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Effects of Low level laser therapy (LLLT) on experimentally induced oral mucositis clinical & immunohistochemistry study.

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In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Dental Science/ Oral Medicine

By

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نَرۡفَعُ دَرَجَٰتٖ مَّن نَّشَآءُۗ وَفَوۡقَ كُلِّ

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صدق الله العظيم

سورة يوسف الاية ٧٦

Declaration

This is to certify that the organization and preparation of this thesis entitled ***Effects of Low level laser therapy (LLLT) on experimentally induced oral mucositis clinical & immunohistochemistry study.*** has been made by the graduated student **BAHAA Al-DEEN ABDULRAHMAN HADI** under my supervision at the College of Dentistry, University of Baghdad in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Dental Science/ Oral Medicine.**

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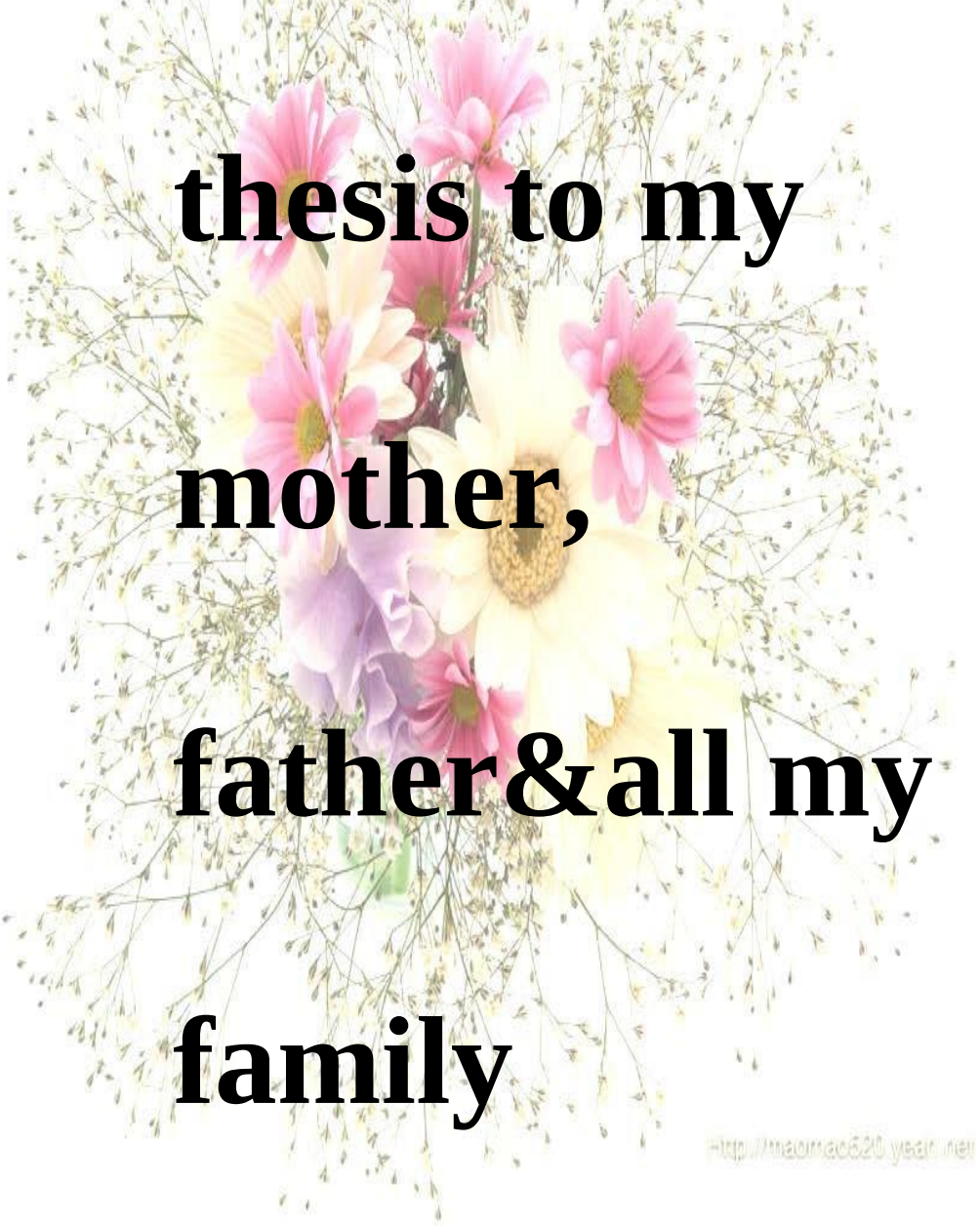
Dedication

I dedicate this thesis to my mother, father&all my family members



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List of abbreviations

Cm	Centimeter
Ct	Cytotoxic
CW	Continues wave
D.A	Dark Agouti rat
Er: YAG	Erbium: Yttrium Aluminum- Garnet
G1	Control non-treated group
G2	Laser 30 mw treated group
G3	Laser **60** mw treated group
Ga-Al-As	Gallium-Aluminum-Arsenide
He-Ne	Helium-Neon
HSCT	Hematopoietic stem cell transplantation
IL-10	Interleukin ten
IL-β	Interleukin-1 BETA

InGaAlP	Indium-Gallium-Aluminum-Phosphide
J	Joule
Kg	Kilogram
LLLT	Low level laser therapy
mg	Milligram
min	Minute
ml	Millilitre
*M*m	Micrometer
mm	Millimetre
MTX	Methotrexate
mW	milli Watt
Nd: YAG	Neodymium: Yttrium -Aluminum- Garnet
NF-κB	Nuclear factor Kappa
Nm	Nanometer
nm	Nanometer
O M	Oral mucositis
PDT	Photodynamic therapy
Sec	Second
TNF-α	Tumor necrosis factor alpha
W	Watt

INTRODUCTION

Oral mucositis severe side effect, caused by treatment with radiotherapy and chemotherapy (CT) for cancer. It is a very common, potentially It can be a limiting factor in the scheduled cancer treatment regimen, leading to suspension or interruption of the programmed treatment, with the consequent decrease in its effectiveness and even in the patient's survival ***(Lalla et al.,2014).***

It has been reported that approximately 40-60% of patients undergoing standard doses of CT will suffer from OM at variable degrees and 80-100% ***(Rodríguez-Caballero et al., 2011)*** of patients undergoing high dose myeloablative CT (e.g. stem cell transplantation patients). The incidence and severity of OM increases when radiotherapy (RT) is used in combination with CT ***(Sadasivan, 2010, Sonis, 2012).***

Cytotoxic CT targets the highly active dividing cancer cells, but unfortunately it also damages other normal rapidly dividing cells in the mucous membranes of alimentary tract, blood cells and bone marrow ***(Bruya and Madeira, 1975).*** Although CT has long been used as a standard treatment option for various neoplastic diseases; the focus on managing and minimizing its mucosal toxicity has only increased dramatically in the past recent years. Many side effects of chemotherapy such as neutropenia and bone marrow suppression are controllable by medications ***(Logan et al., 2007).*** This has allowed the use of higher doses of CT and thus increased the incidence and severity of mucosal toxicity ***(Eilers and Million, 2011).***

Patients with OM often experience intense pain, leading to difficulty with eating and speech. In addition, mucosal barrier injury represents a portal of entry for opportunistic infections ***(Bayder et al.,2005; Jones et al.,2006).*** The pathophysiology of mucositis is dynamic and multifactorial, which

includes five phases: initiation, upregulation and message generation, signal amplification, ulceration, and healing ***(Peterson et al.,2011).***

The initiation phase is followed by both DNA and non-DNA damage. Direct cellular injury targeting the basal epithelial cells occurs simultaneously with the generation of reactive oxygen species (ROS). In the primary damage response (message generation phase) a series of transcription factors are activated and the production of pro-inflammatory cytokines such as nuclear factor-KB (NF-KB), tumor necrosis factor-alpha (TNF-α), interleukin-1, (IL-1β), and interleukin-6, (IL-6), nitric oxide, ceramide, and matrix metalloproteinases occurs, which leads to apoptosis and tissue injury. The inflammatory modulators are activated, and provide a positive feedback loop (signal amplification) that drives the destructive process, so that the oral epithelium eventually breaks down and ulcerates (ulceration phase). The healing phase is also biologically dynamic, with signaling from the submucosal extracellular matrix stimulating the migration, differentiation, and proliferation of epithelial healing ***(Sonis, 2002,2004,2007&2012).*** The exact mechanisms of oral and GI mucositis are not fully understood, yet a lot of progress has been made over the last several years. This is largely due to the development of representative preclinical animal models of mucositis (mouse, hamster, rat) ***(Vanhoecke et al.2015).***

Low-level laser therapy (LLLT) has been used in inflammatory pathologies as a new anti-inflammatory therapy, which, in principle, would not be associated with any side effects ***(Bjordal et al.,2006; Chow.,et al2009).*** Recently, we showed a dual effect of LLLT on anti- and pro-inflammatory cytokines in a model of the acute lung inflammation induced by intestinal ischemia and reperfusion. This result revealed that low-level laser irradiation can exert its biomodulatory effect on different cytokines

(TNF-α and IL-10) independently of each one and at the same time ***(Lima et al.,2013).***

Many medication and approaches have been described to avoid and reduce the severity of mucositis, such as an intensive oral care protocol, antimicrobial agents, antiinflammatory agents, cytoprotective agents, growth factors, natural and homeopathic agents, and local anesthetics ***(Franca et al.,2009; Caballero et al.,2012).*** Clinical trials on these modalities have yielded inconsistent results; therefore, none of them have become a gold standard adjunct with proven efficacy ***(Clarkson et al.,2010; Carvalho et al.,2011).*** Since the simple use, absence of toxicity, low cost of the equipment, and positive results, the use of LLLT has been shown to be a new therapeutic option that can be used for management of OM. Thus, this study described the effect of LLLT on the treatment of the experimentally induced oral mucositis (in vivo) of dark agouti rat treated with 60mg/kg of methotrexate.

AIMS OF STUDY

1- Experimental induction of oral mucositis in rat model by cytotoxic drug (MTX).
2- Clinical evaluation of the oral mucositis among studied groups (laser 30,60mW& non- treated groups).
3- Measurements of tissue cytokines (IL-1 β, TNF-α &1L-10) among three studied groups (laser30,60mW &non-treated control groups).

CHAPTER ONE
REVIEW OF LITERETURE

1.Review of Literature

1.1 Oral mucosa

The alimentary tract (AT) constitutes the major part of the human digestive system. It is lined by a mucous membrane, or mucosa, which provides protective, sensational and secretory functions. The oral cavity is the first part of AT and it is lined by the oral mucosa ***(Underwood and Cross, 2009).***

1.1.1 Anatomical structures and functions of the oral mucosa.

The oral mucosa consists of epithelium and underlying lamina propria. It shows

structural and functional variations at different regions in the oral cavity. The three types of mucosa found in the oral cavity are masticatory mucosa, lining mucos and specialized mucosa. The masticatory mucosa consists of keratinized stratified squamous epithelium which is tightly attached to the underlying collagenous connective tissue. The lining mucosa is non-keratinized and overlies a more flexible and elastic connective tissue. The masticatory mucosa is mainly found covering the gingivae and hard palate, whereas the lining mucosa covers the soft palate, ventral surface of the tongue, floor of the mouth, alveolar, labial and buccal mucosa. The epithelium in the masticatory mucosa is generally thick whilst with the lining mucosa there is regional variation in epithelial thickness ***(Nanci and Ten Cate, 2012).*** The dorsal and lateral surfaces of the tongue are covered by a specialised mucosa, which consist of thick keratinized or non-keratinized stratified squamous epithelium and contain the taste buds and

lingual papillae ***(Nanci and Ten Cate, 2012).***

The digestive tract is lined with epithelial cells from the mouth to the anus which can be usually injured as a side of many cytotoxic remedy regimens. The mucosa acts as a protective bodily and chemical barrier against pathogens that could enter the gastrointestinal device through the mouth or breaks inside the mucosal integrity. The most cancers chemotherapy, radiation, and hematopoietic cell transplantation (HCT), whether or not by myself or in aggregate, causes serious and lifestyles-threatening side consequences in lots of patients. Oral mucositis is among those facet consequences; it's miles related to ache, contamination, nutritional alterationsand diminished first class of existence ***(Yarbro et al.,2011).***

1.1.2 Microscopic structures of the Oral mucosa.

The oral epithelium undergoes constant turnover by the action of the dividing basal cells. These cells differentiate towards the surface in layers at different thicknesses; hence the name stratified squamous epithelium. The epithelial turnover is faster in the lining mucosa than masticatory or specialized mucosa .Immediately beneath the epithelium lies the lamina propria, which consists of cells, blood vessels, neural elements, and fibres embedded in an amorphous ground substance. The cell types in the lamina propria include fibroblasts, endothelial cells, macrophages, mast cells and inflammatory cells. The submucosa lies below the lamina propria and shows variation in structural components at different regions of the oral cavity. It separates the oral mucosa from the deeper muscles and bone. It contains loose adipose or glandular connective tissues and blood vessels and nerves ***(Nanci and Ten Cate, 2012).*** The layers of oral mucosa and submucosa are illustrated in. fig (1.1).

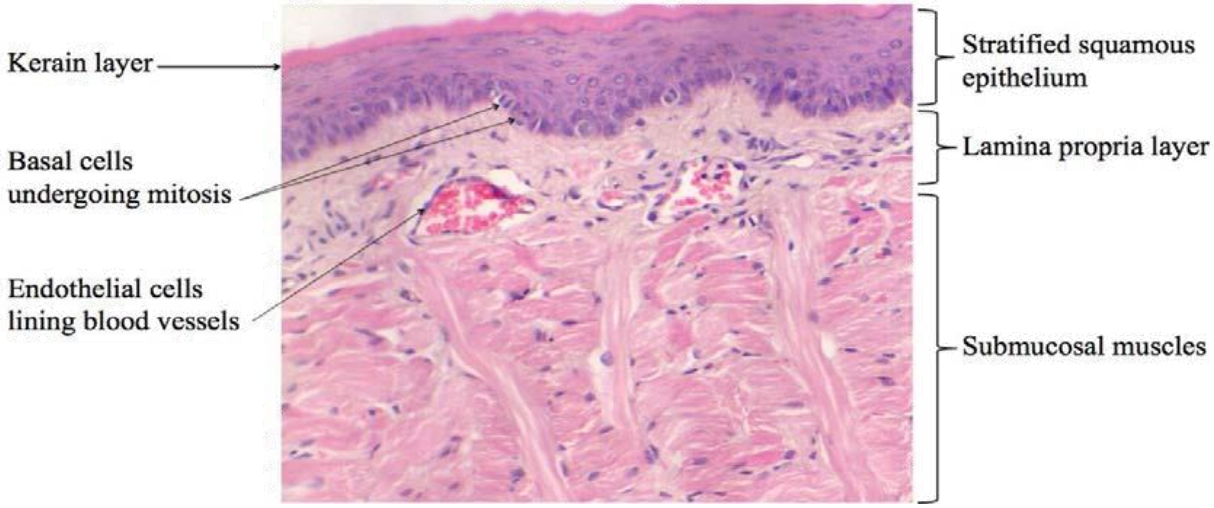

Fig 1.1 Histology of the oral mucosa. photomicrograph of the oral cavity demonstrating the different layers of oral mucosa (Haematoxylin and Eosin stain, original magnification x20) (Nanci and Ten Cate, 2012).

1.2 Mucositis & stomatitis

Mucositis and stomatitis have been used interchangeably to define the inflammation of the mucosa ***(Epstein et al.,2012).***

Mucositis is an inflammatory process that is visually seen in the mucous membranes of the oral cavity and can be present throughout the gastrointestinal tract. Inflammatory diseases of the mouth that include the mucosa and the dentition, periapces, and periodentum are collectively referred to as stomatitis ***(NCI, 2012).***

Mucositis is an inflammatory process this is visually seen in the mucous membranes of the oral cavity and may be gift at some point of the gastrointestinal tract. Inflammatory diseases of the mouth that include the mucosa and the dentition, periapces, and periodentum are together called stomatitis.

Mucositis can begin with erythema and progress to confluent ulceration of the oral mucosa, including gingiva and the tongue, with the ensuing damage ranging from mild to severe ***(Caplinger.,2010).***

Disruption in the integrity of the oral mucosa can have significant effects on the course of treatment such as dose delays and reductions. Many cancer patients report mucositis to be the most distressing side effect of their treatment, especially during radiation or chemotherapy for head and neck cancer. Intact oral mucosa serves as a protective barrier. Mucositis breaks that barrier, allowing for the development of local infection that can progress to life-threatening sepsis. The ability to take in adequate nutrition may be compromised by excessive dryness (xerostomia), oral discomfort and pain, and alterations in taste affecting the ability and desire to eat., ***(Oliveira et al.,2011).***

Disruption in the integrity of the oral mucosa will have enormous outcomes on the route of treatment including dose delays and reductions. Many cancer patients report mucositis to be the most distressing aspect impact in their treatment, especially at some stage in radiation or chemotherapy for head and neck cancer. Intact oral mucosa serves as a defensive barrier .

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The potential to soak up good enough nutrition can be compromised by way of excessive dryness (xerostomia),oral pain and alterations in taste affecting thecapacity and desire to consume .The patient's quality of life may be altered when mucositis interferes with the ability to communicate and decreases oral sensation and pleasure.

There are also financial implications for oral mucositis in terms of increased hospitalization, clinic visits, procedures, and medication costs. In the past two decades, improved antiemetic therapy and use of growth

factors to prevent and minimize bone marrow depression have allowed for continued dose escalation of chemotherapy agents. In turn, severe mucositis that cannot be effectively prevented or minimized has evolved into a dose-limiting side effect. Along with experiencing dose reductions and dosing delays, patients who are immunocompromised can become infected and require hospitalization. Subsequently, patients with severe mucositis have higher healthcare costs and poorer quality of life. ***(Armstrong.,2006).***

1.2.1Etiopathophysiology

Mucositis is a common side effect of anticancer therapy and can be attributed to specific chemotherapeutic and targeted agents as well as radiation to the head and neck. Concomitant chemotherapy and radiation to the head and neck increases the incidence and severity of mucositis as listed in table (1.1). Patients undergoing HCT with high-dose chemotherapy may often experience severe oral and alimentary mucositis ***(Yarbro et al.,2011).***

Table 1.1 Chemotherapeutic Agents With a Tendency to Cause Oral Mucositis (Yarbro et al.,2011)

5-Fluorouracil[a]	Etoposide
6-Mercaptopurine	Floxuridine
6-Thioguanine	High-dose methotrexate
Actinomycin D	Hydroxurea
Bleomycin	Mechlorethamine[a]
Busulfan[a]	Melphalan
Capecitabine[a]	Mitomycin
Cyclophosphamide[a]	Mitoxantrone
Cytosine arabinoside	Paclitaxel
Daunomycin	Procarbazine
Daunorubicin	Thiotepa
Docetaxel	Vinblastine
Doxil[a]	Vinorelbine
Doxorubicin	

1.2.2.Incidence

The National Cancer Institute estimates that 10% of patients receiving adjunctive chemotherapy and 40% of those receiving primary chemotherapy experience mucositis. The incidence of mucositis is higher in patients with cancers of the oral cavity, oropharynx, and nasopharynx than in patients with other cancers ***(NCI, 2013).*** Nearly 100% of patients receiving radiation therapy to the head and neck have some grade of oral mucositis, and 75% to 80% of patients undergoing HSCT develop significant mucositis. Treatment combinations that include both chemotherapy and radiation are also associated with higher incidence of this side effect than either therapy alone, with the most severe grade of mucositis occurring in patients treated with chemotherapy and radiation therapy to the oral cavity. These patients have severe oral mucositis

resulting in the inability to eat solid foods and often require enteral support ***(Lalla,.et al,2008).***

1.2.3Epidemiology

Oral mucositis is among the most common and dreaded toxicities of cancer therapy ***(Lockhart& Sonis,1979).*** It occurs in almost all patients who receive radiation therapy in which areas of the oral or oropharyngeal mucosa are included in the treatment field. Thus, for patients with cancers of the mouth, oropharynx, hypopharynx, larynx, nasopharynx, and salivary glands, clinically significant OM occurs in about 70% of patients ***(Sonis,1990).*** Recipients of conditioning regimens in preparation for HSCT are also considered to be in an especially high risk group for OM ***(Sonis,1999)***. Aside from patient-associated risk factors (see below), the stomatotoxicity of individual conditioning regimens impact OM frequency and severity. For example, the incidence of severe (WHO grade ≥3) OM among patients with multiple myeloma or non-Hodgkin's lymphoma receiving conditioning regimens of high dose melphalan or carmustine, etoposide, cytarabine, and melphalan (BEAM), was reported to be 46% and 42% respectively ***(Sonis,2009)*** In contrast, 98% of patients with hematological malignancies who received a conditioning regimen consisting of cyclophosphamide, etoposide, and total body irradiation developed severe OM ***(Sonis,2007).***

Inconsistencies in reporting oral mucositis .While there is reasonable clarity around the frequency of OM in patients in the categories discussed above, there is wide discrepancy in its incidence in patients with the most common tumor types: breast, colorectal, and lung cancers. In general, the incidence of mucositis in these patients has been underreported ***(Sonis ,2004).*** Among the most common regimens for breast cancer (ie, AC+T –

doxorubicin, cyclophosphamide, and paclitaxel or docetaxel), ulcerative mucositis occurs in about 20% of patients during the first cycle of treatment. If that group of patients receives the same dose of the same drugs in a second cycle, the frequency of OM jumps to 70% ***(Sonis,2009).*** Interestingly, some of the newer regimens for metastatic breast cancer are even more stomatotoxic. The incidence of OM is more than 60% in patients receiving docetaxel and capecitabine, with 15% of patients developing severe
OM ***(Chan et al.,2009).***

There is rarely more inconsistency in the reporting of OM than among patients being treated with the standard 5-fluorouracil- (5-FU) containing regimens for colorectal cancer (CRC). The literature suggests that ulcerative OM occurs with a frequency of somewhere between 15% and 28% in patients receiving the most common 5-FU-based regimens ***(Keefe et al.,2007).*** Yet in a recent study, over 70% of CRC patients noted significant mouth and throat soreness following their treatment ***(Grunberg et al.,2007).*** Furthermore, it appears that women are more likely to develop OM in response to 5-FU than men ***(Sloan et al.,2000).*** The reason for this difference has not been defined. Newer drugs and regimens vary in their stomatotoxicity. Nineteen percent of elderly patients at risk of myelodysplastic syndrome were noted to have ulcerative OM in response to oral clofarabine ***(Faderl et al.,2010).***

In contrast, 70% of patients receiving pralatrexate developed mucositis, with 21% noted to have severe forms of OM ***(Malik et al.,2010).*** The use of the novel microtubule inhibitor vinflunine resulted in about 20% of nonsmall cell lung cancer patients developing OM ***(Krzakowski et al.,2010).*** Mammalian target of rapamycin inhibitors have been approved for the treatment of renal carcinomas and are being investigated as therapy for other cancer types including sarcoma. Mucosal ulcerations are among

the most common toxicities (about 40%) associated with this drug class ***(O'Donnel et al.,2008)*** and are even higher when combination regimens are used. For example, 60% of patients with advanced renal cell cancers who were treated with bevacizumab and everolimus reportedly developed OM ***(Hainsworth et al.,2010).***

1.2.4 The pathophysiology of oral mucositis

1.2.4.1 Historical hypothesis.

Our understanding of the pathogenesis of OM has matured markedly over the past decade. Prior to the late 1990s the prevailing mechanism by which mucositis occurred focused on direct but nonspecific cell death mediated by either chemotherapy or radiation (Figure 1.2) ***(Lockhart & Sonis,1979).*** The concept was simple: since neither chemotherapy nor radiation could differentiate between rapidly dividing (and DNA synthesizing) tumor cells or the rapidly dividing cells of the basal epithelium, these normal "mother" cells were killed, and replenishment of the normally renewing epithelium was eliminated. As a result, the story went, the mucosa would become atrophic and, if there was no replacement of the epithelium, ulceration developed. Ulcers would become secondarily colonized with bacteria, run their course, and then, if there were no extenuating circumstances, go on to spontaneously heal.

Increasing interest in mucositis spurred more in depth studies of its biology primarily as a way to develop targets for treatment ***(Sun et al.,2005).***

1.2.4.2 Etiological complexity of oral mucositis

In the late 1990s a series of studies was published in which the pathobiology of mucositis was studied in animal models that closely duplicated the human condition ***(Elting et al.,2007; Treister et al.,2008).***

The results of these studies revealed findings which, when viewed comprehensively, led to a completely new hypothesis about how mucositis occurs and strongly suggested that the initial damage

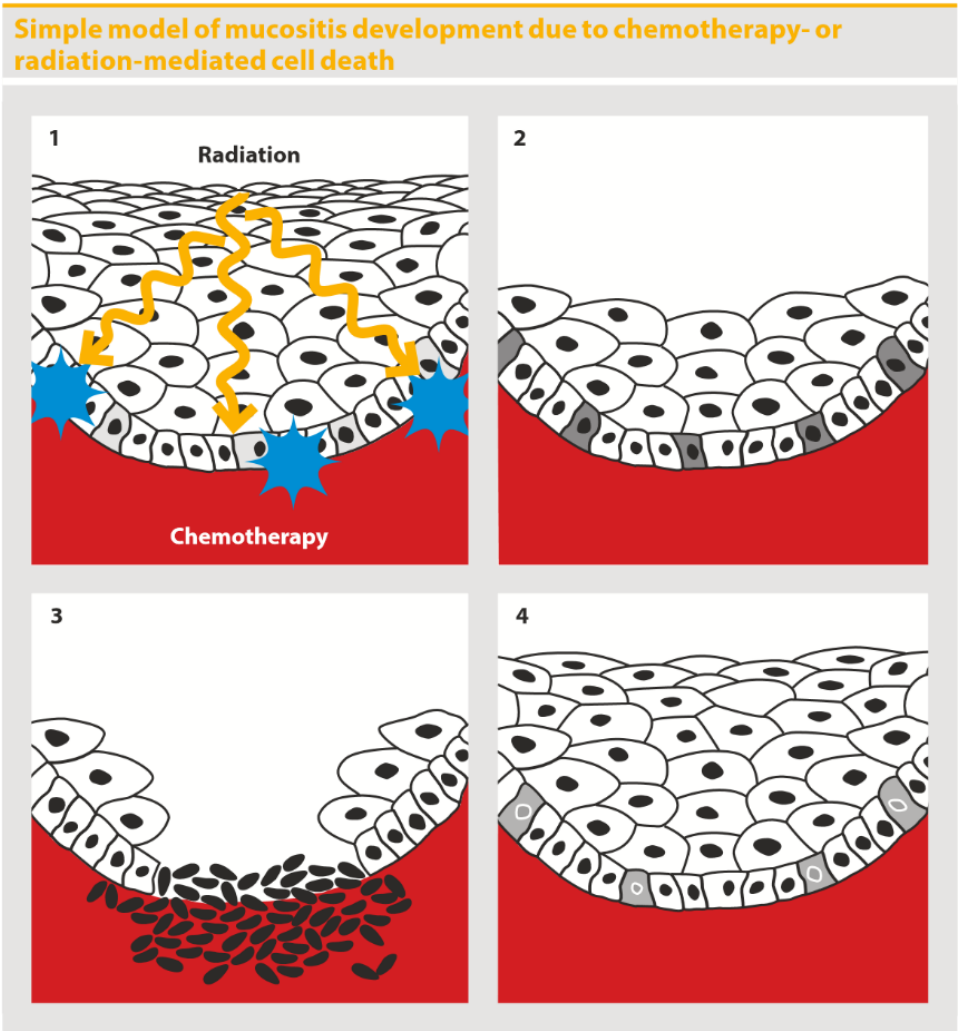

Figure 1.2 Simple model of mucositis development due to chemotherapy or radiation mediated cell death.

Historically, mucosal injury was attributed to the direct effects of radiation and chemotherapy on epithelial stem cells of the mucosa. It was suggested that clonogenic cell death blocked the regeneration of the epithelium. However, the tissue became atrophic and/or ulcerated as illustrated in fig (1.2). Consequent studies have shown that the pathogenesis is much more complex takes place in the cells and tissues of

the submucosa. This injury leads to the generation of signaling pathways that ultimately target the cells of the basal epithelium and leads to their demise. Since the first description of this new hypothesis, supporting data has been obtained from numerous studies ***(Spielberge et al.,2004; Blijlevens e al.,2008).*** These results confirm the concept that mucositis results from the cumulative impact of a number of biological pathways that originate in the submucosa and ultimately target the oral epithelium. These have been summarized in a five-stage schema (Figure 1.3**) *(Spielberge et al.,2004).***

1.2.4.3 Initiation phase.

The initiation phase is characterized by direct DNA injury caused by radiation or chemotherapy and subsequent strand breaks that result in clonogenic death of basal epithelial cells. Even more significant from the standpoint of ultimate tissue damage is the generation of reactive oxygen species (ROS) (Sonis,2004). It has been recently suggested that cells damaged by chemotherapy and radiation may release endogenous damage-associated pattern molecules (CRAMPs), which then bind to specific receptors and contribute to the initiation of stage 2***(Sonis,2009).***

1.2.4.4 Secondary- Primary damage response

Chemotherapy, radiation, ROS, and CRAMPs initiate a series of cascading and interacting biological events, including the activation of a number of transcription factors, such as nuclear factor Kappa-B (NF-κB), Wnt, p53, and their associated canonical pathways ***(Blijlevens et al.,2004)*** .Of the many canonical pathways that contribute to the development of mucositis, the NF-κB pathway is one of the best studied and provides an excellent example of the complexity of the process leading to ulceration.

Chemotherapy and radiation can directly activate NF-κB. Indirectly, it can be activated by ROS or by receptor-bound CRAMPs. As a result, up to 200 genes may be expressed. Among these are genes associated with the production of molecules, which have illustrated activity in the pathogenesis of mucositis including proinflammatory cytokines and cytokine modulators, stress responders (eg, COX-2, inducible NO-synthase, superoxide dismutase), and cell adhesion molecules. Furthermore, cell death (via apoptosis) may occur following NF-κB activation ***(Chan et al.,2009)***.

Other pathways have also been identified as playing significant roles in regimen-related mucosal injuries. Among the most significant are those associated

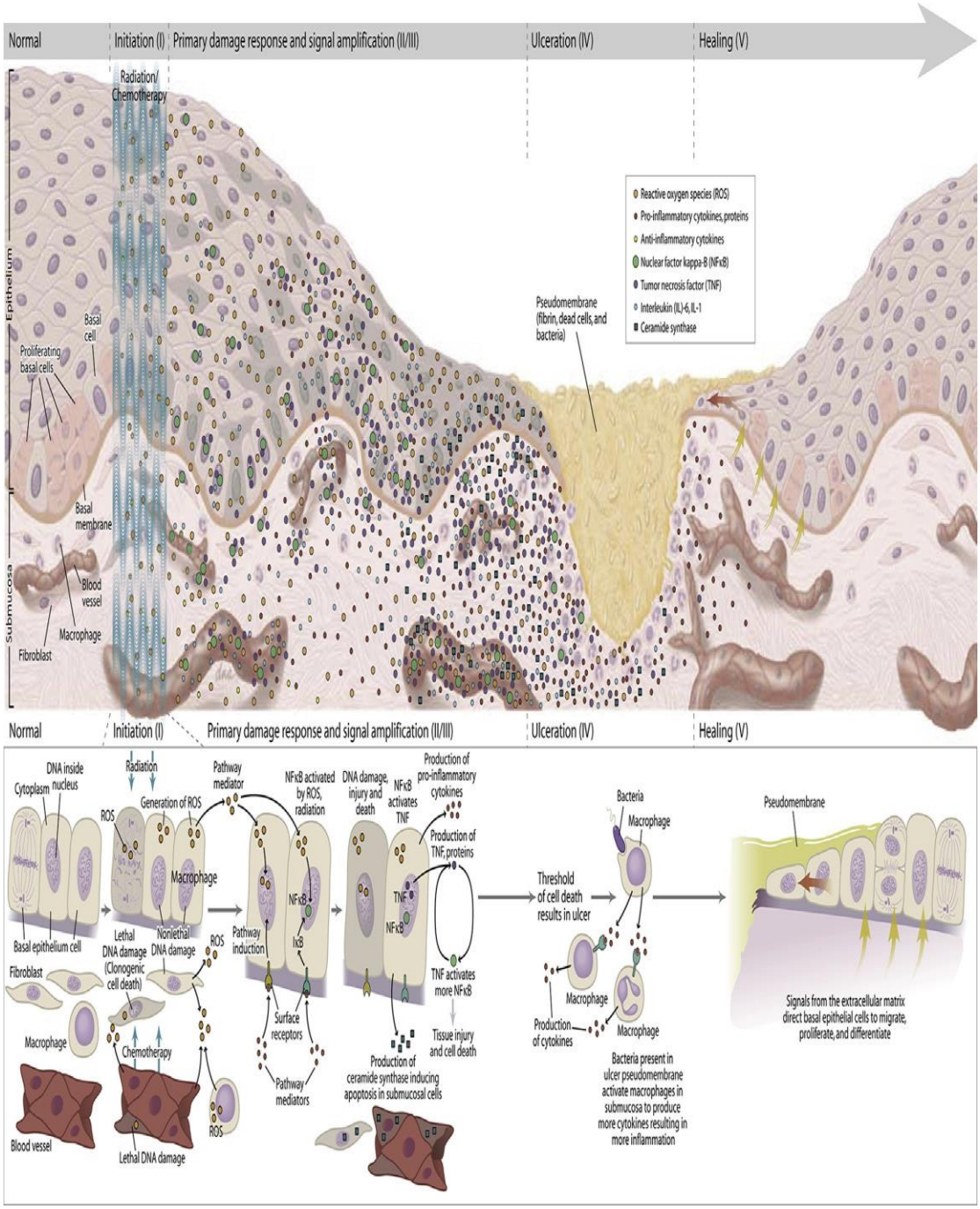

Fig. 1. 3 The five-stage model for the pathobiology of oral mucositis developed by Dr Stephen T. Sonis. The model incorporates a complex interaction among multiple components. These include direct damage to basal epithelial cells from cancer therapy and secondary insult to tissues due to upregulation of proinflammatory factors and products of colonizing microflora. (From Sonis ST. Pathobiology of oral mucositis: novel insights and opportunities. J Support Oncol 2007;5:3–11***(Sonis et al.,2007).***

with nitrogen metabolism, Toll-like receptor signaling, B-cell-receptor signaling, P13K/AKT signaling and mitogen-activated protein kinase

(MAPK) signaling, to name a few (sonis,2004). In addition, other radiation- and chemotherapy-induced mucosal damage is associated with the ceramide pathway and fibrinolysis and the stimulation of matrix metalloproteinases (MMPs) ***(Blijlevens et L.,2004; Keefe et al.,2007).***

The first two phases of mucositis development begin almost immediately after patients receive treatment. The majority of these changes are seen within the cells and tissues of the submucosa and both direct and indirect destruction of epithelial stem cells starts soon thereafter. However, from a clinical standpoint, the impact of all of these destructive activities is not realized for about 4 to 5 days following chemotherapy/radiation therapy challenge. And in the case of fractionated radiation, the precipitating events that lead to extensive mucositis occur in daily increments ***(Keefe et al.,2007).***

1.2.4.5. phase 3 – Signal amplification

Many of the molecules induced by the primary response have the ability to positively or negatively feedback and alter the local tissue response. For example, tumor necrosis factor (TNF) may positively feedback on NF-κB to amplify its response, and initiate MAPK signaling, leading to activation of Jun N-terminal kinase (JNK) signaling ***(Spielberger et al.,2004).***

1.2.4.6 phase 4 – Ulceration

For the patient and the clinician, the most significant stage of mucositis is the development of mucosal ulceration. This is the stage that is most symptomatic, prone to infection, and requisite for increased resource use. Because regimen-related ulceration is the consequence of damage at the basal layers of the epithelium, ulcers transect the full epithelial thickness.

Once formed, ulcers are colonized by both gram positive and gram negative oral bacteria, which spew out cell wall products. These molecules are capable of extending mucosal damage as they stimulate infiltrating macrophages to release additional levels of pro-inflammatory cytokines ***(Blijlevens et al.,2008).***

1.2.4.7 phase 5 – Healing

Ulcerative lesions of mucositis heal spontaneously, although this too is the result of a series of biological signals originating in the submucosa ***(Sonis,2004; Blijlevens et al.,2008).***

Oral mucositis is a significant toxicity of systemic chemotherapy and of RT to the H&N region. The morbidity of oral mucositis can include pain, nutritional compromise, impact on quality of life, alteration in cancer therapy, risk for infection, and economic costs. Management includes general symptomatic support and targeted therapeutic interventions for the prevention or treatment of oral mucositis. Evidence-based clinical practice guidelines are available to guide clinicians in the selection of effective management strategies ***(Lalla et al.,2014).***

Signaling molecules from the extracellular matrix direct the migration, proliferation, and differentiation of the epithelium bordering ulcerative areas. The epithelium extends beneath surface debris, fibrin, and cells to restore the mucosa's continuity ***(Sonis,2004; Blijlevens et al.,2008).***

1.3 The Role of Cytokines in Tissue Inflammation.

They are pleiotropic endogenous inflammatory and immunomodulating mediators that exhibit both negative and positive regulatory effects on various target cells. These cell-derived polypeptides closely orchestrate both acute and chronic inflammatory processes by acting locally or

systemically on the site of tissue infection via autocrine and paracrine pathways. Briefly, inflammation at the site of infected tissue arises from the activation of various resident inflammatory cells such as fibroblasts, endothelial cells, tissue macrophages, and mast cells as well as the recruitment of monocytes, lymphocytes, and neutrophils . This aggregation of inflammatory cells at the site of inflammation is initiated by a number of soluble mediators such as cytokines, inflammatory lipid metabolites such as platelet activating factor (PAF), and derivatives of arachidonic acid such as prostaglandins . Such inflammatory effects can give rise to swelling due to fluid accumulation, increased blood flow and vascular permeability resulting in redness, and pain ***(Papadakis and Targan.,2000).*** As inflammation closely correlates with the production of cytokines, inflammatory events that occur during mucositis development have also been thought to be associated with the generation of cytokine signalling cascade ***(Dinarello.,2000).***

1.3.1 Cytokines AND mucositis

The roles of NF-kB and cytokines in the pathobiology of mucositis have increasingly been reported in the literature. However, the dynamics of inflammatory cytokines in oral mucositis and their particular influence on this process have not been entirely specified. Studies on mucositis using animal models have demonstrated that different types of drugs, such as methotrexate (MTX), irinotecan and 5-fluorouracil (5-FU), can lead to alimentary tract mucositis. However, these studies report differences in the timing of the histological changes as well as in the timing and intensity of the tissue expression of pro-inflammatory cytokines depending on the chemotherapy protocol ***(Logan et al.,2009).***

In humans, studies report an increase in pro-inflammatory cytokines in the blood and/or saliva of patients during cancer treatment ***(Morales.,2012).***

The roles of NF-kB and cytokines within the pathobiology of mucositis have more and more been pronounced in the literature. however, the dynamics of inflammatory cytokines in oral mucositis and their unique have an effect on in this system have no longer been totally particular. research on mucositis the usage of animal models have validated that specific types of tablets, together with methotrexate (MTX), irinotecan and five-fluorouracil (5-FU), can cause alimentary tract mucositis.

However, those research file variations within the timing of the histological changes in addition to inside the timing and intensity of the tissue expression of pro-inflammatory cytokines relying at the chemotherapy protocol.

In people, research record an increase in pro-inflammatorycytokines inside the blood and/or saliva of sufferers during most cancers treatment ***(Logan et al.,2008;;orales et al.,2012).***

1.3.2 Cellur mediators of mucositis.

The kinetics of mucositis development suggest that it is likely that injury results from a series of events in which cellular mediators play a role. Since pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), had long been associated with tissue injury, it seemed reasonable that they might have a role in mucositis. Increased levels of TNF-αand IL-6 were found in the peripheral blood of patients receiving chemotherapy who demonstrated nonhematologic toxicities compared with those who did not

manifest such toxicities ***(Hall et al.,1995).***The magnitude of the difference between the two groups was dramatic. Other studies confirmed this observation. Similarly, increases in pro-inflammatory cytokine levels following conditioning regimens for hematopoietic stem cell transplant were also associated with a number of unfavorable outcomes (Remberger.,1995). Subsequent animal studies confirmed the clinical observations and demonstrated that increased cytokine levels were present, not only in peripheral blood, but, more importantly, within the submucosa, and that genes expressing TNF-α increased within the oral mucosa following radiation Substantiating a role for pro-inflammatory cytokines in the induction of mucositis was the finding that attenuation of TNF-αeffectively blocked the development of radiation-induced mucositis in animals ***(Sonis et al.,2000).***

1.3.3 Tumor necrosis factor

The beneficial roles played by members of the TNF family include inflammatory and protective immune responses as well as being important factors in organogenesis of secondary lymphoid organs and lymphoid structure maintenance. TNF has also been shown to have a host damaging role in the context of sepsis and autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease (IBD). TNF is predominantly produced by activated macrophages, NK cells and T lymphocytes. The two receptors for TNF are expressed either on all cell types (TNF-R1) or only on immune or endothelial cells (TNF-R2) . TNF through the interaction with TNF-R1 causes various cellular events including activation of the caspase cascade which leads to apoptosis. TNF interaction with TNF-R1 also leads to activation of NF-κB. TNF-R2 signaling is less well characterised, however it is known that this receptor does not possess a

death domain and can therefore not directly precipitate apoptosis. The role of NF-κB activation leading to apoptosis via TNF-R2 signalling is unclear . In addition to causing the "classical" caspase-dependent form of apoptosis or PCD, TNF has also been demonstrated to induce necrosis-like caspase-independent PCD Clinically it has been shown that increased serum levels of TNF occur in patients who have undergone bone marrow transplantation and that this event precedes the development of major transplant related complications .Other researchers have demonstrated elevated TNF levels occurring in association with non-haematological toxicities Inhibition of TNF using agents such as pentoxifylline reduced these non-haematological toxicities . With respect to mucositis, various animal and human studies have shown a decrease in the occurrence or severity of mucositis following administration of TNF inhibitors . Interestingly, Orlicek *et al* demonstrated that isolates from viridans streptococci were able to induce TNF production by murine macrophages . These organisms are normal commensal flora in the mouth and respiratory tract, the induction o TNF by these bacteria therefore may be important in the context of mucositis development. This is particularly so in the ulcerative phase of the tissue damage process resulting in further amplification of pro-inflammatory cytokine production and subsequent further tissue damage. It has been demonstrated, using a hamster model of 5-FU induced mucositis, that administration of pentoxifylline and thalidomide, both of which inhibit cytokine synthesis, had a protective effect . These authors concluded that this indicated an important role for TNF in the pathobiology of 5-FU induced oral mucositis. ***(Orlicek et al.,2010)***.

1.3.4 Interleukin-1β

IL-1β is a multifunctional cytokine that has an effect on a wide variety of cell types and also interacts with many other cytokines. IL-1β is part of a family of cytokines which also include IL-1α and IL-1 receptor antagonist (IL-1Ra). The latter molecule binds to each of the two IL-1 receptors. IL-1β has multiple biologic effects which have been demonstrated in *in vitro* and *in vivo* including systemic reactions such as fever and increased gene expression of a range of genes including pro-inflammatory cytokines and pro-inflammatory mediators. IL-1β production can be stimulated by both microbiological and non-microbiological factors. The latter includes, among many things, other cytokines and irradiation .Along with TNF, IL-1β is an important cytokine that is involved in the activation of the NF-κB pathway. In fact IL-1β and TNF have been reported to have a synergistic effect, for example causing induction of endothelial adhesion molecules essential for the initial phases of the inflammatory response. Local tissue levels of IL-1β and TNF have been demonstrated to markedly increase in animal models of radiation-induced oral mucositis concurrently with the development of mucositis . IL-1β may also have a role to play in the healing phase of mucositis development . There is, however, a paucity of data in the literature about the exact role that IL-1β plays in the context of mucositis pathobiology ***(Ninami et al., 2011).***

1.3.5 Interleukin-10

IL-10 controls inflammatory processes by suppressing the expression of proinflammatory cytokines, chemokines, adhesion molecules, as well as antigen-presenting and costimulatory molecules in monocytes/macrophages, neutrophils, and T cells. Early *in vitro* studies demonstrated IL-10 suppresses monocytes/macrophage-derived

proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL- 8, and IL-12 . Additional studies support the notion that IL-10 attenuates TNF-receptor expression and further promotes its shedding into systemic circulation. Together these findings indicated IL-10 is an important immunoregulatory factor that significantly contributes to decreasing the intensity of inflammatory response by downregulating proinflammatory cytokine production at the site of tissue damage. In an attempt to report the effect of IL-10 on NFκB, *in vitro* analysis by Clarke and Colleagues (1998) showed that IL-10 is capable of inhibiting the activation of LPS-induced NFκB in macrophages and pre-B cells . This study supports the evidence that IL-10 mediates anti-inflammatory effects by inhibiting the up-stream NFκB transcription factor, an essential secondary messenger required for inducing proinflammatory cytokine gene expression ***(AL-Azri,2012).***

1.4 Animal Models of Toxicities Caused by Anti-Neoplastic Therapy

Models of Oral Mucositis Induced by cytotoxic Drugs and Radiation.

Oral mucositis is one of the best studied acute toxicities of non-surgical cancer therapy. Since it affects about 40% of all patients being treated for non-cutaneous cancers, the need for a successful intervention remains a high priority ***(Oliva et al.,2013)***.

At Present only a single agent, palifermin, has been approved for this indication in the US and palifermin's applicability is limited to the small cohort of patients receiving stomatotoxic conditioning regimens in preparation for stem cell transplants to treat hematological malignancies (4% of patients at risk for the condition. Clinically, mucositis occurs with

great frequency among patients being treated with radiation therapy, with or without concomitant chemotherapy, for cancers of the head and neck. Virtually 100% of patients with cancers of the mouth or oropharynx will develop mucositis. The incidence is slightly less among individuals being treated for hypopharyngeal or laryngeal tumors. Many of the conditioning regimens for stem cell transplant are stomatotoxic, especially those in which total body irradiation is a component. Lastly, mucositis impacts patients being treated with cycled therapy for the most common solid tumors (breast, colon, rectum, lung). In this group, the overall risk of mucositis in the first cycle of treatment is relatively low (about 15–20%), but if no effort is made to reduce chemotherapy dosing for subsequent cycles, the risk of mucositis increases dramatically, in many cases to more than 60%. The impact of mucositis is profound. Patients suffer marked pain, often requiring opioids, have to modify their diets, lose weight, have increased risk of local and systemic infection, require fluid support, and use consultation and emergency services more than patients who do not develop the condition ***(kapple et al.,2011).***

Clinically mucositis develops in predictable stages. Initially, the mucosa is thinned and hyperemic. Although the tissue is intact, patients note some discomfort, often described as being analogous to a bad food burn. Symptoms can be reasonably controlled at this stage with a combination of topical analgesics and systemic agents such as acetaminophen of NSAIDs. The development of ulceration occurs next. This is the phase that is most symptomatic. Pain increases dramatically, often requiring morphine or fentynal. Eating a normal diet becomes impossible. Patients are limited to very soft or liquid diets and some may not be able to eat anything. Consequently, it is not unusual for nutrition to have to be provided by feeding tubes (gastrostomy tubes) or total parenteral tuition. In the majority of cases ulceration spontaneously resolves ***(Estawer et al.,2015).***

1.4.1Objectives of Animal Models of Mucositis

There are four objectives for an effective animal model of mucositis to provide clinicalmeaningfulness:

1. The manifestations of mucositis should mimic the condition as it occurs in humans in its course, appearance, resolution, and dose response to stomatotoxic therapy. Its presentation should be robust enough as to not require microscopic or surrogate endpoints.
2. The pathogenesis of mucositis in the model should replicate, at the molecular, cellular, and tissue levels, the events that occur in humans.
3. Concurrent toxicities, especially those in which myelosuppression is an element, should occur in a measurable way.
4. The oral environment, especially the microscopic flora, should resemble that of humans and should respond to stomatotoxic therapy in a way that is the same as humans ***(Lalla et al.,2014).***

1.4.2 Current Models

Three species have been and/or are used for studies of oral mucositis: mice, rats, and hamsters.In general, the endpoints used to assess mucositis have relied heavily on histological outcomes since clinical changes tend to be subtle and focus on erythema, rather than ulceration as a primary endpoint. Rats have also been used to assess radiation and chemotherapy-induced mucositis, and both 5-FU and methatrexate have been used to induce mucosal injury, often accompanied by superficial irritation ***(Enmia,2009).***

Lesions in these models tend to be localized. A number of studies focusing on the epithelial biology of oral radiation have been performed

using murine lip, snout, or tongue models. Xu et al. described the effects of single and fractionated radiation schedules on the lip mucosa of mice. They found that acute reactions of the lip mucosa, i.e. focal desquamation, could be reliably scored . Alternatively, ***Kilic et al. 2010*** have used a model in which the ventral surface of the tongues of mice are radiated by guiding the tongues of anesthetized animals through a 3-mm hole in an aluminum block. The dorsal tongue was then fixed with tape and an aluminum plate with a 3 × 3 mm2 window was placed over the target area on the ventral tongue. Importantly, strain-dependent variability in murine vulnerability to radiation injury has been reported. C3H/Neu mice have been used successfully. These models have been useful to define responses to various radiation regimens, including cell repopulation studies, yet the limited anatomic area available for evaluation, challenges associated with the use of topical formulations, and the subtlety of clinical changes have limited their applicability in interventional studies. While the clinical signal noted in murine models may be subtle, the ready availability of syngeneic animals, knock-outs, immune reagents, and gene chips makes the mouse a good choice for answering specific questions associated with the pathogenesis of mucosal injury. Rats have been the species of choice for studies of gastrointestinal mucositis, especially those induced by chemotherapy. Until recently, histological endpoints were mandated. However, we have recently applied endoscopy to assess mucosal injury of the lower GI tract. The rat has also been effective in studying radiation-induced proctitis. ***(Boschi et al.,2012).***

1.4.3 Difficulties of animal models in mucositis research.

While animal models undoubtedly have benefits, they also have difficulties and limitations. The Sonis hamster model has the confounding

issue of wound healing. Hamsters have cheek pouches, and mucositis can be induced by either chemotherapy ***(Sonis.,et al 1990;1997;2000)*** or radiotherapy ***(Sonis et al.,2000).***

However, following administration of the chemotherapy, the cheek pouch needs to be "mechanically" scratched or irritated in order to induce ulcerated lesions. In humans, however, the oral mucosa does not need to be superficially irritated in order to induce mucositis, so this model is not exactly the same as the clinical setting. Additionally, superficial irritation may result in wound-healing mechanisms being initiated. Dose and scheduling issues are also important and cannot be overlooked. The doses used in rats do not automatically translate to humans: There may be species differences in susceptibility to different agents, and the traditional milligram per kilogram dosing of rodents is not often used in humans, where we tend to use (for reasons that are not always logical) body surface area dosing. Despite similarities, animal models are never identical to humans, and there will always be issues with translation from animal to human research. This does not, however, devalue animal research; it just adds an appropriate note of caution. An added difficulty with animal models has been introduced with the development of monoclonal antibodies for treatment of human disease. Fully humanized monoclonal antibodies may not be active in animal models, and toxicities may not develop until translation occurs to the human situation. Difficulties also arise in the DA rat model of mucositis.Unlike the hamster, in the rat visible oral mucositis does not occur due to the highly keratinized nature of the epithelium (D. Wilson and D. Keefe, personal communication) which makes it difficult to successfully investigate oral mucositis.

Furthermore, higher doses of chemotherapy are required to induce mucosal injury in animal models, due to the resilience of the rat AT. Another difference is the presence of squamous epithelium in the rat

stomach, which can lead to reduction in oral intake when KGF, a stimulator of epithelial growth, is used. Rats do not have an emetogenic reflex, and since some vomiting is a manifestation of mucosal injury, this is a disadvantage. However, it is possible to use pica as an indirect marker for nausea ***(Vera G et al.,2006).***

The route of chemotherapy administration has important implications for drug metabolism. In the DA rat model of mucositis, intravenous administration of chemotherapeutic drugs is extremely difficult, with administration into the tail vein being made especially difficult due to the skin pigmentation. As a result, mucositis induced by drugs administered via this route is not routinely investigated. Although all chemotherapeutic drugs cause damage***(Ijiri k&Potten,1983;1987),***the mechanisms by which they do this may be different. Other contributing factors also cause difficulties in animal research; including: stresses in the animals from isolation due to experimental procedures, the need to anesthetize animals on a regular basis and the effect that this has on mucosal homeostasis, and the efficacy of any investigative drugs on tumor load. Toxicities associated with cancer treatment include those that are localized or regional (ulcers, xerostomia, abdominal pain, malabsorption) and those that are more generalized systemic (fatigue, lack of appetite, nausea, cognitive impairment) ***(Sonis et al.,2007)***.

The recent realization of concurrent tissue-based and systemic toxicities has resulted in the new paradigm of toxicity clustering (Nadler et al.,1980). Interestingly, the proof-ofprinciple testing for this new way of thinking was carried out in cancer patients ***(Nadler LM et al.,1980).***

Translational research in the laboratory using animal testing is now occurring to examine in greater detail some of the initial findings. Looking at multiple toxicities in combination will add new knowledge in the area as well as uncover new challenges in applying the models. The final issue

in animal models is strain and sex differences in metabolic enzyme profiles for xenobiotics, particularly CYP family members ***(Bert B et al.,2001;Staack et al.,2004;Kawas et al.,2008;Martignoni M at el 2006)*** .which can have a profound impact on drug clearance, and therefore toxicity, of agents at equivalent doses. Careful consideration of the animal model and the drugs to be administered are paramount for a successful animal trial.

1.5 comparison and assessment of scoring scales for mucositis

For these reasons, we must assess the mucositis severity:

1-To determine the stomatotoxicity of a particular cancer-treatment regimen

2-To help in the management of the patient

3-As a research tool to evaluate the efficacy of a potential mucositis intervention ***(Sonis,2012).***

1.5.1 Harmfulness picture and assessmet.

Scoring scales to describe toxicity are among the most common and include those that use National Cancer Institute Common Toxicity Criteria (NCI-CTC), Radiation Therapy Oncology Group (RTOG), and World Health Organization (WHO) criteria to assess mucositis severity ***(Sonis et al.,2004).*** These scores are then used to describe the overall toxicity of a particular chemotherapy regimen or radiation schedule. To a large degree, these scales are focused on clinician examination of the oral mucosa and

the assignment of a score based on observed clinical changes such as erythema and ulceration. They may also have a component that is based on patient function or use of analgesics ***(Sonis et ,2012).***.

1.5.2 Patient management scales.

Patient management scales tend to be based on a holistic and composite evaluation of the patient's oral health, of which only one element is mucosal damage. They have been primarily developed by nurses for the daily care of their patients ***(sonis,2011)***.

These instruments often include assessments of patient speech, salivary function and quality, gingival health, swallowing, lips, and oral hygiene. While of great value in formulating treatment plans that focus on overall oral cavity health, the evaluation of the oral mucosa is not the primary target of these scales. Examples are the Oral Assessment Guide (OAG), the Western Consortium for Cancer Nursing Research (WCCNR) , the MacDibbs scales and the Nijmegen Nursing Mucositis Scoring System (NNMSS) ***(sonis,2011)***.

1.5.3Research directed scales

Over the years, scales have been developed to be used primarily in mucositis research studies . These tend to provide highly quantitative outputs that are based on a series of strictly defined parameters. The two most commonly cited scales of this type are the Oral Mucositis Index (OMI) and the Oral Mucositis Assessment Scale (OMAS) **(Sonis,2010).** The endpoints for both scales are dependent of clinician assessment. While

the OMAS tends to be very focused on mucosal changes, the OMI has broader criteria.

1.5.4 Mucositis research instruments

-No uniformity in end points

-Wide range of complexity

-Include several variables which are irrelevant to mucositis, so may over-report

-Major value in phase 2 trials and outcome analyses, but of limited value in phase 3 trials

Mucositis research scales were developed in an attempt to provide quantitative, highly objective endpoints for mucositis assessment. They vary widely in complexity from a 34-item Oral Mucositis Index , to a 16-item scale to an Oral Mucositis Assessment Scale that evaluates ulceration and erythema ***(Robitta,.2011).*** These scales all share quantitative outcomes to which statistical analyses can be easily applied. However, the interpretation of data by clinicians is often difficult. Consequently, they are best used in focused applications ***(Sonis,2010).***

Table 1.2 Oral Mucositis Assessment Scale (Sonis,1999).

Oral Mucositis Assessment Scale	
Ulceration	**Erythema**
0=no lesion	0=none
1=<1 cm^2	1=not severe
2=1–3 cm^2	2=severe
3=>3 cm^2	

1.5.5 Mucositis grading staging selected systems .

World Health Organization:

Grade 0 No signs or symptoms

Grade 1 Mild soreness or painless ulcers with oedema or erythema

Grade 2 Pain, erythema, ulcers, ability to eat solids

Grade 3 Pain, erythema, ulcers, requires soft or liquid diet

Grade 4 Alimentation not possible

National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3: Mucositis (clinical exam):

Grade 1 Erythema of the mucosa

Grade 2 Patchy ulcerations or pseudomembranes

Grade 3 Confluent ulcerations or pseudomenbranes; bleeding with minor trauma

Grade 4 Tissue necrosis; significant spontaneous bleeding; life-threatening consequences

Grade 5 Death

National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3: Mucositis (functional/ symptomatic):

Grade 1 Minimal symptoms, normal diet

Grade 2 Symptomatic but can eat and swallow modified diet

Grade 3 Symptomatic and unable to adequately aliment or hydrate orally

Grade 4 Symptoms associated with life-threatening consequences

Grade 5 Death

1.5.6 The ideal mucositis scale

It would be very desirable to have a single scale to describe mucositis severity. At the instant, there are well over a dozen different scoring instruments that are used, and having no consistent and universally used scale is a detriment when comparing regimen toxicities or evaluating new agents. Consequently, although a single scoring system would be ideal ***(Sonis,2012).***

1.5.7 Variability between mucositis scales

Interestingly, the severity of mucositis is not evenly reflected across scales. What may be graded as severe in one scale may be slight or moderate in another. Some real examples will illustrate this point. Two studies were conducted to describe the effect of a new anti-mucositis drug on the course of severe mucositis following the administration of a particular conditioning regimen prior to HSCT. Each study used a different scale to measure mucositis severity. In the first study, the duration of severe mucositis was the same among patients who received the test drug and those who received placebo ***(Dazzi et al.,2003).*** In the second study, not only was the duration of mucositis in placebo patients almost four times that observed in the first study (16.6 days vs. 4.4 days), but the duration in patients being treated with the interventional drug was 11.9 days vs. 4.8 days in the first study ***(Bez et al.,1999).*** Importantly, the *only* difference

between the two studies was the scale used to measure mucositis. In another study, mucositis was measured using two scales in the same patient population . Mucositis was scored using WHO or RTOG criteria. WHO grading is dependent on both objective (ulceration yes/no) and subjective (patients 'ability to eat solids, liquids or nothing) variables. In contrast, RTOG grading is completely reliant on a clinician's ability to judge the size and characteristics of ulceration. In the example below, it is clear that incorporating patients' input into establishing the extent of ulceration markedly impacts scoring. Whereas 93% of patients graded by RTOG criteria were assigned a score of 2 (moderate mucositis), this characterization applied to only 51% of patients when WHO grading was used. Likewise, whereas 49% of subjects had severe mucositis by WHO criteria, the incidence was much smaller (7%) when Rtog criteria were used ***(WHO,2011).***

1.5.8 Minimizing inter-observer variability

A major challenge with any scale that depends on clinical judgment for its determination is the minimization of inter-observer variability. For the assessment of mucositis, a number of factors impact the accuracy of grading and differences in scoring of the same patient by different evaluators. These include the following:

- **Training.** In many instances, clinicians receive little or no formal training on how an oral examination is performed. Consequently the rigor of the evaluation may vary from one person who evaluates all mucosal sites in a systematic way to another who only looks at the dorsal surface of the tongue and palate. Aggressive training in the technique and scoring criteria will help minimize variability.
- **Lighting.** It is difficult to assess the condition of the mucosa if one cannot see easily. Good lighting is essential to assuring an accurate

examination. Since two hands are necessary to adequately perform an oral evaluation, a headlight is desirable. Those available for campers work well and are relatively inexpensive .

- **Clarity of outcome criteria.** The examiner(s) should be absolutely clear as to the criteria by which scoring is done.
- **Standardization of clinical assessment technique** .

All examiners should perform the assessment in the same sequence.

1.6 Laser:

"LASER" is an acronym of Light Amplification by the Stimulated Emission of Radiation. All matter can emit radiation under certain circumstances but only a small proportion of radiation is within the visible area of the electromagnetic spectrum (Jelínková, 2013).

1.6.1 Historical view:

Albert Einstein was the true father of lasers since 1916, who theorized on stimulated emission of radiation as part of his quantum theory. His theories evolved into practice with the development of optical MASER (microwave amplification by the stimulated emission of radiation) by Schawlow and Townes 1953 until 1960 that Theodor Maiman built the first working laser. This used a flash of light to stimulate a ruby crystals, over the next few years other types of lasers have been used such as He-Ne and Nd:YAG lasers in 1961, the Argon laser in 1962 and CO2 laser in 1964,that had been employed as dermatological systems virtually from the time of their introduction (Carruth, 1997; Convissar, 2011; Jelínková, 2013).

1.6.2 Electromagnetic Radiation:

Electromagnetic radiation (EMR) spectrum (figure 1.4) extends from the short wave lengths of X-ray and gamma rays to the long wavelengths of microwaves and radiowaves. The majority of lasers fall in or close to

the visible wavelengths that is usually referred to as light which lie between 400 and 700 nm. This is the range, but it is convenient and intuitively appealing when discussing other parts of EM spectrum also to refer to them as light, even though they are invisible (Herd *et al.*, 1997; Jelínková, 2013).

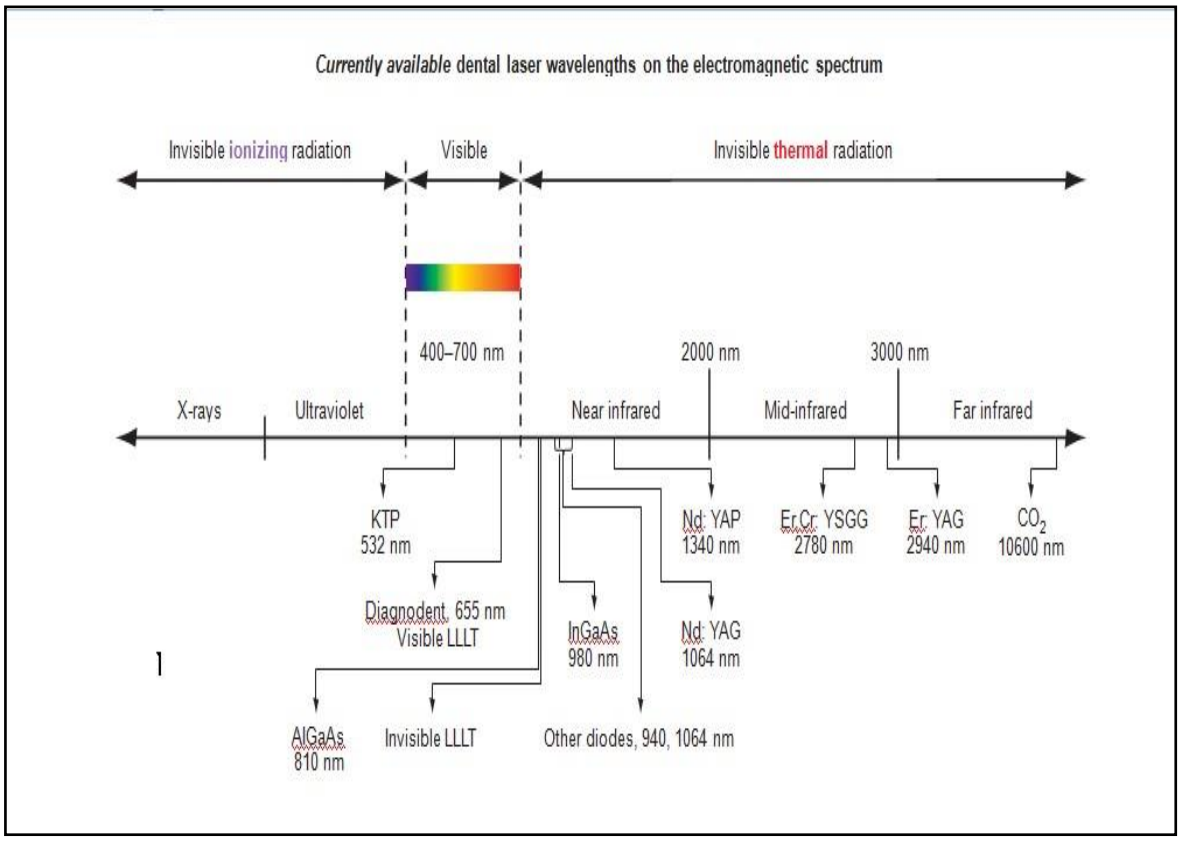

Figure 1.4: Parts of electromagnetic spectrum (Convissar, 2011).

1.6.3 Laser elements:

1.6.3.1The production of laser radiation:

The lasing medium is present within the laser tube that has a fully reflective mirror at the end and partially reflective mirror at other end to allow entrance to the laser beam light. The lasing medium is pumped and excited either by a high energy light source or electrically for creating a population inversion of atoms in a high energy state. The high laser level should have a long lifetime in comparison to the low level if a population inversion is to be achieved stimulated emission then takes place as atoms spontaneously emit photons that on collision with other excited atoms, stimulating these for emitting identical photons travelling in the same way direction as the original stimulating photons. These photons are released in exactly the same axis as that of the laser tube. Then the photons are reflected back into the lasing medium by the mirrors to collide with other excited atoms, which subsequently release their photons in the axis of the tube. Thus, there is cascade effect as a rapid build-up of laser light energy in the tube and the beam is emitted through the partially reflective mirror ***(Carruth, 1997).***

The active medium of laser may be in a solid, liquid, gaseous or semiconductor phase state. The pump source may be electrical discharge, a flashlamp, radio frequency emission, or another laser. The laser radiation may be emitted in a continuous wave (CW) or in pulses due to natural or imposed conditions. The power output of a laser depends on the amount of active medium present in the resonant cavity and the efficiency of matching of the pump source output to the medium, so that a high percentage of the pump source energy goes into exciting the active medium ***(Rosenshein, 1997).***

1.6.3.2 Characteristics of laser beam:

- **Monochromaticity:** all laser rays have same wave length and frequency when they are emitted from the same source.
- **Coherence:** laser light has wave length that spatially and temporally in phase.
- **Collimation:** laser light is nearly parallel and non divergent.
- **Brightness:** The resulted laser beam can be much brighter or more powerful than conventional light source as the coherence of a laser beam allows it to be focused to a very high intensity (Herd *et al.*, 1997; Convissar, 2011; Jelínková, 2013).

1.6.3.3 Parameters*: (Herd, 1997; Convissar, 2011; Jelínková, 2013).*

The most important radiometric terms in the medical laser application:

- **Energy (E):** the energy is work and is measured in Joules (J).
- **Power (P):** it is rate at which work is done and is measured in Watts or J/sec (Joules per second).

$$\text{Power (W)} = \frac{\text{Energy (J)}}{\text{Time (sec)}}$$

However low level laser therapy device outputs are so low, therefore it is expressed in milliwatts (mW).

- **Irradiance** (intensity or power density): laser emits light in a parallel beam, the ratio of the emitted power to the cross sectional area called power density. It is calculated as power per unit area.

$$\text{Irradiance (Power density) (W/cm}^2\text{)} = \frac{\text{power output (W)}}{\text{spot size (cm}^2\text{)}}$$

Spot size is known and can be used to calculate the area by multiplying π * by the impact radius (r) (in cm) taken to the second power, or πr^2.

- **Energy density (fluence):** the energy delivered per unit area, expressed in joules per square centimeter (J/cm^2). It is gained by multiplying the output power of the laser in milliwatts by exposure time in seconds equals the energy has been produce.

In pulse mode laser: Fluence = $\frac{\text{laser output (W)} \times \text{number of pulses} \times \text{exposure time per pulse}}{\text{Area of the treatment site } (cm^2)}$

* $\pi = 22/7$

- **Exposure time:** Time characteristic is a significant parameter of the generated output radiation, because it determines duration of tissue exposition or therapeutic dose, as well as the power of the radiation.

Dose: the most important parameter in low level laser therapy is always the dose.

By dose is meant the energy of light directed during a given session of therapy.

Dose of 1 J = 1 Watt of radiation during 1 second

Dose (J) = average power (Watt) × time of irradiation (sec)

The therapeutic dose is influenced by many factors: the depth of target tissue; type of tissue either mucosa, bone or muscle; another complicating factor is the amount of chromophore in the target tissue, such as melanin. In addition to that hemoglobin in blood in which highly vascular tissue would absorb these certain wavelengths well, and less vascular tissue would absorb these wavelengths poorly ***(Nussbaum et al., 2002; Convissar, 2011).*** That laser light dosimetry is an important part of the cell photostimulation ***(Wilson and Mia, 1993; Frigo et al., 2010).***

1.6.4 Laser safety:

According to safety precautions, lasers are divided into four categories (Smally, 2013):

Class 1: safe under conceivable condition of use in which is viewing without optical aids, but potentially hazardous when using magnification aids (microscopes, loupes, binoculars).

Class 2: Visible wavelengths (400–700 nm). It is safe if viewed for less than 0.25 seconds. Subclass in which visible wavelengths not safe even with optical viewing aids.

Class 3R: Unsafe for viewing of intrabeam of beams with diameters >7 mm.

Class 3B: Unsafe for viewing of intrabeam, causing eye and skin injury from direct, but not diffuse, energy.

Class 4: High power lead to injury of skin and eye from direct and reflected radiation..

1.6.5 Types of laser:

• Laser can be classified according to its state of active medium (Harris and Pick, 1995; Convissar, 2011, Jelínková, 2013).

1. Solid state lasers: for example, Ruby laser, Ho:YAG, Nd: YAG, Er: YAG and alexandrite.
2. Gas lasers: for example, CO2, Helium-Neon, Argon, and Excimer.
3. Liquid laser: for example organic dye laser.
4. Semiconductor lasers: for example, Gallium-Aluminum-Arsenide (GaAlAs) diode laser. Indium-Gallium-Aluminum-Phosphide (InGaALP).

• According to the emission mode. Lasers can be divided into three categories ***(Convissar, 2011; Jelínková, 2013):***

1. Continuous wave (CW): in that mode, lasers work unremittingly and deliver a constant power level.

2. Pulsed mode: periodic alterations of the laser energy in which the laser is emitted in short, high power pulses at a variable pulse repetition rate. Between pulses, no laser energy is emitted. Peak powers are much higher than average powers of CW laser.

3. Free running pulsed mode (true –pulsed mode): large peak energies of laser light are emitted for usually microseconds, followed by a relatively long time in which the laser is off.

1.6.5.1 Semiconductor diode laser:

In diode lasers, the active medium is a semiconductor, that is a material halfway between an insulator and an electric conductor such as gallium and arsenide, and some devices add either indium or arsenide. There were two semiconductors, one of that has a surplus of positive charges (p) and the other with a surplus of electrons that is of negative charges (n). If these materials bring into contact, a junction region is obtained which is in electrical equilibrium that is neutral. Here electrons are pumped that is a short though intense (pulsed) current is applied by bringing a positive electrode into contact with semiconductor (p) and negative electrode into contact with semiconductor (n). Then there is an inversion from the excited state to the ground state with emission of photons. This is a laser radiation born of the electron surplus in (n) which neutralizes the positive charge surplus in (p) thus releasing one photon at every transition ***(Harris and Pick, 1995; Jelínková, 2013).***

Therefore the clinical application of the diode laser in oral and maxillofacial surgical procedures seems to be of beneficial effect for daily practice ***(Romanos and Nentwig, 1999; Convissar, 2011).***

1.6.6 Laser-tissue interaction:

For any laser to have an effect on living tissue, it must be first absorbed. If the energy is reflected from the surface of a tissue, or if it is completely transmitted through a tissue, no biological effect will result. However, when the energy is scattered within a tissue, the effect will be relatively non selective and imprecise (figure 1.5) ***(Bailin et al. 1990).***

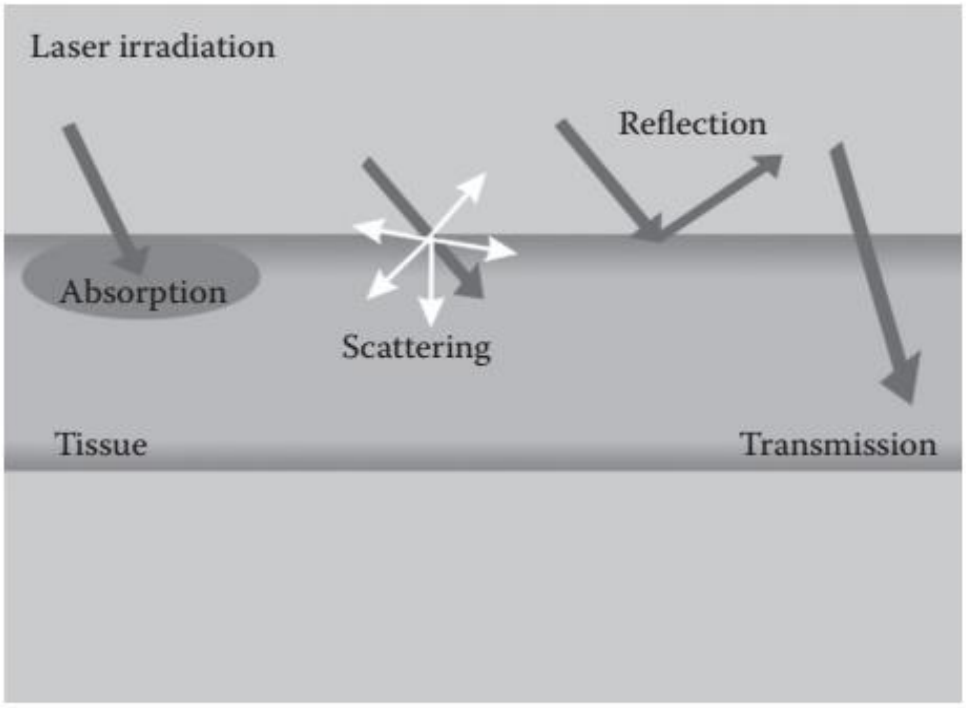

Figure 1.5: Laser-tissue interactions (Convissar, 2011).

The laser tissue interactions are photophysical processes that can be classified in increasing strength of biologic effects, depending on the irradiance, radiant exposure, pulse duration, and wavelength of the laser radiation ***(Rosenshein, 1997).***

1.6.6.1 Biological effect of laser light:

The biological effect of laser light on tissue are ***(Convissar, 2011; Jelinková, 2013):***

Photothermal interactions: this interaction is caused by the change of photon energy (absorbed by tissue fluids) into heat energy that arises as a result of molecular vibration and collisions between molecules. This can lead to photothermal effects on the tissue, such as coagulation, vaporization (thermal ablation) and carbonization or melting.

- **Photoablation:** the photoablation effect is based on the delivery of sufficient energy into the tissue to ablate it in a short time before any heat is transferred to the surrounding tissue. It is caused by molecules with an electron transition from low energy orbital to higher (non- bounded) orbitals absorbing high- energy photons.

- **Laser induced plasma ablation:** plasma induced ablation refers to well defined removal of tissue, without thermal or mechanical damage. If the peak power density of the laser radiation is high enough, localized micro plasma is formed. In the focal volume, free electrons are generated by thermal or multi- photon ionization. These electrons absorb the incoming photons and consequently accelerate. If their kinetic energy is high enough, they ionize colliding molecules and generate new free electrons, repeating the process and starting an avalanche effect leading to the generation of free electrons and ions.

- **Photodisruption:** the high irradiance (may be of the order of megawatts or gigawatts per square centimeter); however it is only to generate such power levels possible within an extremely small volume of

tissue. This is effective on microsurgical techniques like opthalmological surgery.

- **Photochemical interaction:** photochemical reactions are metabolic processes that are activated by low intensity light. Photochemical interactions play a very important role in **biostimulation** processes, therapy using low intensity laser radiation effects directly or indirectly from the electromagnetic interaction of the light with tissue and not from thermal effects.

A second application of photochemical interaction is **photodynamic therapy**, when photosensitizer materials have been used and radiant energy possessing less power and shorter wavelengths.

1.6.6.2 Laser biostimulation:

"Laser biostimulation" is of a photobiological nature, and low-power laser effects can be related to well-known photobiological phenomena (Karu, 1987).

All living cells need energy for growth and metabolism, which supplied by ATP (Adenosine Triphosphate) bond hydrolysis, that is the common energy transfer in living cells (Hebert *et al.*, 1989; Amat *et al.* 2004). The chemoiosmotic theory was that concentration gradients between cell membrane and phosphodiester bonds in ATP were inter-convertible forms of storing energy ***(Lubart et al., 1991; Karu, 2004).***

Respiratory chain components are primary photoacceptors (Karu, 1987).figure (1.6) Photoacceptor pigments in the respiratory chain of cells and porphyrins (flavin and cytochrome) that located in mitochondria they are converted laser energy to electrochemical energy ***(Lubart et al., 1991; Karu, 2004; 2008).***

There is resemblance between photosynthesis in plant chlorophyll and mitochondrial oxidation utilizing cytochromes. Both chlorophyll and mitochondrial cytochromes have conjugated porphyrin ring that is an efficient light absorber. In the living cell, the destructive agent is singlet oxygen which is highly reactive, rapidly oxidizes a great variety of biological molecules, damages DNA and cell destruction. Porphyrins are excellent photosensetizers for single Oxygen. As single Oxygen is photo-produced by porphyrins, the effectiveness of which depends on the frequency of the radiation energy and side chains. In corporations of metal ion into the porphyrin molecule depress or even prevents formation of singlet oxygen ***(Lubart et al., 1991; Friedmann et al. 1991; Ridha et al., 2012).***

Ridha et al., 2012 showed that He-Ne low power laser can improve cell survival for cells damaged when given1hr prior to UV irradiation.

Nevertheless, reaction with various composition of light produces a photobiological response in the terminal oxidases of mitochondrial respiratory chain, which has a complex structure and a complicated absorption spectrum at 400, 450, 605, 760, and 830 nm. In the red spectrum region, flavoproteins and their semiquinone forms have absorption bands, where in the case of the respiratory chain, are represented by dehydrogenases ***(Brunori and Wilson, 1982).***

However, laser-tissue interactions can be further characterized by laser's relative absorption (coefficients of absorption) and a relative distance traversed in tissue before absorption is complete (coefficient of extinction). The relative effect of any laser-tissue impact will depend on a multitude of variable properties, perhaps the most important of which is selective versus non selective absorption. Tissue targets (chromophores) maximally absorb certain characteristic wavelengths of light, mostly depend on color ***(Rosenshein, 1997, Mendez et al., 2004).***

Cellular homeostatis of the mitochondria is affected by laser irradiation, primting a cascade of proceedings in the respiratory chain of, cytochroms oxidase; the terminal enzyme of the respiratory chain; cytochromes and flavin dehydrogenase that permit absorption of light. The reduction-oxidation status of mitochondria and cytoplasm are impacted leading to enhance production of ATP when cellular membranes are exposed to the radiation, the flow of the membrane ion carriers potassium and sodium are changed, affecting the transition of calcium between cytoplasm and mitochondria ***(Karu, 1988; 2004).*** This mechanism initiates a cascade of cell signaling, causing an optimization of body functions ***(Karu, 2004; 2008).***

Cell proliferation, secretion and motility are altered when irradiated with laser that has specific wavelength, dose and intensity ***(Basford, 1993; Reddy, 2004).***

Evident morphological changes in mitochondria of lymphocyte were observed after irradiation of those cells with He-Ne laser ***(Karu, 1992).*** Many researches demonstrated the action of visible light on animal cell and tissue, radiation of isolated liver mitochondria with a He-Ne laser bring about enhanced ATP-ADP metabolism an elevated content of ATP, a growth of electric potential across inner membranes and pH in matrix, in addition to a small changes in the matrix configuration ***(Passarella, 1988).***

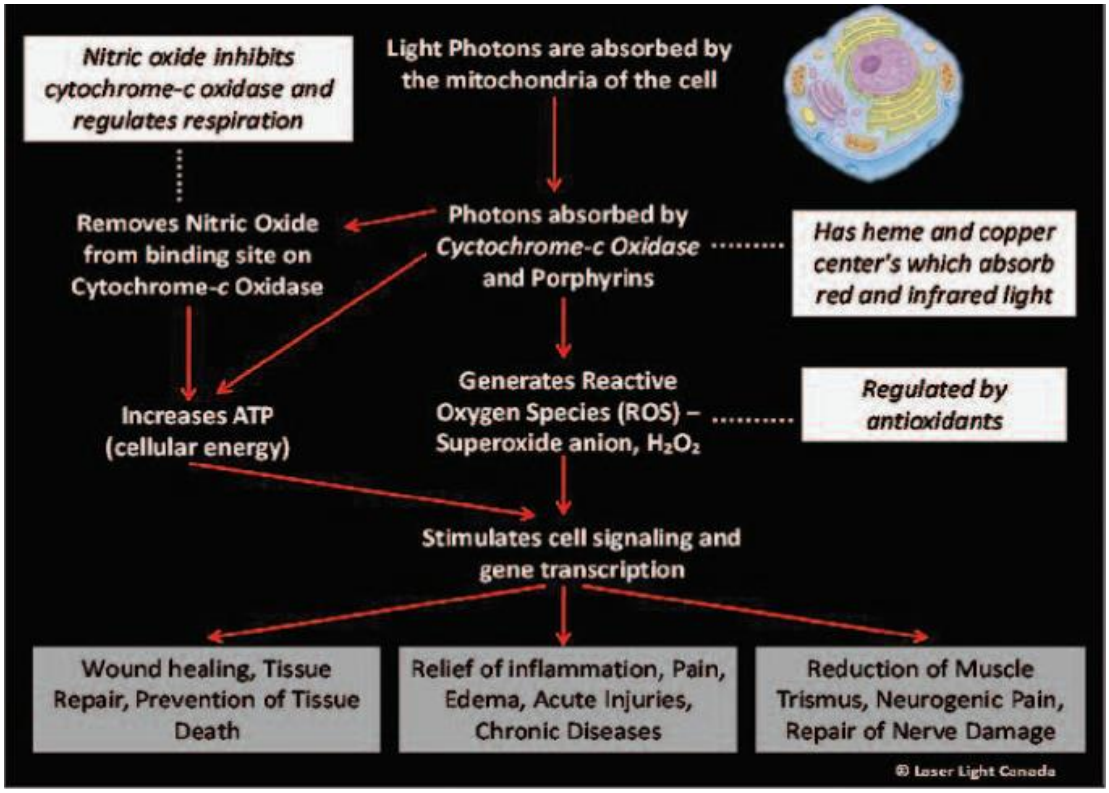

Figure 1.6 Illustration diagram of the primary mechanisms of photobiomodulation (Ross&Ross,2009).

1.6.7 Low level laser therapy:

Lasers can be labeled into two types according to the energy level; (high level lasers) (hard laser) i.e. surgical lasers and another type of lasers called low level lasers (soft laser).

The soft lasers are mainly smaller, less expensive and operate in the range of milliWatt, 1-500. Other names have been given to these lasers, for examples, soft laser whereas the therapy has been called biomodulation that is more appropriate term, since the therapy can stimulate as well as suppress biological processes. Therapeutic lasers mainly operate in the visible and the infrared spectrum, between 600-900 nm wavelengths ***(Convissar, 2011, Mester, 2013).***

Endre Mester; a professor of surgery in Budapest in 1966 pioneered the use of low intensity visible and near infrared laser radiation for therapy. After that many researches shows LLLT works but, out of the thousands

of studies that exist using LLLT, few represent good evidence-based research ***(Myers, 2000; Tumilty, 2010; Mester, 2013).***

As they produce less than 500 mW of energy, low level laser devices was classified as class III lasers with no significant risk by Food and Drug Association (FDA) ***(Convissar, 2011).***

1.6.7.1 Applications of low level laser therapy:

Low level laser radiant energy can produce a positive effect on the biological and biochemical processes of wound repair. AL-Safi (1991) used diode laser and investigated histologically the healing process of wound with laser irradiation, this study has shown enhance the healing process in both single and multiple doses of laser irradiation, however, on fibroblast proliferation, there was no statistical differences between single and multiple irradiation. Mahmud and AL-***Talabani, 1993*** studied the gingival wound healing after low level laser irradiation; a rapid and active healing process of the wound has been found.

In other hand, many other researchers showed negative result in LLLT, laser irradiation after certain periodontal surgery showed no significant differences in the gingival index, healing index and pain reduction ***(Masse et al., 1993).*** Also there were no influence of the inflammatory reaction of the gingiva in gingival inflammation cases ***(Ryden et al., 1994).***

The effect of LLLT on the postoperative pain and swelling after extraction of third lower molar, there were many researches that have controversial in their results, where conclude that low energy Ga-As laser reduces the incidence of dry socket and reduces the severity of postoperative sign and symptoms of non responding sockets but not superior to using tetracycline. ***(AL-Hussaini ,1992; Giovanni et al. ,2003).*** On other hand, Roynesdal *et al.*, 1993 concluded that soft laser treatment

has no beneficial effect on swelling, trismus and pain after third molar surgery.

Ga-Al-P low level laser can be recommended in treatment of Herpes simplex for its evident analgesic effects, as well as for shorter disease duration ***(Zeki, 2010).***

Successful using of LLLT in tendon healing biochemically and biomechanically with no significant differences in compared with ultrasound therapy ***(Demir et al., 2004).*** Other LLLT uses are promoting effect on acceleration of bone healing ***(Ibrahim, 2003; AL-Wattar, 2004; Mustafa et al., 2011).***

‘‘Multinational Association of Supportive Care in Cancer/International Society of oral oncology’’ (MASCC/ISOO), in 2004, the guideline on cancer supportive care and management reported laser therapy as a “possible option” with a mention on the expensive nature of the commercially available devices requiring specialized training due to variations in laser products, procedures and doses. ***(Keefe et al., 2007; Bensadoun, 2012; Migliorati et al., 2013).*** In 2007, MASCC-ISOO ‘evidence-based’ mucositis guidelines have upgraded LLLT as “recommended” methods for the prevention of oral mucositis associated with bone-marrow transplantation or hematopoietic stem cell transplantation ***(Keefe et al., 2007).*** In the international view, World Association for Laser Therapy (WALT) authored existing guidelines in the therapeutic doses of laser for inflammatory cases and diseases but not specific to oral mucositis.

“American Cancer Society” mentioning the evidence behind LLLT as ‘promising’, but with conflicting evidence on large operator and cost variability ***(Bensadoun, 2012).***

1.6.7.2 Histological effect of low level laser therapy

The interaction of laser light with living tissues may lead to different effects depending upon several factors including cell type, laser parameters, and dose rate in which the last one is affected on proliferative response of particular cells ***(Convissar, 2011; Schartinger et al., 2012 AlGhamdi, 2012).***

Many authors studied the effect of LLLT on fibroblasts in *vivo* and in *vitro* ***(Lubart et al., 1991, Frigo et al., 2010; Kaskos et al., 2011; Schartinger et al., 2012).*** Loevschall and Arenholt (1994), study the effect of LLL irradiation on the proliferation of human buccal fibroblasts cultures, they claimed that LLL irradiation can induce increased DNA synthesis.

He-Ne low level laser irradiation also stimulate DNA synthesis of myofibroblasts without any degenerative changes in the organells i.e no modification in cytoplasmic structures ***(Tominaga, 1990).***

In wound healing many changes are seen after treatment with LLLT, include increased granulation tissue, early epithelialization, increased fibroblast proliferation and matrix formation, and enhanced neovascularization ***(Kuliev and Babaev, 1991; Bisht et al., 1994, Kaskos and Al-Hasan, 2011; Colombo, 2013).***

Laser therapy has biostimulatory effects on fibroblasts or keratinocytes cultures as assessed by cell proliferation, adhesion, or migration. Stimulation of epithelial cells. In *vitro*, LLLT increase the motility of human epidermal Keratinocytes ***(Prokhnchukov and Pavlov, 1987; Haas et al., 1990; Al-Wattar et al., 2013)***. This would explain the finding that wound sites treated with LLLT can accelerate healing ***(Becker, 1990).***

In both in *vivo* and in *vitro*, LLLT affects on macrophage function by promoting the secretion of factors, as observed in *vivo*, the enhancement of the phagocytic activity of macrophages in initial phases of the repair response (6 hours post trauma). This is thought to be facilitated

debridement of the wound, and there by establish conditions imported for the proliferative phase healing response to start ***(Petrova, 1992).***

Also LLLT affect on the osteoblastic cells and osteocytes ***(AL-Me'mar, 2002; Ibrahim, 2003; AL-Wattar, 2004).*** Cell proliferation and DNA synthesis were increased by LLLT only when osteoblastic cells were in a phase of active growth laser therapy causes enhanced accumulation of calcium and increased calcification rate in vitro ***(Yamada, 1991; Coombe et al., 2001).*** The observation of intracellular calcium concentration in osteoblastic cells revealed a tendency of a transient positive change after laser irradiation. LLL irradiation was unable to stimulate the osteosarcoma cells utilised for such research at a gross cell population level. Heat shock response and increase intracellular calcium refer that the cells do respond to LLL irradiation ***(Coombe et al., 2001).***

Morrone et al., 2000 estimate the chondrocytes cell viability and level of calcium and alkaline phosphate in vitro and obtained good results and confirmed that GaAlAs laser induces biostimulation without cell damage.
i

AL-Kaisy (2003), studied the effect of diode GaAlAs low energy laser irradiation on the Ultra structures of dental cell of developing rat tooth and assessed the effect of low energy laser irradiation to major elements (calcium and phosphorous) in which obvious changes were seen in cytoarchitecture of odontogenic and pulp cells associated with many alteration in cytoplasmic organelles. In addition to that, this study has been shown abnormal infiltration of inflammatory cells between the ameloblast cell layer and some of odontoblast cells were modified into phagocytic cells. In biochemical part of that study, there was greater increase in concentration of calcium and phosphorous in irradiated rats' teeth.

1.6.7.3 Analgesic effect of low level laser therapy:

Low level laser therapy has ability to exert analgesic effects. Historically, it was a major clinical application of the technique. Laser light has a potent effect on nerve cells which block pain transmitted by nerve cells to the brain. Investigations have shown that laser light enhances the activity of the ATPdependant Na-K pump, thus increases the potential difference through the cell membrane moving the resting potential further away from the firing threshold, lead to decreasing nerve ending sensitivity **(Baxter *et al.*, 1991, Masoumipoor *et al.*, 2013).**

Pain blocking mechanism involves the product of light levels of pain killing chemicals like endorphins and enkephalins from the brain and adrenal gland, as a result of stimulation by laser **(Baxter *et al.*, 1991).**

The neuropharmacological analgesic effects of lasers may be as a result from the releasing of serotonin, acetylcholine at the region and in higher centers **(Baxter *et al.*, 1991)**. Walker (1983) demonstrated increase levels of serotonin in chronic pain patients after treatment with low power He-Ne laser.

There was strong evidence about red and infrared wavelengths of LLLT that can act locally and rapidly during the first hours and days after acute injury to modulate the inflammatory processes in tissue. These anti-inflammatory effects and reduce pain include alteration in biochemical markers, change distribution of inflammatory cells, and reduced hemorrhage, formation of edema, and necrosis by reducing levels of biochemical markers (PGE2, Cox 2, IL-1, TNF-α, mRNA), oxidative stress, neutrophil cell influx, and formation of edema and hemorrhage in a dose dependent manner (median dose 7.5 J/cm2, range 0.3–19 J/cm2). **(Bjordal *et al.*, 2006; Mester, 2013).**

1.6.7.4 Immunological effect of low level laser therapy:

LLLT has immunomodulatory effect **(Aimbire *et al.*, 2006; Badeia *et al.*, 2013; Pezelj-Ribaric *et al.*, 2013; Oliveira *et al.*, 2013; Silva *et al.*, 2015).** Laser light doesn't exacerbate the inflammatory process but rather condenses the time frame from onset to resolution through an acceleration of the process **(Kaskos *et al.*, 2011; Papgeorgiou, 2000; Fenoll *et al.*, 2014).**

Low level laser radiation used to increasing the immune response by stimulating the lymph node action are showed by various changes in the structure of immune cells such as multiplication of the nucleus and cytoplasm cleavage **(Khaleel, 2010; Mester, 2013).**

Low level of laser can increase the phagocytic activity of polymorph neutrophils, and this increase proportional to increase the time of exposure (Khaleel *et al.*, 2010). Laser light 660nm, 820-nm, and 870-nm wavelengths are a useful therapeutic agent by providing a means of either stimulating or inhibiting fibroblast proliferation. In which low level laser can stimulate macrophage cells that in turn released factors that stimulate fibroblasts proliferation **(Young *et al.*, 1989).**

Low energy laser radiation caused obvious reduction in the degree of inflammatory cell infiltration in the site of laser treated area. The reduction in the inflammatory acute phase response, represented by a lower migration of polymorphonuclearneutrophil cells (PMNs) **(AL-Safi, 1991; Boschi *et al.*, 2008).** Latfullin *et al.*, 1994 showed significant positive shifts in the level of Tlymphocytes, serum immunoglobulines, such as IgA and IgM, as well as lysozyme, both in blood serum and saliva under the effect of a He-Ne laser irradiation of the mucous membrane of the oral cavity (radiation power, 2.6 mW; exposure time, 4 min; 3-5 radiation procedures every other day).

Low level laser therapy which applied immediately post-wounding has properties represented by IL-1 β biostimulatory that has one of the most

important proinflammatory interleukins that involved in wound healing **(Bjordal *et al.*, 2006; Al-Wattar *et al.*, 2013).**

The anti inflammatory efficacy of LLLT has been controversial, and some searches have not found any effect from LLLT on inflammation, some findings showed that TNF-α decreased ***(Yamaura et al., 2009; Fukuda et al., 2012, Aimbire et al., 2006, Pezelj-Ribaric et al., 2013)***, but not change the level of IL6 ***(Yamaura et al., 2009; Fukuda et al., 2012).*** Other study approved the reduction in level of IL-6 and TNF-α post therapy ***(Pezelj-Ribaric et al., 2013; Oliveira et al., 2013).*** So, LLLT dose appears to be critical for reducing proinflammatory cytokines ***(Aimbire et al., 2006; Bjordal et al., 2006; Boschi et al., 2008).***

In patients with Candida induces denture stomatitis, laser therapy resulted in a significant decrease in salivary proinflammatory cytokines TNF- α and IL6. These patients were treated by 685-nm GaAlAs diode laser for 5 days a week for four consecutive weeks ***(Simunovic-Soskic et al., 2010).*** On evaluate the LLLT at 660 nm on TNF- α, IL-6 and IL-10 level in skeletal muscle of rats with heart failure at 3J/cm2 and 21J/cm2 doses, findings were the anti inflammatory effect on which TNF- α and IL-6 was decrease while IL-10 increased ***(Hentschke et al., 2011)***. ***Boschi et al., 2008*** concluded that IL10 was reduce where TNF- α and IL-6 same effect after 660 nm LLL and the local application of energy is more efficient than dividing it around the inflammation area.

Treatment of oral mucositis by 35 sessions of 660 nm laser resulted in significant reduction of salivary IL-6. But there was slight reduction in salivary IL-10 after laser irradiation with no significant difference with control group ***(Oton-Leite et al., 2015).*** While 7 days of treatment showed increasing in level of IL-6 ***(Silva et al., 2015).***

Oliveira et al., 2013 revealed immunomodulating effect of LLL on delay type hypersensitivity to mice, as very significant reduction in the density of the inflammatory infiltrate and by a significant reduction in the levels of TNF-α,

INF-γ and IL-10.

1.6.7.5 Effect of low level laser on oral mucositis

Multiple researches have shown that low-level laser therapy (LLLT) can reduce the severity of OM, although the exact mechanism of action is not known. It has been assumed that LLLT may reduce the levels of pro-inflammatory cytokines and/or reactive oxygen species (ROS) which contribute to the pathogenesis of OM. It is difficult to compare the studies due to different laser types and parameters including wavelength and power. However, based on the successful results, the MASCC/ISOO guidelines also suggest the use of LLLT in OM at medical centers that are able to support the required training and technology ***(Raeessi et al.,2014).*** The continued investigation of new treatment modalities to attenuate OM for improving both the efficacy and tolerability of the radiotherapy in head and neck cancer has been introduced by LLLT which is thought to have anti-inflammatory, analgesic and wound healing effects, without any known clinical toxicity. Optimal details of this technology such as type of light source, dose schedule and wavelength are not worked out yet, and its use requires special training and certification. LLLT is atraumatic, non-invasive and well tolerated by patients that explain why the use of this technique in the oral cavity of cancer patients is increasing. Several studies have indicated that the LLLT can minimise the severity and pain in OM.

The outcome produced by the LLLT depends on the capacity to regulate different metabolic pathways, via conversion of the light energy through photophysical and biochemical processes, which convert the laser light

energy into useful energy to the cell. Visible laser light is absorbed in the respiratory cycle of the mitochondria by chromophores that increase the ATP production which results in more cellular proliferation and protein synthesis, facilitating tissue repair. LLLT also increases collagen synthesis and cell activity throughout healing period that ultimately leads to decreased levels of inflammation and pain ***(Cirillo et al.,2015).***

For pain relief, it has been indicated that stimulation of peripheral nerve by laser changes polarisation of the neuron membrane and increases the concentration of ATP, thereby contributing to the maintenance of membrane stability and increasing the pain threshold. Furthermore, the LLLT can raise peripheral endogenous opioid and enkephalin secretion and serum prostaglandin E2. However, pain relief also results in signifcant improvements of basic oral functions, including eating, drinking, swallowing and speaking ***(Brasil et al.,2011; Yildirim et al.,2015).*** Optimum wavelength to promote healing in ulcerative and inflamed tissue is between 680 and 880 nm. Previous investigations have shown that application of He-Ne laser in hematopoietic cell transplantation receiving patients can signifcantly reduce the duration and severity of ulcerations in OM ***(Jadaud et al.,2012).***

Chapter Two
Materials and Methods

2.Samples, materials and methods

2.1 Experimental study of mucositis induction (Pilot study):

A total of sixteen rats were included in this study and divided into three groups: group **(A)** included five rats, group **(B)** included six rats and group **(C)** included five rats.

Group A were injected with a dose of 80mg/kg methotrexate **(MTX)** cytotoxic drug with a single intraperitoneal **(I.P)** injection at base line time (day zero).

Group (B) were injected with a dose of 60 mg/kg MTX (I.P) at day zero,

Group (C) the rats were injected with a dose of 40mg/kg MTX at day zero.

The animals were kept in the cage under standard condition (room temperature, standard rat chow and water drinking). The animals were also kept under strict observation every day to watch their health condition. Deterioration in the health status such as weight loss, diarrhea and oral mucositis were observed and registered to detect the optimum dose for the development of clinically observable oral mucositis as well as on the duration of living to have the investigator to expose them to laser therapy before sacrifices for histopathological study.

2.2 Induction of experimental oral mucositis with 60 mg/kg MTX.

2.2.1 Study sample (Rats and housing).

In this study sixty male dark agouti rats, weighing 220-280 gm were used and the animals were kept under a standard laboratory conditions and maintained on a 12hour light/dark cycle at 20 ± 5°C, fed with a standard rat chow and supplied with tap water for drinking.

2.2.2Experiment design

Sixty-males of dark agouti rat (8 weeks of life; body weight: approximately 220-280 g) were used in this study. The animals were kept in groups of twelve per wire-bottomed cages, with food and water supply.

The animals were randomly divided into three groups:

Group **I**—with MTX (control group) were (**16)** rats included with no laser therapy.

Group **II**— with MTX **(22)** rats were included & treated with **30mw** (LLLT 30 mw).

and Group **III**—with MTX (**22)** rats were included & treated with **60mw** (LLLT 60mw).

The typical clinical signs of drug side effect, such as a decreased food intake, weight loss, and diarrhea, were watched in methotrexate treated rats from the first day until the eleventh day of the experiment.

The MTX 30 mg/kg was administered to each animal intraperitoneally on Day 0 and 30 mg/Kg was administered on Day 3. Based on the pilot study results, it has been demonstrated that this dose and schedule were optimal for producing mucositis with minimal systemic morbidity or mortality, therefore this method was followed in this part of the study.

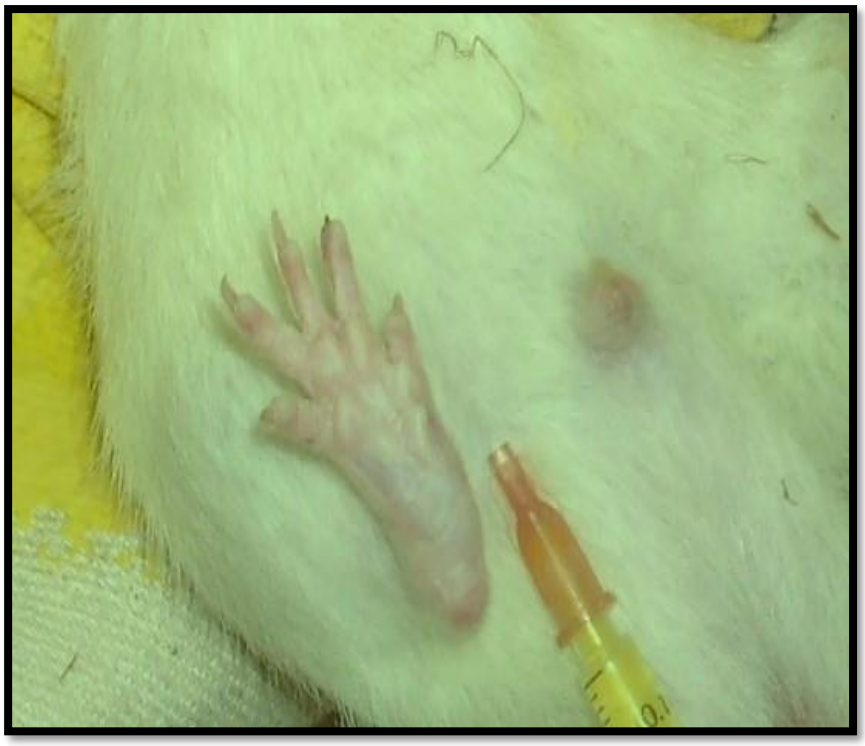

Figure 2.1 Intraperitoneal (I.P) injection of MTX

2.3 Laser protocol (LLLT)

Photolase (I) diode laser 660nm LLLT adminstrated as follow:

The animals were divided into the three groups MTX control group (no treatment or control group) and TLG (two therapeutic laser group) (30mw,60mw). Twenty-two of animals from TLG received laser irradiation by in a punctual (5 points) irradiation mode of the injured area every day. The laser parameters were kept (K= 660 nm, output power 30,60 mw, total energy density 30J/cm^2, exposure time of 40 S, spot size 0.04cm^2).

2.3.1 Laser apparatus:

Photonlase I^R model (DMC EQUIPAMENTOS LTDA) is a patented dental unit, the system is made up of visible red emitting wavelength 660 nm. Emitter useful power (10-100mW), Indium-GalliumAluminum-Phosphide (InGaAlP) diode carrying the optic fiber

beam. The emission may be continuous or pulsed. The unit included one laser protecting goggles for physician.

Technical specification (figure 2.3):

Operation voltage: 127/220 volts

Fuse: 3Ampers

Power: 30 Watts

Classification: B type- class I

The device was calibrated at Institute of Laser for Post-Graduate Studies/ University of Baghdad (figure2.4).

2.3.2 Laser parameters

Photonlase I^R model with a wavelength of 660 nm, 30 ,60mW, and energy density of 10J/cm^2 was used, in punctual (5 points) irradiation mode, delivering a total energy of 30 J. Irradiation time was 8 seconds per point based on the laser beam spot size of 0.04 cm^2.As illustrated in fig.2.2.

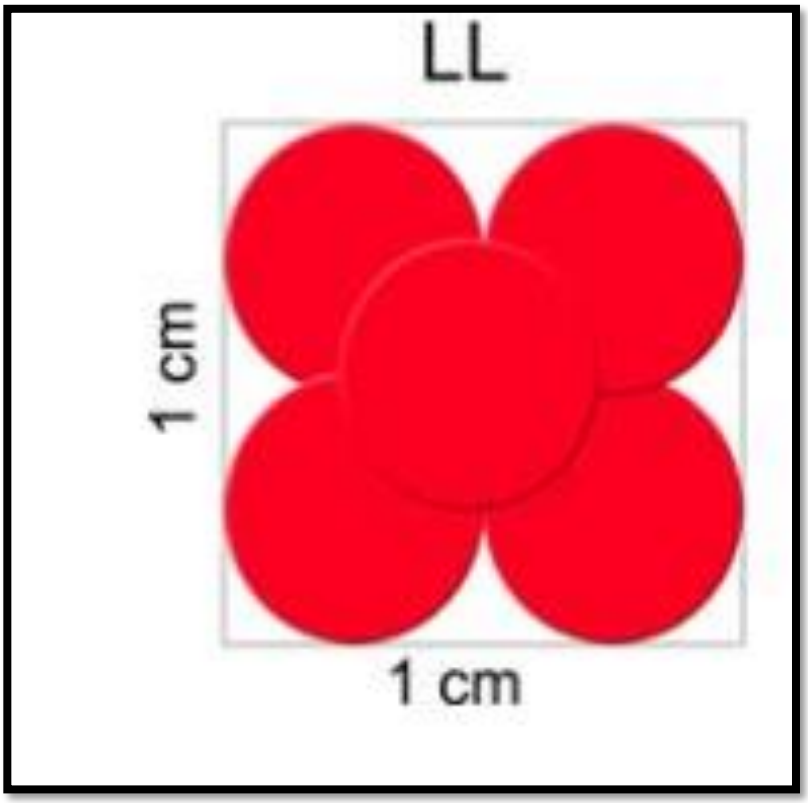

Fig2.2 laser irradiation model

Figure 2.3: specification of Photonlase (I^R) laser devise

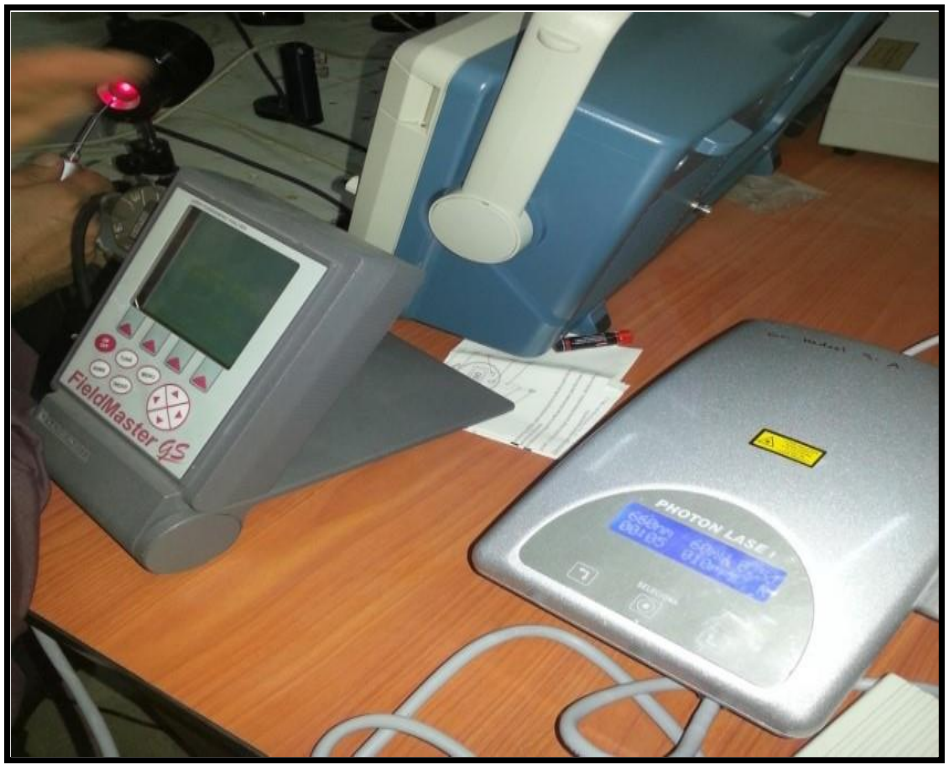

Figure 2.4: Photonlase (I^R) laser device calibration.

2.4 Clinical evaluation

The clinical aspect of the oral mucosa was observed by one calibrated examiner daily, and the degree of OM was evaluated by two specific assessment scales: criteria proposed by World Health Organization (WHO) modified for animals table 2.1. The body mass, unconsumed food and water of each animal were weighed daily ***(Campos et al.,2016; Mumphy,2007).***

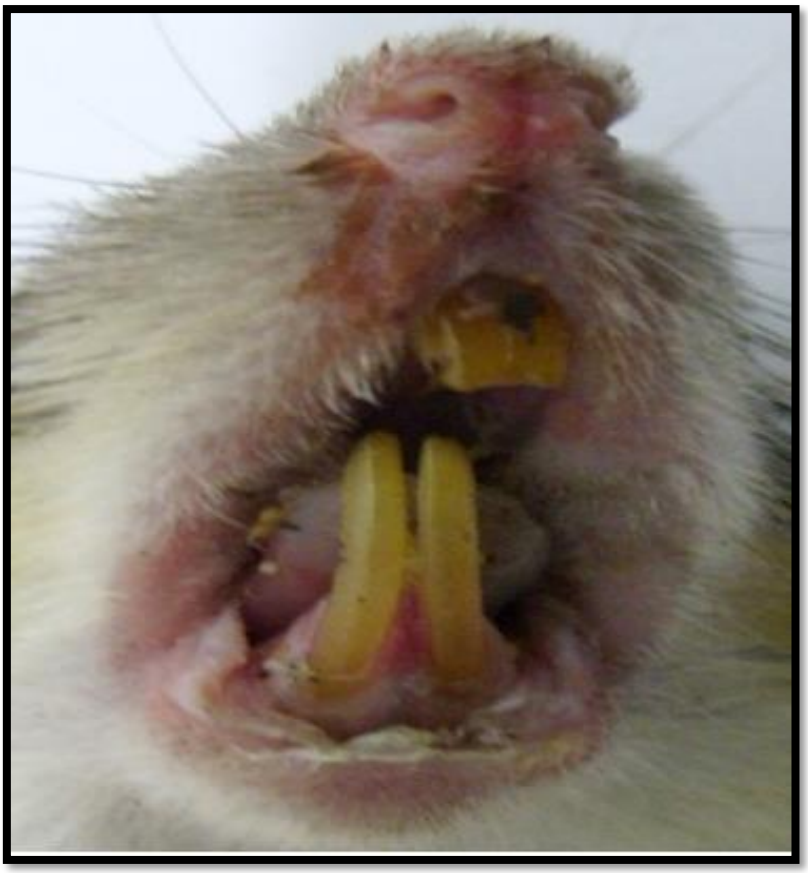

Figure 2.5 clinical photograph of Induced oral mucositis

Table 2.1 Scoring scale used to grade mucositis in the hamster model using outcomes that are analogous to clinical scoring.

Score	Description
0	Pouch completely healthy. No erythema or vasodilation
1	Light to severe erythema and vasodilation. No erosion of mucosa
2	Severe erythema and vasodilation. Erosion of superficial aspects of mucosa leaving denuded areas. Decreased stippling of mucosa
3	Formation of off-white ulcers in one or more places. Ulcers may have a yellow/gray color due to pseudomembrane. Cumulative size of ulcers should equal less than or equal to ¼ of the pouch. Severe erythema and vasodilation
4	Cumulative size of ulcers should equal about ½ of the pouch. Loss of pliability. Severe erythema and vasodilation
5	Virtually all of pouch is ulcerated. Loss of pliability (pouch can only partially be extracted from mouth)

2.5 Histopathological analyses

Sample of the tissues from the oral mucosa of the animals were taken. The specimens were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Sections were obtained for staining with hematoxylin & eosin (H&E) and examined under light microscopy (× 400).

2.5.1. Positive and negative Controls:

Both positive and negative controls were included for each run of IHC. The negative control was obtained by replacing the primary antibodies with PBS buffer. The positive tissue control was included in the present study up on

Table (2.2): The positive tissue control included in this study were according to the Manufacturer's data:

Marker	**Positive control**
1-Anti- TNF-α antibody (ENT4689)	Rat spleen figure 2.7
2-Anti-IL-1βantibody (ENT2322)	Rat liver figure 2.6
3-Anti- IL10 antibody EAP0908	Mouse kidney figure 2.8

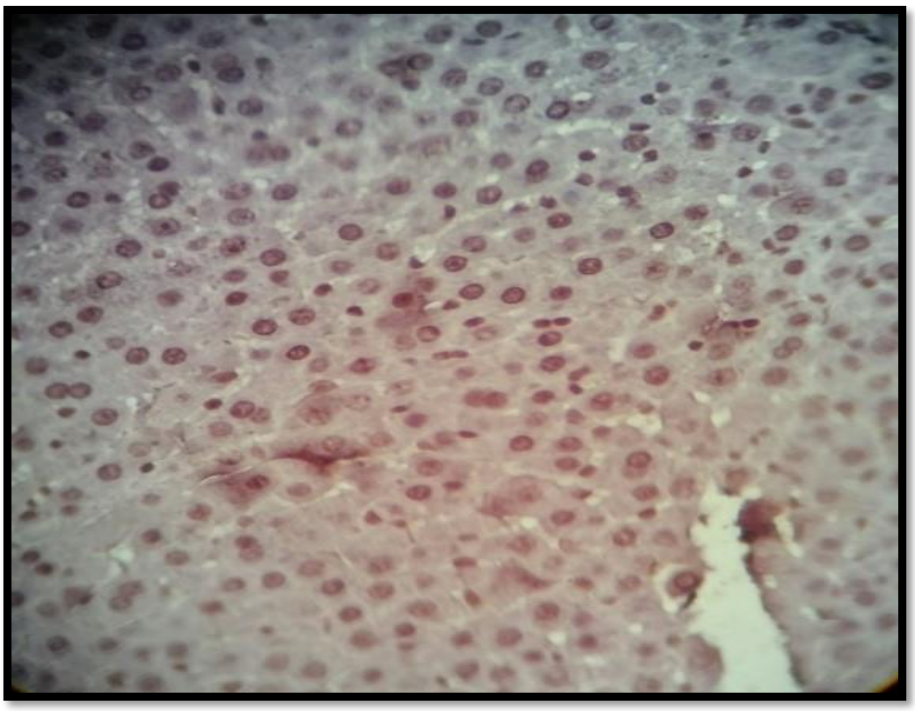

Figure 2.6 nuclear expression of IL1-β in liver tissue of rat.

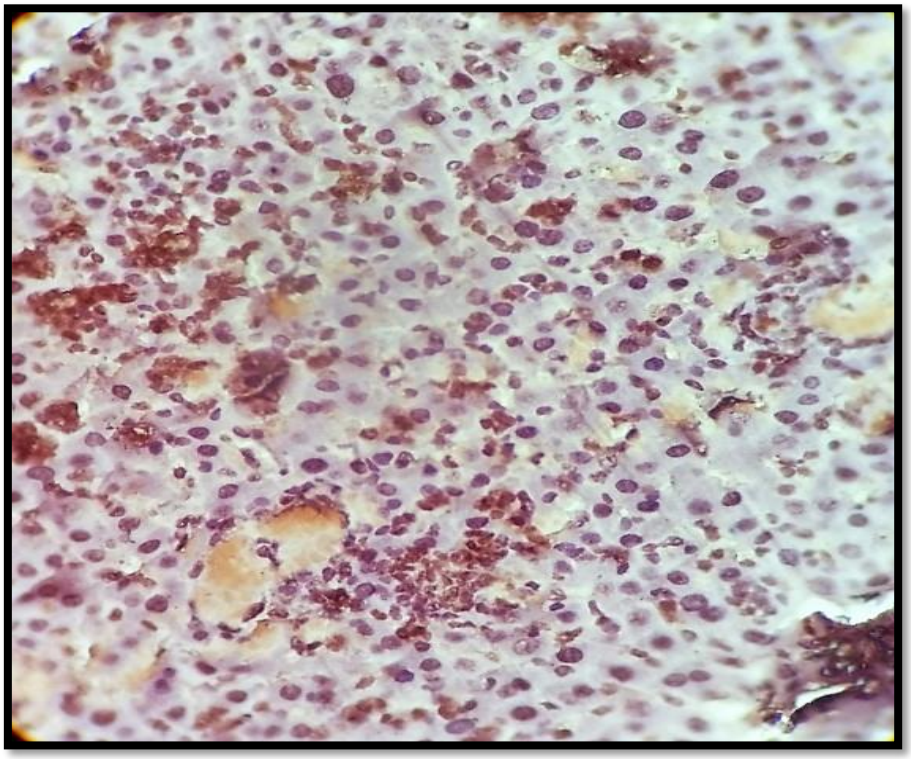

Figure 2.7 Nuclear and cytoplasmic expression of TNF-α in spleen tissue of rat.

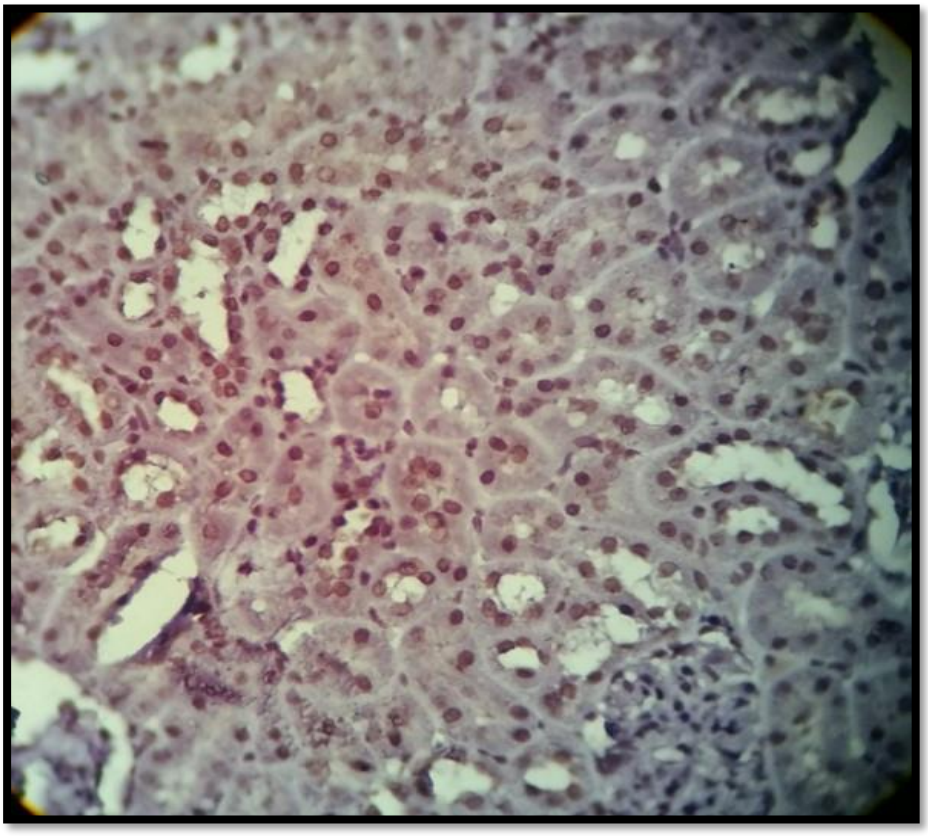

Figure 2.8 Nuclear expression of IL10 in kidney tissue of mouse**.**

2.6 Materials

The following reagents, chemicals and supplies were needed for immunohistochemical procedure and data analysis.

2.6.1 Immunohistochemical detection kit

Expose Mouse and Rabbit Specific HRP/DAB Detection IHC kit (Abcam®, ab80436; 60 ml) was used for the detection of all the primary antibodies. It is an immunoenzymatic, biotin-free antigen detection system. Its technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen that would react with a secondary antibody conjugated to horseradish peroxidase (HRP) and substrate-chromogen (DAB).

Detection	Kit	Component
1-	Protein	Block
2-		Complement
3-	50x DAB	Chromogen
4-	Hydrogen Peroxide	Block
5-	DAB	Substrate.

6- Horseradish peroxidase (HRP) Conjugate.

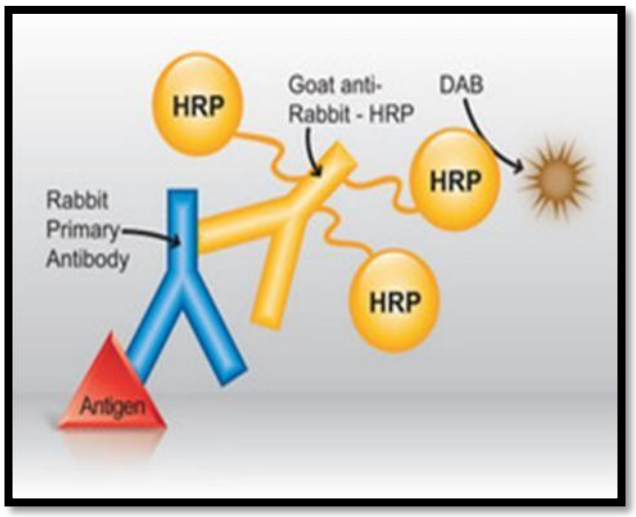

Figure (2.9): Contents of detection kit (Abcam, 2016).

2.6.2 Primary monoclonal antibodies

Three primary polyclonal antibodies, manufactured by Elba science (China) were employed in the study; they are illustrated in table (2.2). (All details are listed in the appendices (1,2,3).

Table (2.3): polyclonal antibodies of the study according to manufacturer's datasheets.

polyclonal antibody	*Manufacturer's Code*	*Isotype*	*Clone/immunogen*	*Host*	*Applied dilution*[1]
Anti-TNF-α antibody	ENT4689	IgG	16 kDa	Rabbit	1:200
Anti-IL-1βantibody	ENT2322	IgG	31 kDa	Rabbit	1:200
Anti- IL10 antibody	EAP0908	IgG	19kDa	Rabbit	1:100

2.6.3 Accessory chemicals and solutions

a. Distilled water (locally produced with a distillator).
b. Absolute Ethanol, analytical grade (AFCO, Jordan).
c. Xylene, analytical grade (Scharlau, Spain).
d. Phosphate buffered saline (PBS) (Syrbio, Syria).
e. Epitope retrieval solution: citrate buffered saline (pH=6.0) (preparation illustrated in a separate paragraph).
f. Mayer's hematoxylin (Chemsworth, India).
g. Mounting medium: Distyrene-Plasticizer-Xylene (DPX) (Syrbio, Syria).

2.6.4 Equipment

- Microscope glass slides
- Positively charged microscopic slides (AFCO, China).
- Micropipette 0.5-10µl (TopPette, DragonMed, China).
- Micropipette 10-100µl (TopPette, DragonMed, China).
- Micropipette tips.

- Cover slips (Citoglas, China).
- Mercurial thermometer.
- Digital timer.
- Glass staining jars.
- Slide holders.
- Absorbent wipes.
- Gloves.
- Hot plate for epitope retrieval.
- Microwave (Toshipa ,Japan).
- Microtome (china).
- Locally made humid chamber to maintain moisture.
- Light microscope (Olympus, Japan).
- Digital sensitive scale.
- Digital camera,20 Mega Pixels (Samsung).
- Personal computer (Intel® Core i5 processor, 500GB of RAM) (HP)

2.6.5 Preparation of citrate buffer solution (epitope retrieval solution) **(pH=6)**.

Stock solutions are prepared as follows:

(A) 0.1 M Citric acid: 19.21 g/l (M.W.: 192.1)

(B) 0.1 M Sodium citrate dihydrate: 29.4 g/l (M.W.: 294.0)

To prepare 100 ml of citrate buffer solution (pH=6.0), 7.2 ml of stock A is added to 42.8 ml of stock B, the volume is brought to 100 ml with deionized distilled water ***(Mohan, 2006)***.

2.6.6 Methodes

Five micrometer thick tissue sections were mounted on, at least, seven positively charged slides for each tissue block to be enrolled in the immunohistochemical procedure. Some custom modifications were considered to the original manufacturer's staining protocol to attain best results.

1. Dewaxing: tissue slides were baked at 60˚C. in a hot air oven for 2 hours then were immersed in two changes of xylene for 5 minutes each.
2. Rehydration: slides were immersed in serial dilutions of ethanol which comprised of two changes of absolute ethanol, 95%, 70% and 50 % ethanol for 3 minutes each then immersed in distilled water till the next step.
3. Heat induced epitope retrieval procedure (HIER): to unmask the antigens' epitopes; the slides were immersed in a citrate buffer solution (pH=6), heated to 90-95˚C on a hot plate for 20 minutes. Afterward, the heating pan with the slides rack was removed from heat source to cool down at room temperature.
4. Slides were rinsed with phosphate buffered saline (PBS) for 5 minutes, blotted and incubated with hydrogen peroxide solution (provided with the kit) for 10 minutes at 37˚C. to block endogenous peroxidase activity.
5. Slide washing was performed in a PBS solution bath twice for 5 minutes and a protein block solution was added to tissue sections and incubated for 10 minutes at 37˚C. to block nonspecific antibody binding.
6. After a single brief wash with PBS, slides were blotted and incubated overnight with the diluted primary antibody in a refrigerator (4-5˚C.) within a humid chamber. Assay dependent

serial dilutions were tried for all antibodies to reach an optimal antigen signal with minimal background, table (2.1). PBS was added to a separate tissue section in each slide along with primary antibody to act as a negative control.

7. Next day, to wash off primary antibody; the slides were immersed in 3 consecutive rinses of PBS for 5 minutes each, blotted and the tissue sections were incubated with the immunohistochemical kit's "complement" solution for 10 minutes at 37˚C. then washed twice in PBS for 5 minutes each.
8. "Conjugate" solution was added to tissue sections and incubated at 37˚C. for 15 minutes. Afterward, the slides were again washed with four rinses of PBS for 5 minutes each.
9. Diaminobenzidine (DAB) chromogen is prepared by adding 30 µl of DAB stock to 1.5 ml of DAB substrate away from light. The chromogen is then added to the tissue sections and left to develop in darkness for 5 to 8 minutes before being rinsed 4 times with PBS immersion baths for 5 minutes each.
10. Tissue sections are then counterstained by with Mayer's hematoxylin for 2 minutes, followed by immersion in running tap water for 5 minutes.
11. Dehydration was performed by immersing the slides in multiple ethanol dilutions (50%, 70%, 95%, 100%, and 100%) for 3 minutes each then cleared with xylene immersion two times for 5 minutes each.
12. Finally, the slides were mounted with DPX and covered with cover slips.

2.6.7 Evaluation of staining results

Immunohistochemical signal specificity was demonstrated by the presence of brown granular DAB staining pattern within the specific subcellular or tissue compartment for a certain antibody in positive control tissue slides according to manufacturer's datasheets, and the absence of the staining in negative controls tissue sections.

At least, five representative fields were selected of each tissue section in all antibodies, visualized and scored microscopically with a 40X objective; a mean positive percentage was recorded for each case. All slides were blindly evaluated without prior knowledge of other parameters. To calibrate the results, an experienced pathologist evaluated the slides independently and the resulting values were statistically correlated to assure acceptable agreement; otherwise, slides were re-evaluated to reach a consensus.

Qualitative immunohistochemistry was performed. Staining was observed using a light microscope. The intensity of staining for was scored as follows: 0, no staining, 1, weak staining, 2, moderate to intense staining. This qualitative staining assessment has been previously validated by published grading systems (Logan et al.,2008).

2.6.8 photomicrographs

A digital camera set to 20 Mega Pixels was approached to the microscope eyepiece at an optimum distance for a clear full field circular view of the microscopic field on the phone display. The camera is adjusted to autofocus and an image was captured when a green rectangle appeared on the screen denoting a perfectly focused image. Multiple photomicrographs were captured for each slide to cover all representative

tissue with a 10X objective and a 10X eyepiece. A total of 100 images were captured for the three aforementioned antibodies.

2.7 Statistical Analysis:

Data were interpreted into a computerized database structure. Then the database was checked for errors utilizing range and logical data cleaning procedures, and inconsistencies were repaired. An expert statistical advice was

looked for. Statistical analyses were performed utilizing SPSS version 23 computer software. The statistical significance of differences in median of such outcome variables between more than 2 groups was checked via Kruskal Wallis test. while between paired combination of groups Mann-Whitney test was used

The level of statistical significance at $P < 0.05$. All analyzed statistical tests of significance were bilateral. The statistical significance, strength as well as direction of linear correlation between 2 ordinal level (non-normally distributed) variables was assessed by Spearman Rank linear correlation coefficient.

Chapter Three

Results

3. Results

3.1 Tolerance of the rats to the chemotherapy methotrexate (MTX) and induction of oral mucositis

A total of sixteen rats were included in this study and divided into three groups: group **(A)** included five rats, group (B) included six rats and group **(C)** included five rats.

Group A were injected with a dose of 80mg/kg methotrexate (MTX) cytotoxic drug with a single intraperitoneal (I.P) injection at base line time day zero. The rats had died four days after injection due to severe diarrhea. Mucosal changes were noticed redness in two rats score (1) only. As shown in table3.1

Group (B) were injected with a dose of 60 mg/kg MTX (I.P) at day zero, we found all rats induced with oral mucositis at day seven with the main scores range (1-2), the rats had died 14 days after injection. As shown in table3.1

Group (C) the rats were injected with a dose of 40mg/kg MTX at day 0, they haven't developed oral mucosal changes and died 16 days after injection. As shown in table3.1

There was a statistical significant negative correlation that correlate the doses of MTX with the mortality duration of rats among the pilot experimental study groups.

We did these pilot experimental study for adjusting the optimal toxic dosage of MTX that promote adequate life duration as long as possible to

study oral mucosal changes (histological and clinical changes) along the course of experimental oral mucositis.

Table 3.1 pilot experimental doses of methotrexate with different mortality time.

Study group/ (N)	**Variables**	**Mortality TIME**	**MTX DOSE**	**Oral mucositis score**
	Mean ± SD	10.40 ± 5.562	64 ±15.776	0.80 ±0.422
	Std. Error	1.759	4.989	0.133
	Median	14	60	1
	Range	17	40	2
	Minimum	4	40	0
	Maximum	21	80	2
	Frequency	(4,21) days	(80,60,40)mg/kg	(0,1,2)
Group A (5)	*p value		(60-80) mg/kg	0.025
Group B (6)	*p value		(60-40) mg/kg	0.025
Group C (5)	*P value		(80,40) mg/kg	0.011

3.2. Sample Description of the experimental groups injected with a dose 60mg/kg of MTX.

Sixty male rats housed for experimental induction of oral mucositis which were divided into three groups

1-Control methotrexate group without laser treatment that contained sixteen rats **(G1).**

2-Laser 30 mw treated group that contained twenty-two rats **(G2).**

2-Laser 60mw treated groups that contained twenty-tow rats **(G3).**

3.2.1 Cytotoxic drug Induction of experimental oral mucositis.

The base line induction was begun with intra-peritoneal administration of 60mg/kg dose of methotrexate on zero day that caused statistically non -significant lesions at day 6 ($P < 0.05$), among all study groups as shown in Fig.(3.1),(3.2),(3.4),(3.6) and Table3.3.A).

The oral mucosal changes had monitored and the clinical scores were registered daily. Our scale for clinical scores registration depend on WHO scale of mucositis assessment modified for animal ***(chow et al.,2009)*** represented by erythema, hemorrhage and ulcers as shown in (Fig.3.1 and Table3.3A&B). On day 11, these alterations were significantly reduced ($P < 0.05$) among the study groups, showing a clinical pattern of moderate hyperemia and erythema, discreet hemorrhage, reduced number of ulcerative lesion as shown in table (3.3B).

3.2.2 Body weight analysis among study groups.

T***he body weight measurements of experimental animals have shown statistical significant differences between control and two laser treated groups at zero, six and eleven days of experiment, as listed in table 3.2***

The body weights of the control group have shown statistical significant differences at three different periods (0day,6day and 11day) $p = (0.003)$.

While the body weights of the laser 30mw groups have shown statistical significant differences at the three measured periods (0day,6day and 11day) p= (0.016).

The laser 60 mw have shown statistical significant differences p>0.001 of the body weights at the three measured periods (0day,6day and 11day).

Table 3.2 Comparative analysis of body weight among experimental groups.

No. rats	*Study group*	*Time (days)*			*F*	*p. value*
		0	*6*	*11*		
16	**Control (G1)**	Mean± SD 239.06±21.773	Mean± SD 207.81±20.081	Mean± SD 184.75±19.157	3.625	0.033
22	**L30** *(G2)*	226.82±24.424	206.68±24.257	207.73±24.927	4.470	0.016
22	**L60** *(G3)*	243.18±20.210	224.91±19.644	219.45±21.231	30.007	<0.001
p. value		0.069	0.097	0.023		
16 G1	**Std. Er**	5.443	5.020	4.789		
(22) G2	**Std. Er**	5.207	5.172	5.314		
(22) G3	**Std. Er**	4.309	4.188	4.526		

***Significant difference; p value <0.05; ANOVA, F test; SD standard deviation.**

3.2.3 Clinical evaluation of experimental oral mucositis

3.2.3.1Clinical scores analysis of experimental oral mucositis among study groups at day six.

The results of the experimental induced oral mucositis of the control group **G1** clinically showed all sixteen animals had induced oral mucositis as follow: two animals with score one; four animals with score two and ten animals with score three. As shown in table (3.3.A) & Figures (3.2), (3.3), (3.4),(3.5) (3.6) &(3.7).

In relation to the laser 30 mw group G2, all of twenty- two animals have been induced oral mucositis with the following frequency: three animals with score one; four animals with score tow and fifteen animals with score three as shown in table (3.3.A).

All animals with laser 60mw G3, developed oral mucositis with clinical scoring in all twenty -two animals as follow: four animals with score one;

nine animals with score two and also nine animals with score three as shown in table (3.3.A).

Both of the experimental laser 30 mw and 60 mw groups (G2&G3) showed statistically non-significant differences when compared to control group G1 (p <0.05) at day six as listed in table 3.3.A.

There were no statistical significant differences among the study groups (p <0.05) at day six.as listed in table 3.3.A.

Table 3.3.A Clinical scores analysis of experimental oral mucositis among study groups at day six.

No. rats	**Study group**	**CLINICAL scoring**	**%**	**P* value (G1, G2)**	**P* value (G2, G3)**	**P* value (G1, G3)**	**P£ Value (G1, G2& G3)**
N 16	G1	SCOR1 (2) rats	12.5%	(0.445)	(0.07)	(0.140)	(0.236)
Mean	2.50						
Std. Error	0.183			**Mean rank**	**Mean rank**	**Mean rank**	**Mean rank**
Median	3.00						

Std. D.	0.730	SCOR2		19	25.25	21.81	46.06
Range	2	(4) rats	25 %				
Minimum	1						
Maximum	3						
		SCOR3	62.5%				
		(10) rats					
N.22	G2		13.6%	19.86	19.75	17.82	22.55
Mean	2.55	SCOR1					27.14
Std. Error	0.157	(3) rats					
Median	3.00	SCOR2	18.2%				
Std. D.	0.739	(4) rats					
Range	2	SCOR3					
Minimum	1	(15) rats	68.2%				
Maximum	3						
N.22	G3	SCOR1	18.2%				
		(4) rats					
Mean	2.23		40.9%				
Std. Error	0.160	SCOR2					
Median	2.00	(9)					
Std. D.	0.752						
Range	2	SCOR3					

Minimum	1	(9)	40.9%				
Maximum	3						

*, Mann-Whitney test, exact significant; £ Kruskall Wallis test.

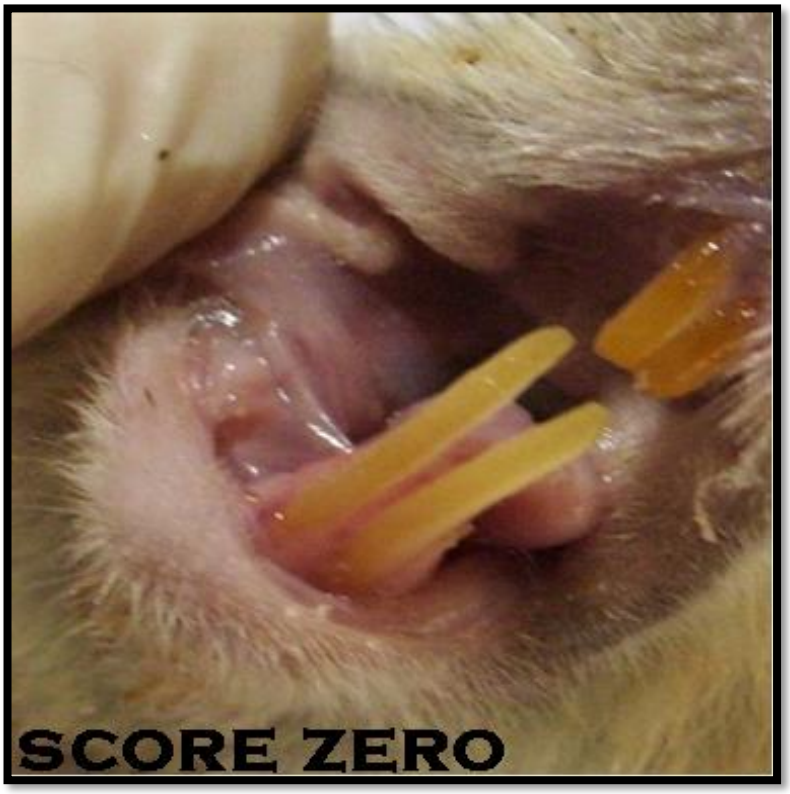

Figure (3.1): Clinical view relevant to the clinical score zero.

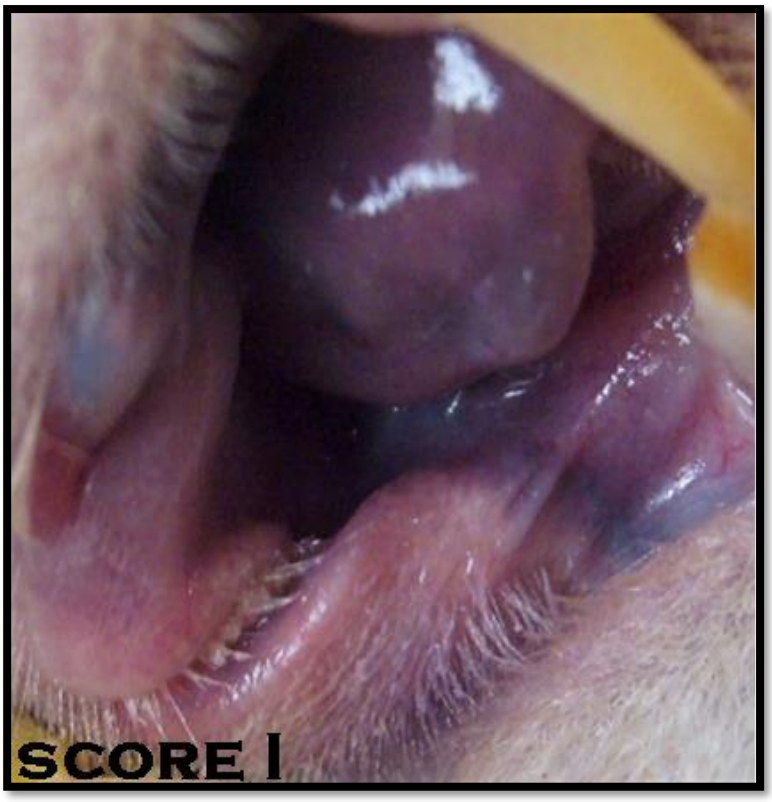

Figure (3.2): Clinical view relevant to the clinical score I.

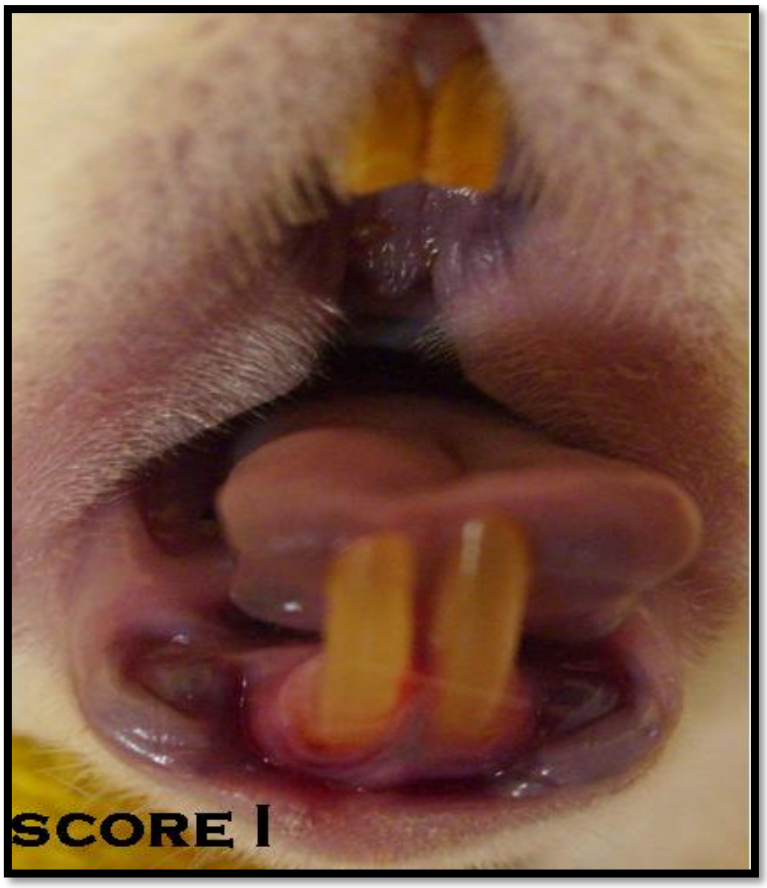

Figure (3.3): Clinical view relevant to the clinical score I

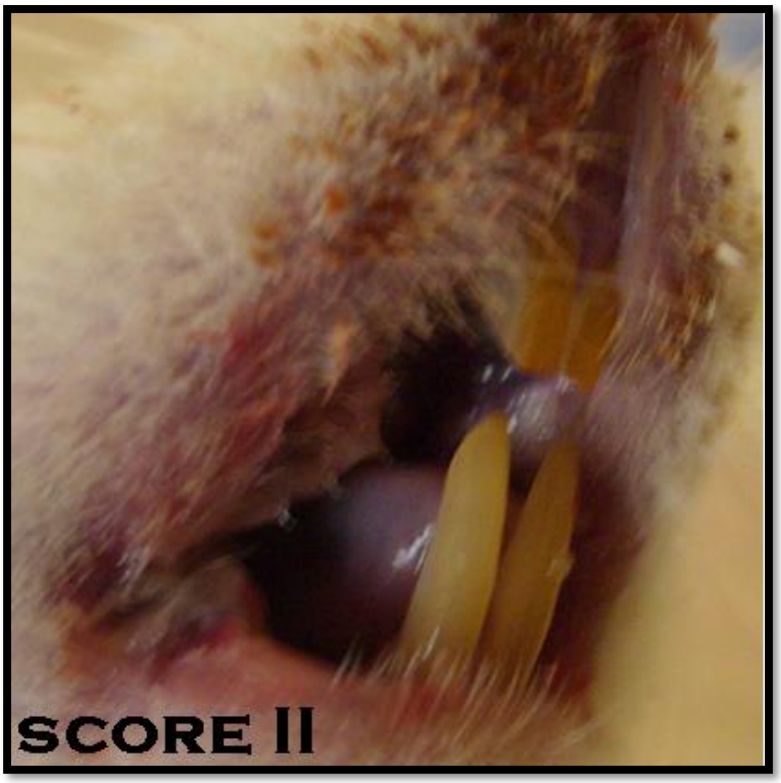

Figure (3.4): Clinical view relevant to the clinical score II.

Figure (3.5): Clinical view relevant to the clinical score II

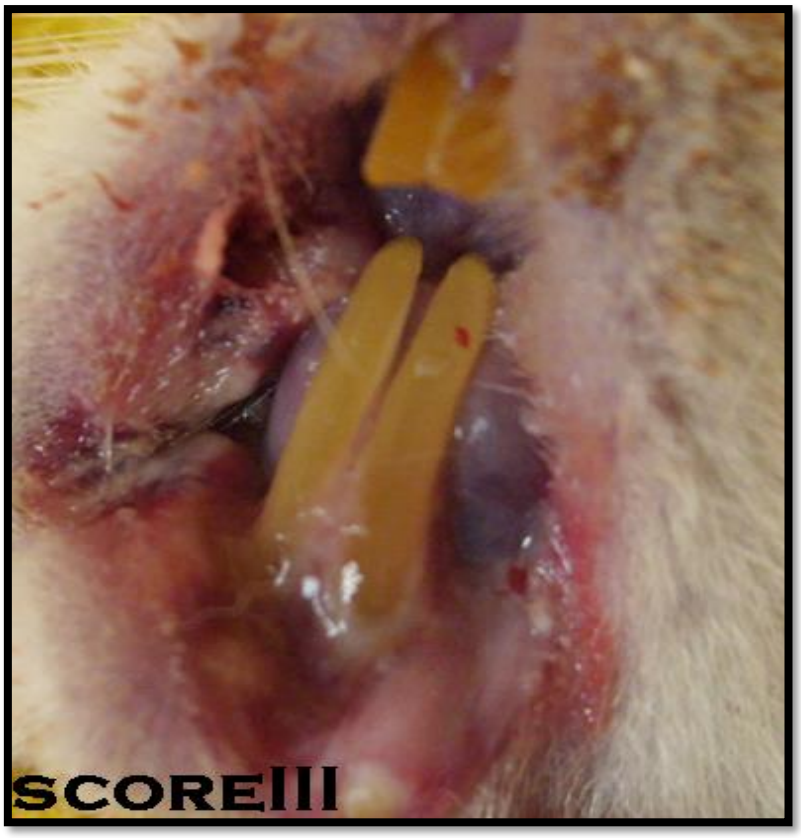

Figure (3.6): Clinical view relevant to the clinical score III

Figure (3.7): Clinical view relevant to the clinical score III

3.2.3.2 Clinical scores analysis of experimental oral mucositis among study groups at day eleven.

Control group G1 had statistical significant decrease in numbers of the affected rats into tow rats with score zero, five rats with score one, four rats with score tow& five rats with score three in comparison with the clinical scoring of G1 at day six, as shown in table (3.3.A&B). their negative non -sig correlations in comparison with the scores at day six. (R=-0.171) (p=0.502)

Clinically Laser 30mw group G2 had included twelve rats and became normal with score zero and ten rats had cure to score two, so they had the lowest clinical scores among the study groups, in comparison with clinical scores of G2 at day six, where as 45.5 % of the animals of G2 clinically appeared with normal oral mucosa at day11, as shown in table (3.3.A&B). these group were showed least scores with range (0-1). Statistically significant negative correlations that explained the decrease of clinical scores among the rats group in comparison with their clinical scores at day six (R= -0.204) (p=0.048).

G3 revealed obvious changes among clinical scores as the following ten rats were score one fig (3.2) (3.3)); five rats were score two fig. (3.4) (3.5) and five rats appeared normal with score zero fig. (3.1). In comparison with clinical scores at day six: there were statistically significant negative correlations (R=-0.055) (p= 0.0089)

The percentile of theses group clinical scores:45.5% score one; 40.9% score two and 13.6% score three. as shown in table (3.3.A&B).

The clinical scores exhibited Statistical significant differences ($p<0.05$) among all study groups as shown in table (3.3.B).

Both of the experimental groups (G2&G3) showed statistically significant differences when compared to the control group G1 (p >0.05) as listed in table (3.3.B).

Table 3.3.B Comparative evaluation of experimental oral mucositis (clinical scoring at day eleven.

No. rats	**Study group**	**Clinical scoring**	**%**	**P* value (G1, G2)**	**P* value (G2, G3)**	**P* value (G1, G3)**	**P£ Value (G1, G2& G3)**
N 16	**G1**	SCORE 0					
Mean	1.75	(2) rats	12.5%	(0.000)	(0.005)	(0.011)	(0.041)

		SCOR1		**Mean rank**	**Mean rank**	**Mean rank**	**Mean rank**
Std. Error	0.266	(5) rats	31.3%				
Median	2						
Std. D.	1.065			26.94		24	42.44
Range	3		25.0%				
Minimum	0	SCOR2					
Maximum	3	(4) rats					
		SCOR3					
		(5) rats	31.3%				
N.22	**G2**	SCORE 0					
Mean	0.45	(12) rats	54.5%		17.86		20.45
Std. E	0.109	SCOR1					
Median	0	(10) rats	45.5%	14.09			
Std. D.	0.510						
Range	1	SCOR 0	22.7%				
Minimum	0	(5) rats					
Maximum	1						
N.22	**G3**	SCOR 1	54.5%		27.14	16.23	31.86

		(12) rats					
Mean	1.00						
Std. Error	0.147	SCOR 2(5)	22.7%				
Median	1.00						
Std. D	0.690						
Range	2						
Minimum	0						
Maximum	2						

*, Mann-Whitney test exact significant; £ Kruskal Wallis test.

3.2.3.4 Assessment of the experimental mucositis degree among study groups (kappa s test):

The Kappa's test score (0.79) between observers showed strong agreement. As listed in table (3.4). It is possible to note that the obtained scores were higher in the control group G1 during all experimental period. Table (3.3.b).

The maximum degree obtained from group 2 group was 3 at day six. On the same group that received laser 30mW treatment, the highest degree of mucositis was of 1 at day 11. At day 11, a statistically significant score differences were observed between groups ($p = 0.041$). Laser groups 30mw and 60mw(G1&G2) presented a mucositis degree significantly lower than the control group (G1).

As shown in table (3.3.b).

Table3. 4 Mucositis degree at day six and eleven among study group.

Kappa tests		***p**	**Std. Error**

Measure of Agreement	0.79	0.041	0.062

3.3. Immunohistochemical cytokines analysis of the experimental oral mucositis among study group. (histological scoring)

3.3.1 Interleukin 1β expression among study group (IL.1β).

The histological scores of interleukin1β in the control group G1 revealed positive expression in the range of (1-2) histological scores among all tissue sections. These scores showed statistically significant differences with both (G2 and G3) scores.as shown in table (3.5) & fig (3.8)A,B,C.

While there was negative expression of the IL1β in seven tissue sections of the G2 and positive expression in the rest of the fifteen sections of the laser 30 mw G2 in range of (0-2) scores as listed in table (3.5).

The expression of IL1β showed one negative and twenty-one positive expression among tissue sections of the laser 60 mw G3 with the range of (0-2) scores.

The experimental oral mucositis tissue scores of IL1β among the study groups had expressed significant differences as listed in table (3.5).

Table 3.5 Comparative immunohistochemical scores of Interleukin 1β among study groups.

IL-1 β SCORES	**STUDY GROUP (N)**			
	G1 (16)	**G2 (22)**	**G3 (22)**	
Mean score ± SD	1.73±.607	0.82+0.66	1.18+0.50	
Mean rank	27.13	19.23	25.38	44.0
	*(G1&G2)	*(G2&G3)	*(G1&G3)	21.68

	13.95	25.77	15.23	29.50 £(G1,G2&G3)
*** £ P value**	*<0.001	*0.048	*0.001	£ p<0.001
-ve	0	7	5	
+ve	4	12	14	
++ve	12	3	3	
Range	2 (0-2)	2 (0-2)	2 (0-2)	
Median	2	1	1	

*,P value; Mann-Whitney test exact significant; £, Kruskal-Wallis test.

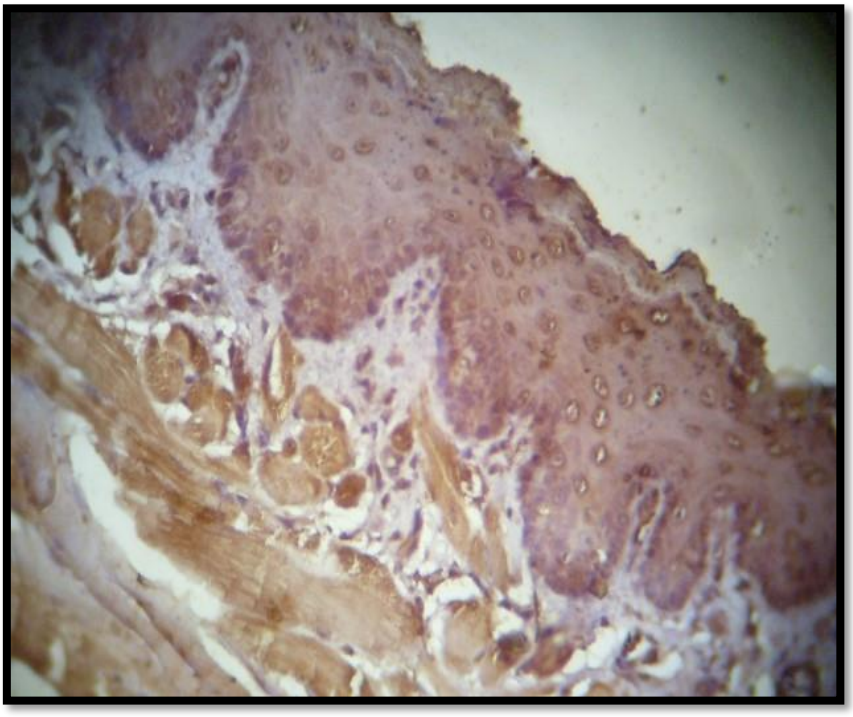

Fig (3.8.A) Immunohistochemical staining of MTX treated oral mucosa. oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against IL-1β. rats of the control group that not received treatment.

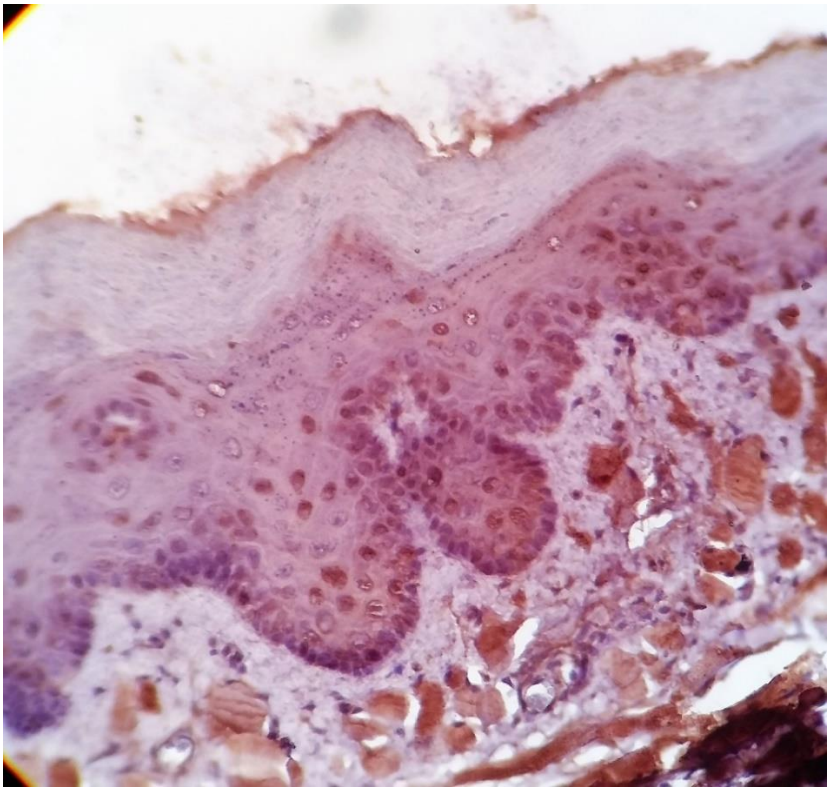

Fig (3.8 B) Immunohistochemical staining of MTX treated oral mucosa.oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against IL-1β. rats subjected to laser therapy(30mw).

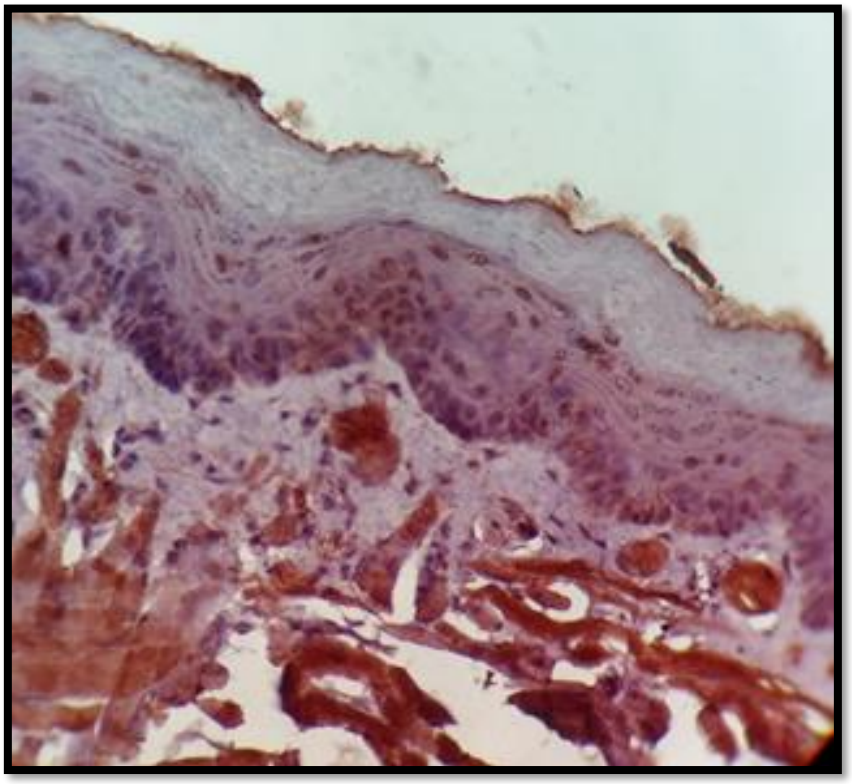

Fig (3.8 C) Immunohistochemical staining of MTX treated oral mucosa.oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against IL-1β. rats subjected to laser therapy(60mw) for six days.

3.3.2 Tumor necrosis factor alpha immunohistochemical scores expression among study group. (TNF-α).

The TNF-α tissue scores of the control group **(G1)** had revealed sixteen positive expression with range (1-2) scores.as shown in table (3.6) & fig (3.9A).

The tissue scores of **TNF- α** of **(G2)** have shown fourteen positive and eight negative expression with range (0-2).as shown in table 3.6 & fig (3.9B).

While the laser 60mw **G3** tissue sections scores of **TNF** had display one negative and twenty-one positive expression in **G3** in range of (0-2).

The TNF- α immunohistochemical scores of the laser groups **(G2)** (laser 30 mw) and (laser 60 mw) G3 had showed statistically significant difference. while there were statistical non-significant differences of **TNF-α** scores in the control group **G1** with the laser 60 mw **G3** (p=0.181) as described in table. (3.6) & fig (3.9)A,C.

Table 3.6 Comparative immunohistochemical scores of TNF-α among study groups.

TNF- α	**Study group (N)**		**G3**	
	G1	G2		
Mean score ± SD	1.44±0.51	0.64±0.49	1.23±0.52	

Mean rank	26.56	17.09	21.59	38.66
				19.95
	14.36	27.91	17.98	34.39 £(G1,G2&G3)
***P value**	*(G1&G2) <0.001	*(G2&G3) 0.001	*(G1&G3) <0.181	<0.001
Negative	0	8	1	
+ve	9	14	5	
++ve	7	0	16	
Range	2 (0-2)	2 (0-2)	2 (0-2)	
Median	1	1	1	

*,P value; Mann-Whitney test, exact significant; £, Kruskal-Wallis test.

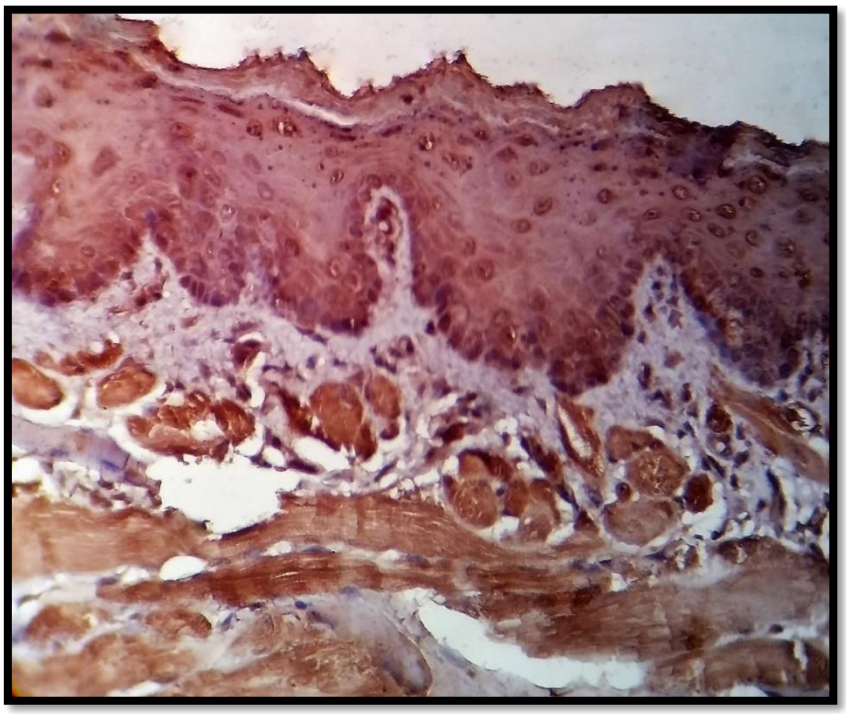

Fig (3.9.A) Immunohistochemical staining of MTX treated oral mucosa. oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against TNF-α. rats of the control group that not received treatment.

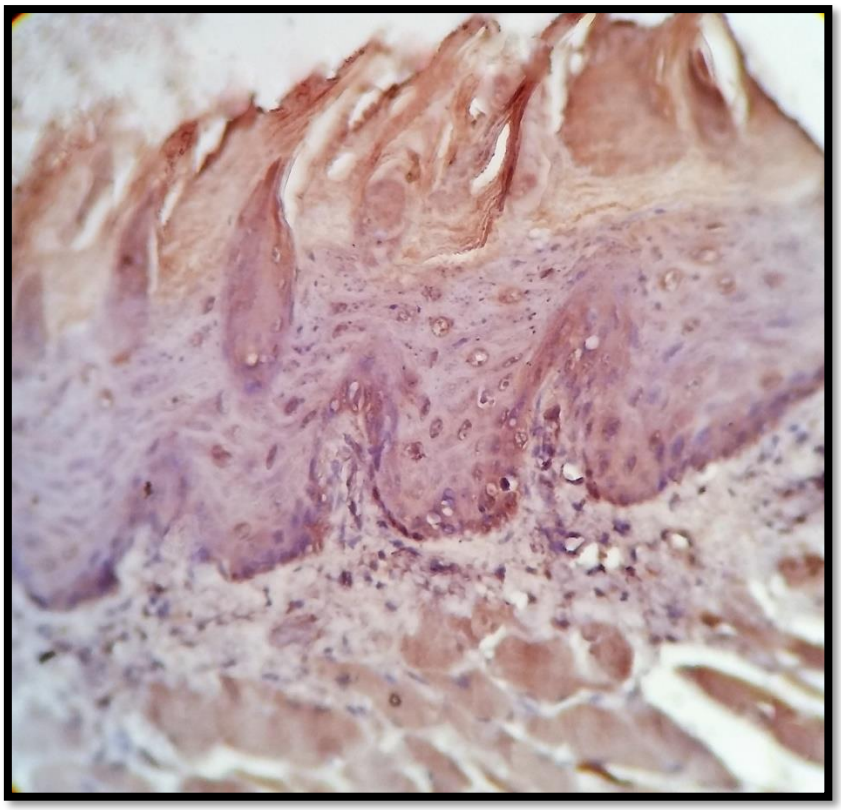

Fig (3.9 B) Immunohistochemical staining of MTX treated oral mucosa.oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against TNF-α. rats subjected to laser therapy(30mw).

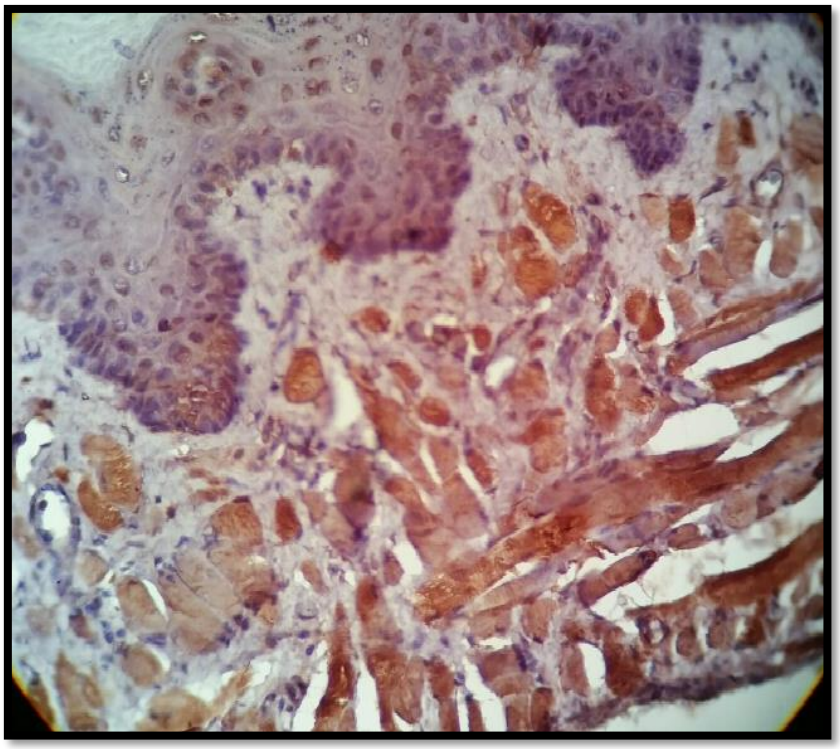

Fig (3.9 C) Immunohistochemical staining of MTX treated oral mucosa.oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against TNF-α. rats subjected to laser therapy(60mw) for six days.

3.3.3 Interleukin 10 expression among study group. IL10

The interleukin10 scores expression in the control group G1showed seven positive and nine negative expression of all tissue sections. As shown in table (3.7)

The G2 tissue sections showed IL-10 positive scores expression in all tissue sections. As shown in table (3.7).

The scoring expression of immunohistochemical IL10 showed eighteen-positive and four negative expression in the laser 60 mw (G3).

The IL10 expression exhibited statistically significant differences among all study groups as listed in table (3.7) and fig (3.10A, B, C). Their correlations had been illustrated in table (3.8). As significant positive correlations with the control group G1.

Table 3.7 Comparative immunohistochemical score of IL10 among study groups.

IL-10 SCORE	**STUDY GROUP (N)**			
	G1 (16)	**G2 (22)**	**G3 (22)**	
Mean score ± SD	0.44± 0.51	1.55± 0.51	0.91± 0.68	
Mean rank	10.38	27.86	15.45	44.0
				29.50
	25.13	17.14	22.44	£(G1,G2&G3)

* £ P value	(G1&G2) * 0.200	*(G2&G3) 0.001	*(G1&G3) 0.023	£ p<0.001
-ve	9	0	6	
+ve	7	10	12	
++ve	0	12	4	
Median	0	2	1	
Range	2 (0-2)	2 (0-2)	2 (0-2)	
Median	2	1	1	

*,P value; Mann-Whitney test ,exact significant ; £, Kruskal-Wallis test.

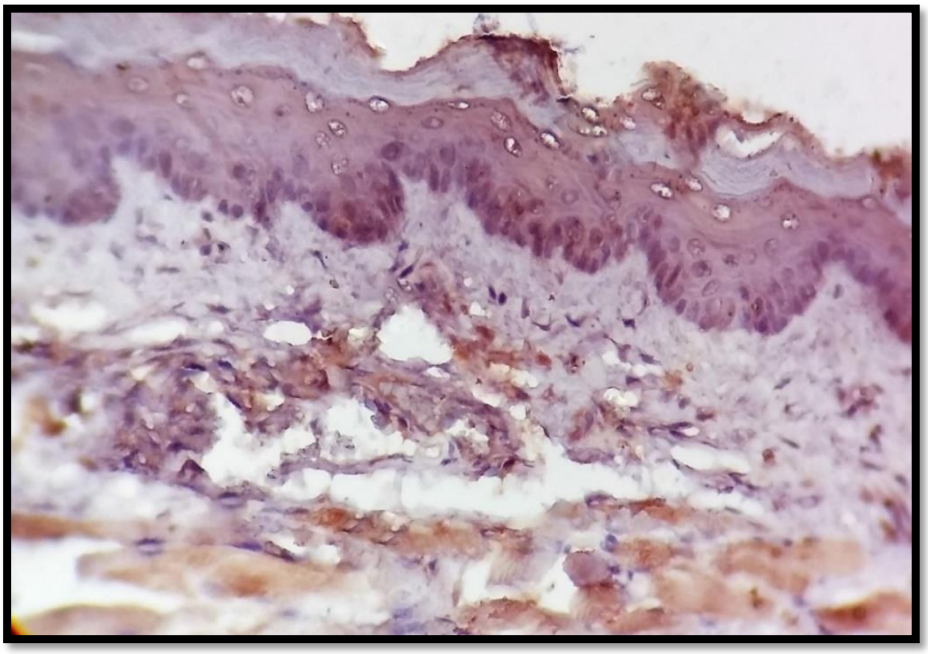

Fig (3.10.A) Immunohistochemical staining of MTX treated oral mucosa. oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against IL-10. rats of the control group that not received treatment

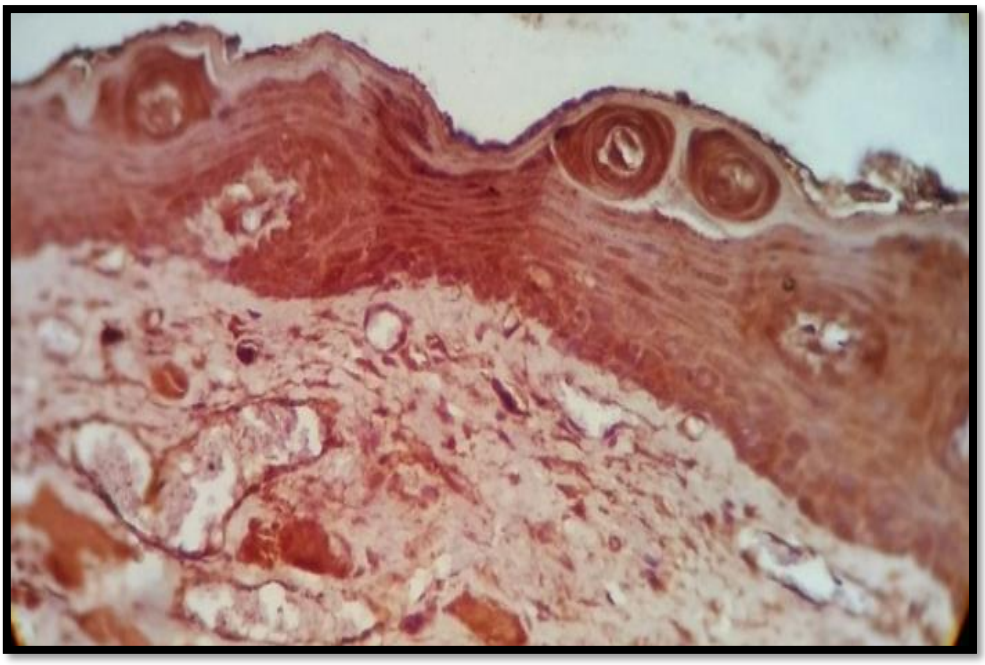

Fig (3.10 B) Immunohistochemical staining of MTX treated oral mucosa.oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against IL-10. rats subjected to laser therapy(30mw).

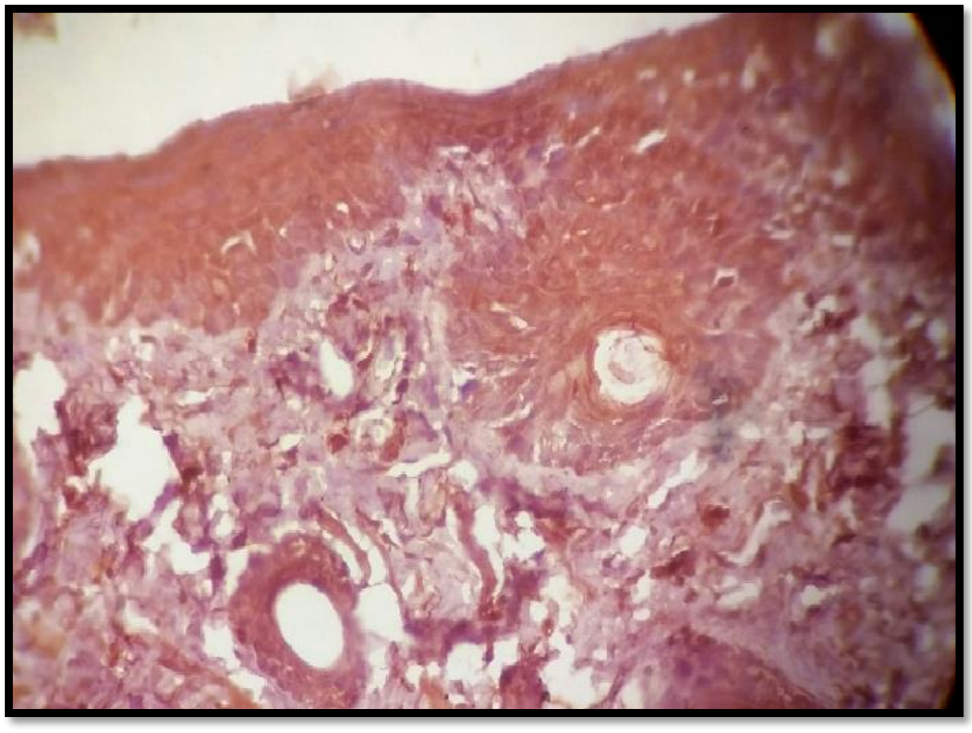

Fig (3.10 C) Immunohistochemical staining of MTX treated oral mucosa.oral mucosa was collected 11 days later. (Original magnification x40). Sections of oral mucosa were stained with antibodies against IL-10. rats subjected to laser therapy(60mw).

3.4 Immunohistochemical and clinical scores of experimental oral mucositis Correlations among the study groups

3.4.1(IL-1β,TNF&1L-10) Immunohistochemical scores correlations among study the groups.

The immunohistochemical scores of IL-1β in the control group G1 had illustrated significant positive scores correlations with TNF-α, and non-significant positive weak correlations with IL-10 as shown in table (3.8)

The immunohistochemical scores of IL1β in G2 had shown positive non -significant negative correlation with TNF-α and significant-negative correlation with IL-10 as shown in table (3.8)

The immunohistochemical scores of IL1β in G3 revealed non-significant positive correlations with TNF- α and non -significant negative with IL-10 as shown in table (3.8)

The immunohistochemical scores of TNF-α in the control group G1 had exhibited significant positive -correlation with IL-1β, while it had shown statistical significant negative correlations with IL-10. As shown in table (3.8).

The TNF-α immunohistochemical scores had display statistical significant positive correlation with IL1β in G2 where as these group under laser therapy 30mw while it showed statistically significant negative correlation with IL-10. As shown in table (3.8)

The TNF immunohistochemical scores were expressed as statistical positive non-significant correlation in the laser 60 mw group G3 with and negative non-sig correlation with IL-10. As shown in table (3.8).

In concern to IL-10 immunohistochemical scores trend toward negative non -significant correlation with TNF-**α** and IL-1β in the control group G1,

while in the 30mw G2 the IL-10 immunohistochemical scores revealed significant negative correlate with IL1β and TNF-**α**.

The IL-10 immunohistochemical scores in the 60mw group G3 expressed statistical non-sig positive correlate with IL-1β and TNF-**α**. As shown in table (3.8).

Table (3.8): Spearman correlations coefficients among immunohistochemal scores of the study groups**.**

STUDY GROUP VARIABLES	**IL-1β**	**TNF- α**	**IL-10**
G1(16) **IL1β**	R=1 P<0.001	R=0.277 P=0.023	R=0.218 P=0.0741
TNF-α	R=0.277 P=0.023	R=1 P<0.001	R= 0.132 P= 0.507
IL-10	R=0.218 P=0.417	R=0.132 P=0.507	R=1 P <0.001
G2(22) **IL-1β**	R=1 P<0.001	R=0.223 P=0.042	R=-0.320 P=0.022
TNF-α	R=0.223 P=0.042	R=1 P<0.001	R=-0.129 P=0.031
IL10	R=-0.320 P=0.022	R=-0.129 P=0.031	R=1 P<0.001

G3(22) **IL-1β**	R=1 P<0.001	R=0.492 P=0.077	R=-0.122 P=0.364
TNF-α	R=0.492 P=0.077	R=1 P<0.001	R=-0.254 P=0.142
IL-10	R=-0.122 P=0.064	R=-0.254 P=0.142	R=1 P<0.001

3.4 .2 Clinical scores correlations of experimental oral mucositis at six and eleven days of follow up among study groups.

The clinical scores of the control group (G1) expressed significant positive correlations with IL-1 β and TNFα at day six and eleven, while there were non-significant -positive correlations with IL-10 at day six and significant negative correlation at day eleven. as shown in table (3.9).

The clinical scores of laser 30mw G2 showed statistical significant -negative correlation with IL-1 β and TNFα and statistical significant positive correlation with IL-10 at day eleven while these clinical scores had statistical non-significant -correlate with IL1β and TNFα at day eleven. as shown in table (3.9).

Clinical scores of laser 60mw G3 were expressed significant positive correlation with IL1-β and TNFα and non-significant positive correlation with IL-10 at day six, while at day eleven there were non-sig negative correlations with IL1-β and TNF α and statistically non- significant positive correlate with 1L-10 as shown in table (3.9).

Table 3.9 Clinical and immunohistochemical scores correlation among study groups.

Study groups	**Variable**	**Clinical scores at day six**	**Clinical scores at day eleven**
G1	IL-1β	R=0.653 P=0.051	R= 0.432 P=0.010
	TNF-α	R= 0.413 P=0.012	R=0.327 P=0.051
	IL-10	R=0.317 P=0.083	R=-0.436 P=0.81
G2	IL-1β	R=0.317 P=0.095	R=-0.425 P=0.02
	TNF-α	R=0.317 P=0.083	R=-0.012 P=0.013
	IL-10	R=0.317 P=0.072	R=-0.654 P=0.033
G3	IL-1β	R=0.248 P=0.079	R=-0.361 P=0.049

	TNF-α	R=0.384 P=0.028	R=-0.110 P=0.059
	IL-10	R=-0.153 P=0.061	R=0.842 P=0.003

Table (3.10): spearman correlation coefficients of the clinical and immunohistochemical scores in experimental induced oral mucositis among study groups.

G1 N (16)	**IL-1β**	**TNF α**	**IL-10**	**Clinical scores at day 6**	**Clinical scores at day 11**
IL-1β (Immunohistological scores)	R=1 P<0.001	R=0.277 P=0.023	R=0.218 P=0.417	R=0.582 P=0.040	R=-0.432 P=0.010
TNF α (Immunohistological scores)	R=0.277 P=0.023	R=1 P<0.001	R= 0.132 P= 0.507	R=-0.413 P=0.112	R=0.423 P=0.102
IL-10 Immunohistological scores)	R=0.218 P=0.417	R=0.132 P=0.507	R=1 P <0.001	R=-0.317 P=0.231	R=0.327 P=0.217
Clinical scoring At day six	R=0.182 P=0.500	R=0.413 P= 0.112	R=0.317 P=0.231	R=1 P<0.001	R=-0.171 P=0.502
Clinical scoring at day eleven	R=0.432 P=0.102	R=0.327 P=0.217	R=0.323 P=0.121	R=-0.171 P=0.502	R=1 P<0.001
G2 N (22)					
IL-1β Immunohistological scores)	R=1	R=0.223	R=-0.320	R=0.317	R=-0.425

	P<0.001	P=0.042	P=0.022	P=0.095	P=0.02
TNF α Immunohistological scores	R=0.223 P=0.042	R=1 P<0.001	R=-0.129 P=0.031	R=0.317 P=0.083	R=-0.012 P=0.013
IL-10 Immunohistological scores)	R=0.320 P=0.022	R=-0.129 P=0.031	R=1 P<0.001	R=-0.012 P=0.013	R=-0.654 P=0.033
Clinical Scores **At day six**	R=0.317 P=0.095	R=0.317 P=0.083	R=0.317 P=0.072	R=1 P<0.001	R=-0.204 P=0.048
Clinical scores at day eleven	R=0.425 P=0.02	R=-0.012 P=0.013	R=-0.654 P=0.033	R=-0.204 P=0.048	R=1 P<0.001
G3 N (22)					
IL-1β Immunohistological scores)	R=1 P<0.001	R=0.492 P=0.077	R=-0.122 P=0.364	R=0.248 P=0.079	R=-0.361 P=0.049
TNF-α Immunohistological scores)	R=0.492 P=0.077	R=1 P<0.001	R=-0.254 P=0.142	R=0.384 P=0.028	R=-0.110 P=0.059
IL-10 Immunohistological scores)	R=0.122 P=0.364	R=-0.254 P=0.142	R=1 P<0.001	R=-0.653 P=0.061	R=-0.842 P=0.003
Clinical scoring **At day six**	R=0.161 P=0.093	R=0.384 P=0.028	R=0.653 P=0.061	R=1 P<0.001	R=-0.055 P=0.0089
Clinical scoring at day eleven	R=0.361 P=0.049	R=0.110 P=0.059	R=-0.842 P=0.083	R=-0.055 P=0.0089	R=1 P<0.001

CHAPTER FOUR
DISCUSSION

4. Discussion

Mucositis is a major oncological problem. The entire gastrointestinal and genitourinary tract and also other mucosal surfaces can be affected in recipients of radiotherapy, and/or chemotherapy. Major progress has been made in recent years in understanding the mechanisms of oral and small intestinal mucositis, which appears to be more prominent than colonic damage. This progress is largely due to the development of representative laboratory animal models of mucositis ***(Vanhoecke et al.,2015).***

Methotrexate (MTX) is one of the antimitotic drugs. These drugs act by blocking deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis, inducing apoptosis and slowing down or stopping cell proliferation in rapidly proliferating cells such as tumors, bone marrow and gut mucosa cells ***(Soares et al.,2011).***

Although the effects of LLLT have been widely demonstrated, it is important to highlight that the parameters have been highly variable in laser therapy studies related to anti-inflammatory results, with a wide range of energy doses being used by different authors using different tissues ***(Darossa et al .,2012; Assis et al .,2012)***. Some studies have compared different parameters of laser therapy and different results in the same tissues and conditions have been observed ***(Medalha et al.,2012).***

The experimental OM model adopted in this study was performed according to the methodology proposed by earlier pilot study findings. The clinical signs resulting from CT observed in all animals treated, such as diarrhea, decreased food intake and water consumption with consequent weight loss confirmed standardization of the methodology.

Various pharmacological and nonpharmacological agents have tried in preventing and treating oral mucositis. Despite some positive outcomes, it
has not be proven to be completely effective in preventing oral mucositis on
its own. Till now, there are no single intervention acts on all phases of oral mucosotis ***(Bjordal et al., 2011).***

Low Level Laser Therapy (LLLT) is a local application of a nonchromatic. However, narrow-band coherent light source used for the photostimulation of biological tissue is recommended as a treatment options
for oral mucositis ***(Abramoff et al ., 2008; Bjordal et al ., 2011).***

Moreover, recent publication from the Multinational Association for Supportive Care in Cancer (MASCC) and the International Society of Oral Oncology (ISOO), recommended the administration of LLLT in patients receiving HSCT, conditioned with high dose chemotherapy with or without
total body irradiation ***(Lalla et al ., 2014; Oberoi et al ., 2014).***

From this point of view, the present study aimed to evaluate the impact of LLLT in the management of the experimentally induced oral mucositis.

This data showed statistically significant difference between the studied groups in terms of the clinical scores to the day eleven (the end of the experiment) .

Oral mucositis does not merely represent direct damage to epithelial cells, but is associated with a wide range of local tissue reactions including damage from reactive oxygen species, inflammatory cytokines, and damage to submucosal connective tissues and vasculature ***(Sonis,2004).***

In the present study, LLLT(30&60mw) that applied daily had a therapeutic effect on the chemo-induced oral mucositis in DA rats, as it was associated to low mucositis scores in both the clinical evaluation and immunohistochemical analysis (IL-1β&TNF-α, IL-10). These results are important, as the development of non-invasive effective treatment for the control of oral mucositis, that is of high priority in cancer patient care.

The clinical efficacy of LLLT can no longer be questioned and is supported by well-controlled evidence; however, the basic mechanisms by which LLLT produces the desired clinical effects in the oral mucosa remain unclear. In the present investigation, an animal model was used to broad our knowledge about the mechanism that involved in oral mucositis mediated by MTX at two different output power (30&60Mw).

4.1 The pilot study fitting tolerant dose of MTX in experimental rats:

These pilot study was designed in order to expose the optimum dosage of MTX that is well tolerated by dark agouti rats. One of the most extensively used models to investigate chemotherapy-induced mucositis is that employs the use of the female dark agouti rat. This model has been demonstrated to effectively parallel the development of mucositis that occurs in humans. Various cytotoxic chemotherapy agents have been investigated with this model including irinotecan, methotrexate and 5-FU ***(Logan 2009, Al-Azari et al., 2012).***

Since the main problem associated with thats rats model were suffered from high toxicity degree encompass of MTX dosages. Regarding these challenge, five rats were injected intraperitoneally with a dose of 80mg/kg MTX at base line time and monitored thoroughly, after MTX injection these animals suffered from less motility and appetite since rat chow

weighted daily. They have diarrhea, GIT bleeding to some degree and respiratory tract infection then they had died after four days could be due to diarrhea, severe dehydration, renal & liver toxicity might have speculated.

The other six rats were injected with 60mg/kg of MTX at base line time ,they had continues feeding and motility for seven days, then they had oral mucositis with varying degrees, so their feeding& drinking would be less than daily consumption range after seven days from the evolution of oral mucositis their health began to deteriorate could be due to diarrhea or anemia.

The last five rats were injected with 40mg/kg at base line time also they had no signs or symptom only less motile for few days and then return to their health with the same feeding rate and not induce any obvious clinical sign of oral mucositis. The present study revealed the superiority of 60mg/kg among 80mg/kg,40mg/kg. from the clinical point of view, oral mucositis induced at this dose and showed lower mortality rate with quite precise span of life that might thoroughly monitored for the clinical and histological collection of data. Since some of the limiting factors associated with the cytotoxic drug that used in this experiment are higher GIT toxicity, difficulty in attaining homogenous exposure and the need for sophisticated instruments. in spite of these limiting factors we use it since their availability and they can induce oral mucositis with varying degrees ***(Bowen et al., 2011).***

The results published by sultan et al. showed that the animals that received 10 mg/kg, 20 mg/kg, and 40 mg/k of MTX, there were increase in the thickness of the epithelium and the number of sub epithelial inflammatory cells and congested blood vessels, so that the present results could be proved regarding absence of clinical oral mucosal changes at 40mg/kg of, MTX ***(Sultan et al.,2014).*** The differences between the

present results and other results in the methodology used prevent a direct comparison between them.

The present work coincided with the results that obtained from Lotfy & Zayed,2009 have showed histological view of the buccal mucosa of treated rats by 80 mg/kg MTX showed a significant decrease in the thickness of the epithelium. The direct inhibitory effects of chemotherapy on DNA replication and mucosal cellular proliferation result in reduction in the renewal capacity of the basal epithelium. In the initial phase, the chemotherapy causes the release of cytokines from the epithelium and the connective tissues which initiated an inflammatory response that may result in increased sub epithelial vascularity ***(Naidu et al.,2004).***

4.2 Experimental induction of oral mucositis with MTX dose 60mg/kg among study groups.

MTX had serious effects on quality of patient's life once used in high dosage

(Sugita et al.,2012). Since that their experimental contribution should be strictly adjusted in order to obtain meaningful results. the present study used 60mg/kg for induction of oral mucositis as tested by preceded pilot study results.

Oral mucositis is a common side effect of methotrexate therapy (Yasbella et al.,2015). When MTX is used in high dose, it has a substantial clinical impact on patient's quality of life ***(Barasch& Epistein ,2011).*** Alternately, dose modification or discontinuation of the treatment, would likely cause an extended stay in hospital ***(Sugita et al.,2012).***

Rat's keratinized stratified squamous oral epithelium, with highest mean epithelial thickness and columnar shaped basal cells of control group,

served as a standard condition to compare mucosae of experimental animals under intervention conditions ***(Soleimani et al.,2011).***

The present study illustrated the predominant ulcerative lesions (62.2%,68.2%,40.9%) in combined with erythematous lesions (25%,25%,40.2%) among the study group at day six. The experimental study of Ahmed &Kazi,2016 have showed moderate to severe inflammation with predominant macrophages along with neutrophils and lymphocytes. Ulcers were also seen in few animals as a corroborating support for damaged oral epithelium. Jenson et al .2008 made similar findings upon clinical observation and reported that oral mucositis was present in 44% of the patients as a sequel of chemotherapy ***(Jensen et al .,2008).***

Stratified squamous epithelium of experimental rats, who took MTX, showed atrophy along with flattening or shortening of rete ridge, something not visible in control group since the epithelial thickness was much higher there along with intact multilayered keratinocytes. Decreased epithelial thickness is attributed to reduction in proliferative potential of epithelium by methotrexate ***(Munaretto et al .,2011).***

Munaretto et al.,2011 reported similar findings where mice were immune suppressed with the sub-cutaneous injections of 2.5 mg/kg MTX for 3 consecutive days. The epithelial thickness of the tongue mucosa decreased as duration of study increased, however, inflammation and ulceration were not found in their study ***(Munaretto et al.,2011).***

4.3 Body weight analysis among the study groups.

Weight loss could be an indicator of discomfort and pain while eating and drinking ***(Sonis et al.,1997; Franca et al.,2009).*** Thus, the weight of the animals was determined at baseline time, day six and day eleven among all study groups. The greater weight losses were expected in the control

group. This might have occurred since the animals in this group were excluded from daily irradiation by LLLT of their oral mucosa.

It was possible to describe the overall health status of the animals via daily measurement of their body weight. Animals that received Chemotherapy for several days and after 60mg/kg injection of MTX showed less resistance to handling and were clearly debilitated, causing impairment to their feeding abilities. The clinical evolution of oral mucositis.

This study support ***(Franca et al.,2009; Lopez et al.,2013; Campos et al.,2013)*** in their literature whose reported an increasing body weight loss in animals that received chemotherapy. However, these authors relate this phenomenon first to feeding impairment and second to oral mucositis. It has been observed in that study even with improved oral mucositis severity, animals of the experimental groups continued to lose body weight and their food intake did not increase. Thus, it can be assumed that this persistent clinical deterioration may be secondary to damaging effects of Chemotherapy drugs.

In contrast to that debated thoughts, the present study was indicated significant body weight loss in respect with control group in comparison with two LLLT treated groups.

The current study groups didn't reveal significant weight loss differences among them in the same measured period. This study coincided with Lopez et al .,2013. Likewise, it was observed in this study that even with improved OM severity, animals of the experimental groups continued to lose body weight. Thus, it can be assumed that this persistent clinical deterioration may be secondary to damaging effects of chemotherapy drugs. This probably excessive manipulation could have caused greater stress, which may also explain the weight losses observed in the animals of these groups. ***(Cruz et al .,2015).***

This results were reject, these hypotheses since the two laser groups regain some of their weights at the end of the experiment.

Other clinical signs linked to oral mucositis were observed in this study. Eating difficulties and weight loss are common in irradiated patients and can be related to pain caused by oral mucositis. ***Currza et al.,2015*** observed weight loss exclusively in 7- day groups. In these animals, there was also tongue-limited movement, which was not seen in the animals evaluated at 5 days. This finding suggests that situation was crucial for weight loss, and this was not influenced by the presence or absence of lesion or even use of the test products, but by the time after irradiation ***(Currza et al.,2015).***

4.4 Clinical evaluations of LLLT on mucositis outcome among groups:

LLLT using the visible red spectrum has been found to reduce the severity of oral mucositis lesions as well as pain scores ***(Schubert et al.,2007).***

Furthermore ***Cruz et al. (2007)*** reported that the LLLT appears to be a simple, non-traumatic technique for the prevention and treatment of radiation induced mucositis.

The ability of different molecules (and thus tissues) to absorb LLL energy is known to be dependent on wavelength. Consequently, the shorter wavelengths (632–660 nm) have been shown to deposit most of their energy in the superficial layers of irradiated tissues, while longer wavelengths (780–901 nm) will penetrate much deeper. This characteristic does not appear to be merely a function of absorption. Thus, the shorter wavelength lasers (632–660 nm) would be predicted to more effective in preventing mucositis than the longer wavelength lasers (over 780 nm), and

this was shown by this study regarding 660nm (30mw,60mwLLLT). ***(Cowen et al .,2010).***

For these reasons, a laser emitting in the visible red (660 nm) range was used in the present study, one protocol and two different out power (30mw,60mw) to test possible therapeutic effects. This study investigated clinical features, including the animals weight, performed a clinical analysis of the oral mucosa in which mucositis was induced by MTX followed by local trauma, and also evaluated the tissue proinflammatory and anti-inflammatory cytokines of the oral lesion at the end of the experimental period.

A meta-analysis by ***Worthington et al., 2011*** showed significant beneficial evidence for prevention or reduction of oral mucositis in cancer treatment from cryotherapy, keratinocyte growth factor and sucralfate. Interestingly,

these did not include LLLT as a treatment tool fighting out for mucositis. The present study provides strong evidence for the utility of LLLT as a rescuing agent in treatment of oral mucositis ***(Worthington et al., 2011).***

LLLT has effects only on stressed or diseased tissues. This therapy is able to restore the regular metabolic potential of stressed cells (Marques et al.,2004). With this in mind, it could be posited that the initial irradiation on an already stressed tissue as occurred in the treated group may have led to a prompt response to the LPT, whereas the tissue that started to be irradiated when it was in a regular metabolic condition could have been inhibited using a relatively high dose of daily irradiation ***(Lopez et al.,2013).***

The findings of the present study also demonstrate the positive effect of LPT in reducing the severity of mucositis when this therapeutic protocol was used. On Day 11, following MTX injection, when an increase in the clinical and histological scores was expected, this group reduced its initial

scores, which were significantly lower than those of all the other groups at the same period and in comparison, with other their scores at day ***(Lopez et al.,2013).***

In other studies, this result was observed only on Day 15, when, in fact, it was already expected, because by this time, i.e., after the conclusion of chemotherapy, the oral mucositis would be self-resolved. (Lopez 2009,2010) Thus, our study revealed something new.

However lower output power (30mw) exhibited significant positive therapeutic effects with less clinical scores than 60mw output power study group.

In respect with animals that irradiated with laser 60mw group GaAlP, diode laser at 660nm wavelength has been used from six till eleven day. the present study expressed statistically significant decrease in clinical scores of experimental O.M at day eleven with 22.7% score zero (p=0.048).

Similarly, other studies reported the use of high doses of radiation for this purpose, but such radiation has better effects in promoting analgesia (Simoes et al .,2009; Freitas et al .,2014). Regarding tissue repair, the use of high doses of radiation is related to an apparent delay of the process ***(Corazza et la.,2007; Lopes et al .,2009).***

In contrast, Lara et al (2007) concluded that application of LLLT (GaAIAs) on animal model (rats) of oral mucositis, showed that GaAIAs was not effective in comparison to topical dexamethasone.

Unlike the 35mW laser, the 100mW laser did not have an effect on the severity of clinical mucositis, indicating a more pronounced anti-inflammatory effect of the low level laser treatment, through inhibition of COX-2. This reinforces the concept that the laser-use parameters are of critical importance. In particular, the total time for which irradiation is done could be of importance. During irradiation in vivo, in addition to the

local area being targeted, the blood that circulates in this area during exposure also receives irradiation, and the amount of irradiated blood is proportional to the time of irradiation ***(Lopez 2009).***

Dissimilar findings by Schubert et al.,2007 whose used treatment parameters, output powers in the range of 50–100 mW would seem to be the most practical to provide appropriate fluency and clinically practical treatment times. The ideal treatment period and timing of LLLT treatment. The current study looked to extend the total treatment period by providing laser therapy starting on the sixth day of experiment and continuing daily with the total number of treatments six days at all ***(Schubert et al.,2007).***

Group with LLLT 30MW maintained lowest degrees of injury at the end of the experimental period. However, it had milder degree of OM when compared with both laser 60mw and non-laser treated group, indicating that this output power may be the best option, nevertheless it could be an alternative in the OM treatment. Similarly***, Campos et al.,2016*** concluded the same thing.

The interaction of laser radiation with biological systems probably occurs at the cellular level, but the mechanisms involved are still unknown ***(Aimbire et al., 2006; Convissar, 2011).*** It has been reported that tissue absorbs certain amount of laser radiation per volume and transforms it into a certain amount of energy, the amount of energy absorbed depends on the exposure time used, the type of tissue irradiated and the wavelength of the laser ***(Mortiz et al., 1997; Mahmud and Arif, 2000; Nussbaum et al., 2002; Maver-Biscanin, 2004; 2005; de Souza et al., 2006; Pereira et al., 2011; Kazem and Maki, 2013; Rossoni et***

al., 2014). The choice of wavelengths and dose in this study was pragmatically
based on the range commonly used by similar researches, that is, red 660 nm
(Wilson and Mia, 1993; Nussbaum et al., 2002; Pereira et al., 2011; Kazem and Maki, 2013). Response to laser is affected by exposure period and energy density
(Wilson and Mia, 1993; Kazem and Maki, 2013).

4.5 Immunohistochemical analysis of (IL1BETA, TNF-α &IL-10) among study group.

4.5.1 proinflamatory cytokines IL-1BETA and TNF -α expression among study groups.

There has been substantial literature published on various management strategies to treat oral mucositis [11, 14, 22, 78, 88, 101, 104]. However there has been a paucity of data reporting the mechanistic aspects of the development or pathobiology of oral mucositis, particularly in the clinical setting. Logan study examined the expression of IL-1β&TNF-α in the rat oral mucosa following cytotoxic chemotherapy. Although preliminary, also
demonstrated that NF-κB and COX-2 were elevated following chemotherapy even when histologically, there appeared to be little difference between the pre- and post-chemotherapy appearance of the tissue.

Cytokines are regulatory proteins produced by immune cells and other

cells

of the body. Cytokines may exert proinflammatory and anti-inflammatory effects. The abnormalities of various cytokines may reflect the imbalance among different immune cell subsets contributing to pathogenesis of disease ***(Dongari-Bagtzoglou and Fidel, 2005, Schroeder, 2014).***

The cytokines investigated in this study were chosen based on the fact that they represent important members of proinflammatory (IL-1β&TNF-α) and anti-inflammatory cytokines (IL-10).

In recent years, it has been demonstrated that various cytokines, with a strong emphasis on pro-inflammatory cytokines, play key roles in the pathogenesis of mucositis. Studies have clearly shown that elevated serum levels of pro-inflammatory cytokines, in particular TNF-α, IL-1β and IL-6 are excellent markers of the inflammatory response induced by various chemotherapeutic agents with elevated levels generally occurring after histopathological changes.

The exception was seen with 5FU where serum pro-inflammatory cytokine levels peaked prior to histopathological damage (Logan et al.,2008). Based on these findings, Logan and colleagues determined that serum pro-inflammatory cytokines were not effective biomarkers of regimen-related toxicities. They hypothesised that this was due to critical time constraints with detectable serum changes and histological damage ***(Logan et al .,2008; Gibson & Bowen et al .,2011).***

It has been definitely shown that MTX therapy causes oral and intestinal mucositis in patients. Rats as well, are suitable models for the MTX-induced gastrointestinal mucositis (GIM) ***(Howarth et al ., 1996).*** In this present study, diarrhea was observed in all of the study groups that underwent MTX therapy ***(Kaynar et al .,2012).***

The anti-inflammatory efficacy of LLLT has been controversial. While some searches have not found any effect from LLLT on inflammation,

some findings showed that TNF-α decreased ***(Yamaura et al ., 2009;Fukuda et al., 2012, Aimbire et al ., 2006, Pezelj-Ribaric et al ., 2013),*** but not change the level of IL-6 ***(Yamaura et al ., 2009; Fukuda et al ., 2012).*** However, others showed increase in IL-6 accompanied with increasing TNF-α ***(Silva et al ., 2015).***

Pezelj-Ribaric et al ., 2013 used the same parameters of ***Simunovic-Soskic***

et al study and dosimetry in treatment of burning mouth syndrome and the study reported the same results of highly significant decrease of salivary IL-6 level. The significant reduction of IL-6 was shown in ***Oton-Leite et al ., 2015*** study, that used 25 mW power output, 660 nm laser for 35 sessions for treatment of oral mucositis. The controversy of this study with others is probably related to the critical dose of LLLT that appears reducing proinflammatory cytokines,

related by lower levels of IL-6 would suggest less damage to the oral mucosa

(Aimbire et al ., 2006; Bjordal et al ., 2006; Boschi et al ., 2008; Convissar, 2011;Oton-Leite et al ., 2015).

Singh et al., 1991 looked at the toxicity profiles of all the disease modifying anti-rheumatics and found the methotrexate group gave the highest levels of mucosal ulceration, 87 events per 1,000 patient years (mean dos 10.7 mg per week). The level of IL-1β&TNF-α showed statistically significant increase in the oral mucosal of animals not receiving laser, compared with LG groups; however, these parameters were lower in oral mucosal lesion of LLLT (30,60mW) groups.

Bowen et al 2005 have been showed one of the most extensively used models to investigate chemotherapy-induced mucositis is that that employs the use of the female Dark Agouti rat. This model has been demonstrated

to effectively parallel the development of mucositis that occurs in humans ***(Bowen et al .,2005,2007; Howarth et al .,2006).***

The current study used MTX with 60mg/kg as induction chemotherapy for experimental oral mucositis with less mortality rate than the other doses of MTX as proofed by the preceded pilot study, with varying clinical scores range from (0-3) as follow: Control MTX non-laser group score I (12.5%), score II 25% and score III 62.5%, laser 30mw treated group 27.3% score I, 54.5% score II and score III 18.2%. laser 60 Mw

Inflammatory cytokines have been considered to play a critical role in the development of mucositis induced by chemotherapy and radiotherapy ***(Ong et al .,2010; Sonis,2011; Sultani et al .,2102).*** Particularly, TNFα, IL-1β, and IL-6 (Long et al .,2007; Ong et al .,2010). have been implicated in mucositis and have been the targets of inhibition. IL-1β is responsible for mucositis induced by the gut specific deletion of β -transducin repeat-containing protein, an E3 ubiquitin ligase. ***(Kanarek e al.,2014)*** Noticeably, IL-1β is derived from epithelial cells rather than from inflammatory cells after DNA damage via an unknown mechanism, and the secreted IL-1β causes mucositis by disrupting epithelial tight junctions. ***(Kanarek e al.,2014).***

However, ***Kanarek e al.,2014*** study demonstrated that NF-κB activation is not involved in mucositis. The secreted IL-1β following DNA damage induces a mucosal barrier breach in an NF-κB-independent manner. Moreover, the tissue damage caused by mucosal barrier disruption is exacerbated in the absence of NF-κB, because of failure to express the endogenous IL-1β receptor antagonist IL-1RA, and thereby NF-κB inhibition exacerbates mucositis rather than inhibits the source of inflammation. Therefore, it needs to be reexamined whether NF-κB mediates mucositis induced by chemotherapy and radiation or whether NF-κB-related gene expression merely coincides with the mucositis

phenomenon. The role of NF-κB in mucositis needs to be further elucidated in the future. ***(Kanarek e al.,2014).***

Th current study were showed statically significant increased tissue expression of IL1 β& TNF-α in the control group in comparison with the two laser treated groups. That were coincided with the previous studies.

Logan et al.,2007demonstrated that NF-κB, thought to be a key driver of mucositis, and pro-inflammatory cytokines (TNF-α, IL-1β and IL- 6) were expressed in different sites along the alimentary tract following the administration of irinotecan in rats and this coincided with histological evidence of tissue damage at early time points. These observations were supported the hypothesised role of NF-κB activation and subsequent elevation of tissue levels of TNF-**α**, IL-1β and IL-6 prior to clinical evidence of mucositis manifesting.

Logan et al .,2008, proinflammatory cytokine protein levels (TNF-**α**, IL-1β) in the epithelium throughout the gastrointestinal tract were upregulated after chemotherapy. However, another study demonstrated an increase in the protein expression of IL- β in the oral submucosa and not in the epithelium following radiotherapy ***(Sonis et al .,2000).***

Ong et al .,2010, study was concluded the novel fractionated radiotherapy induced mucositis model has allowed the characterization of pro-inflammatory cytokines IL-1BETA, IL-6 and TNF in the jejunum and colon of the rats following radiotherapy, thus confirming the importance of these cytokines in the development of mucositis. The present study provides additional support that may illustrated the crucial role of IL1 BETA and TNF alpha in understanding the evolution of experimental oral mucositis.

Also, in the context of mucositis following MTX administration, production of TNF by mucosal T cells and macrophages is increased in response to LLLT derived from commensal gut flora. This indicates that

the immune cells within the mucosa may, in themselves, contribute to mucositis development (Koning.,2006).

The current results were showed statistically significant ($p<0.001$) decreases of IL1 BETA and TNF alpha expression in both laser treated groups (30mw &60mW) output as compared with the control group (non-treated) .

The photobiological effect of low level laser light on cells and tissues depends on the cell type and wavelength of light source. At low radiation dose photoreceptors propagate cellular responses will be activated. The light will be absorbed by endogenous chromophores such as porphyrins and cytochromes (Hwang et al .,2010). Regarding the pro-inflammatory cytokines TNF-α, IL-1β, the LLLT was efficient in reducing almost all in groups of animals challenged with collagenase ***(Oliveira et al 2011).***

He-Ne and Ga-Al-As lasers (LLLT) were used to stimulate mitochondrial membrane potential (MMP) ***(Moussa et al .,2012).*** The irradiated cells were found to have a higher mitochondrial activity than nonirradiated cells. This may be due to enhance cells size which provides a higher respiratory demand at cellular level translated into higher activity of mitochondria. This results were agree with previous findings that found a positive relation between cell size and mitochondrial activity ***(Al-Rubeai,2009).*** From these hypothesis, we can support our data about the regression of proinflammatory cytokines between two laser treated groups (30mw,60mw).

After statistical analysis, the Campos et al .,2016 results showed LLLT therapy were efficient treatment for oral mucositis, decreasing TNF-α concentration on day 7 ($p < 0.05$) and completely healing the mucosa on day 10. ***(Campos et al .,2016).***

4.5.2 Anti-inflamatory cytokine (IL-10) analysis among study groups.

IL-10 is the important cytokine with anti-inflammatory properties. In monocytes and macrophages, IL-10 diminishes the production of inflammatory mediators and inhibits antigen presentation, although it enhances their uptake of antigens ***(Sabat et al .,2010)***. This study clarified that both laser treated groups (30mw,60mw) showed significantly increased tissue levels of IL-10 in contrast with their expression in the control group. may be due to significant reduction of IL-1 β and TNF-α tissue levels.

IL-10 is an anti-inflammatory cytokine that inhibits the release of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) from monocytes/macrophages, thus preventing subsequent tissue damage ***(****Pajkrtet al.,1997)****.***

The current study contra directed findings by ALazzawy & Ahmed,2016 that showed, salivary IL-10 was slightly reduced after laser irradiation, but the result did not reach statistical significance between baseline and after day 8 of treatment (after 5 doses of LLLT). It was coincided with Oton-Leite *et al.,* 2015.and Silva *et al.,* 2015 that used 660 nm LLLL in treatment of oral mucositis for seven sessions continuously in comparison to six sessions for the current study. Other study used the same wave length (660) nm in skeletal muscles that concluded rising in IL-10 concentration ***(Hentschke et al., 2011).***

Oliveira et al., 2013 and Boschi et al., 2008 also dissimilar by their findings that local IL-10 was reduced after laser irradiation with the same 660nm.

De lima et al.,2014 findings highlighted the potential importance of the imbalance between proinflammatory and anti-inflammatory cytokines in

acute lung inflammation, which is corroborated by the ratio of IL-10 and TNF-α in the lung ***(De lima et al.,2014)***. This results showed the trend of LLLT significantly induced an increased in the tissue expression of IL-10 in animals subjected to L660nm (30mw,60mw) reinforcing its anti-inflammatory role.

These results are in agreement with those reported by Souza and Teixeira, which evidenced that LLLT increases the levels of IL-10 in animals submitted to LLLT ***(Souza and Teixeira,2005).***

Similarly adopted notion by Xavier et al.,2010 also said that LLLT acts as anti-inflammatory mediator by reducing the classical features of tendinitis by increasing the IL-10 concentration in inflamed tissue ***(Xavier et al.,2010).***

Sultani et al .,2012 concluded that a huge gap in the knowledge to recognize whether anti-inflammatory cytokines such as IL-4, IL-10 are essential tools in downregulating the inflammatory response associated with mucositis. Lack of this knowledge which ties pro- and anti-inflammatory cytokines together within the complex yet interesting cytokine milieu leaves an incomplete image of immune response associated with mucositis ***(Sultani et al .,2012).***

Since this data showed significant negative correlation of IL-10 with both IL1-β and TNF-α among study groups exclusively in two laser treated groups, thus we could add a new evidence about the crucial anti-inflammatory role of IL-10 in the development of experimental oral mucositis.

Furthermore, there is no evidence in literature that interprets the net balance of the subclass of cytokines in accordance with different phases of mucositis development. Moreover, the underlying mechanisms of action of that anti-inflammatory cytokine in chemotherapy induced mucositis remain under researched ***(Sultani et al .,2012).***

Obviously, there are limitations in this study in that it is not longitudinal; however, a true longitudinal study using serial biopsies from the same rat taken at different time points is not practical and would pose increased risk of mortality from procedures and compromise results.

4.6 Low level laser therapy parameters used for treatment of the experimental oral mucosaitis.

Phototherapy with LLL is used in numerous areas of life sciences to encourage tissue revival of injured tissues (*Walsh, 1997)*. This treatment modality produce pain-relieving, anti-inflammatory and biomodulatory effects ***(Reddy 2004; Silveria et al 2007 and Barros et al 2008)***. The laser light within the red visible and near infrared wavelengths matches to the energy absorption spectrum of the respiratory chain components, increasing the cellular metabolism under stress conditions ***(Hawkins and Abrahamse 2006 and Silveria et al 2007).***

The selection for the wavelength was up on the target tissue – oral mucosa. It is accepted and proven that penetration depth is a wavelength dependent property. Higher wavelengths are more resistant to dispersion than lower ones and deeply penetrate the skin ***(Kolárová et al 1999).*** It has been reported that 632.8 nm laser light penetrates 0.5-1 mm before losing 37% of its intensity. On the other hand, infrared wavelengths penetrate 2 mm before losing the same percentile of energy ***(Basford 1995).*** As a result, visible red laser light (630 nm) is indicated for superficial lesions while infrared laser light (830 nm) is used for deeper tissues ***(Brugnera et al 1991; Genovese 2000 and Enwemeka 2003)*** and thus it wasn't used.

Visible lasers have been the most widely used for wound healing, but, the development of low costs diode lasers have provided a new option for treatment of these wounds. Previous studies showed differences of effects between close wavelengths. ***Al-Watban et al .,2001*** observed the effects of different wavelengths on the healing process and evaluated the transmission of the laser light throughout the skin, suggesting that 632 nm laser light (20 J/cm2) was more effective than the other wavelengths used and the increase of transmission of the laser light throughout the skin is not related to biomodulation.

These preliminary results indicate that LLLT improved cutaneous wound repair and that the effect is a result of an inversely proportional relationship between wavelength and intensity. The treatment is more effective combining higher intensity with short wavelength or lower intensity with higher wavelength

(Do Nascimento et al.,2004).

Increasing intensity to 15 mW resulted in more intense neovascularization than their controls since this aspect is strongly associated with intense fibroblastic proliferation. This result represents a positive effect of LLLT on endothelial cells and increased release of several mediators of cellular proliferation. These results also confirmed previous reports that lower wavelengths have stronger effects on both collagen deposition and distribution as the presence of a connective tissue rich on collagen fibers was markedly present when 670 nm laser light was used ***(Do Nascimento et al .,2004).***

The current study used two out power 30 mw& 60mw in order to differentiate their treatment efficacy. Regarding, these parameters from the above data. LLLT 660nm with 30mw produce statistically significant clinical improvement of the oral mucositis outcome scores, in comparison with non-treated and LLLT 660nm with 60mw treated group. On other

hand the 60mw can depressed the severity of these oral mucositis score but in less percentage.

In concern to histological changes the output 30mw yields significant reduction of proinflammatory cytokines (IL-1β &TNF-α). Histological findings in the biopsies that analyzed.

Based on the conditions of ***(Ghaleb,2016),*** they concluded that the low-level laser inhibit the apoptotic process and increase mitochondrial membrane potential (MMP) ***(Ghaleb,2016).***

Rodrı´guez-Caballero et al ., 2012 were published their conclusion, to date, no intervention has been able to prevent and treat oral mucositis on its own. It seems necessary to combine interventions that act on the different phases of mucositis. There are currently an alarming number of treatments, but there is no gold-standard protocol that is prominently better than the rest ***(Rodrı´guez &Caballero et al ., 2012).***

CHAPTER FIVE
CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions:

1- MTX at 60 mg/kg can be used in experimental induction of oral mucositis in dark agouti rat with minimal mortality rate and representative clinical grading of oral lesions that may synonymous human oral mucositis.

2- LLLT 660nm (30 & 60 mW) can be used for treatment of experimental oral mucositis with 10J dose intensity, with hiegher therapeutic effects on power 30mW.

3- The present immunohistochemical study reveals an increase in pro-inflammatory cytokines with the development of mucositis in the control non-treated group, while level of the pro-inflammatory cytokines decreased in laser treated groups (30&60mW).

5.2 Suggestions:

1- Further *in vivo* studies involving this animal model with specific oxidative stress and inflammatory biomarkers analysis to uncover pathophysiology & the benefcial effects of LLLT in controlling oral mucositis.
2- Clinical applications of LLLT in Iraqi oncologic centers for treatment of oral mucositis as a safe & costless photomedicine that proved and used in the world oncologic centers.
3- Clinical randomised controlled study for establishment of the perfect laser protocol parameters (wave length, output power& dose intensity) that can be standard for supportive cancer care usage.
4- In view of this study findings, author believe that the results obtained in this study can also be reproduced in humans, but detailed and tightly controlled methods should be used to avoid possible biases interfering with the results.
5- More extensive clinical &immunological analysis of tissue and serum cytokines as a biomarkers before and after LLLT with the same laser treatment parameters.
6- Repeat this study with a larger samples size of animals for tissue collection at variable duration before and after LLLT irradiation in comparison with the control group (non -irradiated animals).
7- Randomized, double-blinded, clinical trials involving humans, including a sufficient number of patients, should be carried out to reach clinically relevant conclusions regarding the use of LLLT 660nm (30&60mw) for oral mucositis management.

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Appendix A

Primary antibodies manufacturer's datasheets

Elabscience®
www.elabscience.com

Elabscience Biotechnology Co.,Ltd

DATASHEET

Product Name:	IL-1 β Polyclonal Antibody
Catalog No:	ENT2322
Source:	Rabbit
Concentration:	1mg/ml
Synonyms:	IL1B; IL1F2; Interleukin-1 beta; IL-1 beta; Catabolin
Purify:	Antigen affinity purified
Buffer:	PBS with 0.02% sodium azide, 50% glycerol, pH7.3.
Storage:	Store at -20℃ or -80℃ . Avoid freeze / thaw cycles.

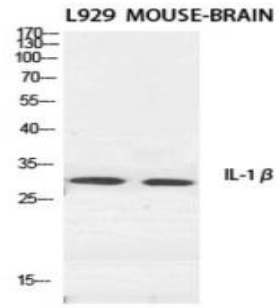
Applications

Tested Applications:	WB,IHC,ELISA
Specificity Reaction:	Human,Mouse,Rat
Recommended Dilution:	WB 1:500-1:2000,IHC 1:50-1:200,ELISA 1:5000-1:20000

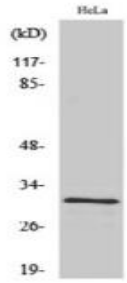
Immunogen Information

Immunogen:	Synthesized peptide derived from the Internal region of human IL-1 .
Calculated MW:	31 kDa
Observed MW:	31 kDa

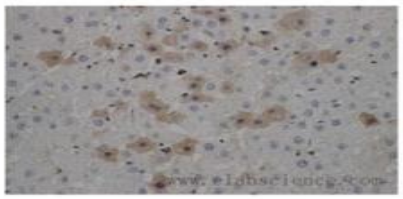
Images



Western Blot analysis of L929 MOUSE-BRAIN cells using IL-1β Polyclonal Antibody



Western Blot analysis of HeLa cells using IL-1β Polyclonal Antibody



Immunohistochemistry of paraffin-embedded mouse liver using IL-1 β antibody at dilution of 1:200

Tel:86-27-87805095
Fax:86-27-87645690
Email:antibody@elabscience.com

Elabscience
www.elabscience.com

Tel: 86-27-87385095 Fax: 86-27-87645690
Email: antibody@elabscience.com

DATASHEET

Product Name:	TNF-α Polyclonal Antibody
Catalog No:	ENT4689
Source:	Rabbit
IsoType:	IgG
Synonyms:	TNF; TNFA; TNFSF2; Tumor necrosis factor; Cachectin; TNF-alpha; Tumor necrosis factor ligand superfamily member 2; TNF-a
Purify:	Antigen affinity purified
Concentration:	1mg/mL
Buffer:	Liquid in PBS containing 50% glycerol, 0.5% BSA and 0.02% sodium azide.
Storage:	Store at -20℃. Avoid freeze / thaw cycles.

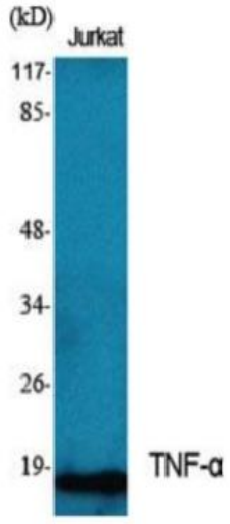
Applications

Tested Applications:	IHC,IF,ELISA
Specificity Reaction:	Human,Mouse,Rat
Recommended Dilution:	IHC 1: 50-1: 200, IF 1: 50-1: 100, ELISA 1: 5000-1: 20000

Immunogen Information

Immunogen:	Synthesized peptide derived from the Internal region of human TNF-α.
Calculated MW:	26 kDa
Observed MW:	16 kDa

Images



Western Blot analysis of Jurkat cells using
TNF-α Polyclonal Antibody

Elabscience
www.elabscience.com

Tel: 86-27-87385095 Fax: 86-27-87645690
Email: techelabscience@gmail.com

DATASHEET

Product Name:	IL10 Antibody
Catalog No:	EAP0908
Source:	Rabbit
IsoType:	IgG
Synonyms:	IL10; CSIF; GVHDS; IL-10; IL10A; TGIF; Interleukin-10; Cytokine synthesis inhibitory factor;
Purify:	Antigen affinity purified
Concentration:	1mg/mL
Buffer:	PBS with 0.1% Sodium Azide, 50% Glycerol, pH7.3.
Storage:	Store at -20℃ (regular) or -80℃ (long term). Avoid freeze / thaw cycles.

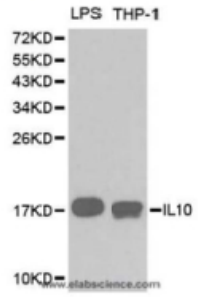
Applications

Tested Applications:	WB IHC
Specificity Reaction:	H M R
Recommended Dilution:	WB (1:500-1:2000); IHC (1:50-1:200); IF (1:50-1:200)

Immunogen Information

Immunogen:	Recombinant protein of human IL10
Calculated MW:	
Observed MW:	19kDa

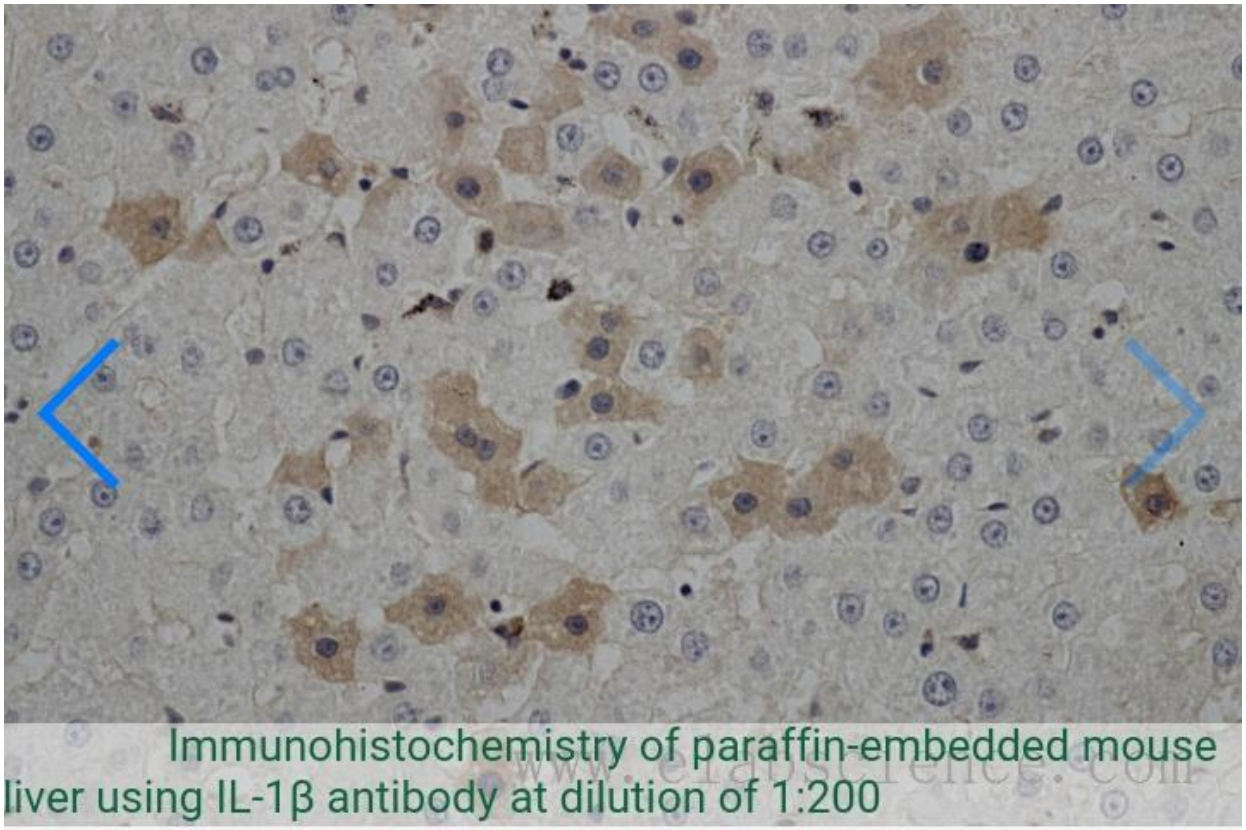
Images



Western blot analysis of extracts of various cell lines, using IL10 antibody.

Appendix B

Immunohistochemical analysis of paraffin impeded animals tissue.



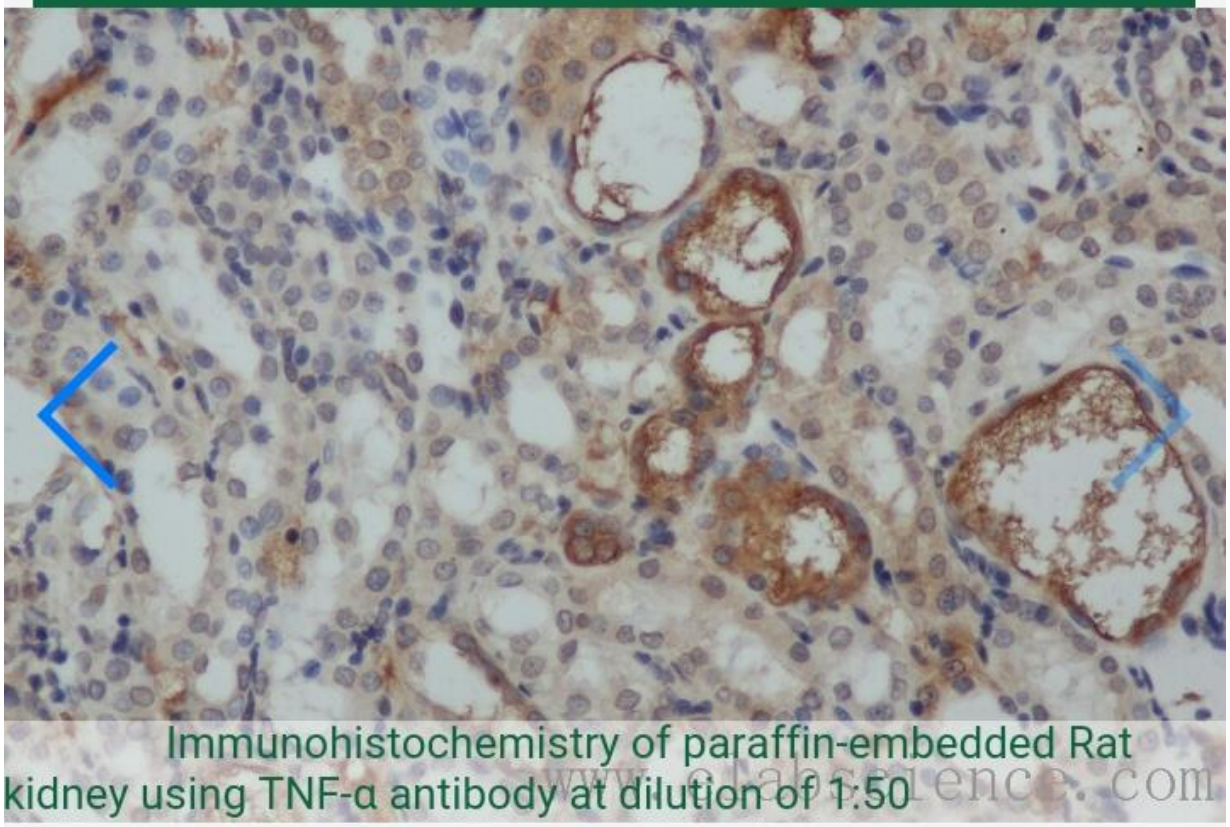
Immunohistochemistry of paraffin-embedded mouse liver using IL-1β antibody at dilution of 1:200

Cat.No

ENT2322

Reactivity:

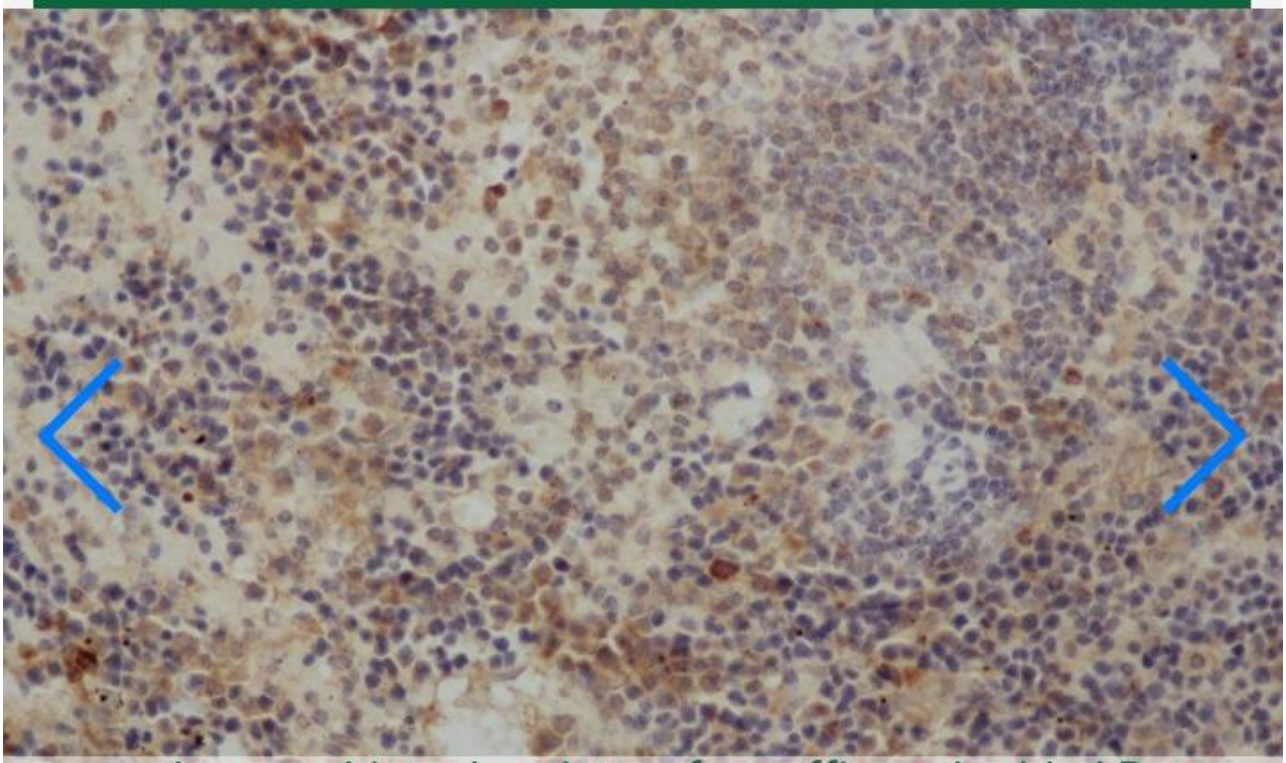
TNF-α Polyclonal Antibody



Immunohistochemistry of paraffin-embedded Rat kidney using TNF-α antibody at dilution of 1:50

Cat.No

IL10 Polyclonal Antibody



Immunohistochemistry of paraffin-embedded Rat spleen using IL10 antibody at dilution of 1:50

Cat.No

تاثيرات الليزر واطئ الطاقه على التهاب يطانة الفم بالحقن التجريبي بالعلاج الكيميائي،دراسه سريريه، ونسيجيه مناعيه

اطروحة مقدمة

إلى كلية طب الأسنان- جامعة بغداد
كجزء من متطلبات نيل درجة دكتوراه فلسفة في
في علم طب الفم

من قبل

بهاء الدين عبد الرحمن هادي
ماجستير طب الفم

إشراف
الأستاذ الدكتور
جمال نوري أحمد
ماجستير طب الفم/ دكتوراه طب الفم

١٤٣٨هـ

٢٠١٧م

المقدمه:

التهاب الغشاء المخاطي الفموي لا يزال من الآثار الجانبية الحادة شيوعا وشديدا من العديد من العلاجات الأورام، خاصة في المرضى الذين يعالجون من سرطان الرأس والعنق. قد تؤثر على نوعية الحياة، ويحتاجون إلى رعاية داعمة وتخطيط العلاج التأثير وفعاليته. حاليا، لا توجد علاجات التقليدية المتاحة لمنع التهاب الغشاء المخاطي للفم والعواقب على نظم الرعاية الصحية لا تزال واسعة النطاق .أحرز تقدما كبيرا في السنوات الحالية في فهم آليات التهاب الغشاء المخاطي الفموي، الذي يبدو أنه أكثر إسقاط من الضرر القولون. ومن المقرر أساسا لتقدم نماذج حيوانية مختبر تمثيلية من التهاب الغشاء المخاطي هذا التطور. يبدو العلاج بالليزر منخفض المستوى لتعزيز تخفيف الآلام ويقلل من حدوث

التهاب الغشاء المخاطي للفم وحدته.

أهداف الدراسة:

الحث التجريبي للالتهاب الغشاء المخاطي الفموي من علاج الميثوتركزيت السام للخلايا.التقييم السريري لالتهاب الغشاء المخاطي الفموي لعلاج الليزرواطيء الشده٣٠ ،٦٠ مللي واط معاملة، مقارنة بمجموعه غيره معالجه بالليزر (مجموعةالسيطره).التقييم النسيجي المناعى للمتغيرات (TNF- α)، IL-1 β، (IL-10 بين مجموعتي العلاج

الليزرواطيء الشده٣٠ ،٦٠ مللي واط ومجموعة السيطرة غير المعالجة.

المواد وطرق العمل:

تم استخدام 60 جرذان داكنة أغوطي، وزنها 220-280 غم، وتم الاحتفاظ بالحيوانات تحت ظروف المختبر القياسية. وقد استخدمت ستة عشر الفئران في الدراسة التجريبية للعثور على

الجرعة السمية القصوى من متكس. تم تقسيم 60 من فئران أغوتي الداكنه إلى ثلاث مجموعات تم حقنها داخل الصفاق مع 30ملغم / كغ في (0 و 3) أيام: المجموعة الأولى مع علاج متكس (مجموعة السيطرة) (16) الفئران مع عدم وجود علاج بالليزر. المجموعة الثانية مع متكس (22) الفئران تم تضمينها ومعالجتها مع 30 ميجا واط (لت 30 ميجاواط) ليزر واطيء الطاقه، كثافة الطاقة 10 جل / سم 2، ليزر ديود في 660 نانومتر، وضع مدببة مستمرة يوميا من يوم 6 حتى اليوم أحد عشر لمدة 5 جلسات. إي مع متكس (22) الفئران وشملت ومعالجتها مع 60ملواط. في يوم أحد عشر جميع الحيوانات التضحية وخزعة المخاطية الشدق أخذت من كل حيوان لتحليل المناعية من السيتوكينات المدروسة (IL-1β,TNF-α,IL-10).

النتائج:

كشفت هذه الدراسة الهامة فقدان وزن الجسم بين مجموعات الدراسة في (0،6 و 11) يوما من القياسات وإنما كانت هناك فروق ذات دلالة إحصائية بين مجموعات الدراسة في الفترة نفسها من التقييم. أظهرت التهاب الغشاء المخاطي عن طريق الفم التي يسببها تجريبيا الحد الأقصى التسامح في ٦٠ملغم / كغم في المجموعات الثلاث درس في اليوم السادس. لا توجد فروق ذات دلالة إحصائية بين مجموعات الدراسة (ع (0.05>في اليوم السادس .عشرات السريرية أظهرت فروق ذات دلالة إحصائية (ف (0.05>بين جميع مجموعات الدراسة في اليوم الحادي عشر. ومع ذلك، كل من المجموعات التجريبية G2) و (G3 أظهرت فروق ذات دلالة إحصائية عند مقارنة G1 المجموعة الضابطة .(P> 0.05) بالمقارنة مع عشرات من السريرية في اليوم السادس: وجود ارتباط ملموس السلبية (R = -0.055) ع =.(0.0089) الحكمة على الأرجح، كان هناك انخفاض ملحوظ إحصائيا من علامات سريرية بين مجموعات الدراسة بالمقارنة مع المقاييس الخاصة بهم في اليوم السادس. ان التعبير النسيجي لل IL1βو TNF- αأعربت زيادة ذات دلالة إحصائية من نتائجهم في المجموعة التي تلقت العلاج السيطرة غير الليزر بالمقارنة مع انخفاضها في كل من الليزر ٣٠ ،٦٠ مللي واط .المجموعات المعالجة. في حين أن التعبير النسيجي لل IL-10 كشف الصور المتناقضة بين مجموعة الدراسة. حيث انخفضت في السيطرة على المجموعة بالمقارنة مع مجاميع الليزر٣٠ ،٦٠ مللي واط .

الاستنتاج:

٦٠ ملغم / كغم من علاج الميثوتركزيت يمكن أن تستخدم في الحث التجريبية على التهاب الغشاء المخاطي الفموي في الفئران مع معدل وفيات بالحد الأدنىبالدرجات السريرية تمثيلية من الآفات الفموية التي يمكن أن تحاكي التهاب الغشاء المخاطي الفموي البشري يمكن أن نستخدم ,الليزر واطيء ٦٦٠نانومتر 30 الكفأه ميلي واط لعلاج التهاب الغشاء المخاطي الفموي تجريبية بجرعة. ١٠جول /سم ٢ليزر واطيء الشده ٦٦٠ نانومتر ، ١٠جول / سم ٢و ٦٠ ميغاواط يمكن استخدامها أيضا مع الآثار العلاجية أقل عرضة لعلاج التهاب الغشاء المخاطي للتنمية عن طريق الفم.