Faculty of Dentistry
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LEVELS OF MATRIX METALLOPROTEINASE-8 IN GINGIVAL CREVICULAR FLUID AFTER INTRAPOCKET APPLICATION OF CYMBOPOGON CITRATUS GEL ADJUNCTIVE TO NON-SURGICAL TREATMENT IN PATIENTS WITH MODERATE PERIODONTITIS.

Protocol for M. Sc. Degree

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CONTENT

ABSTRACT

Background: The gold standard therapy in treating moderate periodontitis is mechanical removal of dental biofilm. Using local delivery drugs as adjunctive to scaling and root planing is widely used to modulate inflammatory host response and eradicate microbes. Nowadays, gingival crevicular fluid (GCF) biological molecular markers located in the exact area of contact between bacterial biofilm and host tissues are a suitable way to assess and predict periodontal disease progression. Collagenase-2 (MMP-8), which breaks down collagen of periodontal ligament, is associated with the onset of inflammation and is indicative of the severity of the non-specific inflammatory response.

Study objective: To evaluate gingival crevicular fluid levels of matrix metalloproteinase-8 (MMP-8) in the non-surgical management of moderate periodontitis after the adjunctive intra-pocket application of *Cymbopogon citratus* (lemon-grass) gel.

Materials and Method: This randomized controlled trial will include forty patients with moderate periodontitis, divided equally into two groups. Group-I will be managed by scaling and root planing with intra-pocket application of 2% lemongrass oil gel. Group- II will be managed by scaling and root planing with intra-pocket application of a placebo gel. Gingival crevicular fluid will be collected from both groups at baseline before treatment, one week, and at twelve weeks after treatment, respectively. Then analysed by Enzyme-linked Assay (ELISA) technique.

Results: Results will be tabulated and statistically analysed.

Keywords: Enzyme-linked immunosorbent assay (ELISA). Gingival crevicular fluid Lemongrass oil, Matrix metalloproteinase -8 (MMP-8)

INTRODUCTION

Periodontal disease is an inflammation of the supporting tissue that surrounds the teeth. The major etiological factor for initiating and progressing of this disease is dental biofilm, which contains bacteria that have virulence factors that cause reversible or irreversible destruction of connective tissue and alveolar bone. [1] Although gingivitis is a completely reversible condition and can return to health by removing local factors, it can extend into alveolar bone to cause periodontitis, which is irreversible destruction of supporting tissues of the teeth, in case causative agents are not removed. [2]

Caton and his colleague (2018) [3] introduced that periodontitis has two categories. According to ***Tonetti, Greenwell and Kornman (2018)***, [4] first category consists of four stages based on severity and complexity of management. Stage I: initial periodontitis, stage II: moderate periodontitis, stage III: severe periodontitis with potential for additional tooth loss, and stage IV: severe periodontitis with potential for loss of the dentition. Second category consists of three grades based on risk of rapid progression and expected treatment response. Grade A: slow rate of progression, grade B: moderate rate of progression, and grade C: rapid rate of progression.

The prevalence of periodontitis remains high globally, especially severe periodontitis which is the sixth most prevalent disease of humanity. [5] In case of severe periodontitis, teeth may be lost which has an impact on function and aesthetics. Different forms of periodontitis could affect any age. [6]

Appropriate diagnosis can identify the severity, activity and progression of periodontitis which is important for reaching the best treatment plan. There are several diagnostic procedures such as probing depth, bleeding on probing, mobility assessment, plaque index and radiographs to assess alveolar bone level. [7] ***Goodson (1992)*** [8] emphasized that all previously mentioned methods are easy to use, inexpensive, and relatively non-invasive. However, they could not assess the current disease status and show only the history of disease.

Nowadays, novel methods of diagnosis can identify periodontal risk and predict its progression and thus allow earlier detection of the disease. [9] Those methods include microbiological analysis and biomarkers in saliva, [10] and gingival crevicular fluid. [11] Biologic markers are substances that are measured as an indicator of normal biologic and pathogenic processes, or pharmacologic responses to a therapeutic intervention [12]. ***Srivastava, Nayak, and Rana (2017)*** [13] indicated that prostaglandin E2, tumour necrosis factor-α, matrix metalloproteinase (MMP-8, 9 and 13) are biomarkers that not only help in diagnosis and predicting the progression of many periodontal diseases, but also help in predicting treatment outcomes.

According to ***Teles and his co-worker (2006)***, [14] there are many protocols for treatment of periodontitis. Nonsurgical treatment includes mechanical instrumentation alone or in conjunction with host modulation or antimicrobial therapy. Removing bacterial biofilm,

calculus, and toxins from periodontally involved root surfaces is the main goal of periodontal therapy to stop progression of bacterial infection. It also reduces the levels of inflammation, probing pocket depth and helps in clinical attachment gain. Although removing dental biofilm mechanically is still the gold standard therapy, there are many factors that reduce its effectiveness such as severity of periodontal disease, rate of progression and response to therapy. Therefore, systemic host modulation and antimicrobial therapy are used as adjunctive therapy to nonsurgical treatment to overcome these limitations. [15] Despite controlling or eradicating many pathogens, systemic antibiotics cause several side effects to patients, including the development of resistant species. [16]

Localized intra-pocket drug delivery systems were developed to reduce these side effects. Local drug delivery systems have controlled release ability for extended periods of time. Moreover, they provide high concentrations of active ingredients at the targeted site. However, the use of local delivery antibiotics did not overcome antimicrobial resistance. [17,18] Thereafter, phytotherapics were advanced as an alternative option to antibiotics to reduce side effects. Phytochemicals are derived from plants that have therapeutic anti-inflammatory, anti-collagenase, antimicrobial, antioxidant, and antiseptic properties. Nowadays, periodontists use local herbal drugs as adjuncts to conventional periodontal therapy. [19]

Phytochemicals (herbal) drugs such as *Aloe vera* gel, [20] *Emblica officinalis* gel, [21] a bark of *Mimusops elengi* (Bakul) and a bark of *Vachellia nilotica* (Acacia arabica), [22] and *Cymbopogon citratus* (lemongrass) essential oil gel were investigated in several studies. [23,24,25,26]

Cymbopogon citratus (lemongrass) is native plant to Asia, Africa, and the Americas, but it is widely planted in moderate and equatorial regions of the world. [24] Generally, lemongrass is recognized as safe (GRAS) in the US. According to Federal Regulations Section in 1982, lemon grass plant extract/essential oil is safe for human consumption. Pharmacological study on lemon grass, two months daily administered orally in male and female rat, in dose up to 20 times larger than the estimated corresponding human dosage. Results showed no inducing to any effect which could be taken as evidence of toxicity. [25] Another study on the assessment of eventual toxic, hypnotic and anxiolytic effects of lemon grass herbal tea on human. It had taken for two weeks of daily oral administration. The tea has created no changes in urea, serum glucose, creatinine, alkaline phosphates, cholesterol, indirect bilirubin, lipid total bilirubin, total protein and albumin, but there was slight raise of amylase and direct bilirubin in some of the volunteers but without any clinical manifestation. [26] It has been used in ancient India as a therapy to inflammation, food flavouring agent, for pharmaceutical application, and as ornamental plant. [27,28] It is used as a popular medicine for throat infections, catarrh, coughs, bronchitis, asthma and sinusitis associated with respiratory system. [29,30] In addition, it can be used for headache, leprosy, and malaria. [31,32,33,34] Moreover, it used for arthritis and inflammatory disorders. [35,36,37]

*Cymbopogon citratus*essential oil can be added to ointments, creams and in massages to treat topical inflammation because of the citral, main component of *Cymbopogoncitratus,*

has shown a high inhibitory effect to inflammatory mediators. [38] Accordingly, it was used as an adjunctive mouth wash in nonsurgical treatment of periodontitis. [39]

Many researchers stated that lemongrass oil at concentration 2% or less than 2% when applied subgingivally with scaling and root planning shows more probing depth reduction and inhibits the growth of several kinds of microorganisms including periodontal pathogens, especially the reference strains *Actinomyces naeslundii* and *Porphyromonas gingivalis*. [18,23,40,41] Semisolid formulations consisting of mucoadhesive polymers such as hydroxypropyl cellulose, polyvinylpyrrolidone, polycarbophil, and carbopol have been added to increase the contact time of agent to the tissue, in the periodontal pocket. [41,42] In a longitudinal study in 2018, the effectiveness of lemongrass oil gel was more than chlorhexidine gel. [18]

To assess the response to periodontal therapy, there are several molecular biomarkers in plaque, saliva, and gingival crevicular fluid (GCF). [7,43] Gingival crevicular fluid is located between the bacterial biofilm and host tissues. This location is the most suitable place to monitor inflammatory changes. [44] Matrix metalloproteinases (MMPs), are a group of enzymes responsible for the degeneration of most extracellular matrix proteins and basement membrane (BM) components. They are assessed by collecting gingival tissue extracts, gingival crevicular fluid and saliva. [45,46]

There are twenty four genetically separate types of matrix metalloproteinases which are divided into subgroups, such as collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), membrane-type MMPs (MMP-14,-15,-16, -17,-24, and -25), and others. Collagenases show cleavage of type I collagen. The major collagenases in periodontitis are human collagenase-2 (MMP-8) accompanied by gelatinase B (MMP-9). [7]

Matrix metalloproteinase-8 (MMP-8) is considered the main collagenolytic matrix metalloproteinase detected in gingival tissue and oral fluids and is used as a periodontal biomarker. The major cellular source for this marker is neutrophils (PMN), but it can be released from gingival sulcular epithelial cells, fibroblasts, endothelial cells, monocyte/macrophages and plasma cells. [7] Matrix metalloproteinase-8 (MMP-8) has been related to the diagnosis, severity of inflammation, progression, and treatment follow-up of periodontitis. [47]

The null hypothesis of this research is that there will be no difference in GCF matrix metalloproteinase-8 (MMP-8) levels in moderate periodontitis patients treated by the non-surgical treatment and *Cymbopogon citratus* (lemongrass) gel in comparison to those with non-surgical treatment only.

AIM OF THE WORK

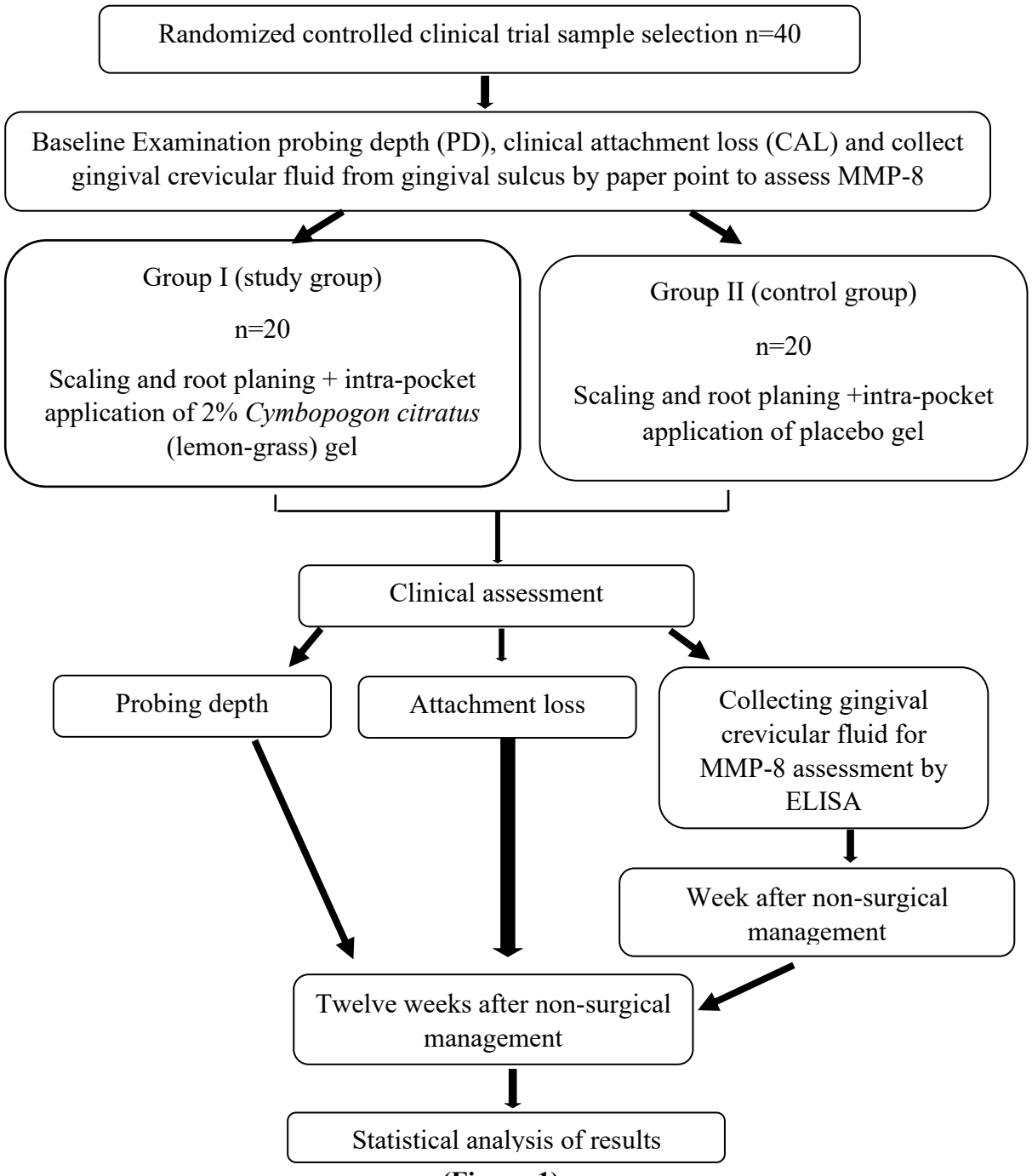
The present work aims to evaluate gingival crevicular fluid levels of matrix metalloproteinase-8 after intrapocket application of *Cymbopogon citratus* gel adjunctive to non-surgical treatment in patients with moderate periodontitis.

PLAN OF THE STUDY

I. Materials

i. Study design:

This study will be a randomized controlled design following CONSORT guidelines. [48] Assess and compare the effect of adjunctive intra-pocket application of *Cymbopogon citratus* (lemon-grass) gel to placebo gel on the matrix metalloproteinas-8 (MMP-8) in the gingival crevicular fluid in the non-surgical management of moderate periodontitis. (Figure-1)



(Figure-1)

ii. Study sample and setting

A total number of forty moderate periodontitis patients will be included in the study. They will be recruited from the outpatient clinic of the Department of Oral Medicine, Periodontology, Diagnosis and Oral Radiology Faculty of Dentistry, Alexandria University.

Sample size calculation

A sample size of twenty patients per group (number of groups = 2) (total sample size = 40 patients) is the sufficient required sample as statistically significant with 80% power. [41,49] The sample size was calculated using G Power version 3.1.9.2. [50]

Inclusion criteria

(1) Patients of both sexes having moderate periodontitis (stage II), CAL 3-4mm. [4]
(2) Patients' age between 25 and 45 years old.
(3) Systemically healthy patients.
(4) No history of periodontal therapy (surgical and non-surgical) or taken any antibiotic therapy for the past six months.

Exclusion criteria

(1) History of smoking.
(2) Patients having previous adverse reaction to the products (or similar products) used in this study.
(3) Grade C category that has rapid rate of progression. [4]
(4) Pregnant and lactating women.

iii. Materials

a. *Cymbopogon citratus* (lemongrass) gel

Preparation of gel will be at the Department of Pharmacology; Alexandria University. 2% Lemongrass essential oil gel will be prepared by a method like other anti-inflammatory gel formulations. For this preparation, certain quantity of carbopol * 934 will be soaked in water for a period of two hours. Carbopol will be then neutralized with triethanolamine (TEA)† by stirring. In a pre-weighted amount of propylene glycol and ethanol, 2% lemongrass essential oil will be dissolved. The solvent blend will be transferred to carbopol container and will be agitated for an additional twenty minutes. Then, the dispersion will be allowed to hydrate and swell for sixty minutes. pH will be adjusted with 98% triethanolamine (TEA) until it will reach (6.8-7). During pH adjustment, the mixture will be stirred gently with a spatula until a homogeneous gel will be formed. [18]

*Kind gift from Alexandria company for pharmaceuticals and chemicals industry.
†Kind gift from Alexandria company for pharmaceuticals and chemicals industry.

b. Placebo gel: (Carbopol®-Based pH-Sensitive Gels)

Preparation of gel will be in the Department of Pharmacology; Alexandria University. For this preparation, certain quantity of carbopol 934 will be soaked in water for a period of two hours. Carbopol will be then neutralized with triethanolamine (TEA) by stirring. Then, the dispersion will be allowed to hydrate and swell for sixty minutes. pH will be adjusted with 98% triethanolamine (TEA) until it will reach (6.8-7). During pH adjustment, the mixture will be stirred gently with a spatula until a homogeneous gel will be formed). [51]

c. Human Matrix metalloproteinase 8/Neutrophil collagenase (MMP-8) ELISA Kit *

*Bioneovan Co., Ltd., Beijing, China.

II. Methods

i. Randomized Controlled Clinical Study

Patients diagnosed as having moderate periodontitis requiring non-surgical management after clinical and radiographic examination will be included in this study. The purpose and nature of the study will be explained, and an informed consent will be obtained from patients who agree to participate in this study prior to any procedure.

Grouping and Randomization

The selected forty patients will be randomly assigned to the following groups:
Group I: twenty patients will be treated with scaling and root planning and intra-pocket application of 2% lemongrass oil gel.

Group II: twenty patients will be treated with scaling and root planning and intra-pocket application of placebo gel.

Allocation concealment

Simple randomization of subjects into two groups will be carried out using a computer-generated list of random numbers of the patients, and the two treatment modalities used in the study and will be performed by an expert statistician. The list will be generated using random allocation software. Each allocation will be represented by a code and sealed in sequentially numbered opaque envelopes that will be only opened at the time of intervention. [52,53]

ii. Clinical examination includes:

1. Bleeding on probing. [54]
2. Plaque index, and probing depth (PPD) and clinical attachment loss (CAL). [55,56]
3. Collection of gingival crevicular fluid by paper point to analyses MMP-8. [60]

iii. Preliminary Phase

All the patients who have emergencies such as dental or periapical lesion will be treated, and nonrestorable teeth will be extracted. [57]

iv. Non-surgical Phase

1. Oral hygiene instructions will be given to the patients which include tooth brushing using proper technique twice daily.
2. Scaling and root planing will be performed.
3. Coronoplasty will be done when required. [58]
4. The gels (lemongrass oil gel and placebo) will be administered by means of a syringe with a bent, blunt-end needle. The needle will be carefully inserted into the periodontal pocket and the gel will be applied in the test sites in a gentle probing manner, attempting to fill the full extent of the pocket. The gel will be applied up to the gingival margin and the excess gel will be removed with sterile gauze.

5. Periodontal dressing will be applied after delivery of the drug, and they will be also asked not to eat for thirty minutes. [59]
6. After placement of the gel *in situ*, patients will be instructed to follow strict oral hygiene protocol and not chew hard or sticky foods at the gel placement sites for rest of the week.

Sample collection and analysis

1. The sites to be sampled will be isolated with cotton rolls and gently air-dried.
2. Gingival crevicular fluid will be collected by paper point which will be gently placed for 30 seconds into the pocket until a minimum of resistance was felt. [60]
3. The paper points will be inserted into tubes containing 500 µl phosphate-buffered saline and will be eluted at 4°C overnight, then will be centrifuged at 400 g for 4 minutes. [44,60]
4. The samples will be frozen immediately to the temperature of –20°C and stored until the analysis. [60]

v. Follow up

Clinical evaluation

a. Patients' oral hygiene status will be reassessed after first week, four weeks, and twelve weeks post-non-surgical follow up. [18, 23]

b. Collection of gingival crevicular fluid by paper point to analyses MMP-8 after first week and twelve weeks. [61]

c. Probing depth (PBD) and clinical attachment loss (CAL) after twelve weeks. [18, 23]

vi. Outcomes

The primary endpoint with respect to efficacy in moderate periodontitis will be achieving CAL gain 3.20 ± 0.75 mm and PBD reduction 2.40 ± 0.73 mm baseline to 12 weeks as measured by Michigan 'o' probe after twelve weeks. [18, 23] The GCF concentration of MMP-8 will be decreased after therapy but reduced even more dramatically (approximately 50%) following a 3-month period of maintenance. [61]

ROLE OF SUPERVISORS

1. Prof. Dr. Ahmed M Hommos.

He will supervise managing patients and collecting samples.
He will also help in interpreting results and revising the thesis.

2. Dr. Souzy Kamal Anwar.

She will supervise managing patients and collecting samples.
She will also help in interpreting results and revising the thesis.

3. Dr. Riham Mohamed El-Moslemany.

She will supervise preparing the medicine drugs (2% *Cymbopogon citratus* oil gel and placebo gel).
She will also help in interpreting results and revising the thesis.

4. Dr. Neveen Lewis Mikhael.

She will supervise analyzing the matrix metalloproteinases-8 gingival crevicular fluid levels.
She will also help in interpreting results and revising the thesis.

ETHICAL CONSIDERATIONS

The purpose and nature of the study will be explained to the patients and an informed consent will be obtained from patients who agree to participate in this study prior to any procedure of the study. (Appendix -1) Treatment will be in accordance with the principles of the modified Helsinki code for human clinical studies (2013). [62]

The clinical study will be conducted following the ethical guidelines for conduct of research on human subjects, by the Faculty of Dentistry, Alexandria University (IRB NO:00010556 - IORG 0008839).

DATA MANAGEMENT AND STATISTICAL ANALYSIS

The data will be processed and analysed using Statistical Package for Social Sciences program SPSS (20.0) software*. The study will include descriptive and analytical data. A P-value of less than 0.05 will be considered statistically significant.

Armonk, NY: IBM Corp, USA.

DURATION OF THE STUDY

Estimated time: Nine Months.

Study activities/ Months	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Materials collection and preparation	✓											
Patient selection		✓										
Clinical study			✓	✓	✓							
Data management and statistical analysis						✓						
Writing thesis							✓	✓				
Thesis submission									✓			

BUDGET

Item	**Cost in LE**
Lemongrass essential oil and other ingredients	1000
Miscellaneous instruments and materials	6,000
Human Matrix metalloproteinase 8/ Neutrophil collagenase (MMP-8)ELISA Kit	7,500
Statistical Analysis	700
Thesis printing and Binding	5000
Publication	2000
Total	22,200 EL

PROBLEMS ANTICIPATED

Availability of indicated patients who have moderate periodontitis with satisfactory oral hygiene and are not medically compromised. Cooperation of the patients participating in the study.

PUBLICATION POLICY

This study will be sent for either national or international Journal for publication, in the following order:

- Nadein Abd Elnasser El. Sharif.
- Prof. Dr. Ahmed M Hommos.
- Dr. Souzy Kamal Anwar.
- Dr. Riham Mohamed El-Moslemany.
- Dr. Neveen Lewis Mikhael.

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APPENDIX 2

IRB NO: 00010556 – IORG 0008839
http://www.hh.gov/ohrp/assurances/index.html

جامعة الإسكندرية
ALEXANDRIA UNIVERSITY
كلية طب الاسنان
FACULTY OF DENISTRY
كليـــــه معتمـــــدة

Informed Consent

- **Name of the Researcher:** .....
- **Name of the Department:** .....
- **Title of the Research:**

.....
.....
.....

- **Name of the Patient:** .....

	ETHICAL CONSIDERATIONS	YES	NO
1	Statement that it is a research		
2	Explanation is given to the subject of the research in clear understandable words about the procedure & its duration		
3	The benefit of the research to the subject & others is described		
4	The subject is informed about liable reasonable risk or discomfort		
5	The subject is informed about any alteration in procedure if needed		
6	Confidentiality is assured		
7	The subject can quit at any time without any penalty		

Signatures

• **Signature of the Patient:**	
• **Signature of the Researcher:**	
• **Date:**	

Researcher pledge

The researcher is responsible to fulfill this attached consent form that involving the ethical considerations for each patient during the period of the study.

Signature of the Researcher

_____

شارع شامبليون الازاريطه الاسكندرية مصر تليفون : ٤٨٦٨٠٦٦/٢٠٣ - ٤٨٦٩٦٩٠ فاكس ٤٨٦٨٢٨٦/٢٠٣
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