradiol level

Estradiol (E2) (pg/ml)	N	Mean	Std. Deviation	Minimum	Maximum	F	P
Clomid Induction	19	32.211	15.0465	16.0	77.0	3.474	0.037
Laser Induction	36	31.222	8.7541	15.0	57.0		
Control	17	24.000	6.8099	13.0	33.0		
Total	72	29.778	10.7824	13.0	77.0		

N: Number; F: ANOVA test; P: significance.

There was the statistically significant difference between the three study groups regarding serum Estradiol (P = 0.037) (Table 2).

Table 2: Comparison between the three study groups as regard serum Progesterone level

Progesterone (ng/ml)	N	Mean	Std. Deviation	Minimum	Maximum	F	P
Clomid Induction	19	30.977	12.2515	13.0	52.0	8.183	0.001
Laser Induction	36	25.299	9.0915	12.0	51.4		
Control	17	18.353	5.2433	7.0	27.0		
Total	72	25.157	10.2544	7.0	52.0		

The cutoff the value of serum Progesterone in ovulation is 3.6ng/ml.

There was the statistically significant difference between the three study groups regarding serum Progesterone (P = 0.001) (Table 3).

Table 3: Comparison between laser & Clomid groups as regard serum Estradiol & serum progesterone levels

	GROUP	N	Mean	Std. Deviation	Std. Error Mean	t	P
Estradiol (E2) (pg/ml)	Clomid Induction	19	32.211	15.0465	3.4519	0.309	0.759
	Laser Induction	36	31.222	8.7541	1.4590		
Progesterone (ng/ml)	Clomid Induction	19	30.977	12.2515	2.8107	1.949	0.057
	Laser Induction	36	25.299	9.0915	1.5152		

N: Number; t: independent test; P: significance.

There was no statistically significant difference between laser-treated group & Clomid induction group regarding serum Estradiol levels (P = 0.759).

There was no statistically significant difference between laser-treated group & Clomid induction group regarding serum Progesterone levels (P = 0.057) (Table 4).

Table 4: Pearson Correlation between serum Estradiol & serum Progesterone levels in control group

GROUP = Control		r
Estradiol (E2) (pg/ml)	Pearson Correlation	1.00
	N	17
Progesterone (ng/ml)	Pearson Correlation	0.585*
	P	0.014
	N	17

There was a significant positive correlation between serum Estradiol & serum Progesterone levels in control group (P = 0.014) (Table 5).

Table 5: Pearson Correlation between serum Estradiol & serum Progesterone levels in clomid group

GROUP = Clomid Induction		r
Estradiol (E2) (pg/ml)	Pearson Correlation	1.00
	N	19
Progesterone (ng/ml)	Pearson Correlation	0.579**
	P	0.009
	N	19

There was a significant positive correlation between serum Estradiol & serum Progesterone levels in clomid group (P = 0.009) (Table 6).

Table 6: Pearson Correlation between serum Estradiol & serum Progesterone levels in the laser group

GROUP = Laser Induction		r
Estradiol (E2) (pg/ml)	Pearson Correlation	1.00
	N	36
Progesterone (ng/ml)	Pearson Correlation	0.206
	P	0.228
	N	36

Non-significant positive correlation was found between serum Estradiol & serum Progesterone levels in laser group (P = 0.228).

Discussion

This new modality of low power laser (LPL) biostimulation was compared with the conventional medical therapy using clomiphene citrate that depends on upregulation of hypothalamic-pituitary-ovarian axis to induce ovulation, and with non-interventional controls.

The purpose of this study is to assess and compare the levels of serum Estradiol and serum Progesterone between the three studied groups, exactly at the same phase of the oestrous cycle (metestrus = diestrus 1) [27].

This is the 1st time that low power laser has been used as a biostimulant to normal (non PCO) ovaries and used to induce ovulation.

The only work done in this field, was carried out by two studies, the first study was conducted by Al-Watban and Andres [24], where they used He-Ne laser (632.8 nm) and solcoseryl in "in vitro" biostimulation on Chinese hamster ovary (CHO) and human skin fibroblast (HSF), using an optimum power density 1.25 mw/cm^2 and cumulative doses of 60-600 mj/cm^2, with average 180 mj/cm^2 given for three consecutive days, resulted in significant increase in cloning efficiency of CHO and HSF cells.

The second work was carried out by Sherein and Hossam Eldein [25] who compared the effect of low power laser biostimulation with clomiphene citrate, in the induction of ovulation with non-interventional controls in female Wistar Albino rats with polycystic ovarian disease (PCO). In this study induction of polycystic ovaries was done by receiving oral letrozole 0.5 mg/kg body weight-daily for 20 days in 60 rats. Rats were divided into three groups, 20 rats in each group.

Clomid group received clomiphene citrate 20 μg/rat orally-daily for 2 consecutive days.

Laser group received general anaesthesia, where their abdomen was opened, and their ovaries were exposed to diode laser 650 nm, using a dose of 150 mj/cm^2 (power density was 30 MW/cm^2), using an apparatus - Intelite R 650-250, daily for 2 consecutive days.

All animals then sacrificed on the 24th day from the 1st day of letrozole administration. Whole blood was collected for serum progesterone assay, and the ovaries were taken for histopathology. This study concluded that low power laser is a new encouraging method for induction of ovulation, and it

is more effective with less complication compared to clomid.

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 [28].

The mitochondria are sensitive to irradiation with monochromatic visible and near-infrared light. It increases adenosine triphosphate synthesis and consumption of oxygen [29], as well as RNA and protein synthesis in the mitochondria [30].

In our study, we used diode laser 660nm wavelength, since its apparatus forms are small, portable&easily modulated. Also, 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, when compared to laparotomy and surgical mobilization of ovaries, to be directly exposed to laser beam and re-exposure on the 2nd day, this increases the animal loss to be six rats, i.e. 23% fatality, as done by Sherein and Hossam Eldein [25]. Mechanical manipulation of the ovary during laser application is not physiologic and may have a role in abnormal ovarian response, also using fibre optic can be suitable for application in women by introducing the fibre through an egg-pickup needle socket (used for in vitro fertilisation = IVF) under ultrasound guidance.

Our study shows the statistically significant difference as regard serum Estradiol levels between the three studied groups (P value less than 0.05).

The clomid induction group has higher serum Estradiol values (mean 32.21 ± SD 15.04), compared to the control group (mean 24 ± SD 6.81).

This is in contrast with the study of Kilic-Okman et al., [26] that was carried out to compare the effect of clomiphene citrate and letrozole on ovarian follicles, endometrium and hormone levels in rats. This study found no significant difference between clomiphene citrate treated rats and that given placebo as regard serum Estradiol (P value more than 0.05).

The laser induction group has higher serum Estradiol levels (mean 31.22 ± SD 8.75), compared to the control group (mean 24 ± SD 6.81).

The mean serum Estradiol was not statistically significantly higher in the clomid group compared with laser group (P value more than 0.05).

Also, this study shows the significant difference as regard serum progesterone levels between the three studied groups (P value less than 0.05) statistically.

The clomid induction group has higher serum progesterone values (mean 30.97 ± SD 12.25), compared to the control group (mean 18.35 ± SD 5.24).

The result is in contrast with the study carried out by Sherein & Hossam Eldein [25], which found no significant difference between clomid and control groups as regard serum progesterone (P value more than 0.05)

The laser induction group has higher serum progesterone levels (mean 25.29 ± SD 9.09), compared to the control group (mean 18.35 ± SD 5.24).

This result is by Sherein&Hossam Eldein [25], who found the highly significant difference (P less than 0.001) in serum progesterone towards the laser-treated group.

While in comparing serum progesterone levels between clomid and laser-treated group, the difference was found non-significant (p more than 0.05), this is in contrast with Sherein & Hossam Eldein [25] who found statistically significant difference towards laser stimulated group (p less than 0.05).

Significant positive correlation between serum Estradiol & serum progesterone levels was found in the control group (r = 0.585, P = 0.014). Significant positive correlation between serum Estradiol & serum progesterone levels was found in clomid treated group (r = 0.579, P = 0.009). Non-significant positive correlation between serum Estradiol & serum progesterone levels was found in the laser treated group (r = 0.206, P = 0.228).

In conclusion, this study shows that ovarian laser biostimulation is a new encouraging method for induction of ovulation, at least in animals. This had been proven biochemically by a significant increase in serum Estradiol (that assess the endogenous estrogenic hormonal activity) and serum Progesterone (a good indicator for ovulation and corpus luteum function).

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