2018

Laser light interaction and their influence on DNA and cell under in vitro conditions

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LASER LIGHT INTERACTION AND THEIR INFLUENCE ON DNA AND CELL UNDER *IN VITRO* CONDITIONS

By

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A thesis submitted to Plymouth University
in partial fulfilment of the degree of

DOCTOR OF PHILOSOPHY

School of Computing, Electronics and Mathematics
Faculty of Science and Environment

April 2018

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Abstract

Laser photobiomodulation (PBM) or low-level laser therapy (LLLT) is a form of phototherapy recognized worldwide for its expansive use in medicine. PBM/LLLT has the ability to enhance enzymatic activity and mitochondrial transmembrane potential that increase the availability of energy and the signal transduction, which promotes cell proliferation. This study primarily investigates the appropriateness of a range of treatment parameters, including light wavelength, irradiance (doses) and exposure time of PBM/LLLT in proliferation of cultured human monocytic leukaemia cell line THP-1(Tamm-Horsfall Protein 1), as well as DNA under *in vitro* conditions. A secondary objective was to exploit the beneficial effect of PBM/LLLT to reduce the harmful impacts caused by exposure to ultraviolet (UV) radiation. Cells were irradiated with near infrared (NIR) diode laser at 850 nm with doses ranging from 0 – 26.8 J/cm^2. After irradiation, cells were incubated for 12 hr and 24 hr to allow time for proliferation. Comet assay was conducted to evaluate genotoxicity of the irradiated cells. Trypan blue exclusion test and MTT assay (3-4-5-dimethy-2.5 thiazol-2.5 diphenyl tetrazolium bromide) were used to estimate cytotoxicity, Exposure to NIR diode laser revealed the beneficial effect of PBM/LLLT at low doses (< 5 J/cm^2), which increased the viability and proliferation of THP-1 cells, which conformed by increasing ATP synthesis and the activity of mitochondria. Comet assay showed no significant difference between irradiated and non-irradiated cells at low doses and showed no DNA damage. The photobiomodulation effect of low doses, in particular 0.6 J/cm^2 and 1.2 J/cm^2, managed to reduce the damage to THP-1 cells after exposure to UV radiation by decreasing cell apoptosis, accelerate DNA damage repair and increase cell survival. While, the exposure for high doses (> 5 J/cm^2), showed different results involved decrease

in the number of THP-1 cells and viability, in addition to a considerable increase in DNA damage, which emphasised by decrease in the level of ATP synthesis and reducing the mitochondrial activity. These findings demonstrated the photobiomodulation effect of NIR diode laser through modulating various pathways such as ATP synthesis and mitochondrial activity in monocyte cells and DNA.

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Abbreviations, Symbols and Acronyms

Abbreviation	Glossary
(6-4)pp_s	(6-4) pyrimidine-pyrimidon photoproducts
$^1O_2$	Single oxygen
8-OHdG	8-hydroxy-2′-deoxyguanosine
AlGaInP	Aluminium-Gallium-Indium-Podiminum
ANOVA	Analysis of variance
ATP	Adenosine tri phosphate
BAX	Bcl-2 assosiated X protein
BELTP	Biological effect of laser therapy properties
BER	Base excision repair
BRCA1	Breast cancer gene
BRCA1	Brest cancer 1
$^{\circ}C$	Degree centigrade
Ca^{2+}	Ionized calsium
CCO	Cytochrom c oxidase
Cis-syn	Configurational isomerism-syn
Cm^2	Square centimetre
CO_2	Carbon dioxide
CPDs	Cyclobutane pyrimidine dimers
CSCs	Cardiac stem cells
CuA	Cooper A centres
CuB	Cooper B centres
CW	Continuouse wave
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbeccos phosphate Buffer Saline
DSB	Double strand break
EDTA	Ethylene- Di-amine Tetra-Acetic acid
ERK	Extracellular regulated kinase
ETC	Electron transport chain
Fasl	First apoptosis signal ligand

FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fe^{2+}	Ferrous
FLIPL	FLICE-Like Inhibitory Protein Long form
GaAlAs	Gallium-Aluminium-Arsenide
GaAs	Gallium-Arsenide
H_2O_2	Hydrogen peroxide
He-Ne	Helium-Neon
HR	Homologous Recombination
Hr	Hour
HUVECs	Human umbilical vein endothelial cells
InGaAlP	Indium-Gallium-Aluminium- Podiminum
IR	Infrared
IRED	Infrared emitting diode
J	Joule
J/cm^2	Joule per square centimeter
J/s	Joule per second
JAKs	JAnus kinases
KJ/m^2	Kilo joule per square meter
L	Litre
LED	Light Emitting Diode
L-glutamine	Levo (left)-glutamine
Linc RNA-p21	Long intergenic non-coding RNA-p21
LLLT	Low level laser therapy
LMPA	Low melting point agarose
LMPC	Laser microdissection and pressure catapulting
LPT	Laser phototherapy
M	Molarity
mA	milliamper
Min	Minutes
Ml	Millilitre
mm^2	Square millimetres
MSCs	Mesenchymal stem cells
mW	Milliwatts

n	Number of test organisms in sample (treatment)
Nd:YAG	Neodymium yttrium aluminium garnet
NER	Nucliotide excision repair
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells B
NHEJ	Non-homologous end Joining
NIR	Near-infrared
Nm	Nanometre
NMPA	Normal melting point agarose
NO	Nitric oxide
ns	nanosecond
O_2^-	Superoxide
$O_2^{\cdot}$	Anion radical
$OH^{\cdot}$	Hydroxi al radical
P	Probability
p21	Protein21
p53	Phosphorotein53
PBM	photobiomodulation
PBS	Phosphate buffer saline
PDT	Photo dynamic therapy
pH	potential Hydrogen
PI3K	Phosphatidylinositol 3-kinase
R	Pearson’s product-moment correlation coefficient
R^2	Coefficient of determination
RLU	Relative light units
ROS	Reactive oxygen species
Rpm	Revolution per minutes
RPMI	Roswell Park Memorial Institute
S	second
SCGE	Single cell gel electophoresis
SD	Standard deviation
Sec	second
SEM	Standard error of the mean
SOS	Super oxide dismutase
SSB	Single strand break

$t_{1/2}$	Half-time
TAE	Tris-acetic acid EDTA
THP-1	Tamm-Horsfall Protein-1
TNF-α	Tumor necrosis factor-Alpha
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
V	volts
v/v	volume/volume
Vs	Versus
W/m^2	Watt per square meter
µg/ml	Microgram per mililitre
Ml	Microliter
µM	Micromole

Publications & Conferences

Publications:

MUSSTTAF, R., D Jenkins, A Jha. (2017) The photo-stimulatory effect of low level laser therapy on the proliferation rate of human monocytic leukaemia cells. *IET Nanobiotechnology,* **12,** 175- 18. (Conference paper)

MUSSTTAF, R., D Jenkins, A Jha. (2018) Genotoxic and cytotoxic potential influence of solar UV radiation on human monocytic leukaemia cells and DNA damage *in vitro*. (Unpublished).

MUSSTTAF, R., D Jenkins, A Jha. (2018) Assessing the impact of low level laser therapy on biological systems: A review. (Unpublished).

Conferences

2015:

July, Plymouth, UK. UKEMS Annual Meeting

Poster presentation:

Assessing the impact of low level laser therapy on biological system.

2016:

June, Plymouth, UK. UKEMS Annual Meeting

Poster presentation:

The Photo-stimulatory effect of low level laser therapy on the proliferation rate of cell culture.

2017:

June 2017, Plymouth University, UK

Attendance: International Symposium, current trends in nanotoxicology: implications for environmental & human health

October, Chicago, USA

Poster presentation:

Photobiomodulation effects of near infrared radiation on DNA damage in UV-irradiated human monocytic leukaemia THP-1 cells.

Acknowledgement

First of all, I would like to thank the Almighty God, Allah, without whose blessings none of this would have been possible. It is with utmost sincerity that I express my gratitude to all of those who have supported me throughout my PhD research.

First and foremost, I would like to express my special appreciation and thanks to my supervisor Dr. David Jenkins for his guidance, continuous support and approachability throughout the project. I would like to extend my huge gratitude to my supervisor Professor Awadhesh Jha for his encouragement, giving me precious opinions and suggestions and helping me accomplish my research project. Without my supervisors help, this dissertation would not be what it is now. This unique experience has greatly expanded my knowledge and skills for further PhD research.

Personally, I wish to express my deepest thankfulness to my beloved “Mum”, your prayer for me was what sustained me thus far. You were, are and always will be a continuous source of unconditional love, tremendous support, encouragement and inspiration not only in my graduate study but in everyday life.

My sincere gratitude to my loving husband “Hayder” for putting up with me, always being there and provided unconditional support and encouragement throughout the journey. Thanks for helping me make my dreams possible. I am forever grateful for the years we have spent together so far and look forward to what the future brings. I would also like to thank my kids “Basma & Mohammad” the angels of mine. I know PhD stole our special moments together, I apologise to you for working late hours, weekends and holidays during this journey. Please forgive me for ever having said “I am busy, I cannot listen now or I cannot read bed time story”. Thank you for being the smile, the hope and the greatest loves of my life. I wish to express my deepest thankfulness to my brother “Firas” and sister “Shurook” for their motivation and encouragement throughout my career.

I would also like to extend my thanks to Prof. Waleed Al-Murrani for his support, encourage and his help in discuss the analysis program “Minitab”. My deep thanks for Dr. John Moody for his generosity and help me in an important part of

the project. To my colleague, Dr. Kamrul Islam for his help, you are one of the most generous, helpful and welcoming people I have ever met, and my thankful for Dr. Lorna Dallas for her help whenever I need her and many thanks for Dr. Rana Moyeed for his help to draw some figures in “R”. I want to thank Mrs Samantha Brown in library and Digital Support for her kindness, helpful and generosity and come to help me to prepare the references of the thesis.

I would like to thank the technicians in Davy building, Dr William Vevers and Lynne Cooper for many discussions on my experiment, and the technician in Brunel Lab Mr. Nick Fry.

Also, I would like to thank my friends, Dr. Abbas Al-Shabany “May the God have mercy on him and placed him into his havens”, for his helpful, generosity and my deep thanks for my best friend Dr. Zainab Dangana for her encourage, support, generosity and help whenever I need her. I must also express my very profound gratitude to my country, the Ministry of the Higher Education and Scientific Research, Republic of Iraq, for providing necessary funding, and the School of Biological Sciences of Plymouth University for giving me the opportunity to study for this Ph.D.

Finally, I would thank those who directly and indirectly involved in this project for their supports and encouragements all through my studies. To each of the above, I would like to extend my deepest appreciation.

Dedication

This thesis is dedicated to my Father`s soul, to my Mother, to my brother and sister, to my dearest Husband: Hayder and to my lovely kids: Basma and Mohammad for all their love, support and encouragement.

Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment. The research in this thesis is original and represents the author's own work.

This study was financed with the aid of Ministry of Higher education and Scientific Research/Iraq.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; several papers were prepared for publication. One paper has been published in refereed journal.

Word count of main body of thesis: (45,797 words)

Signed:

Date:

CHAPTER 1

GENERAL INTRODUCTION

1 General Introduction

1.1 Introduction

Laser therapy or low-level laser therapy (LLLT) has been widely used for over 50 years (Ginani et al., 2015). Evolutionary, it emerged in its modern form after the invention of the laser in 1960 by Endre Mester in Hungary (Sousa et al.) becoming a widespread treatment modality in a variety of clinical applications (Karu, 1989b, Kreisler et al., 2003, Posten et al., 2005). Investigators introduced a diverse set of terms to describe this potentially beneficial treatment tool (Lucas et al., 2002). Initially, expressions such as ‘photobioactivation’ and ‘biostimulation’ frequently relative to the stimulation effect of low level lasers were used (King, 1989, Wu et al., 2012b). Subsequently an inhibitory effect of this radiation was also noted, which led them to coin the term ‘biomodulation’ (Schindl et al., 2000). Recently a consensus decision was taken to use the terminology “photobiomodulation” or “PBM”. Where some researchers gave LLLT a status of subjectivity, and it is limited for actual laser specific interactions, this is not a requirement for in-coherent light emitting diodes (LEDs) which can work equally well (Hamblin, 2017). On the contrary, other researchers reported that although LLLT is a well-established researchable, and for much time used by clinician and researchers, but it is not optimal. It is a broad term that could include photodynamic therapy (PDT) and optogenetics, these techniques use lasers and LEDs with low dose and require exogenous chromophores, unlike LLLT that utilise endogenous chromophores with low dose of light delivered at the target site. However, they also suggest using photobiomodulation (PBM), since it is more ideal, has specific definition for this application of light to be more accurate and can confirm its scientific principle (Anders et al., 2015). Specialists of medical field successfully used photobiomodulation in treating many health conditions when other

methods had had limited success, such as healing-resistant wound, chronic diabetic ulcers, injuries of spinal cord and nervous system and pain management (Tuner and Hode, 2004). Nevertheless, photobiomodulation is not considered as a part of mainstream medicine as still not standard treatment (Karu, 2013). Photobiomodulation treatment has evolved over the years and is being developed as a sophisticated tool for therapeutic procedure and utilized clinically for several different ailments (Chung et al., 2012). The therapeutic treatments are based upon three principles; to minimize inflammation, edema, and chronic disorders of joints by targeting brain, skin, joint etc. (Bjordal et al., 2003), to promote wound healing of superficial and deeper tissues, neurological damage etc. (Posten et al., 2005, Gigo‐Benato et al., 2005), and to treat neurological disorders and pain (Chung et al., 2012). Recently, many studies on PBM therapy at infrared IR wavelengths, in particular from 700 nm up to the near infrared NIR (Barrett and Gonzalez-Lima, 2013, Salehpour and Rasta, 2017, Xuan et al., 2014), which was shown to produce more benefit impacts than red light in many medical conditions, including neural stimulation (by triggering direct activation of neural tissue) (Salehpour et al., 2017), photoaging (where IR radiation evidently has a biphasic effect), anti-tumor action (IR radiation is capable of inhibiting the proliferation of cancer cells and enhances chemotherapy efficacy, and brain neuroprotection (treatments for stroke, Traumatic brain injury (TBI) *in vivo* models) (Salehpour et al., 2017, Naeser et al., 2011), and neurodegenerative disorders for Alzheimer's and Parkinson's diseases. These are given, in addition for many other diseases in Table 1. Therefore, a better understanding of the mechanisms using IR radiation could support improved therapeutic effectiveness via new strategies of PBM therapy at IR wavelengths (Tsai

and Hamblin, 2017) that can be employed in several different ways to treat many ailments (Chung et al., 2012), (Table 1).

Laser is a device which produces intense, monochromatic, coherent, and highly collimated beam of light (Fonseca et al., 2010). Laser light has quite pure frequency, which makes it useful for biomedical applications (Ratkay-Traub et al., 2001). Laser therapy involves visible red and near infrared (NIR) portions of the electromagnetic spectrum (390–1600 nm and 10^{13}–10^{15} HZ) , because researchers have shown that these portions of the spectrum have been absorbed highly by the biological systems and bring about a beneficial therapeutic effects in living tissues (Hawkins et al., 2005). According to the portion of the spectrum (wavelength) that strikes the tissue and the intensity (power density or irradiance) of laser radiation, the photobiological impacts of laser therapy on tissue are different that lead to divide the laser therapy into two classes (Hawkins and Abrahamse, 2006a). Class I, which refers to radiation of wavelengths ranges (<390 nm) and (>1600 nm) and high power and intensity levels, are used for ablation, cutting and sterilization, because of its thermal effect. Class II, which refers to radiation of wavelengths ranges (390 -1600 nm), levels of power (10^{-3} to 10^{-1} W) and intensity (10^{-1} to 10^{0} W/cm^2) and a dose of 10^{-2} to 10^{2} J/cm^2 (Posten et al., 2005).

Whereas there is some agreement on the best wavelengths of light and appropriate dosages to be used (irradiance and fluence), there is no agreement on the emission mode of laser light; whether continuous wave (CW) or pulsed light is more suitable for the various applications of PBM. However, pulsed lasers in PBM therapy are used widely in clinical research (Fonseca et al., 2010, da Silva Sergio et al., 2012a) and for medical treatment (Vasheghani et al., 2009, de Meneses et al., 2015, Bayat et al., 2016, Ahrari et al., 2014) Two types of pulsed laser are used for PBM therapy,

a super-pulsing gallium-arsenide (GaAs) diode laser, which has a wavelength in the region of 904 nm and pulse duration in the range of 100–200 ns, and the semiconductor super-pulsing indium-gallium-arsenide (In-Ga-As) diode laser, which emits light at a similar wavelength (904-905 nm), producing very short pulses of light (200 ns) in the range of kilohertz (kHz) frequencies (Hashmi et al., 2010b). Therapeutically, the super-pulsed GaAs and In-Ga-As lasers are capable of deep penetration without the undesirable influences associated with continues wave lasers (CW) (such as thermal damage), as well as allowing for shorter treatment periods. Pulsed lasers offer potential benefits, attributed to the pulse OFF times (pulse quench intervals) following the pulse ON times, so that pulsed lasers can deliver less tissue heating.

Low intensity laser radiation is clinically well accepted tool in medicine and dentistry [(Amid et al., 2014);Table 2]. It is featured by its ability to incite a thermic, non-damaging photobiological action (McDaniel, 2015). Unlike hard high power laser, LLLT provides low energy only sufficient to induce stimulation response of body tissue, and has a wavelength-dependent manner able to change the cellular function in the absence of considerable heating (Surendranath and Arjun, 2013). Hence, LLLT is also called soft laser therapy or cold laser, as low energy laser has no thermal effects (Nelson, 1993, Chung et al., 2012).

Table 1. 1: Review of published studies using LLLT to treat different diseases

Study No.	Type of laser	Wavelength (nm)	Power (mW)	Energy density (J/cm^2)	Power density (mW/cm^2)	Emission model CW / Pulse	Types of diseases	Reference
1	Diode laser	810	10 W	3 and 30	5 and 50	CW	Zymosan-induced arthritis	(Castano et al., 2007)
2	He – Ne	632.8	10	3, 5, 10, 20, 25 and 50	64.6	CW	Neurodegenerative	(Song et al., 2012)
3	He – Ne	632.8	10	0.5, 1, 2 and 4		CW	Alzheimer's disease	(Meng et al., 2013)
4	Nd:YAG	1064	1.25 W			CW	Dental/Tooth extraction	(Vescovi et al., 2013)
5	GaAs	904	10	5.4	20	CW	Musculoskeletal diseases	(Bjordal et al., 2006)
6	Diode laser	830	30	1.1		Pulse	Painful stomatitis control	(Toida et al., 2003)
7	Diode	810	30	0.9	30	CW	Diabetic wounds	(Dancáková et

	laser							al., 2014)
8	Diode laser He – Ne	830 632.8	30 20			CW	Chronic diseases of inner ear	(Wilden and Dindinger, 1996)
9	Diode laser	660	50	2		CW	Chronic lichenoid graft-vs.-host disease (cGVHD)	(Chor et al., 2004)
10	Diode laser	810		3	20	CW	Cortical neurons	(Huang et al., 2014)
11	He – Ne	632.8	400	1		CW	Alzheimer's Disease	(Farfara et al., 2015)
12	GaAlAs	860	30 60	3	3000	Pulse CW	Osteoarthritic (OA) pain	(Brosseau et al., 2005b)
13	GaAs	808			10 and 20	CW	Traumatic brain injury (TBI)	(Oron et al., 2007)
14	GaAlAs	830	60	45	4000	CW	Lumbago	(Ohshiro and Shirono, 1992)
15	Diode	660	30	7.5		CW	Lung neutrophils	(Aimbire et al.,

	laser							2008)
16	Diode laser	660	40	20		CW	Burning mouth syndrome	(Santos et al., 2011)
17	Diode laser	665, 730 810 and 980		36	150	CW	Traumatic brain injury (TBI)	(Wu et al., 2012a)
18	Diode laser	660	24			CW	Periodontal disease	(de Almeida et al., 2008)
19	Diode laser	820	300	3		CW	myofascial pain (MP) dysfunction syndrome	(Öz et al., 2010)
20	GaAlAs	780	50	7.5		CW	Rheumatoid arthritis	(Ekim et al., 2007)
21	Diode laser	810		0.03, 0.3, 3, 10 and 30	25	CW	Cortical neurons	(Sharma et al., 2011)
22	GaAlAs	830	70	6		CW	Peripheral nerves regeniration	(Midamba and Haanaes, 1993)
23	GaAlAs	810	1 W	4.8 24	80	CW	Orofacial granulomatosis	(Merigo et al., 2012)
24	Diode	830	100	3		CW	Chronic periodontitis	(Makhlouf et al.,

	laser							2012)
25	Diode laser	780 830	30 500	6.3 100		CW	Temporomandibular joint pain	(Chang et al., 2014)
26	He - Ne	632.8	10	0.18 - 27		CW	Indolent ulcers	(Schindl et al., 1992)
27	Diode laser	808			110 165	CW	Hearing loss	(Tamura et al., 2015)
28	Diode laser	532 635	7.5			CW Pulse	Hearing loss	(Goodman et al., 2013)
29	Diode laser	650	5			CW	Complaints of Tinnitus	(Salahaldin et al., 2012)
30	InGaAlP	660	10	2.5		CW	Acute zymosan-induced arthritis	(Carlos et al., 2014)
31	GaAs	904	20	2 - 20	11.2	Pulse	chronic myofascial pain syndrome (MPS) in the neck	(Gur et al., 2004)
32	GaAs	904		29.5	246	Pulse	SalivaryGlands (Xerostomia)	(Lončar et al., 2011)
33	Diode	630	– 10–100	2, 3 and 4		CW	Oral mucositis due to	(Bensadoun

	laser	670 – 780 – 830					cancer therapy	and Nair, 2012)
34	Diode laser	660, 810 and 980		36		CW	Traumatic brain injury (TBI)	(Wu et al., 2010)
35	GaAlAs	670	5	2		CW	Chronic periodontitis Diabetes mellitus (DM)	Obradovic et al. (Obradović et al., 2013)
36	Ga-AsI-Al	780	22	7.7	100	CW	Rheumatoid arthritis (RA)	(Alves et al., 2013)
37	Diode laser	810		36	50	CW	Traumatic brain injury (TBI)	(Xuan et al., 2015)
38	Diode laser LED	685 640 – 685	200	2		CW	Reynaud's phenomenon	(Hirschl et al., 2004)
39	Diode laser	810			50	CW	Parkinson's disease (PD)	(Trimmer et al., 2009)
40	Diode laser	790	120	6		CW	Burning mouth syndrome	(Kato et al., 2010)
41	IR laser	830	35	3		CW	Lung inflammation	(Oliveira et al.,

								2014)
42	GaAs	904	150	6		Pulse	Carpal tunnel syndrome	(Dakowicz et al., 2011)
43	AlGaAs	780	30	22.5	750	CW Pulse Pulse	Renal Interstitial Fibrosis	(Oliveira et al., 2012)
44	GaAlAs	830	60	18	3000	CW	Knee Osteoarthrosis	(Trelles et al., 1991)
45	AlGaAs	785	70	3		CW	Rheumatoid arthritis	(Meireles et al., 2010)
46	Diode laser	670	50	3		Pulse	Temporomandibular disorder (TMD)	(Núñez et al., 2006)
47	GaAs	904	45	5		CW	Muscle trauma	(Rizzi et al., 2006)
48	GaAlAs	980	300	4	1500	CW	Mucous membrane pemphigoid	(Cafaro et al., 2012)

49	Diode laser	660	5	4.5		CW	Acut Lung inflammation	(de Lima et al., 2011)
50	GaAs	980	10 80 W	2-4		CW Pulse	Chronic low back pain (LBP)	(Hadi et al., 2009)
51	GaAlAs	980	300	4	1000	CW	Oral lichen planus	(Cafaro et al., 2014)
52	GaAlAs	660	30	57.14	428	CW	Periodontal disease (PD)	(Garcia et al., 2011)
53	InGaAlP	660	40	2	1000	CW	Ulcers in patients with leprosy sequelae	(Barreto and Salgado, 2010)
54	GaAlAs	815	250	12		CW	inflammation in retrodiscal tissues in patients with temporal mandibular joint	(Kucuk et al., 2010)
55	GaAlAs	808	500	5	1.8	CW	Bisphosphonate Related Osteonecrosis of Jaws	(Altay et al., 2014)
56	AsGaInP	660	50 100	12.5 25	1.25 2.5	CW	Third-Degree Burns	(Brassolatti et al., 2016b)

It was observed that the broad range of laser therapy included molecular, cellular and tissue level effects and the modes of action of LLLT may vary with different confounding factors and applications (Chung et al., 2012). To produce photo-biological action, photon absorption of laser radiation must occur (Hawkins et al., 2005). Endogenous or exogenous chromophores are the initial photoacceptor molecules (i.e. molecules that can absorb light at certain wavelengths) absorb the incident photon energy (Bjordal et al., 2001). A photochemical conversion of the photon energy absorbed by a photoacceptor has been demonstrated (Brondon et al., 2005). The absorbed energy of photon can be transferred to another molecule, which can then cause chemical reaction without alteration in temperature in the surrounding tissue (Brondon et al., 2005, Mochizuki-Oda et al., 2002). Some native component can be activated in the irradiated cell at certain wavelength, and consequently, biochemical reaction as well as cellular metabolism might be altered (Karu, 1999).

Several studies suggested that mitochondria is the most sensitive component of cell to visible and near infrared light (Karu, 1999), (Karu et al., 2001), that result in increased production of adenosine triphosphate (ATP), increased deoxyribonucleic acid (DNA) synthesis, modulation of reactive oxygen spaces (ROS) and nitric oxygen species (NOS) and the induction of transcription factors (Hamblin and Demidova, 2006b). Moreover, PBM at red and NIR wavelengths stimulate increasing intracellular calcium Ca^{2+} (Karu, 2008, de Freitas and Hamblin, 2016, Irvine and Schell, 2001a, Santana-Blank et al., 2005), however recent studies emphasised that blue (420 nm) and green (540 nm) lights are more effective in increasing Ca^{2+} when applied at the same doses (Wang et al., 2016). Many researchers suggested that the response of some cells to blue or green light interacting by light-gated ion channels,

which enable light to control electrical excitability, intracellular acidity, calcium influx and other cellular processes (Roska and Lagali, 2018, Kulbacka et al., 2017, Roska and Juettner, 2017). The most likely ion channel is light-gated channel rhodopsin, because the action spectra of the channel rhodopsin family displays peaks in the blue-green spectral region (Schneider et al., 2015).The precise mechanism of laser-tissue interaction has not been completely explained, therefore there is no ability to offer a clinical treatment protocol at present (Amid et al., 2013).

The review of the available literature suggests that the variety of studies have been mostly *in vitro*, using a range of cell lines for different types of LLLT and varying some of their parameters, as summarised in Table 3. It is possible to select wavelength, power density, laser beam intensity profile, polarisation and exposure time. The available information suggests both positive and negative outcomes with respect to different parameters (Table 2).

It could be concluded that conflicting results have been published which may be attributed to a disparity in study design, including the use of different laser wavelengths, and numerous illuminated parameters, in addition to different confounding factors which influence the determination of different biological parameters.

Table 1. 2: Parameters involved in LLLT applications.

Irradiation Parameters	Unit of measurement		Diffenitions
Wavelength	nm	390– 1600	An electromagnetic radiation travels in discrete packets that also has a wave-like property.
Power	W	10^{-3} – 10^{-1}	It is the amount of energy consumed per unit time, and can be calculated as: Power (P) = Energy (J) /Time (sec)
Power density	W / cm^2	10^{-1} – 10^{0}	Often called Irradiance, or Intensity, is the power transmitted per unit area, and calculated as: Power density = Power (W) / Area (cm^2)
Energy density	J / cm^2	10^{-2} – 10^{2}	Energy density is the common expression of LLLT dose The dose is the most important parameter in laser Phototherapy, and is usually calculated as Power / Beam Area x Time = J/cm².
Total irradiation time	sec	10 – 3,000	The allowed interval through which the energy has delivered to the target system.

Table 1. 3: Review of published studies evaluating the effect of LLLT on different cell lines.

study No	Cell Types Used	How the cells are grown	Type of LLLT	Quality of Laser Used	Biological Effects Determination	References
1)	Human skin fibroblast cells	Cultures in minimum essential medium with Earl's balanced salt solution & incubated in 37℃ in 5% & 85% humidity	He-Ne Laser	λ: 632.8 nm Energy density (ED) 5J/cm^2	1) Non irradiated Hydroxyuria (HU) treated cells had a reduced number of cells in the central scratch compared to non-irradiated non treated cells, suggesting that HU inhibited cellular proliferation. 2) Irradiated HU treated cells showed an increased number of cells in the central scratch compared to non-irradiated treated cells. This increase was due to the stimulatory effect of irradiation with 5 J/cm2. The addition of HU had no significant effect on cell viability. 3) The Trypan blue exclusion test showed no significant difference in percent viability between treated and non-treated cells. 4) Irradiated non treated cells showed a significant increase in the formazan dye, which is as a result of cleavage of XTT by the mitochondrial succinate dehydrogenase in actively proliferating cells, compared to non-irradiated non treated cells.	Mbene et al. (Mbene et al., 2006)

					5) Cell viability, proliferation and DNA integrity assays showed that irradiated and non-irradiated N cells were not significantly affected at both 1 and 24 h post irradiation. 6) There was a significant decrease in damage at 24 h compared to 1 h incubation due to the activation of DNA repair mechanisms.	
2)	E.Coli AB1157, BW527, BW9091 and BW375	Cultures in exponential and stationary growth phase. *E. coli* suspensions (1-2 × 10^8 cells/mL, in 0.9% NaCl solution)	Laser HTM Compact model, AlGaInP	Power:10 mW λ: 658 nm	1) There is no alteration of survival fractions of these E. coli cultures when exposed to laser. 2) I was indicate that laser exposure induces filamentation in exponential E. coli AB1157, BW527, BH20, BW375 and BW9091 cultures at all emission modes.	da Silva et al. (da Silva Sergio et al., 2013a)
					3) Laser – induced stimulation of cell replication in E.coli cultures depends on the culture conditions, determining the particular metabolic state necessary for the division.	

3)	Stem cells	Does not maintion the culture procedure	He-Ne Laser Gallium-Aluminum-Arsenide (Ga-Al-AS)	λ: 632.8 nm λ:600 nm Energy density: 0.5 - 4.0 J/cm^2 Power 1- 500 mW	1) LLLT can increase enhance the proliferation rate of various cell lines. 2) The stimulation of cellular proliferation is dependent on the doses of laser irradiation, as lower doses increase the cell proliferation rate and other cellular functions, while higher doses of LLLT have negative effects.	(AlGhamdi et al., 2012)
4)	Mesenchymal stem cells (MSCs) & Cardiac stem cells (CSCs)	Cell cultured at 1.3 × 10^6 cm^2 in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 m mol/L Glutamine, 100 U/ml pencillin, 100 U/ml stroptomycin CSC cultured in a class 2 flow hood.	Diod (Ga-As)	λ 804 nm Power density: 50 mW/cm^2 Energy density: 1 & 3 J/cm^2 Exposure time: 20 sec or 60 sec	1) CSCs of (1J/cm^2) 1 and 2 weeks post LLLT irradiation significant increase of sevenfold and twofold respectively in the number cells compared to control. 2) Significant increase in the number of cells at the energy density 3 J/cm2 after 1 week. 3) The number of MSC_S increased post LLLT of 50 mW/cm^2 for 20 sec and 60 sec	(Tuby et al., 2007)
5)	Fibroblast of skin cells, buccal mucosa and gingival			λ: 540 nm 600 – 900 nm Energy density: 0-56 J/cm^2	1) Increased proliferation, maturation and locomotion as well as transformation to myo-fibroblasts. 2) Reduced production of pro-inflammatory prostagland in E2	(Walsh et al., 1997)

		3) Increased production of basic fibroblasts growth factors. 4) Increased proliferation at low doses and suppressed at high doses.
Macrophages		1) Increased ability to act as phagocytes, and greater secretion of basic fibroblasts growth factors. 2) Macrophages resorb fibrin as part of the demolition phase of wound healing more quickly with LLLT, because of their enhanced phagocytic activity during the initial phases of the repair response.
Lymphocytes	λ: 660 nm 820 940 nm	Lymphocytes become activated and proliferate more quickly
Epitheli		These cells become more motile and are

	al cells			able to migrate across wound sites with accelerated closure of defects.	
	Endothelium cells			Endothelium forms granulation tissue more quickly. Relaxation of vascular smooth muscles	
6)	Human Gingival Fibroblasts (Hgf3-Pi 53 NCBI code C50)	The cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS). This medium was also supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100μg/ml streptomycin.	(Ga–Al–As) diode laser	λ: 810 nm Power: 50 mW Energy density: 4J/cm^2 Exposure time: 32 sec	1) The differences between the case and the control groups were statistically significant on 48 hr and 72 hr after irradiation. 2) The results of this *in vitro* study revealed that good levels of cell proliferation could be achieved if enough time has been given to the cells to show the effect of laser irradiation on cell proliferation rate.	(Frozanfar et al., 2013)

7)	HeLa cells	They were grown as monolayers in scintillation vials	He-Ne laser	λ: 632.8 nm Power density: 10 W/m^2 Exposure time: 10 sec Energy density: 100 J/m^2	1) When the cells exposed to laser radiation for 60 min before exposure to γ-radiation, substantial differences was seen between the survival curve and the curve representing the survival of γ-irradiated cells. 2) Increased the number of cells after stimulation with He-Ne in the exponential phase of growth than that for the control.	(Karu et al., 1994b)
8)	Yeast, HeLa		He-Ne laser	λ: 632.8 nm Power density: $I \geq 2\times10^{11}$ W/cm^2	The activity of some enzymes was determined and shows that the growth stimulation is accompanied by the respiratory activity increase with no accumulation of toxic intermediates of oxygen metabolism and by synthetic processes in cell predominance over degenerative once. The data indicated that the irradiation causes a cell metabolism rearrangement, the light playing the role of	(Karu, 1988)

					a trigger controller of the cell metabolism.	
9)	Human B-lymphoblasts	Human B-lymphoblast cells (NC 37) were grown in suspension in RPMI 1640 medium (Sigma, Germany) with 10% fetal calf serum at 37°C in a 5% CO2 atmosphere. The cells were sub-cultured twice weekly in fresh RPMI 1640 medium.	He-Ne laser	λ: 632.8 nm Power: 10 W Diameter of beam: 0.75cm Doses ranging 0.5-2.7 kJ/m^2	1) The cell viability measurement shows no significant change of the cell survival. 2) He-Ne lasers alone do not result in any DNA damage.	(Dube et al., 2001)
10)	Human alveolar bone	Cells were cultured in α-Minimum Essential Medium	GaAlAs diode laser	λ: 780 nm Power:	1)Cell growth was affected by time only in LLLT group	(Petri et al., 2010)

	fragments	(Gibco), supplemented with 10% fetal bovine serum (Gibco), 50 µg/mL gentamicin (Gibco), 0.3 µg/mL fungizone (Gibco), 10-7 M dexamethasone (Sigma, St.Louis, MO, USA), 5 µg/mL ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma)		70 m W Diameter of beam 0.2 cm Energy density: 3 J/cm^2 Exposure time: 9 min	2)From day 10 to 14, LLLT treated cultured showed an increase of cell growth	
11)	human gingival fibroblasts	A cell line of human gingival fibroblasts named LMF was grown in DMEM with either	Diode laser	λ: 670 nm, 780nm, 692nm 786nm	1)The irradiated cell number of cell cultured in 5%nutrition deficit more than that control cell cultured in idial conditions 2) In the same fluence, IR laser induced a	(Almeida - Lopes et al., 2001a)

		5% nutritional deficit or 10% (FBS)		Energy density (fluence) 2 J/cm^2 Exposure time: 9 min	higher cell proliferation than visible laser when the output powers are different. 3) Lasers of equal output power presented the similar effect on cell growth independently of their wavelength.	
12)	Human Macrophages	The macrophage J774 cell line was grown in (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C and in a wet environment with 5% CO2. Cell growth was assessed every 24 hours using an inverted phase microscope	Diode laser	λ: 780 nm Power: 70 mW Energy density: 3 J/cm^2 λ: 660 nm Power: 15 mW Energy density: 7.5 J/cm^2	1) After 1 day of culture, activated and 780 nm irradiated macrophages showed lower mitochondrial activity (MA) than activated macrophages, but activated and 660 nm irradiated macrophages showed MA similar to activated cells. 2) After 3 days, activated and irradiated (660 nm and 780 nm) macrophages showed greater MA than activated macrophages, and after 5 days, the activated and irradiated (660 nm and 780 nm) macrophages showed similar MA to the activated macrophages.	(Souza et al., 2014)

13)	MG-63	Cells were maintained it in Dulbecco's modified Eagle medium (DMEM) with 100 IU/ml penicillin , 50 µg/ml gentamicin, 2.5 µg/ml amphotericin B , 1% glutamine and 2% HEPES {(4-(2-hydroxyethyl)-1-piperazineethanes ulfonic acid)} , supplemented with 10% fetal bovine serum.	Diode laser	λ: 940 nm Energy outputs: 1-5 J Intensities: 0.5, 1, 1.5 and 2 W/cm^2	Pulsed low-level laser with low-energy density range appears to exert a biostimulatory effect on bone tissue.	(Huertas et al., 2013)

		Cultures were kept at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. .				
14)	Osteoblastic (MC3T3) cell line	Cells were grown in sterile Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM = F-12) (Invitrogen, Mount Waverley, Australia) supplemented with heat-inactivated fetal bovine serum (FBS) (Cambrex, East Rutherford, NJ), and 200 ml penicillin + 200 mg =ml streptomycin (Invitrogen)	Diode laser	λ: 830 nm Power: 30mW Energy density: 10 J/cm^2	Reduction in cell proliferation compared to non-irradiated controls.	(Renno et al., 2010)

15)	Human osteoblast cell line	Cells were maintained in sterile medium (Dulbecco's Modified Eagle's Medium): Nutrient Mixture F-12 (DMEM= F-12) (Invitrogen, Mount Waverley, Australia) supplemented with heat-inactivated fetal bovine serum (FBS) (Cambrex, East Rutherford, NJ), and 200 ml penicillin+ 200 mg =ml streptomycin (Invitrogen)	He-Ne laser 632 nm	λ: 632 nm Power:10mW Energy density: 0.43 J/cm^2	LLLT promotes proliferation and maturation of human osteoblasts *in vitro*, and a significant 31–58% increase in cell survival	(Stein et al., 2005)
16)	Human monocytic THP-1 cell line	THP-1 were maintained, in RPML-1640 complemented with 10% foetal bovine serum FBS, 5ml of L-glutamine and 5ml penicillin/streptomycin.	Diode laser 850 nm	λ: 850 nm Power: 9.5mW Energy density: (0.6-27) J/cm^2 Power density: 29.6 mW/cm^2	PBM promotes proliferation of human monocyte *in vitro*, and a significantly increased cell survival due to increasing membrane integrity and mitochondrial activity	(MUSSTTA F et al., 2017)

		THP-1 cells were grown in a 75 ml culture flask, containing 20ml of medium plus cell, at 37˚C with 5% CO2 in a humidified incubator.				
17)	Stem cells from exfoliated deciduous teeth (SHED)	Cells were maintained in Eagle's minimum essential medium alpha modification supplemented with 10% FBS and 1% penicillin and streptomycin solution (penicillin–streptomycin, Gibco, Invitrogen) at 37 °C and 5% CO2 in incubator.	InGaAlP red laser	λ: 660 nm Energy density: (1.2- 6.2 J/cm2)	Improved cell viability and proliferation of SHED after laser irradiation, except for 1.2 J cm−2.	(de Souza et al., 2018)

1.2 Optical sources and biological interactions

Low level laser irradiation has been used in clinical practice causing biostimulation. A number of diseases and physical conditions are mentioned to respond to laser therapy (photobiostimulation) (Basso et al., 2013). At the cellular and molecular level, there is still significant argument regarding the effectiveness of lasers in producing the desired practical responses (Basso et al., 2013).

To illustrate the therapeutic effects, through optical stimulation processes, we introduce here briefly the available light sources and their potential to interact at the cellular and molecular level. Currently these are not well supported by the literature.

Laser light is generated on the principle of light amplification of stimulated emission of radiation (Koutná et al., 2003). The beam energy of laser light is powerful because it is highly coherent (waves are all in phase), polarized, focused and monochromatic (a single wavelength). It was first used in ophthalmological field in the early 1960s, although, the basic principle of laser was proposed by Einstein as back as in 1917 (Koutná et al., 2003). Lasers are commonly designated and named by the type of lasing material employed. The laser medium can be a solid state semiconductor, a gas, a liquid or a solid, as in Nd:YAG lasers which employ a Nd:YAG rod as the lasing medium (Thompson, 1988) .

Laser light is characterised by its single wavelength, although some lasers, such as dye laser, can be tuned over a wide range of wavelengths (Singh et al., 2012). Lasers are also classified according to their intensity and if they are pulsed or continuous wave (CW), in order to identify the risk of harm to the patient (Karu et al., 2004). In the medical field, lasers are classified as high power surgical lasers and low power therapeutic lasers (Mbene, 2008). Non-invasive or ‘soft’ lasers were

introduced into medicine in the 1980s, and since then, have been seen as useful light sources for medical application (Koutná et al., 2003). The wavelengths of laser radiation used, have been investigated to show their therapeutic use (Smith, 1991).

LLLT or photobiomodulation is a form of phototherapy, which is designed to apply low levels of red and near- infrared light with wavelengths in the region of 390-1600 nm and output powers up to 500 mW (AlGhamdi et al., 2012). LLLT is effective in a number of clinical situations where the wavelength of red and near-infrared region are effective in such therapies. However, both of these two wavelength spectra are different in their photochemical and photophysical properties (Smith, 1991).

LLLT refers to the use of photon energy at low levels to alter biological activity with no-thermal reactions because there is little increase in the temperature of the irradiated tissue (AlGhamdi et al., 2012). Lasers of low level intensity are suggested to be non-toxic, non-allergic and because of their ease of application, these techniques have gained wide application in many fields of health care (Koutná et al., 2003),Table1. Phototherapy has been found to have significant effects on a variety of pathological conditions including pain attenuation, inflammation and induction of wound healing in non-heating effects (AlGhamdi et al., 2012).

From observations, it appears that LLLT has beneficial effects at the molecular, cellular, and tissue levels (Tafur and Mills, 2008). It has been found that medical treatment with LLLT at various intensities has stimulatory effect on cellular processes (Avci et al., 2013a). Recently, it has been reported by several investigators that at low –levels of red or near-infrared light illumination, LLLT can prevent cell apoptosis (AlGhamdi et al., 2012, Huang et al., 2009), stimulation of mitochondrial activity, increased cell turnover, recruitment and proliferation, modulation of the cellular

metabolites (Di Giacomo et al., 2013). It was suggested that LLLT might promote changes in the cellular redox state, playing an important role in sustaining cellular activities, and induce photobiostimlative processes (Silveira et al., 2009). In addition to the above, pre-exposure of PBM had a protective effect against many external agents such as hydrogen peroxide, H_2O_2, and UV radiation (Sergio et al., 2015, Canuto et al., 2015). There is an evolutionary standpoint confirm that NIR pre-exposure protect cells from the hazard impacts of UV exposure, and the re-exposure for NIR radiation could be important for protection maintenance (Continenza et al., 1993, Lettnin et al., 2016)

1.2.1 Optical properties of tissue

When the laser light strikes biological tissue, part of this light is absorbed, part is reflected, refracted or scattered, and the rest transmitted.

Refraction phenomenon is produced due to a change in refractive index of air and tissue. Snell's law can be used to explain this phenomenon:

$$\frac{Sin\ \theta_1}{Sin\ \theta_2} = \frac{n_2}{n_1} \dots\dots\dots\dots (1.1)$$

Where θ_1 is the angle between the incident light and the surface normal in the air, θ_2 is the angle between the ray and the surface normal in the tissue, n_1, n_2 are the refractive index of air and tissue respectively (Niemz, 2013).

Most of the light is absorbed by the tissue because the energy state of molecules is quantized; therefore, photonic absorption occurs only when its energy equals the energy difference between such quantized states. Absorption is key for the desired impact on tissue healing. The magnitude of optical absorption is described in terms of the absorption coefficient μ_a, in units of cm^{-1}(Jacques, 2013). The depth of penetration (mean free path) into the absorbing medium is defined by the inverse, l_a (Chung et al., 2012).

The primary step for tissue interaction is scattering behaviour of light in the biological tissue, which is followed by absorption, it is also important because it determines the magnitude distribution of light intensity in the tissue. Scattering of a photon is synchronous with a change in the propagation direction without loss of energy. Analogous to absorption, scattering is expressed by the scattering coefficient μ_s (cm^{-1}) (Niemz, 2013, palan, 2007). The length until next scattering occurs is 1/ μ_s (cm). Scattering is not isotropic, having a physical property that has the same value when measured in different directions. Forward scattering prevail in biological tissue. This physical characteristic is expressed by the anisotropy factor giving absolute values for isotropic scattering (g = 0) to forward scattering (g = 1). In biological tissue, g can differ from 0.8 to 0.99, and can have a considerable role in a reduced scattering coefficient, μ_s' (cm^{-1}), which can be defined as:

$$\mu s' = \mu s\,(1 - g) \ldots\ldots\ldots (1.2)$$

The sum of absorption coefficient (μ_a) and scattering coefficient (μ_s) is called the total attenuation coefficient, that the beam is "attenuated" (weakened) as it passes through the medium. Attenuation coefficient of the volume of a material characterizes how easily it can be penetrated by a beam of light, in other words, the fraction of an incident beam of photons that is absorbed or scattered per unit thickness of the target absorber, μ_t (cm^{-1}):

$$\mu t = \mu s + \mu a \dots\dots\dots (1.3)$$

1.2.2 Light distribution in laser-irradiated tissue

Most of the recent evolutions in describing the transfer of light energy in tissue are based on transport theory (Chandrasekhar, 1960) (radiative transfer), the physical phenomenon of energy transfer in the form of electromagnetic radiation. The propagation of radiation through a medium is affected by absorption, emission, and scattering processes (Chandrasekhar, 1960, Lenoble, 1985). According to transport theory, the radiance L(r, s) of light at position r traveling in the direction of unit vector s is reduced by absorption and scattering, but it is increased by light that is scattered from s′ direction into direction s. Radiance is a radiometric measure that refers to the amount of light that passes through or is emitted from a particular area, and drops within a given solid angle in a particular direction. Then, the transport equation which describes the light interaction is:

$$s.\nabla L\,(r,s) = -(\mu a + \mu s)L(r,s) + \mu s \int p(s,s')L(r,s')d\omega' \dots\dots\dots (1.4)$$

Where $d\omega'$ is the differential solid angle in the direction s', and $p(s,s')$ is the phase function (Chung et al., 2012, Cheong et al., 1990).

Determining the distribution of light in an irradiated tissue is based on the transport equation requiring μ_s, μ_a and p. An exact solution for transport equation is often difficult therefore, several approximations have been made concerning the illustration of the radiance and phase function. The approximate calculations of distributed light in tissue are related to the type of light irradiation (diffuse or collimated) and the optical boundary conditions (matched or unmatched refractive indexes) (Cheong et al., 1990).

1.3 The mechanism of laser-sub-cellular and cellular interaction

It is being suggested that the key underlying mechanism of action for most of the physiological effects attributed to LLLT is the stimulation of mitochondrial activity (Di Giacomo et al., 2013), (Hashmi et al., 2010a). The first law of photobiology states that photons of low power light must be absorbed by electronic absorption bands belonging to chromophores to produce significant effects on living biological systems (Huang et al., 2009). A chromophore (or photoacceptor) is a molecule of a compound, which imparts some colour to the compound (Huang et al., 2011).

According to the theory of quantum mechanics by Max Planck (1900), light energy consists of photons or discrete packets of electromagnetic energy. The individual photon energy depends on the wavelength; therefore, the dose energy of light depends on the number of photons, their wavelength and surface area through spot-size of the laser (Hamblin and Demidova, 2006b).

When photons from a laser are incident on living tissue, it can be locally absorbed or could scatter. Scattered photons are reflected or transmitted (Hamblin and Demidova,

2006b). Absorbed photons interact with the chromophore molecule located within the tissue. The absorption of light leads to excitation of electrons to higher energy levels. The delocalized electrons of the energized molecule which are excited rise from the ground state to an excited stat. This excited molecule must lose its extra energy, which must be conserved according to the first law of thermodynamics. Three possible pathways occur when LLLT is delivered into tissue (Smith, 1991).

Pathway 1: The commonest pathway that occurs is called internal conversion, the excited singlet state of chromophore transport from a higher to a lower electronic state. This transition takes place without photons emitting, known as non-radiative decay (Hamblin and Demidova, 2006b). The energy of the electronically excited state is coupled to rotational and vibrational modes of the molecule. Thus, this interaction increases the kinetic energy of the molecule, such that the excitation energy is transformed into heat. This process would not be expected to cause chemical changes to the molecule (Smith, 1991).

Pathway 2: The second pathway that can occur is fluorescence. Fluorescence is re-emission of light by a substance that has absorbed light. It is a form of luminescence. The excited molecule tends to return to its stable state by emitting photons with a longer wavelength (i.e., lower energy than the absorbed photon) (Smith). The resultant heat (from molecular vibrations) arises from the energy difference between the absorbed and emitted photons.

Pathway 3: The third pathway that can occur after the absorption of low level laser light by a tissue photo-acceptor representing a number of photochemical processes. Although, covalent bonds cannot be broken by low energy photons, the energy is however sufficient for electrons to go from the first excited singlet state to the triplet

state of the photoacceptor through intersystem crossing. Increasing the reaction rate allows transforming such as ground state molecular oxygen (a triplet) to singlet oxygen state (reactive oxygen species). Alternatively, the long-lived triplet of the chromophore may undergo electron transfer to form a radical anion, which can transfer an electron to oxygen to form a superoxide (Hamblin and Demidova, 2006b).

The photochemical pathway is the separation of a non-covalent bound ligand from a binding site on a metal in an enzyme. Cytochrome c oxidase of the mitochondrial respiratory chain is the candidate enzyme for a photoacceptor (chromophore), a molecule imparts a color to a compound, mediating the transfer of electrons from cytochrome c to molecular oxygen. After absorbing red or near-infrared light, cytochrome c oxidase undergoes photochemical processes through the dissociation of binding of nitric oxide from the iron-containing and copper-containing redox centres in the enzyme (Hamblin and Demidova, 2006b). There is a growing body of evidence which suggests that cytochrome c oxidase could act as a photoacceptor of light in the near-infrared spectral range (Silveira et al., 2009). It is also considered as the photosignal transducer in the region of visible and IR-A region (Karu, 2010). This reactivity is due to four redox active metal centers: the bi-nuclear CuA, CuB, heme a, and heme a3, all of which have strong absorbency in the red to IR-A range (Karu, 2010, Piazena and Kelleher, 2010, Smith, 2007).

Many studies on the biological influence of LLLT have compared the action spectrum, a plot of the relative effectiveness of different wavelengths of light in causing a particular biological response, and under ideal conditions it should follow the absorption spectrum of the specific molecule, and whose photochemical alteration

causes the biological effect attributed to the absorption spectra. These studies have suggested cytochrome c oxidase as the primary photoacceptor (chromophores) (Smith, Desmet et al., 2006).

Cytochrome c oxidase is the fourth enzyme in the inner membrane of cellular mitochondria (Di Giacomo et al., 2013, Habash et al., 2006), as shown in Figure 1, that plays a pivotal role in Adenosine tri phosphate (ATP) synthesis (Silveira et al., 2009). Excitation of cytochrome c oxidase components with infrared light energy accelerates the rate of electron transfer and in turn increases the ability of mitochondria to produce ATP, which accelerates cellular metabolic processes (Silveira et al., 2009). Moreover, signal transduction to other parts of the cell has occurred, including cell membranes (Woodruff et al., 2004). Photobiological responses are the result of photochemical and /or photophysical changes after the absorption of non-ionizing electromagnetic radiation (Smith, 1991).

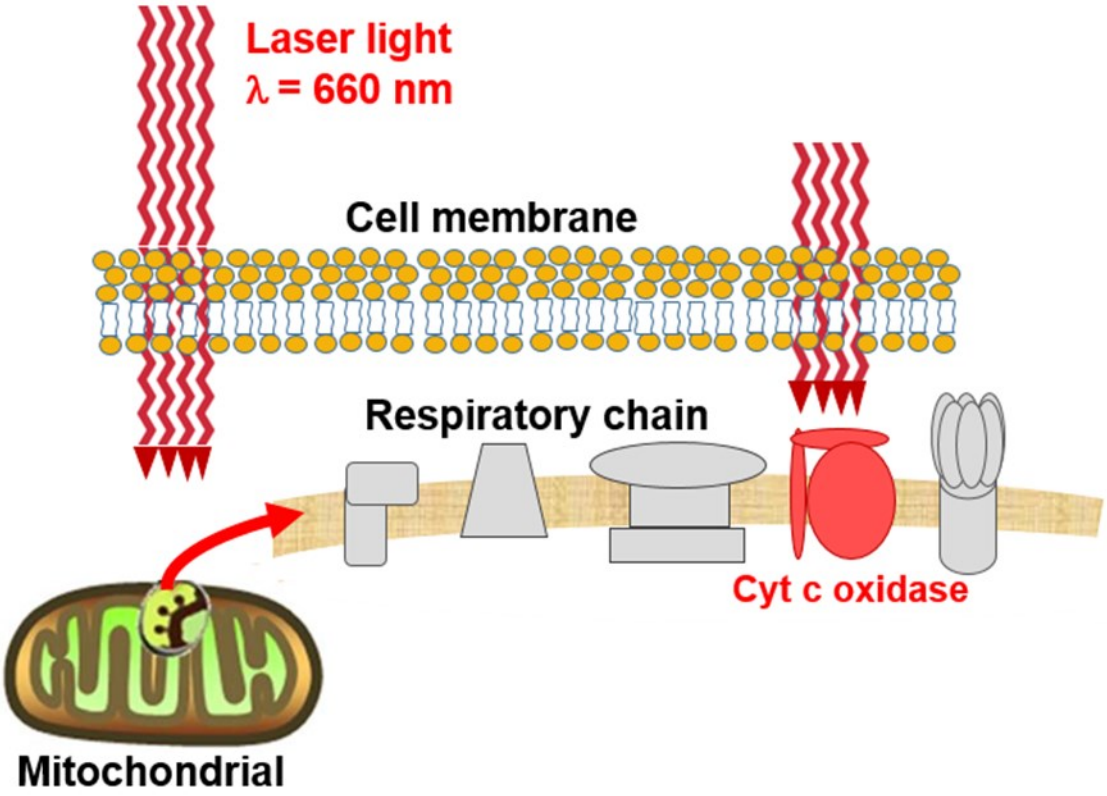


Figure 1. 1. The absorption of laser light photon by mitochondrial respiratory chain enzyme cytochrome c oxidase as a chromophores molecule highly absorb light at red visible and near infrared wavelength. (Figure adapted from Huang et al.(2011)

Production of nitric oxide (NO) in mitochondria especially in injured or hypoxic cells can inhibit respiration by binding to cytochrome c oxidase and displace oxygen (Brown, 1995). This binding is proposed to dissociate by the PBM or LLLT effect, and reverse the mitochondrial inhibition of respiration due to excessive NO binding (Lane, 2006). The photobiomodulation effect of LLLT is able to occur a shift in the overall cell redox potential in the direction of greater oxidation by generating reactive oxygen species (ROS) and inhibiting reactive nitrogen species (RNS) (Alexandratou et al., 2002; Lavi et al., 2003; Lubart et al., 2005; Zhang et al., 2008; Cotler et al., 2015). The excited mitochondrial cytochrome c oxidase after absorbing NIR radiation

photon generates ROS that causes changing the oxidation state of the mitochondrial membrane (Gilmore, 2006b).

For the phototherapeutic effect to be observed, the appropriate wavelength of light and dose (fluency) of radiation are needed (Woodruff et al., 2004). However, phototherapy will not be effective on every system and in every situation. Karu (1989) (Karu, 1989b) has emphasised that the magnitude of the phototherapy effect depends on the physiological state of the cell at the moment of irradiation (Karu, 1989b).

1.4 Light Emitting Diodes (LEDs)

A light emitting diode (LED) is a semiconductor light source (Han et al., 2011). Henry J Round was the first who reported of light emission from carborundum (raw silicon carbide) in 1907. Oleg Losev, as a lot of people today believe, was the actual inventor of LED. He published his first paper in 1927 on emission of silicon carbide diodes. Losev set up the current threshold for the onset of light emission from the contact point between a silicon carbide crystal and a metal wire and recorded the spectrum of this light (Desmet et al., 2006). A LED is formed by p-n junctions (p-positive, n-negative), but not all semiconductors are suitable for use as LEDs (de Abreu Chaves et al., 2014). The physical mechanism by which LED emits light is spontaneous emission(de Abreu Chaves et al., 2014). They emit near-monochromatic, incoherent light (Ishida, 2005), in a process called electroluminescence (Molinaroli, 2001). LEDs are small, robust devices that emit a

narrow band of electromagnetic radiation from the ultraviolet to the visible and infrared parts of the spectrum, from around 240 nm up to around 950 nm, according to their electronic structure (Ishida, 2005).LEDs have been publicised as a comfortable, potentially highly selective light-based therapy for many indications (McDaniel et al., 2003). LEDs are also very controllable as light sources for non-thermal applications, acquiring a broad area of medical applications (Avci et al., 2013a).

1.4.1 Laser light vs light emitting diode (LED)

Not all lights are the same or have equal medical benefit (LED or Laser therapy). Recently, controversy has arisen around the comparison between low level laser therapy and light emitting diodes, which have completely different biological effects (Agnol et al., 2009). A number of studies compared the effectiveness of LLLT to LED light (Figure 1.2), and the majority found, although lasers have small focused spots so only a small area of tissue (< 1 cm^2) is exposed to light; on the other hand LEDs usually have a large area (100 cm^2) so much more tissue is exposed to light however, lasers are far more effective (Agnol et al., 2009). Laser therapy can achieve much greater and deeper stimulative and therapeutically beneficial effects Laser beams are easily manipulated using Gaussian beam optics, a simple analytical tool, to enable a laser beam to be fully controlled spatially, position, size etc. While a LED is difficult to control in terms of position and spot size, and so it is limited for treatment of superficial tissue only. Nevertheless, it is believed that LED light can have a photo-modulation effect on certain cellular and sub-cellular

receptors. In addition, they have greater choice of wavelengths, are low cost and suitable for acute and chronic conditions (Darren Starwynn, 2004).

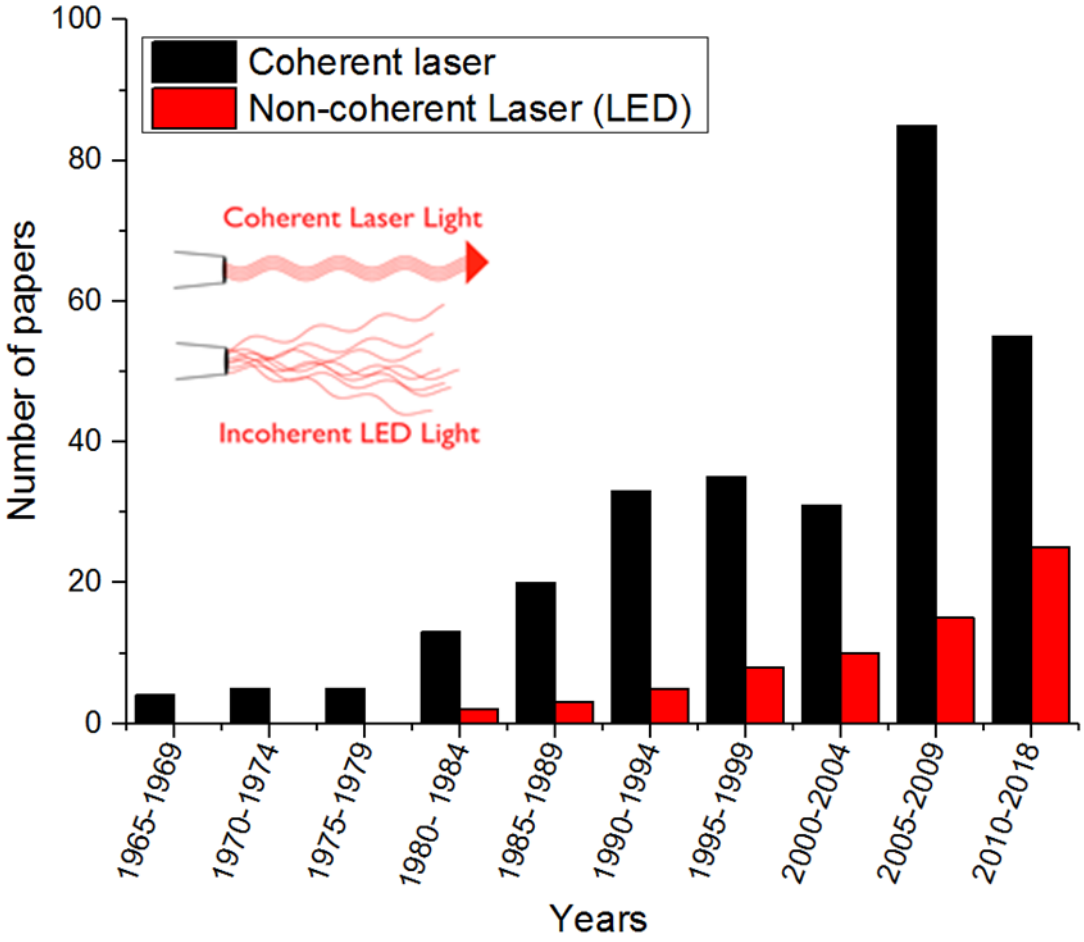


Figure 1. 2. Coherent sources and non-coherent (LED) of LLLT in clinical and laboratory studies on the effect of LLLT on cell and DNA from 1965-2018

A number of studies have been published comparing these two modalities:

Kubota and Ohshiro (Kubota and Ohshiro, 2004) treated rat skin flaps with an 830 nm GaAlAs laser and an 840 nm infrared LED. They found an increasing flap survival area in a rat model after being irradiated with 830 nm laser. Flaps treated with the laser had better perfusion, a greater number of larger blood vessels, and significantly enhanced flow rates. While, flaps treated with an 840 nm IR LED showed no difference from the control group (Kubota and Ohshiro, 2004).

Berki et al. (Berki et al., 1988) used a HeNe laser to stimulate cell activation *in vitro*. They observed increasing phagocytic activity along with immunoglobulin secretion, but this effect was not seen after irradiation of the cell cultures with LED light of the same wavelength and doses (Berki et al., 1988).

A comparative study has been performed by Haina et al. (Haina et al., 1982) to show the effectiveness of HeNe, coherent laser compared with incoherent light of the same wavelength. Experimental wounds were 'punched out' in the muscle fascia of 249 Wister rats. They reported increasing granulation of tissue in the HeNe treated group, whereas there was less granulation in the incoherent light therapy group (Haina et al., 1982).

Rockhind and colleagues (Rochkind et al., 1989) conducted a study comparing five different wavelengths lasers. They gave a single transcutaneous irradiation dose to injured peripheral nerves. They observed reduced subsidence in functional activity following crush injury after HeNe laser irradiation. While the 830 nm IR laser was less effective, the 660 nm incoherent light was even less effective; 880 nm and 950 nm incoherent lights were completely ineffective (Rochkind et al., 1989). Laasko et al. (Laakso et al., 1994) treated patients with chronic pain using an 820 nm IR laser at 25 mW, a 670 nm laser at 10mW and a 660 nm LED. They found an elevated level of ACTH and beta endorphin in the laser therapy groups but not in the LED group (Laakso et al., 1994).

The effect of HeNe laser and incoherent LED light on leukocytes in migration inhibition assays has been studied by Lederer et al. (Lederer et al., 1982). They reported that irradiation with HeNe laser light affected leukocytes. While, incoherent

light of the same wavelength and power density showed no influence (Lederer et al., 1982). al. et al. (al., 1989) investigated the role of coherent laser therapy in wound healing. They noticed that HeNe lasers with a dose of $1J/cm^2$ produced an acceleration of the healing process, but incoherent light of the same wavelength and dose was less favourable (al., 1989).

Other studies have indicated many reasons which could lead to a preponderance of LED light than to laser light. NASA has stepped into developing LED light therapies for accelerating wound healing, photodynamic cancer treatment and much more. According to NASA: “The near-infrared light emitted by these LEDs seems to be perfect for increasing energy inside cells. This means whether you’re on Earth, in a hospital, working in a submarine under the sea or on your way to Mars inside a spaceship, the LEDs boost energy to the cells and accelerate healing”(Darren Starwynn, 2004, Sommer et al., 2001) . Oliveira and colleagues (Oliveira Sampaio SC, 2012) studied the effect of low level light therapy on the healing of cutaneous wound and their impact on fibroblastic activity during wound healing. They showed an increasing number of healthy animals after irradiation with laser light, and a higher increase was seen when irradiated with LED. They concluded that using LED light caused a considerable bio-modulation of fibroblastic proliferation on anaemic animals. While laser light was more effective on increasing proliferation on non-anaemics (Oliveira Sampaio SC, 2012). A clinical study by Esper and colleagues (Esper MA, 2011) was carried out to show the effect of two phototherapy protocols on pain control in orthodontic procedure. They found that LED light therapy had a significant effect in the reduction of pain levels compared to laser light therapy. LED

therapy showed a significant reduction in pain sensitivity (an average of 56%), when compared to the control group (Esper MA, 2011).

Dall et al. (Dall Agnol MA, 2009) performed a comparative analysis of coherent laser light versus incoherent (light emitting diode) light for tissue repair in diabetic rats. They found that the coherent and incoherent lights produced similar effects during a period of 168 hr after the lesions had been made. For the control group composed of diabetic animals, 72 hr after creation of the lesion, it was observed that the therapy with LEDs had been more efficient compared with the laser for the reduction of the healing period (Dall Agnol MA, 2009). Similar findings have been obtained by Klebanove and colleagues (Klebanov GI, 2005) in a comparative study of the effect of laser and light emitting diode irradiation on healing and functional activity of wound exudate leukocytes (Klebanov GI, 2005). They deduced that coherent laser and incoherent light-emitting diode radiation have very similar effects on wound healing and activity of wound exudate leukocytes, and that the coherence of light is not required for this activity (Klebanov GI, 2005). Another study by Klebanove and colleagues (Klebanov GI, 2006) has been carried out to explore the comparative effects of laser light and light emitting diodes on the production of superoxide dismutase and nitric oxide in wound fluid of rats. The study indicated that dose-dependent changes in superoxide dismutase activity and production of nitrites in wound fluid after irradiation with visible coherent laser and incoherent LED and the radiation coherence does not play any significant role in the changes of superoxide dismutase activity or nitrogen oxide formation (Klebanov GI, 2006).

The rapid evolution of light emitting diodes makes feasible the use of LEDs for medical treatment and light therapy (Yeh et al., 2010). The single frequency laser does not diffuse, whereas the LED light does. This diffusion allows the cell to be in control of the treatment (Ghuloom, 2013). Moreover, LED light therapy has been considered non-significant risk by the FDA (Desmet et al., 2006). For this reason it was published that using light emitting diodes for treatment is much safer than laser therapy (Ghuloom, 2013).

Given the above information, and from recently published studies (Oliveira et al., 2013, Lee et al., 2007), it has been shown that lasers have an important role in many medical conditions with many positive research results (Avci et al., 2014, Cotler et al., 2015, Bell and Stout, 2018), as well as LEDs which are also important in many cases of disease (Corazza et al., 2007, Xavier et al., 2010). Nevertheless, in most comparative studies that used laser and LED with the same qualities (wavelength, doses, intensity), it is concluded confirmed LASERs offer many advantages compared to LEDs (Leal Junior et al., 2009).

1.5 Effect of LLLT at cellular level

To assess the influence of low level laser therapy at the cellular level, cell culture is one of the best biological systems used to find out the effect of laser irradiation on cell proliferation rate. Various studies, which have used different types of laser therapy with a variety of cells, have been designed to improve understanding on the effect of LLLT at the cellular level (Figures 1.3 & 1.4). More recent studies have studied the bio-stimulatory effect of low level laser on cell proliferation processes.

Early work by Karu and colleagues (Karu et al., 1994a) have reported that the cytotoxic response of Hela cells to ionizing radiation can be influenced by irradiation with He-Ne laser 632.8 nm with an energy density 100J/m^2. They observed that there was a substantial difference between the survival curve of Hela cells treated with He-Ne laser for 60 min before exposure to γ- irradiation and the curve representing the survival of untreated γ-irradiated cells. Moreover, an increase in the number of cells has been observed after stimulation with a He-Ne laser compared to the control group (Karu et al., 1994a).

Pereira and colleagues (Pereira et al., 2002) examined a 632.8 nm He-Ne laser with an energy fluence of 0.053 to 1.89 J/cm^2 and a 904 nm (GaAs) laser with an energy fluence of 1.94×10^{-7} to 5.84×10^{-6} J/cm^2 on fibroblast cell cultures, which determined by using the Trypan blue dye exclusion assay. No difference in cellular proliferation for fibroblast cells exposed to a He-Ne laser versus untreated fibroblast cells could be found. On the other hand, with GaAs laser, a decrease in cellular proliferation of fibroblast cells compared to controls was observed. However, both He-Ne and GaAs lasers induced procollagen production (Pereira et al., 2002).

It was noted that with exposure to a 670 nm GaAlAs laser, an increase in myofibroblasts and collagen deposition was observed (Medrado et al., 2003). Furthermore, an increase in gingival fibroblasts after exposure to diode lasers (670, 692, 780, and 786 nm) was also found (Posten et al., 2005).

Bouma and colleagues (Bouma et al., 1996) examined human monocytes and human umbilical vein endothelial cells (HUVECs) with a 904 nm GaAs laser at 40.18 mW/cm^2 power density. They found no difference in the cytokines level such as tumour necrosis factor TNFα, interlukin-6 and -8, E-selectin, intercellular adhesion

molecule 1, and vascular cellular adhesion molecule 1 (Bouma et al., 1996). Schindl and colleagues (Schindl et al., 2003) reported that HUVECs irradiated with a 670 nm diode laser with a dose of 2 to 8 J/cm^2 resulted an increase in the proliferation of these cells, that determined by using a haemocytometer (Schindl et al., 2003). An *in vitro* study by Hass and colleagues (Haas et al., 1990) showed an increase in human keratinocytes mortality, that observed by inverted phase microscopy after exposure to He-Ne laser and found no change in proliferation or differentiation (Haas et al., 1990). While, Grossman and colleagues (Grossman et al., 1998) observed an increase in proliferation rate of keratinocyte cells, which counted microscopically using a counting chamber after exposure to a 780 nm continuous-wave diode laser with a dose from 0 to 3.6 J/cm^2 (Grossman et al., 1998)

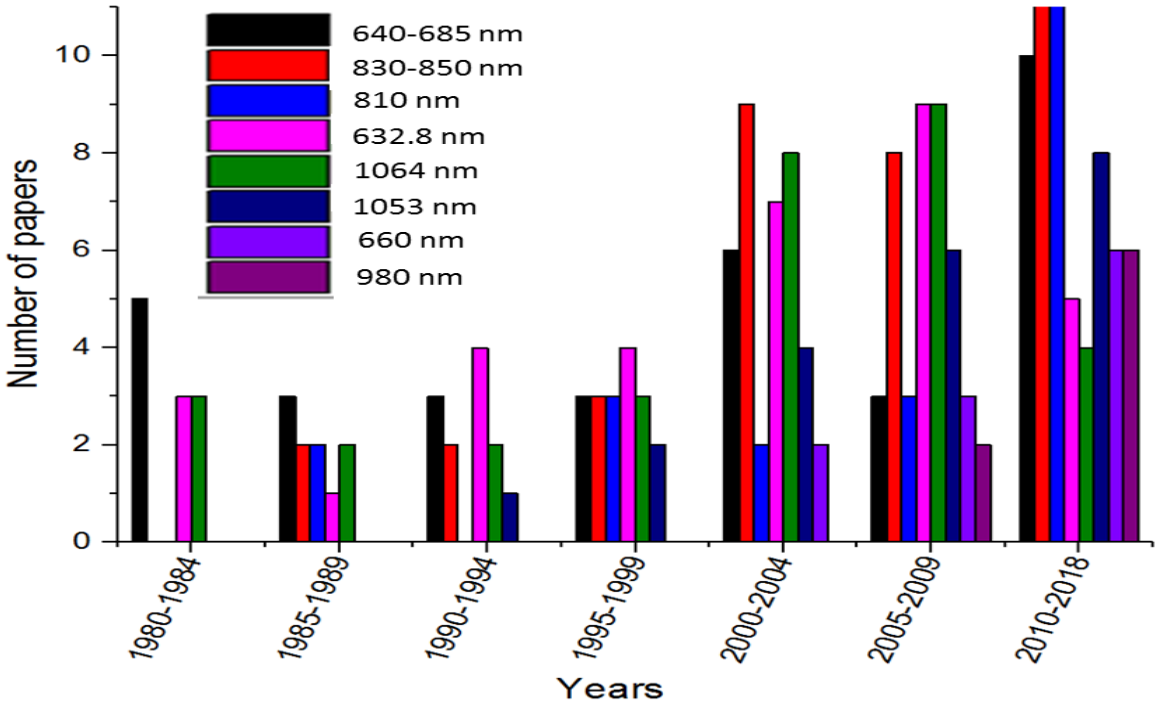


Figure 1. 3. Light sources used in clinical and laboratory studies on the effect of LLLT on cell functions from 1965-2018

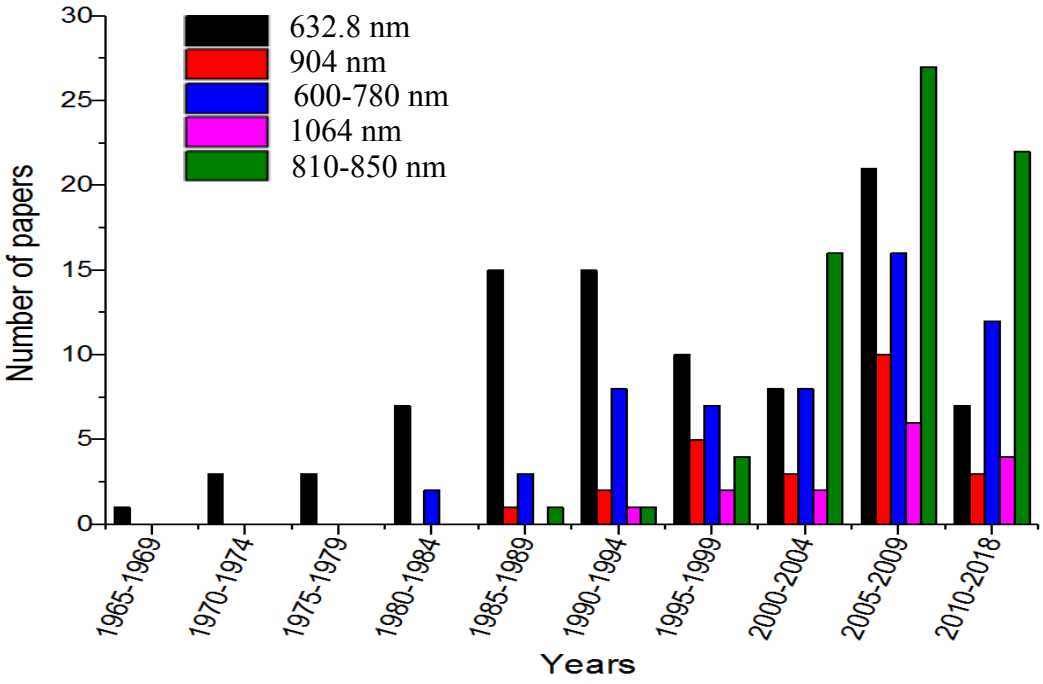


Figure 1. 4. Light sources used in clinical and laboratory studies on the effect of LLLT on cell functions from 1965-2018

Researchers pointed out that using low laser therapy with low doses can increase the proliferation rate of cultured cells when compared to high doses. Beyond a certain dose level, which is cell type dependent, high dose levels have a detrimental effect on cell proliferation rates. AlGhamdi and colleagues (AlGhamdi et al., 2012) have examined stem cells with a He-Ne laser at 632.8 nm and a GaAlAs at 600 nm, with a range of energy densities (doses) from 0.5 – 4.0 J/cm^2 and power densities from 1-500 mW and found that LLLT can increase the proliferation rate of various cell lines. They have confirmed that the stimulation of cellular proliferation is dependent on the dose level of laser irradiation. They concluded that lower doses increase the rate of cell proliferation and other cellular functions, the determination of cell count was achieved by using Trypan blue stain. Whereas, higher doses of low level laser therapy have negative effects, where the high doses caused a significant

decrease in cells count and the percentage of cell viability (AlGhamdi et al., 2012). Similar results have been obtained by Walsh and colleagues (Walsh, 1997), when they irradiated fibroblasts of skin cells, buccal mucosa and gingival cells with semiconductor lasers at 540 nm and 600-900 nm and energy densities 0-56 J/cm^2. Walsh noted increased cell proliferation at low doses, which measured by using Trypan blue dye exclusion assay, and repressed at high doses. They, also observed increase maturation and locomotion, transformation to myo-fibroblasts, and increased production of basic fibroblasts growth factors.

Walsh and colleagues (Walsh, 1997) used the same laser with the same energy densities to examine macrophage cells. They observed convergent results, greater secretion of basic fibroblasts growth factors, increased ability to act as phagocytes, and resorption of fibrin by macrophages. Walsh in another study used semiconductor lasers of 660, 820, and 940 nm to treat human lymphocytes cells. They showed activated lymphocytes and high proliferation rate. With the same wavelengths, Walsh noted the increased motility of epithelial cells and an ability to migrate across wound sites with quickened closure of defects.

Unlike AlGhamdi and Walsh, Petri and colleagues (Petri et al., 2010) found that cell growth, as measured by MTT assay, was affected by time with LLLT after exposing human alveolar bone fragment cells to a GaAlAs diode laser of 780 nm with power of 70 mW and energy density 3 J/cm^2 (Petri et al., 2010). Recently, Forouzanfar (Forouzanfar, 2014)has support Petri' s results when examining human gingival fibroblasts with a Ga-Al-As diode laser at 810 nm, output power 50 mW and energy density 4 J/cm^2 . Forouzanfar noted that both good levels of cell proliferation and secretion of macromolecules can be regulated if enough exposure time of low level laser therapy has been given to the cells to determine whether LLLT could induce a

bio-stimulatory effect on human cells. As well, they have found a significant difference between the case and control groups on 48 and 72 hr after irradiation (Forouzanfar, 2014).

Tuby and colleagues (Tuby et al., 2007) obtained a positive result when they exposed mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) to a GaAs diode laser at 804 nm with an energy density between 1 and 3 J/cm^2 and an output power 50 mW. The results showed a significant increase of seven-fold and two-fold in the number of CSCs after 1 and 2 weeks post irradiation of 1 J/cm^2 for 20 sec exposure and increased the number of MSCs and CSCs after 1 week post irradiation of 3 J/cm^2 compared to the control (Tuby et al., 2007).

Almeida and colleagues (Almeida‐Lopes et al., 2001b)used diode laser with 670, 692, 780, and 786 nm wavelengths and fluence (energy density) of 2 J/cm^2 to show the comparison of LLLT effects on the proliferation rate of cultured human gingival fibroblast cells. They found that in the same fluence and with different output powers, infrared lasers induced a higher proliferation rate of cells compared to visible laser. Whilst lasers of equal output power were shown to have similar effect on cell growth independently of their wavelengths(Almeida‐Lopes et al., 2001b).

In this study THP-1 cell lines were used to study the effect of LLLT on the proliferation rate and the viability of these cells. These cell lines have been established thirty years ago. THP-1 cell is a human leukaemia monocytic-like cell line derived from leukaemia from a one year old boy, this cell line had Fc and C3b receptors, but no surface or cytoplasmic immunoglobulins (Chanput et al., 2014, Qin, 2012). This cell line can provide continuous culture, grown in suspension and do not adhere to culture plate surfaces(Tsuchiya et al., 1980). THP-1 cells have the ability

to differentiate into macrophage cell. They are phagocytic and show increased CO_2 production on phagocytosis (Bremner et al., 1999).

The number of studies that using LLLT and its biological influence on THP-1 cell line is very limited. Most of them have been included the effect of LLLT on monocyte/macrophage functions, mechanisms and signalling pathway (Hwang et al., 2015). Low-level light therapy (LLLT) has been widely known to regulate inflammatory reaction (Dos Santos et al., 2014), and investigate the anti-inflammatory effect of LLLT at a range of wavelengths (405, 532 and 650 nm) on macrophage-like THP-1 cells (Hwang et al., 2015, Kim et al., 2008) While few of the rest have assessed the effect of LLLT on proliferation, viability and proliferation-induced growth factors. Figure 1.5 highlights the sources of laser therapy used in clinical and laboratory studies on the effect of LLLT on THP-1 functions.

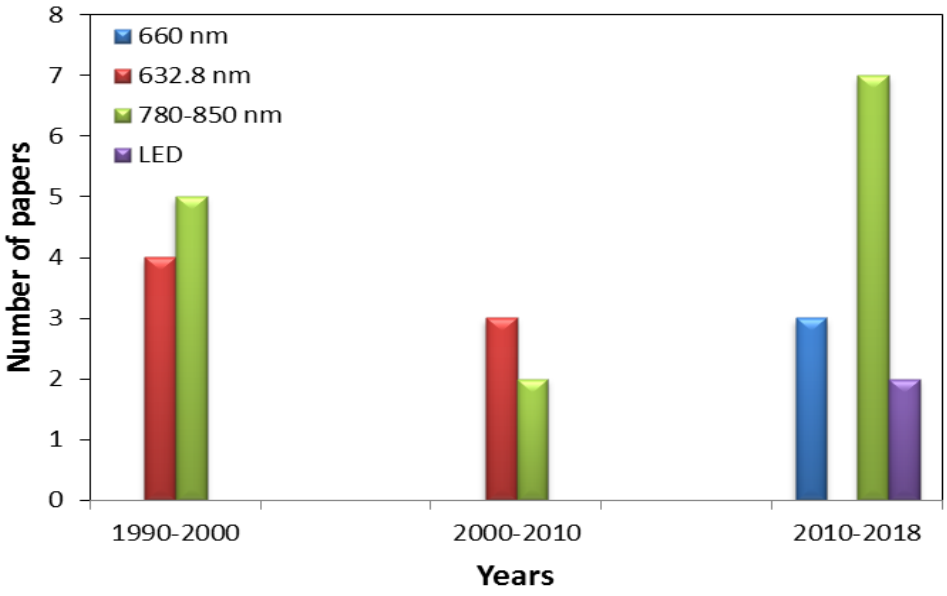


Figure 1. 5. Sources of laser therapy used in clinical and laboratory studies on the effect of LLLT on THP-1 functions.

1.6 Effect of LLLT at molecular level

LLLT has been in existence for more than four decades. It has been found beneficial in a wide variety of therapeutic applications (Mbene, 2008). However, the possibility of induced DNA damage has now arisen; even though, this damage could be repairable (Mbene, 2008). Although, phototherapy is used in the biomedical treatment of many diseases, the mechanisms of laser-molecule interaction remain unclear and the deleterious effects of laser irradiation are still controversial (Kujawa et al., 2004).

LLLT is usually performed with visible red or near infrared laser light and with typical accumulated doses. Since employing wavelengths within the red side of the optical spectrum, which is likely to be less damaging to DNA than sun light, it is assumed that the doses per area of LLLT are safe when corresponding to the DNA damaging effects of a few minutes sunlight (Kujawa et al., 2004). If such irradiation induces DNA breaks, these breaks are likely to be repaired immediately; otherwise unrepaired damage could lead to mutations consequently leading to development of cancer in the long run (Albertini et al., 2008).

Different studies in eukaryotic and prokaryotic cells have reported adverse effects on cells and DNA damage after exposure to low power laser therapy (Kong et al., 2009), (Figure 1.6). Experimental data about the effect of these light sources with different power, wavelengths, and emission modes on DNA are however scared (Karu, 2010). A study by Zhang and colleagues (Zhang et al., 2003) using microarray technologies indicated that low intensity laser exposure (red light) at therapeutic doses has been demonstrated to promote expression of DNA repair genes following DNA lesions induced by free radicals (Zhang et al., 2003).

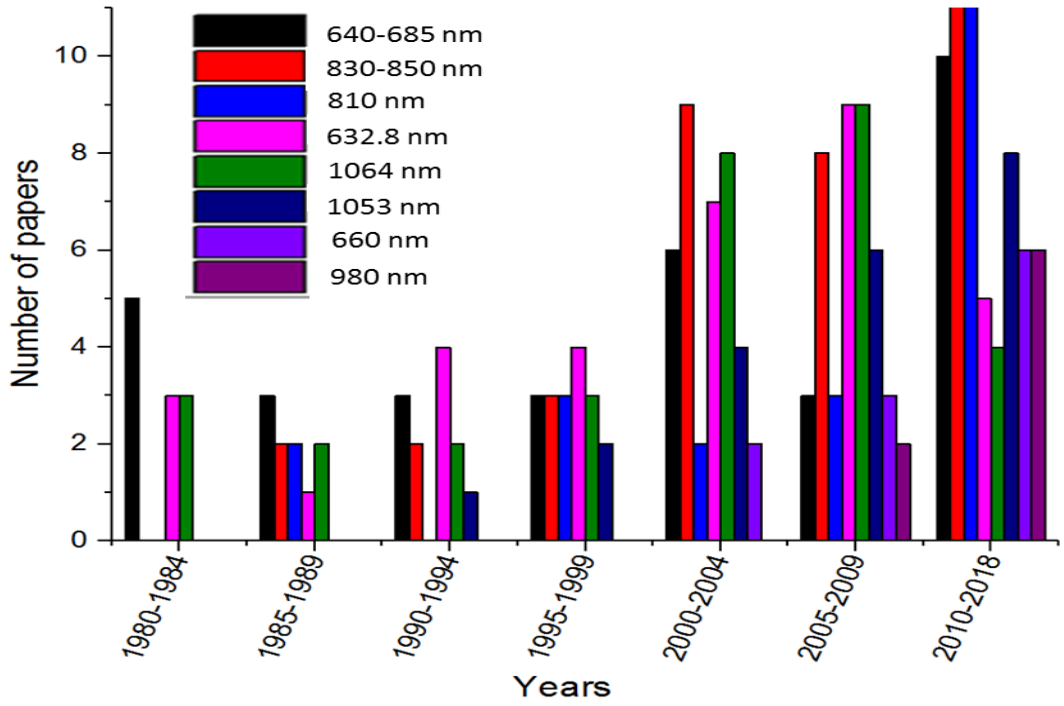


Figure 1. 6. Light sources for LLLT used in clinical and laboratory studies on the effect of LLLT on DNA from 1980-2018

It has been reported that the photo-reactivating enzyme (DNA photolyase) distinguishes one type of DNA damage as its substrate (i.e. the cyclobutane-type pyrimidine dimer), and combines with these dimers in the dark (Smith, 1991). However, when exposing the enzyme-substrate complex to visible light, the enzyme uses the absorbed energy of light to split the dimer to produce repaired DNA. Mbene (Mbene, 2008) treated wounded human skin fibroblast cells by He-Ne laser with 5 J/cm^2 and 16 J/cm^2 doses. Irradiation with 5 J/cm^2 and 16 J/cm^2 showed insignificant change in DNA damage, as determined by alkaline comet assay, at 1h when compared to their respective controls. However, a significant decrease in DNA damage at 24h incubation due to the mechanism of DNA damage repair was shown (Mbene, 2008).

Fonseca and colleagues (Fonseca et al., 2010) irradiated E.coli cells with low intensity (AlGaInP) red laser with a power of 10 mW and with different fluencies (1, 4 and 8 J/cm^2). It was suggested that low-level red laser light induces DNA lesions as a result of the generation of free radicals. They suggested that biological effects induced by low level laser fluence could occur due to the generation of free radicals. They suggested that considerable importance should be given to low-level lasers for their potential to induce DNA repair and changes in gene expression profile of the irradiated cells (Fonseca et al., 2010).

A study by da Silva and colleagues (da Silva Sergio et al., 2012b)used an AlGaInP laser with a power output of 10 mW, and with continuous or pulsed mode of irradiation. They found that low-intensity red laser radiation could induce DNA lesions via oxidative mechanisms. Moreover it was found that the survival mechanism against harmful radiation could be activated or induced after irradiation with monochromatic red light (da Silva Sergio et al., 2012b). Kohli and colleagues (Kohli et al., 2001) examined E.coli cells with a He-Ne laser at 632.8 nm. They observed that irradiation with low level He-Ne lasers induces photolyase gene (*phr*) and DNA repair genes investigated by *phr* gene expression assay. The magnitude of induction relies on fluence rate of the He-Ne laser and the time of incubation post irradiation. The study concluded that the stimulation of DNA repair may explain the higher survival cell against UV radiation (Kohli et al., 2001).

Dube and colleagues (Dube et al., 2001) studied the effect of He-Ne laser 632.8 nm pre-irradiation on UVA induced DNA damage in the human B-lymphoblast cell line, as measured by comet assay. They found a decrease in UVA-induced DNA damage. Whereas, the control cells showed higher DNA damage, the same rate of DNA damage in He-Ne laser pre-irradiated cells. The results suggest that He-Ne laser

irradiation plays an important role in protecting the cells from UVA-induced DNA damage primarily through an influence on processes of preventing an initial damage of DNA (Dube et al., 2001).

Dillenburg and colleagues (Dillenburg et al., 2014) triggered epithelial cells with laser phototherapy (LPT) of energy density 4 J/cm^2 and 20 J/cm^2. They observed that laser phototherapy at a low energy density of 4 J/cm^2 did not induce DNA damage or genomic instability, that determined by comet assay. Interestingly, a low energy of LPT induced nuclear influx of the BRCA1 protein of DNA repair, which is a genome protective molecule that effectively takes part in DNA repair. Importantly, these findings suggest that LPT of low dose induces a safe level of reactive oxygen species (ROS), which accelerate healing (Dillenburg et al., 2014).

Ridha and colleagues (Ridha et al., 2012) used a He-Ne laser 632.8 nm to irradiate human lymphocytes. They concluded that the effect of low red laser light in maintaining cell survival may be attributed to the induction of endogenous radioprotectore and improvement of DNA repair due to induce enzymes involved in repair process (Ridha et al., 2012). More recently, Trajano and colleagues (Trajano et al., 2014) stated that at therapeutic fluences, exposure to red visible laser therapy alters the expression of genes related to the base excision and nucleotide excision pathways of DNA repair during wound healing (Trajano et al., 2014).

Although, most of the aforementioned studies have been appeared to show the effect of LLLT on cell proliferation, conflicting results have been published. As well, studies tried to explain the induction effect of LLLT on repair mechanisms of DNA damage showed variance results. All these contrasts may be related to a disparity in

study design, including the use of different lasers and/or variations in parameters such as energy densities, wavelengths, exposure time, output power etc.

1.7 DNA damage

DNA is the genetic material of organisms, and its integrity is essential for the maintenance of life (Bohr et al., 1987). DNA exclusively serves as the repository for the genetic information in each living cell and its integrity and stability are of much greater consequence than other cellular components, such as RNA and proteins (Acharya, 1972).

DNA damage is an alteration in the chemical structure of DNA, such as a break in a strand of DNA, a base missing from the backbone of DNA, or a chemically changed base such as 8-OHdG (Lomax et al., 2004). DNA is under constant onslaught from different sources from either intrinsic or extrinsic agents. Oxygen and light are major causes of DNA damage (Guengerich, 2014).

1.7.1 Types of DNA Damage

According to the sources of attack, DNA damage can be subdivided into two main types: endogenous and exogenous damage, the vast majority of DNA modifications are endogenous in origin (Friedberg et al., 2005, De Bont and Van Larebeke, 2004). Endogenous damage is, due to normal metabolic and spontaneous hydrolytic processes inside the cell (Lindahl, 1993), include alkylating agents (e.g. s-adenosylmethionine), oxidants (reactive oxygen and nitrogen species), and electrophilic products arise from oxidative and other intracellular reactions

(Guengerich, 2014, Friedberg et al., 2005). Figure 1.7 shows the endogenous sources of DNA damage.

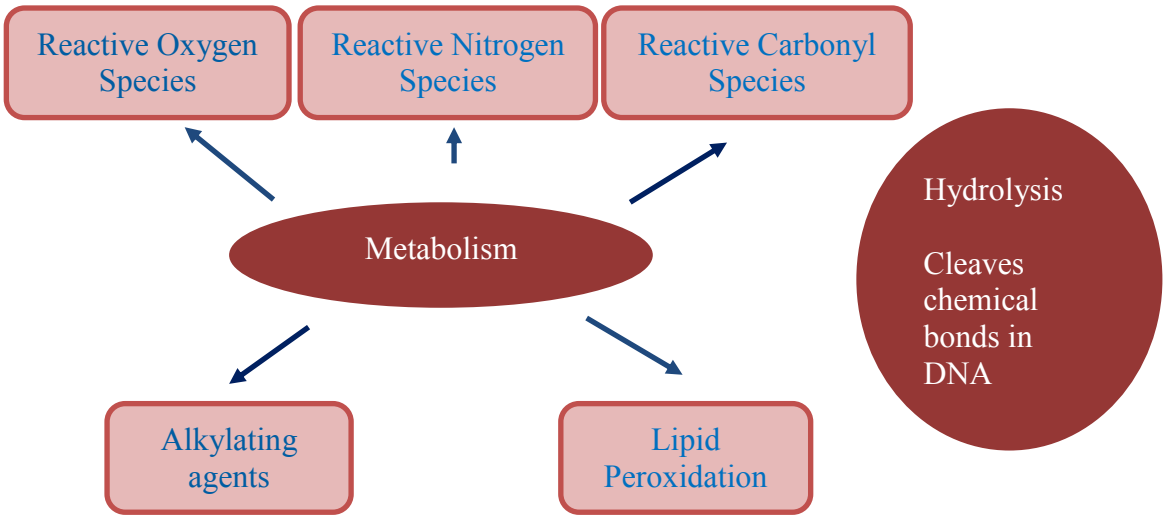


Figure 1. 7. The essential endogenous sources of DNA damage, metabolism and hydrolytic processes

Exogenous damage is due to environmental factors include physical agents such as ultraviolet (UV) light, and other radiation, chemical agents such as pollution, carcinogens in food, and chemotherapeutic agents (Guengerich, 2014, Setlow, 1995), Figure 1.8 demonstrates the general extrinsic and intrinsic causes of DNA damage.

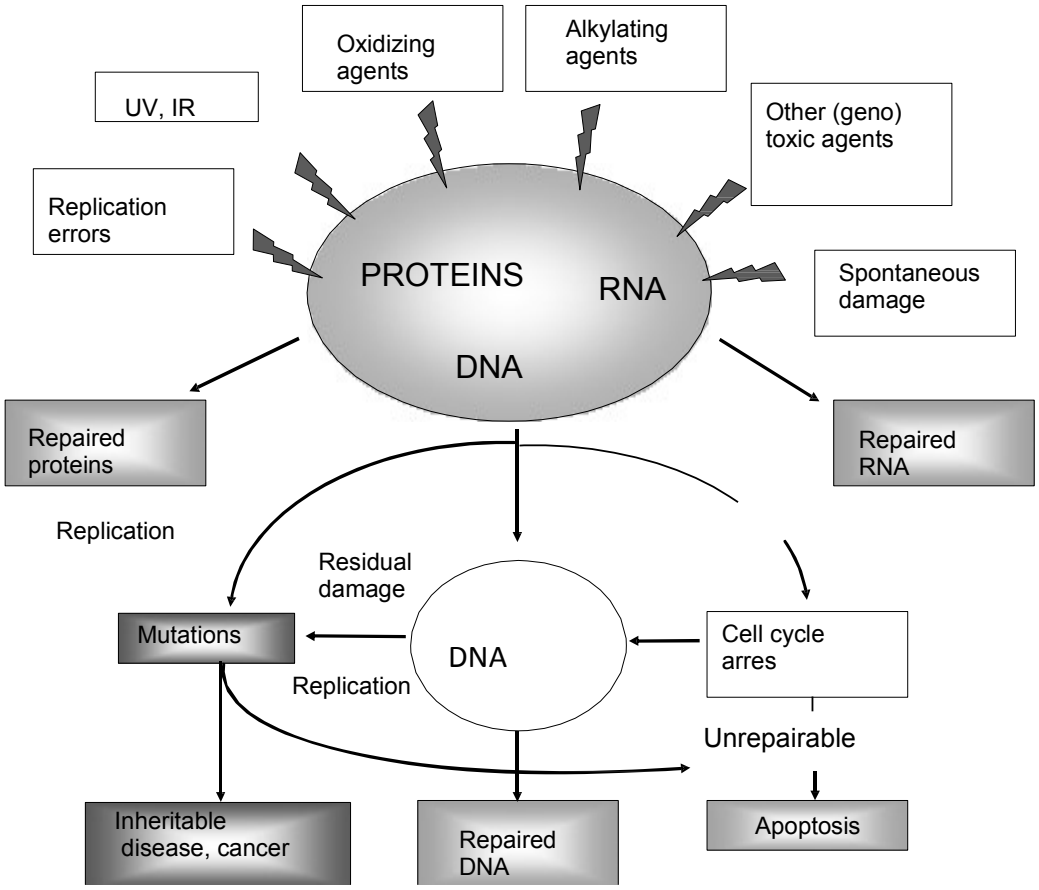


Figure 1. 8. Scheme of the general extrinsic and intrinsic factors affecting on cellular macromolecules and their impacts. The Figure is adapted from Degterev and Yuan, (2008).

There are several types of damage to DNA intracellular and that damage caused by extracellular agents comes in many forms, Figure 1.9 shows variety of damaged DNA.

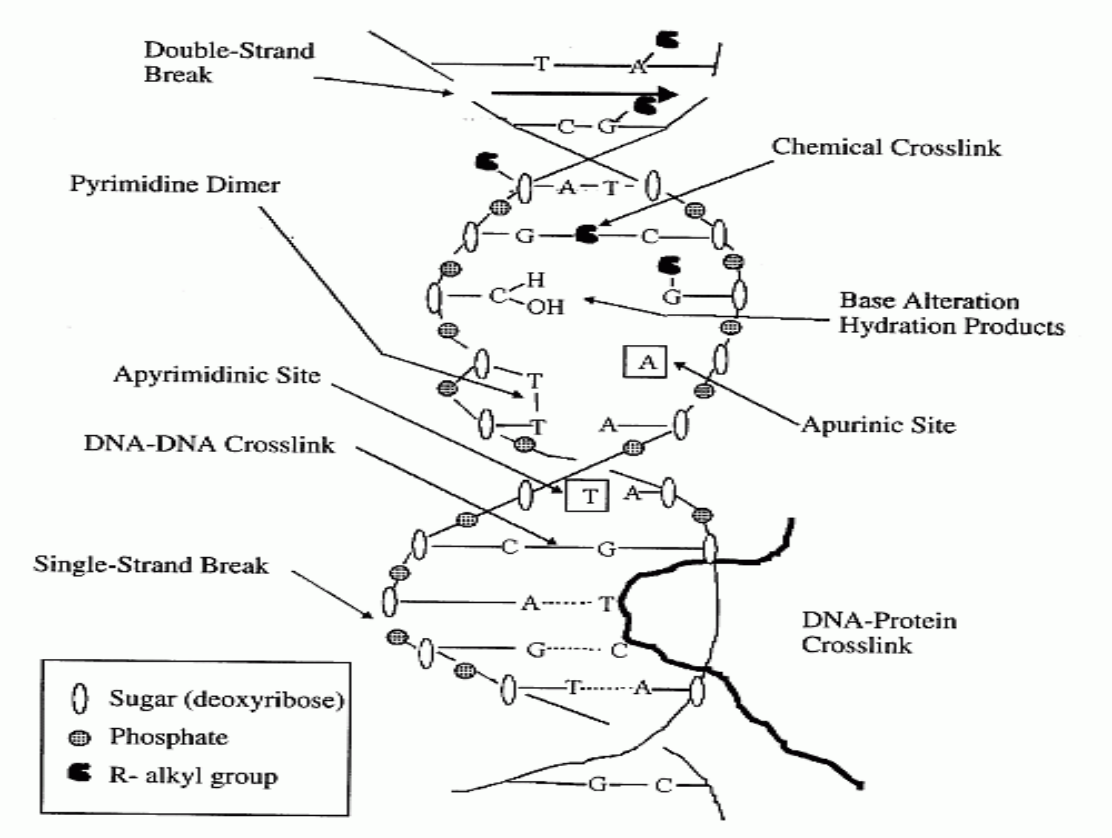


Figure 1. 9. General types of damage in DNA molecule. The Figure is adapted from Yaar and Eller, (2002).

Levels of DNA damage in the present study have been measured in cells using comet assay. A single cell gel electrophoresis (SCGE) known as the comet assay, a method was initially developed in 1984 (Ostling and Johanson, 1984), and then modified to detect single strand breaks by Singh (Singh et al., 1988). Comet assay has rapidly become one of the most popular methods used in genetic toxicology. This method allows detection and quantification at the single-cell level of a variety of DNA lesions (Tenopoulou et al., 2005, Benhusein et al., 2010) .

1.7.2 DNA repair mechanisms

It is estimated that each of the ~10^{13} cells within the human body endures tens of thousands of DNA-damaging events per day (Lindahl and Barnes, 2000). DNA damage can intermediate with main cellular processes, such as transcription or replication, and can settle the viability of the cell. Specific DNA lesions can also stimulate mutations that cause cancer or other diseases as well as aging (Hoeijmakers, 2009). Thus, cells have evolved a network of DNA repair mechanisms to remove different types of DNA damage (Sancar et al., 2004), Figure 1.10 clarify different types of DNA damage and their specific repair processes.

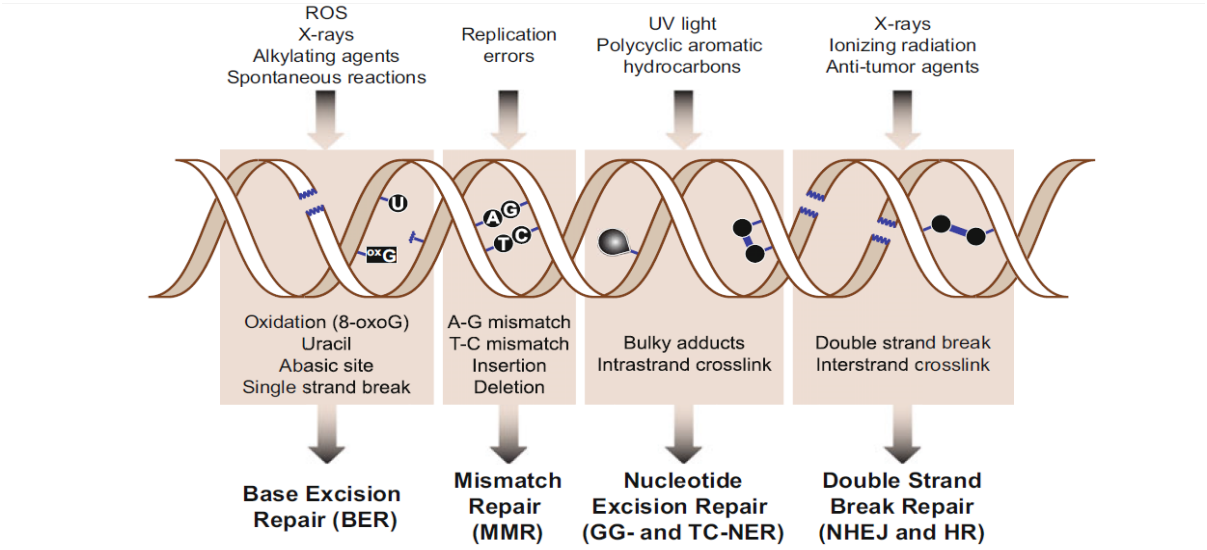


Figure 1. 10. DNA damage and repair mechanisms. The diagram illustrates common DNA damaging agents, examples of DNA lesions caused by these agents, and the relevant DNA repair mechanism responsible for their removal. The Figure is adapted from Boland et al. (2005).

DNA repair is a set of processes due to cellular responses associated with the identifies and corrects damage to the DNA molecules that encode its genome and restoration of the correct and regular nucleotide sequence and stereochemistry of

DNA following damage (Cleaver, 1968). Cells cannot function well if DNA damage corrupts the integrity and accessibility of fundamental information in the genome (Campisi and di Fagagna, 2007).

Damage to DNA changes the spatial arrangement of the helix, and the cell has the ability to detect such changes. Once damage is detected and localized, specific DNA repair molecules bind at or near the location of damage, promoting other molecules to connect and form a complex that induce the actual repair to occur (Friedberg, 2003). Cells use the unmodified complementary strand of the DNA or the sister chromatid as a template to recover the original information. Without access to a template, unsuccessful recovery mechanism takes place known as translesion synthesis (Watson et al., 2008).

The repair process of damaged DNA is constantly active as it responds to damage in the helical structure of DNA. But irreparable DNA damage may occur, including double-strand breaks and DNA cross linkages, when failing normal repair processes and then cellular apoptosis may occur (Roos and Kaina, 2006), (Acharya, 1972). Figure 1.11 illustrates the results of the occurrence and the lack of DNA damage repair.

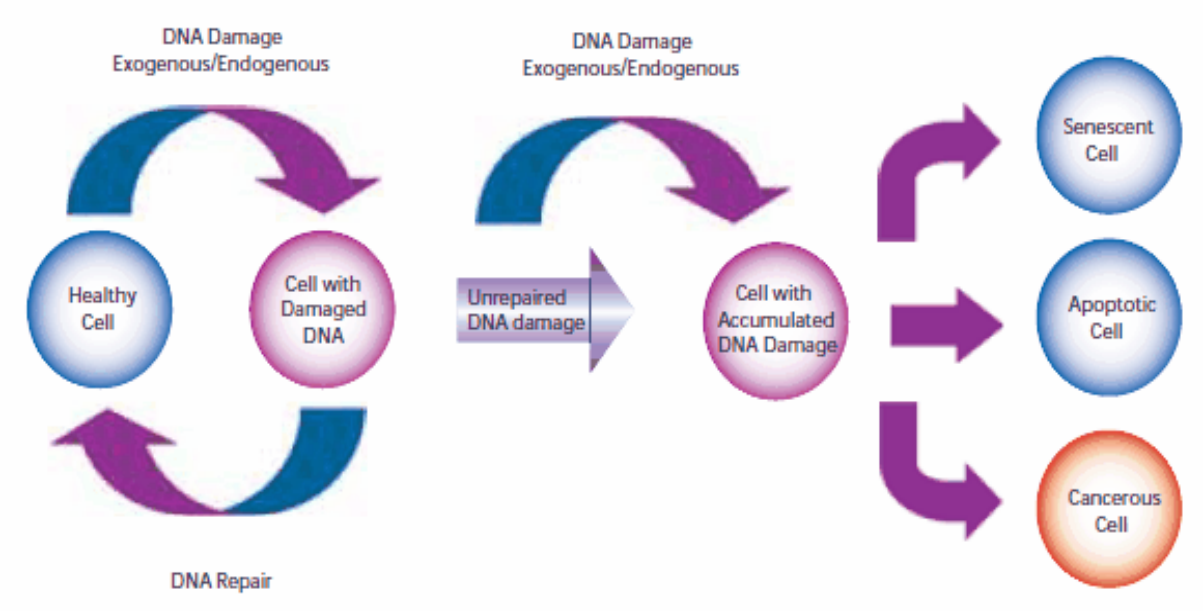


Figure 1. 11. DNA damage and the potential results with and without repair process. The Figure is adapted from Ward (1991).

The repair of damaged DNA dependent on type and age of the cells and the extracellular environment (Roos and Kaina, 2006).

The enzymatic pathways and the strategies involved in DNA repair to restore lost information are differing with the type of damage inflicted on the DNA `s double helical structure, (Figure 1.10). The most prevalent mechanism is excision-repair which is responsible for the removal of many types of lesions, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair system and double-strand break repair, which includes both homologous recombination (HR) and non-homologous end joining (NHEJ) as well as UV-induced cyclobutane pyrimidine dimers and bulky chemical adducts (Carrier, 1964, Boyce and Howard-Flanders, 1964). The time course of repairing damaged DNA has been studied and is fairly easily measured, however each type of DNA lesion has specific time to be repaired for example single strand breaks (SSB) is re-joining rapidly, with a half-time ($t_{1/2}$) for repair of a few minutes in normal cells. While double strand breaks (DSB)

repair is slower, ($t_{1/2}$) is greater than 1 hr, evaluation of the rate is vary, depending on the method used to measure them (Collins et al., 1995).

1.8 Reactive oxygen species (ROS) as main inducer of cytotoxicity and genotoxicity

Reactive oxygen species (ROS) is a term used to represent a number of reactive molecules and free radicals formed basically from molecular oxygen, so it is called (the oxygen based radicals) (Apel and Hirt, 2004). ROS molecules are produced as by-products during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalysed oxidation. These molecules participate in cellular signalling processes (Yang et al., 1998).

ROS were first implicated in cytotoxicity based on the similarity observed between oxygen poisoning and radiation toxicity (Gerschman et al., 2001). ROS could be responsible for the modulation of various cellular functions (Remacle et al., 1995), and because of their high reactivity, ROS can inducing irreversible damage to cellular macromolecules like fatty acids, proteins and DNA (Ziech et al., 2010). An exacerbation of ROS inevitably causes cell degradation and death (Remacle et al., 1995).

Reactive oxygen species have various species such as the short-lived superoxide anion ($O_2^{\cdot}$) and more stable hydrogen peroxide. The highly reactive hydroxyl radicals ($OH^{\cdot}$) are formed by these molecules in the presence of ions of transition metal (Dumont et al., 1999).

1.8.1 ROS induced DNA damage

DNA damage induced by ROS is a considerable intermediate in the pathogenesis of human conditions such as cancer and aging (Lee et al., 2002). ROS generate over one hundred different oxidative DNA adducts, such as base modification, deoxyribose oxidation, single- or double-strand breakage, and DNA- protein cross-links (Cadet et al., 1997).

Increased ROS production and its accumulative is suggested to stimulate oncogenesis by changes in pathways of redox regulated signalling, these events proposed the critical role of redox state in signal transduction, cellular proliferation, differentiation and apoptosis (Ziech et al., 2010). Oxidative stress has the ability to induce a diversity of influences including oxidation, increased levels of oxidative DNA damage and decreased concentrations of circulating antioxidants(Cadet et al., 1997).

The mechanisms by which ROS promot lethal effects are identified according to many factors such as the free radical nature involved and the properties of the targets. Within one cell type, ROS become cytotoxic when their generation exceed a threshold in the cellular antioxidant capacity, which is linked to parameters including the antioxidant level, the differentiation stage, the growth rate, and cell age (Remacle et al., 1995). This threshold can be diminished or elevated inducing the cell to become more or less sensitive to free radical damage via increasing or decreasing the level of the antioxidant defences. Within the cell, small increases in the ROS generation which are not lethal can be observed in several activated cells and such activity shifts the redox balance towards the oxidative state (Dumont et al., 1999).

A basal level of production is predominant through normal cell metabolism, either during the process of respiration or through many enzymatic or chemical reactions. In order to counter balance ROS-mediated injury, intracellular antioxidant defence systems exist and function through quenching and clearing intracellular ROS activity and aggregation and sustaining the redox state equilibrium (Leist et al., 1996).

In other words, the presence of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase, scavenger molecules, repair enzymes and removing the altered molecules by turnover can exceedingly compensate the transient increases in the redox state (Cooke et al., 2003). Also, the non-enzymatic endogenous antioxidants (Vitamins E and C, coenzyme Q, β-carotene and glutathione) have the ability to quench ROS activity. Whereas, the high levels of ROS are encountered either in experimental or in certain pathological situations like the inflammatory response (Ziech et al., 2010, Indo et al., 2007).

1.9 Overarching aims and objectives

Having identified the knowledge gaps outlined above, this thesis intends to further the current knowledge base by addressing the following major aims and objectives:

(1) Establish the effects of photobiomodulation therapy PBM/LLLT on human monocytic cells at cellular and molecular levels, utilizing a variety of endpoints across multiple levels of biological arrangement by using a new designed system help to use different characteristics of laser for current study and for future researches (Chapter 4). This approach serves to validate our choice of energy densities and exposure time (in terms of their validity to enhance cell survival) and act as a pilot study for Chapter 5.

(2) Establish the deleterious effect of exposure to UV radiation, and emerge the effect of UVA and UVB and UVC band on human monocytic cells using a variety of endpoints across multiple levels of biological arrangement (Chapter 3).

(3) Estimate the influence of PBM/LLLT at near-infrared wavelength (NIR) for different energy densities in reducing the risk impacts of UV radiation exposure (Chapter 5).

CHAPTER 2

GENERAL MATERIAL AND METHODS

2 Materials and methods

2.1 Materials

All chemicals and reagents used throughout this study, and their sources are listed in table 2.1.

Table 2. 1. Chemicals and buffers used in the current study, and their suppliers.

Media and chemicals	Supplier
RPMI 1640 L-glutamine Trypan blue Penicillin-Streptomycin	Lonza Bioscience Ltd, UK
Foetal Bovine Serum (FBS)	Labtech.com, UK
Phosphate buffered saline (PBS) tablets	Melford Laboratories Ltd., Ipswich, U.K.
Dulbecco's PBS (DPBS)	Gibco Life Technologies
Low melting point agarose (LMPA), 1.5% in TAE buffer (0.04M Tris-Acetate, 0.001M EDTA, pH 8) Normal melting point agarose (NMPA) Ethylenediaminetetracetic acid (EDTA) Dimethyl sulfoxide (DMSO) Hydrochloric acid (HCl) (37%) N-lauroyl-sarcosine Ethidium bromide	Sigma-Aldrich, UK 1.5% in TAE (0.04M Tris-Acetate, 0.001 M EDTA, pH 8)

Tris Base Triton X-100	
Sodium hydroxide (NaOH) Sodium chloride (NaCl)	Fisher chemical, UK
Hydrogen Peroxide (H_2O_2)	VWR Chemicals Prolabo, UK
Dichloro-dihydro-fluorescein diacetate (DCFH-DA)	Molecular Probes, USA

2.2 Methods

2.2.1 Cell maintenance

The human monocytic leukaemia THP-1 cell line, a permanent cell line, is derived from the peripheral blood of a one year old male with acute monocytic leukaemia. THP-1 were maintained, as described by Chen et al. (2014), in RPMI-1640 (Roswell Park Memorial Institute medium) complemented with 10% foetal bovine serum FBS, 5ml of L-glutamine and 5ml penicillin/streptomycin. THP-1 cells were grown in a 75 ml culture flask, containing 20ml of medium plus cell, at 37℃ with 5% CO_2 in a humidified incubator. The cells were sub-cultured approximately every 4 days by changing the media at the ratio of 1:4, and were used routinely between passages 10 and 26. The cell density was counted after every subculture.

2.2.2 Storage of THP-1 cells

For long–term preservation frozen THP-1 cell stocks were stored at -80℃.THP-1 cells were grown in 75 cm^2 flask to 80% confluence. Cell suspension was centrifuged at 1200 rpm for 5 min at room temperature, and the cell pellet re-suspended in freezing medium containing 90% FBS and 10 % (v/v) dimethyl sulfoxide (DMSO), and gently pipettes up and down to re-suspend cells. The suspension was aliquoted into 1.5 ml and transferred to cryovial to be kept at -80 ℃ freezer or under liquid nitrogen.

2.2.3 Reviving frozen cell stocks

Cells are usually frozen in 10% DMSO, which is toxic to cells at temperature above 4 ℃, therefore its removal is critical to maintain high cell viability. Cryovial-frozen cells were rapidly thawed at 37 ℃, in hand grip. Then cells were transferred to a 50 ml falcon tube and supplemented with 9 ml culture media, and finally centrifuged at 1200 rpm for 5 min. The pellet of cells was re-suspended in 10 ml fresh culture media by gentle pipetting and seeded into a 6 well plate for cell culture and incubated at 37 ℃ at CO_2, as described above.

2.2.4 Cell count and proliferation

The proliferation of the cultured THP-1 cell line was determined microscopically using the Trypan blue exclusion test. 20 µl cell suspensions transferred into eppendorf and mixed with 20 µl of 0.4 M Trypan blue solution, and 10 µl of the mixture was taken to be counted in a Neubauer haemocytometer chamber. The haemocytometer consists of nine 1 mm^2 squares, each square representing a volume of $1x10^{-4}$ ml. An inverted microscope used to count cells in the four corner squares, and by taking the average, we got the number of cells per 1 mm^2 square. The density of cells was calculated by applying the following formula:

$$C = \frac{N}{V} \dots \dots \dots (2.1)$$

C: Cell density in cells/ml

V: Volume counted = $1x10^{-4}$ ml.

N: Average number of cells per mm^2

So: C=N×10^4 cells/ml.

2.2.5 Assessment of cell viability

Cellular viability is an important assessment of the acute and chronic toxicological effects that include reduced integrated metabolic function and impaired ability to proliferate. A suite of several assays have been used in this study to find out the toxicological effects of different external factors spanning mitochondrial function and membrane integrity of THP-1 cells.

2.2.5.1 Cell viability by trypan blue

Cell viability was assessed in order to determine cytotoxic events taking place in the cell as described by Louis and Siegel (Louis and Siegel, 2011). THP-1 cell lines were tested for cell viability prior to use, the standard methodology was followed for using trypan blue exclusion dye. This method depends on membrane integrity to recognize the viable and non-viable cells: viable cells can exclude the dye, while non-viable cells become stained with blue dye. 20 µl of cell suspension was mixed with 20 µl of 0.4 M Trypan blue solution and incubated for 10 minutes at room temperature.

The viability of alive (clear) and dead (blue colouration) cells were detected under light micro-scope, and a haemocytometer used for cell count to determine the cell number. The percentage of cell viability was calculated by the following equation:

$$\text{cell viability} = \frac{\text{Number of viable(alive)cells}}{\text{Number of total cells}} \times 100\% \ldots\ldots\ldots (2.2)$$

Only cell samples with >90 % viability were used for further tests.

2.2.5.2 Cell viability by MTT assay

Cytotoxicity was evaluated by assessing the cell viability of irradiated cultured cells using MTT (the 3-4-5-dimethy-2.5 thiazol-2.5 diphenyltetrazolium bromide). This method is depended on the cellular mitochondrial activity. 200 µl of cell suspension were plated in a 96-well microplate at a density of $1x10^6$ cells/well in PBS. 20 µl of MTT (5 mg MTT in PBS) was added to each well, and incubated for 3h at 37 ℃ in incubator. After incubation, 200 µl of DMSO was added to each well, and covered with foil and kept at room temperature overnight. The absorbance was read at 540 nm using the Versa Max plate reader (Molecular Devices, Sunny Vale, CA, USA) and the software processes the data was Soft max Pro version 2.4.1.

2.2.6 Single Cell Gel Electrophoresis (Comet Assay)

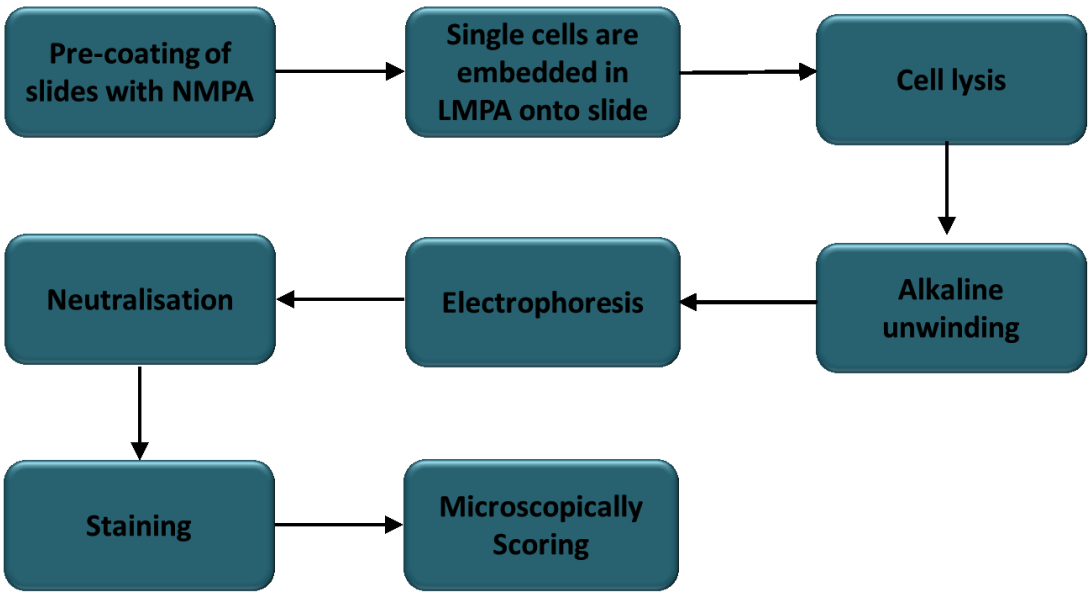


Figure 2. 1. Essential steps of comet assay

Comet assay, as commonly known or single cell gel electrophoresis (SCGE) is a technique that permits the detection of DNA damage in single eukaryotic cells (Collins, 2004). It is considered the most simple, sensitive and rapid tool for quantifying DNA damage and assessing the genotoxic potential of chemicals and many environmental agents (Jha, 2008; Drlickova et al., 2017). The main measurement of DNA damage by comet assay is DNA strand breaks, which are caused by cytotoxicity, excision repair or direct genotoxic effects (Langie et al., 2015). Therefore, comet assay can be used as a tool in risk assessment for hazard characterization (Flamand et al., 2006). The basic principle of the comet assay is that the smaller molecules of damaged DNA migrate faster in an electric field than larger molecules, because DNA molecule has negative charge makes the fragment of DNA attract and migrate fast toward anode (Benhusein et al., 2010). In brief, the

main steps of comet assay protocol are summarized in Figure. 2.1. Representative images of THP-1 cells after SCGE are shown in Figure. 2.2. Through electrophoresis, cells with elevated levels of DNA damage display the distinctive ‘comets ’ due to negatively charged DNA fragments moving away from the nucleoid towards the anode.

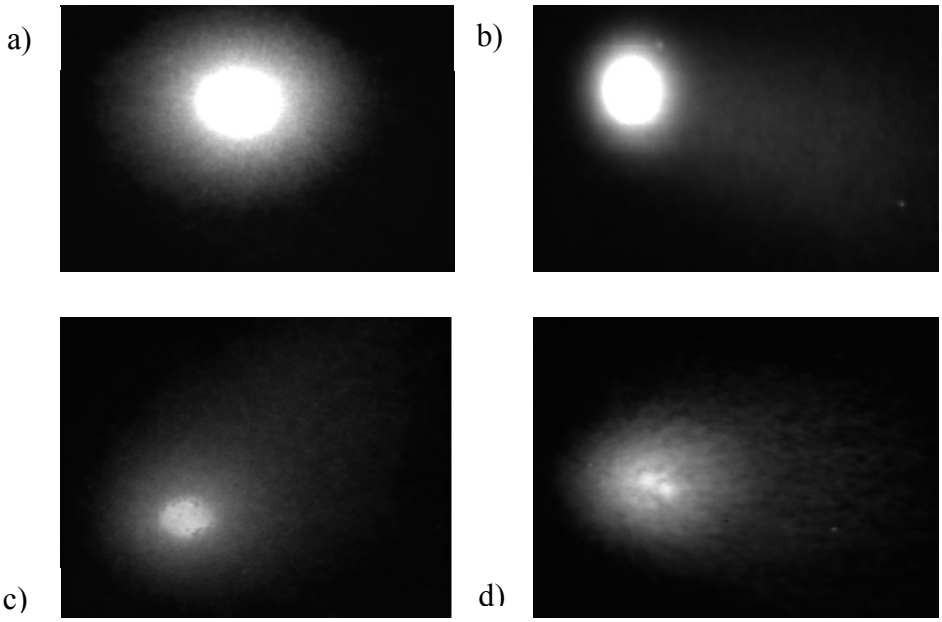


Figure 2. 2. THP-1 cell lines showing increasing damage of DNA as detected by the SCGE (Comet assay); (a) Non-damage DNA make a circle around the nucleus of control cells ; (b) Cell response for external factors initiate DNA damage migration; (c) Increased cell response raises migration of DNA damage; (d) Complete damage and maximum migration.

The comet assay was performed as described by Raisuddin and Jha. (Raisuddin and Jha, 2004), with some modifications. Microscope slides were pre-coated with normal melting point agarose (NMPA; 1.5% in TAE (0.04M Tris-Acetate, 0.001 M

EDTA, pH 8)) and kept in 37℃ incubator before use. 10 µl (10,000 cells) of cell suspension was transferred into eppendorf and suspended in 75 µl of low melting point agarose (LMPA; 0.75% in PBS) and two 75 µl drops were dispensed onto each marked slide and cover slipped (to produce two replicate microgels), slides were left in cold room at 4°C for 1h to allow gels to set. Removing the coverslips gently and arranging the slides back to back into coplin jar containing lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% N-lauryl-sarcosine, 1% Triton X-100, 10% DMSO, pH adjusted to 10 with NaOH) and incubated for more than 1 h at 4°C. Following lysis, allowing time for DNA to unwind, slides were transported to an electrophoresis chamber, filled with 545 ml of chilled electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH 13) at 4 °C for 20 min. The chamber is switched on (21 V/ 620 mA) to allow electrophoresis to run for 25 min. After electrophoresis, slides were transported to a new jar containing neutralisation buffer (0.4 M Tris, adjusted to pH 7 with HCl) for 2 min, twice, and finally rinsed twice with distilled water and left to dry before scoring.

To ensure unbiased scoring, cells on the slides were scored within 24 hr, and always within 1 week, using comet image analysis software program. 20 µl of 20 µg/ml ethidium bromide was added onto each replicate microgel to be stained, and 50 cells per microgel (100 per slide) were scored randomly. Scoring of slides were performed using an epifluorescence microscope using an epifluorescence microscope (DMR; Leica Microsystems, Milton Keynes, UK) and imaging system (Comet IV, Perceptive Imaging, UK). Comet assay software packages record a number of different parameters, with % tail DNA considered the most reliable. Hence, comet assay results are reported as mean % tail DNA (Kumaravel et al., 2009).

2.2.6.1 Optimization of the comet assay with hydrogen peroxide

The comet assay was carried out as described in section 2.2.6, with the following modifications. Alkali (pH > 13) unwinding and electrophoresis of DNA were optimised by investigating various unwinding (10, 15 or 20 min) and electrophoresis times (15, 25 or 40 min) respectively. Preliminary experiments were conducted to optimise the comet assay. In order to determine the unwinding and electrophoresis times for THP-1 cell line, a range of hydrogen peroxide (10, 50, 100 and 500 μM) were used as positive control and the cells were exposed to these concentrations for 10 min. Different unwinding (10, 15 and 20 min) and electrophoresis (15, 25 and 40 min) times were investigated. Percentage tail DNA (% Tail DNA) migrated has been investigated to measure DNA damage. Mann-Whitney U test has been performed by using Minitab to identify any significant differences between unwinding and electrophoresis times and between hydrogen peroxide concentrations for different unwinding and electrophoresis times. There were statistically no significant differences between % tail DNA calculated over different concentrations of H_2O_2 ($p > 0.05$) for unwinding times (10, 15 and 20 min) for 15 min (Figure 2.3abc) and for 40 min electrophoresis (Figures 2.3ghi). While, significant differences between % tail DNA measured over various concentrations of H_2O_2 ($p < 0.05$) for unwinding times (10, 15 and 20 min) for 25 min electrophoresis (Figure 2.3def). These findings indicated that THP-1 cells are affected by the electrophoresis time more than unwinding time.

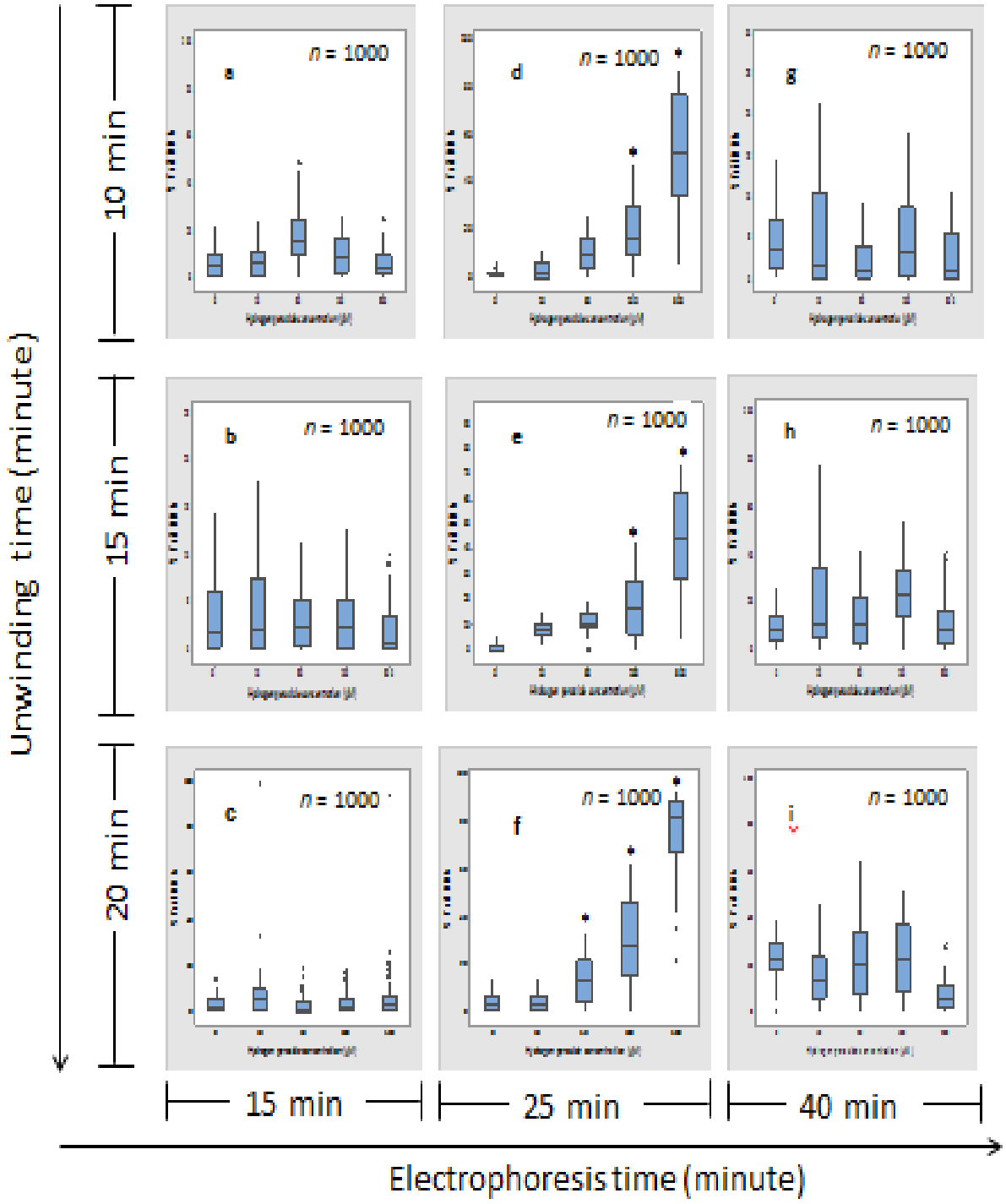


Figure 2. 3. The effect of hydrogen peroxide concentrations (0, 10, 50,100 and 500 µM) with unwinding times (10, 15 and 20 min) and 15 (figure 3.4 a, b, c), 25 (figure 3.4 d, e, f) or 40 min (figure 3.4 g, h, i) electrophoresis times on the % Tail DNA migrated in THP-1. Significant differences ($p < 0.05$) between the untreated and treated cells with hydrogen peroxide (*). Significant differences ($p < 0.05$) between the hydrogen peroxide concentrations for different unwinding and electrophoresis times.

In respect of finding a suitable unwinding time of alkaline comet assay for THP-1 cells, different periods of unwinding have been used (10, 15 and 20 min) for different electrophoresis times. Figure 2.4 showed that the determined % tail DNA (DNA damage) in THP-1 cell line showed no significant change for different unwinding times and different concentrations of H_2O_2 (a-e) for 15 min electrophoresis time (Kruskal-Wallis test; $p > 0.05$). Similar results were obtained for % tail DNA for different unwinding times and various concentrations of H_2O_2 for 40 min electrophoresis (Figure 2.6).

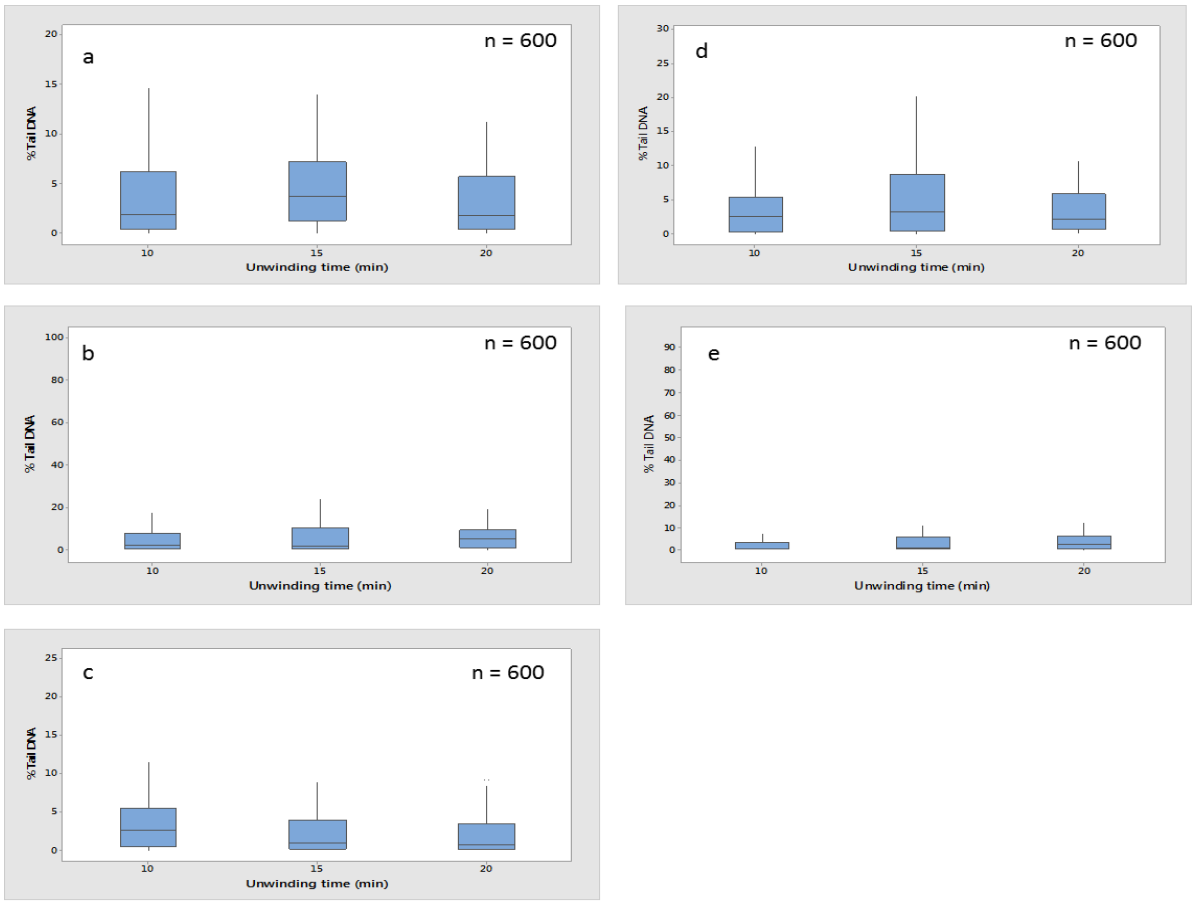


Figure 2. 4. Effect of unwinding times (10, 15 and 20 min) with 15 min electrophoresis time for (a) untreated, (b) 10, (c) 50, (d) 100 or (e) 500 µM hydrogen peroxide concentrations on % Tail DNA migrated in THP-1 cells. There were significant differences ($p < 0.005$) between unwinding times for all H_2O_2 concentrations except 10 µM (b), ($p > 0.05$).

While, a significant changes was found between the % tail DNA for the different unwinding periods for 25 min electrophoresis time at all concentrations of H_2O_2 (Kruskal-Wallis test; $p < 0.05$). The most measurable value of % tail DNA was found at 20 min unwinding for diverse concentrations of H_2O_2 (Figure 2.5).

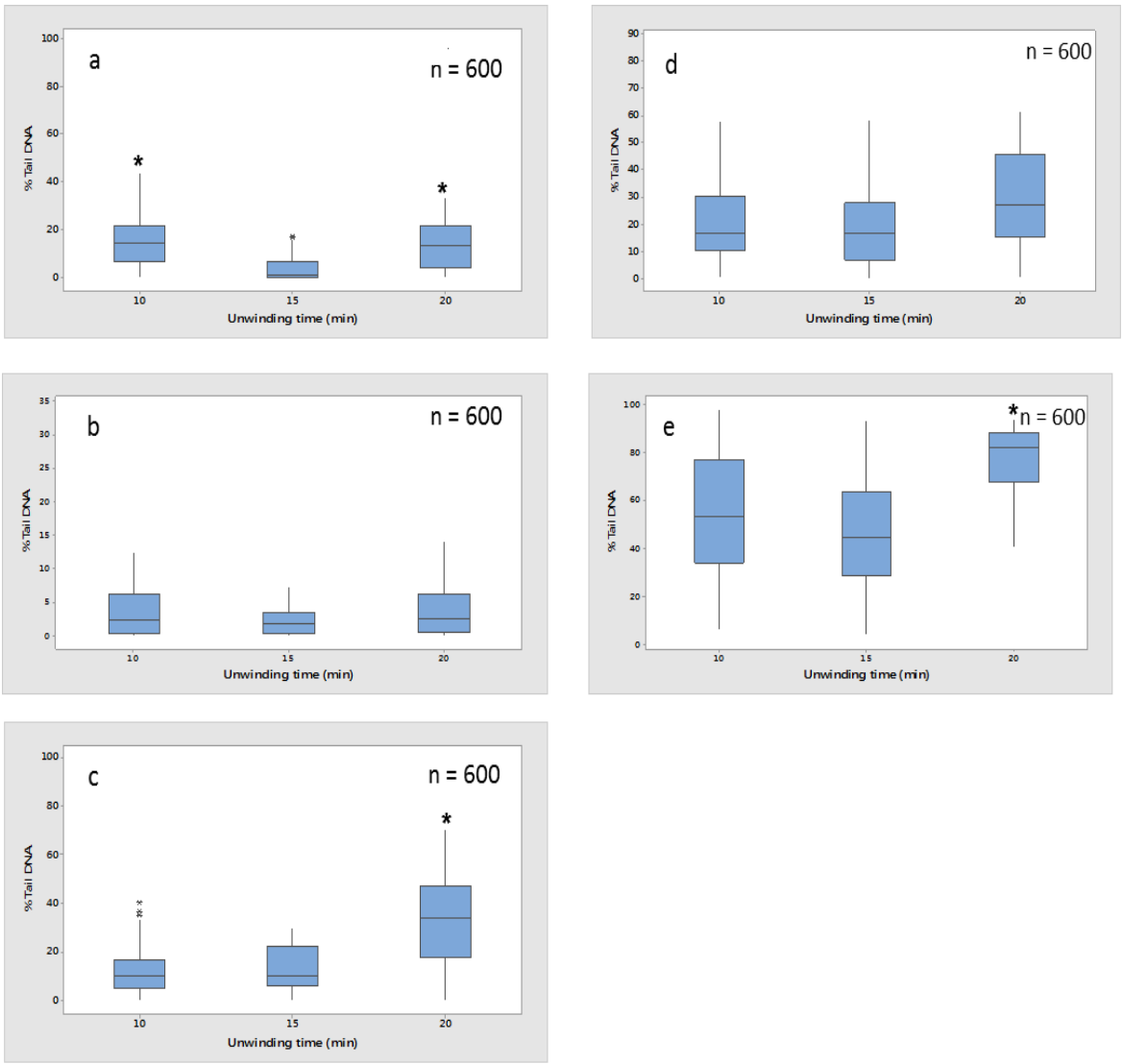


Figure 2. 5. Effect of unwinding times (10, 15 and 20 min) for (a) untreated, (b) 10, (c) 50, (d) 100 or (e) 500 µM hydrogen peroxide concentrations with 25 min electrophoresis time on % Tail DNA migrated in THP-1 cells. There were significant differences ($p < 0.005$) between unwinding times for all H_2O_2 concentrations except 10 µM (b), ($p > 0.05$).

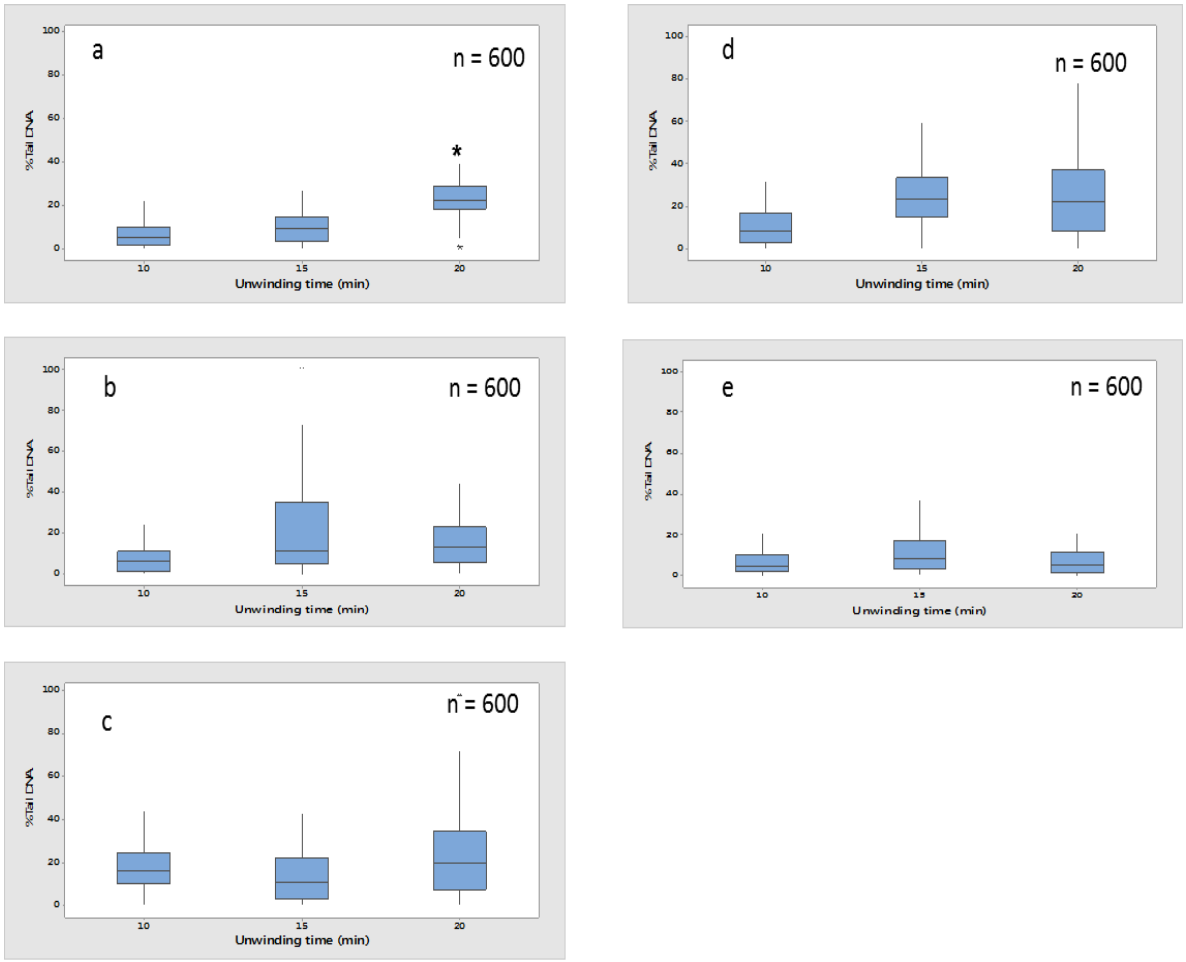


Figure 2. 6. Effect of unwinding times (10, 15 and 20 min) for (a) untreated, (b) 10, (c) 50, (d) 100 or (e) 500 µM hydrogen peroxide concentrations with 40 min electrophoresis time on % Tail DNA migrated in THP-1 cells. Insignificant differences ($p > 0.05$) between unwinding times for all H_2O_2 concentrations, and significant for untreated cells ($p < 0.05$).

This indicated that 10 min and 15 min unwinding times were not enough for double helix strands of DNA to unwind, but 20 min appeared to be enough for DNA double helix strands to unwind at 25 min electrophoresis time.

In addition to unwinding time for comet assay optimization, a range of electrophoresis periods were investigated, in order to find the optimum time period for comet assay and DNA damage measurement. Although, there was no

significance between DNA damage (% tail DNA) between electrophoresis times of 15 min and 40 min as elucidated in Figures 2.7, 2.8 and 2.9 (Kruskal-Wallis test; $p > 0.05$). Nevertheless, 25 min electrophoresis time gave statistically measurable value of % tail DNA over the most concentrations of H_2O_2 for the different unwinding times in particular at 20 min unwinding time (Figure 2.9; $p < 0.05$)

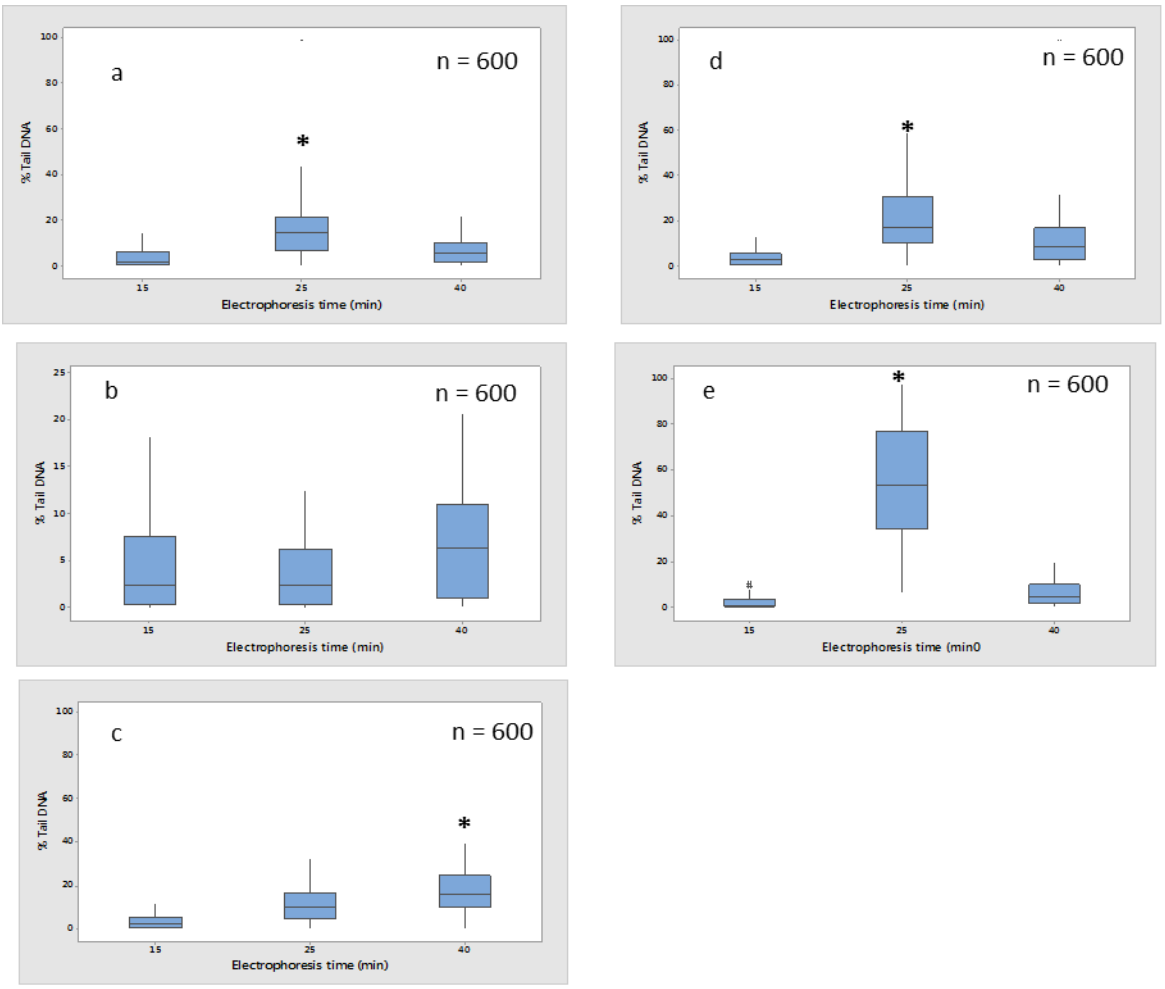


Figure 2. 7. Effect of electrophoresis times (15, 25 and 40 min) for untreated (a), 10 (b), 50 (c), 100 (d) or 500 µM (e) hydrogen peroxide concentrations with 10 min unwinding time on % Tail DNA migrated in THP-1 cells. Significant differences ($p < 0.005$) between electrophoresis times for all H_2O_2 concentrations except 10 µM (b), ($p > 0.05$).

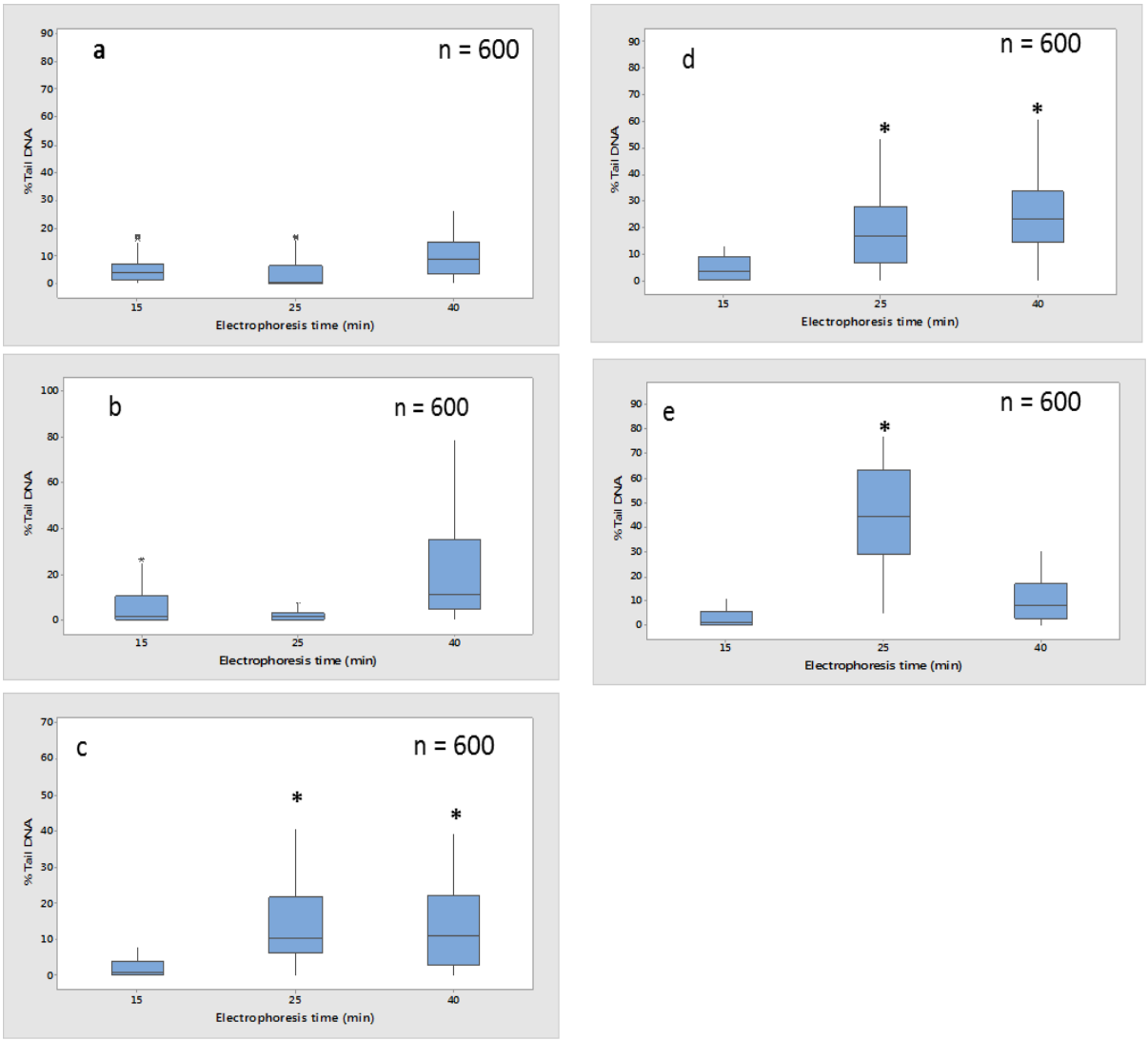


Figure 2. 8. Effect of electrophoresis times (15, 25 and 40 min) for untreated (a), 10 (b), 50 (c), 100 (d) or 500 μM (e) hydrogen peroxide concentrations with 15 min unwinding time on % Tail DNA migrated in THP-1 cells. Significant differences ($p < 0.005$) between electrophoresis times for all H_2O_2 concentrations except 10 μM (b), ($p > 0.05$).

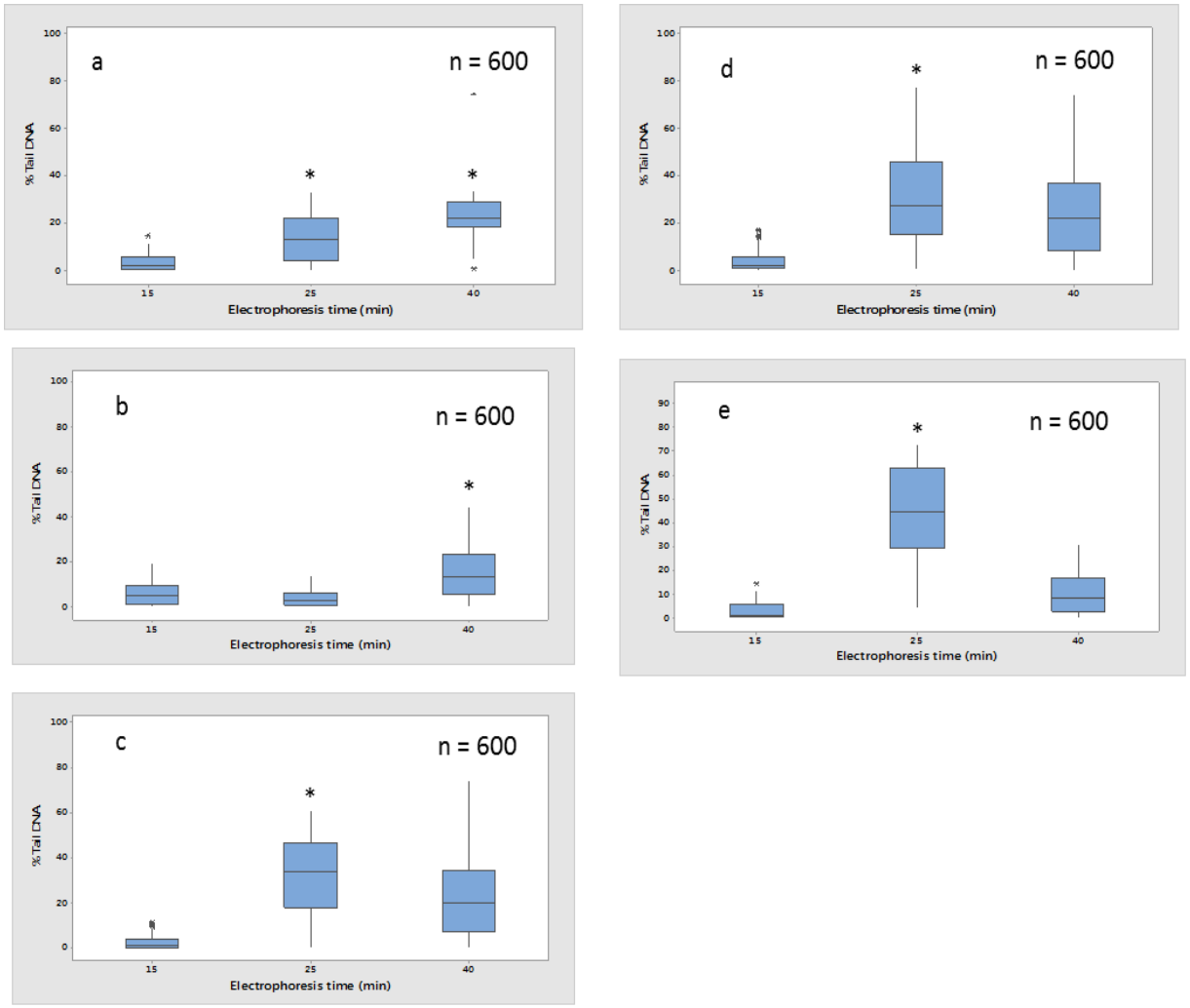


Figure 2. 9. Effect of electrophoresis times (15, 25 and 40 min) for untreated (a), 10 (b), 50 (c), 100 (d) or 500 µM (e) hydrogen peroxide concentrations with 20 min unwinding time on % Tail DNA migrated in THP-1 cells. Significant differences ($p < 0.005$) between electrophoresis times for all H_2O_2 concentrations except 10 µM (b), ($p > 0.05$).

To find the optimization of comet assay and DNA damage of untreated THP-1 cell line, a range of unwinding times (10, 15 and 20 min) and electrophoresis times (15, 25 and 40 min) were used. The results clarified in Figure 2.10 that the unwinding time (20 min) gave the highest calculated % tail DNA for the periods 25 min and 40 min electrophoresis (Figure 2.10bc). And the electrophoresis time (25 min) gave highest value of % tail DNA for the three unwinding periods 10, 15 and 20 min (Figure 2.10 abc). Therefore, the comet assay for measuring DNA damage in THP-1

cell line was performed with 20 min unwinding time and 25 min electrophoresis period.

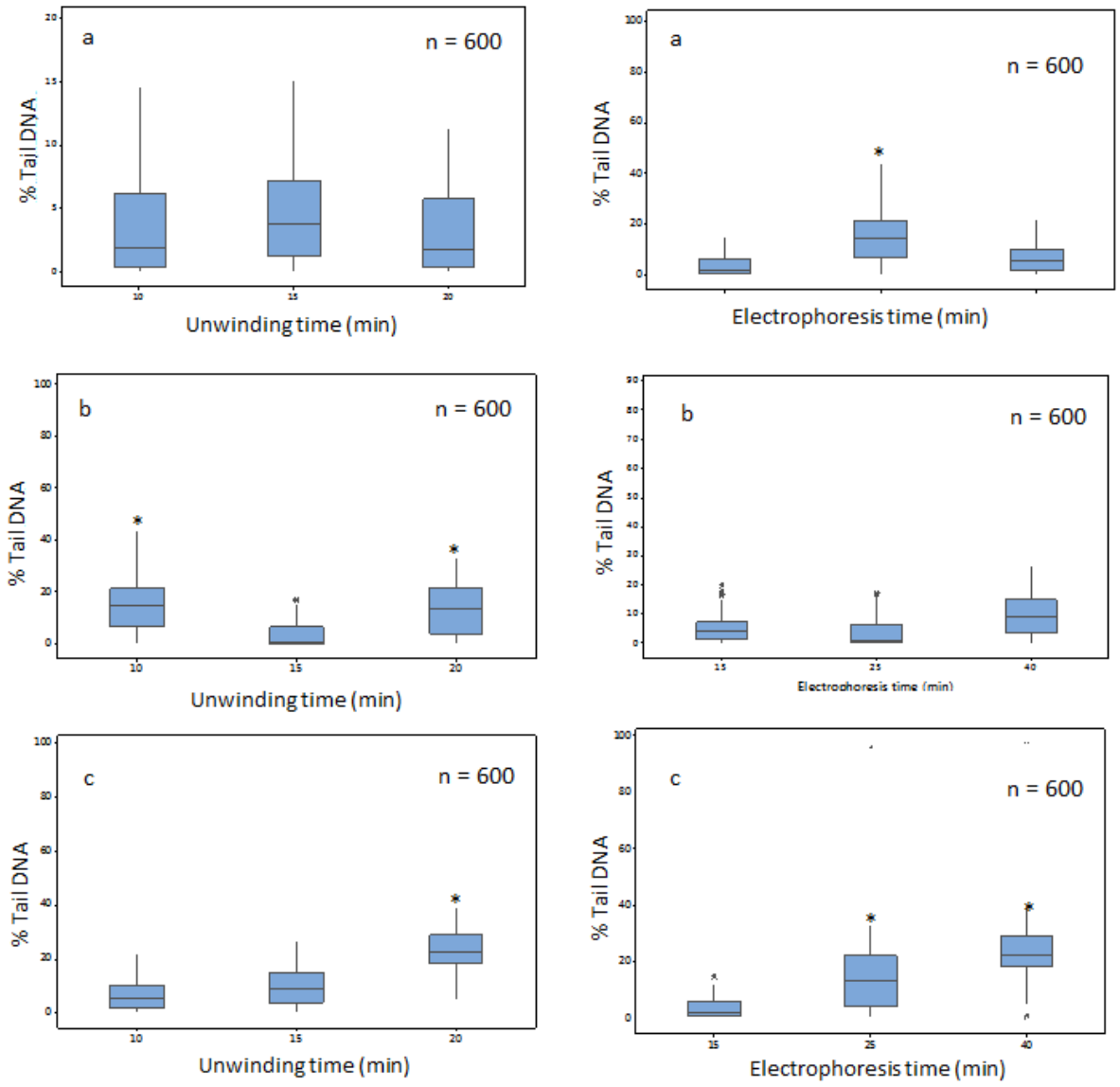


Figure 2. 10. Effect of unwinding times (10, 15 and 20 min) with 15min (a), 25min (b) and 40min (c) electrophoresis time on % tail DNA migrated in untreated THP-1 cells (left). Effect of electrophoresis times (15, 25 and 40 min) with 10min (a), 15min (b) and 20min (c) unwinding time on % tail DNA migrated in untreated THP-1 cells (right). Significant differences ($p < 0.005$) between unwinding times for electrophoresis times, and significant differences ($p < 0.005$) between electrophoresis times for unwinding times for control.

2.2.6.2 Validation of the comet assay with hydrogen peroxide

To obtain reliable comet data with genotoxic agents, it is imperative to validate the comet assay depending on the type of cells and type of damage anticipated. Hydrogen peroxide (H_2O_2) has been widely used to validate the comet assay in previous studies; therefore it was used as a reference genotoxic agent to validate our comet assay procedures. This was conducted by *in vitro* exposure of THP-1cells to a range of H_2O_2 concentrations. 10 µL (10,000 cells) of cell suspension was transferred into siliconized eppendorf, and exposed to 100 µL hydrogen peroxide (0, 50, 100 or 500 µM). All marked eppendorfs of cells and H_2O_2 were imbedded in ice for 5 min at dark, then the eppendorfs were spun at 3000 rpm for 5 min, (the total exposure time 10 min), the suspension is removed and the samples processed through the comet assay as in section 2.5 (Figure 2.11).

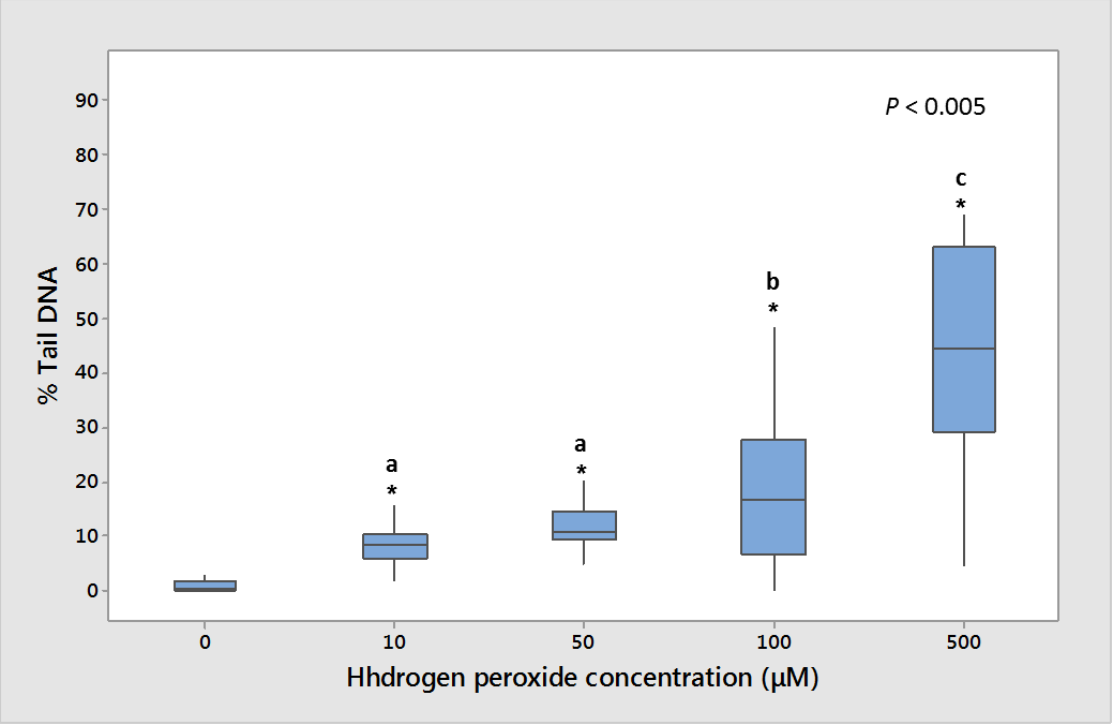


Figure 2. 11. Percentage tail DNA in THP-1 following *in vitro* exposure to hydrogen peroxide. Asterisks (*) indicate a significant difference between the exposed cells and the control ($p < 0.05$); mismatched lower case letters indicate significant differences ($p < 0.05$) between treatment group. n =3.

2.2.7 Detection of ROS by Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay:

Dichlorofluorescin (DCFH) is widely used to measure oxidative stress in cells. Hydrogen peroxide (H_2O_2), which is the more stable species of reactive oxygen species (ROS), is considered an appropriate model for ROS and causing the oxidative stress. The main principle of fluorometric assay is the use of the chemically reduced, non-fluorescent dye that is oxidized to the parent dye molecule, causing a considerable increase in fluorescence intensity. 5 ml of cell suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and 5 ml of PBS was added. One group was untreated was used as a control, cell-free PBS group used as blank and four groups were treated with different concentrations of hydrogen peroxide H_2O_2 (10, 50, 100 and 500 µM). 10 µl of DCFH-DA dye was added to each group, 10 min later, the microplate was read by fluorescing microscope. The oxidation of the non-fluorescent DCFH-DA by using different concentrations of hydrogen peroxide, measured as increased fluorescence intensity at 522 nm, is shown in Figure (2.12). ROS generation (fluorescence intensity) was significantly increased (p <0.005) with increasing H_2O_2 concentrations.

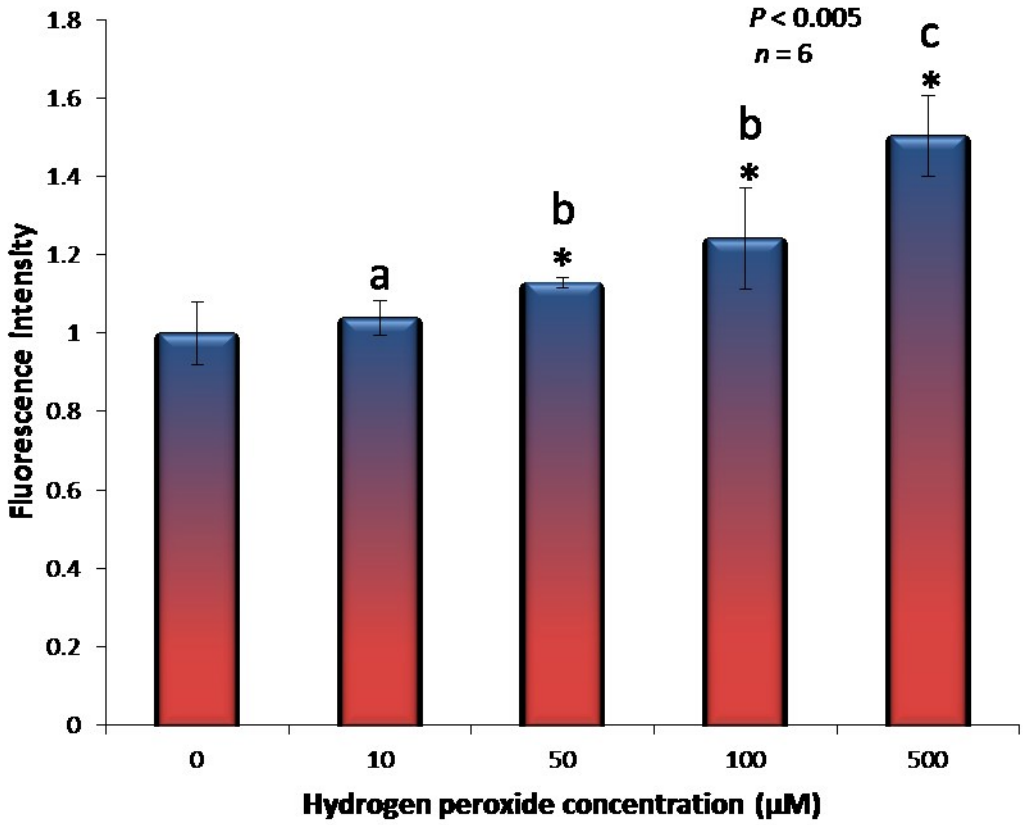


Figure 2. 12. Fluorescence intensity of cells during reaction of DCFH-DA with different concentrations of hydrogen peroxide H_2O_2. Asterisks (*) indicate a significant difference between the exposed cells and the control ($p < 0.05$); mismatched lower case letters indicate significant differences ($p < 0.005$) between treatment groups, similar letters refer to insignificant differences ($p > 0.05$). Data are representing mean ± SE, n=6.

2.2.8 Ultraviolet irradiation

2.2.8.1 Test THP-1 cell line in PBS and DPBS

To irradiate with UV light, THP-1 cells were re-suspended in Phosphet buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl and 10 mM phosphate, pH 7.4 by OXOID, UK), and DPBS (Dulbeccos phosphate Buffer Saline free $CaCl_2$ and $MgCl_2$ Gibco by Technology , UK) (Figure. 2.13). Cell suspension was centrifuged at 1000 rpm for 5

min with centrifuge, and after removing the supernatant, the cells were re-suspended in PBS and DPBS. 1 x 10^6 cells/ml cells density of each solusion were added to a 12 well plate in addition to cell suspension of the same density. Trypan blue was used to find the sustainability of cells suspended in PBS and DPBS. In general, the sustainability of cells was reduced with increasing time, however cells in DPBS were more endurance than in PBS. So, cells have been re-suspended in DPBS to exposure to UV light.

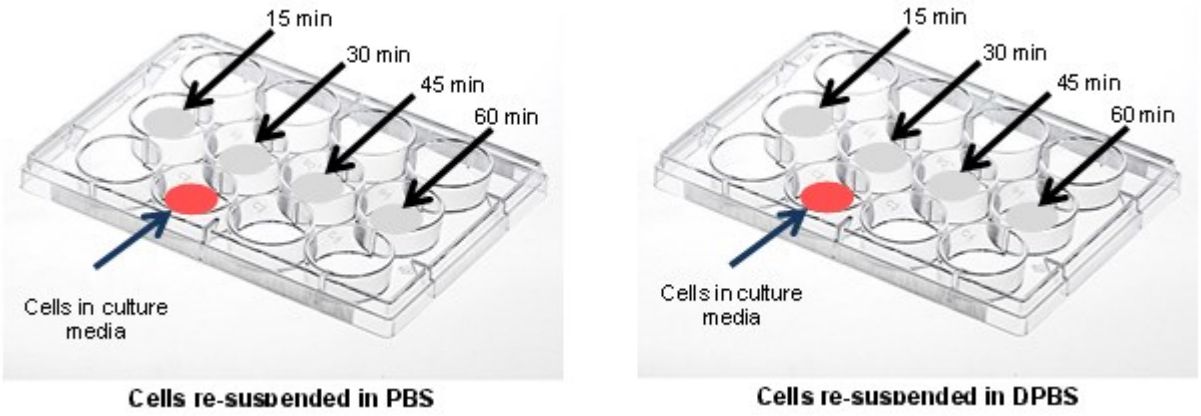


Figure 2. 13.Testing the sustainability of THP-1 cells in PBS and DPBS

2.2.8.2 Irradiation with UVA

THP-1 cells were irradiated as freshly harvested suspensions. The cells were centrafuged at 1000 rpm for 5 min and re-suspended in DPBS after two washes. 1 x 10^6 cells / ml were put in a plastic petri dish and placed on ice before and after irradiation with 0, 260.2, 520.4 and 1040.76 J/m^2 for (0, 15, 30 and 60 min) UVA radiation, UVA dose received by the person in Denmark expressed as a percentage

of the corresponding ambient UV dose is about 325-464 (Thieden 2004) . The intensity of UVA radiation (0.2891 W/m^2) has been calculated by integrating the intensity between 320 - 400 nm (peak intensity of 360 nm) by a Macam spectroradiometer (Macam SR9910, Livingston, UK) (Fig.2.14). The exposure was performed using commercially available fluorescent bulbs (Spectro-line XX-40, USA). The temperature of the sample was measured with each period of exposure.

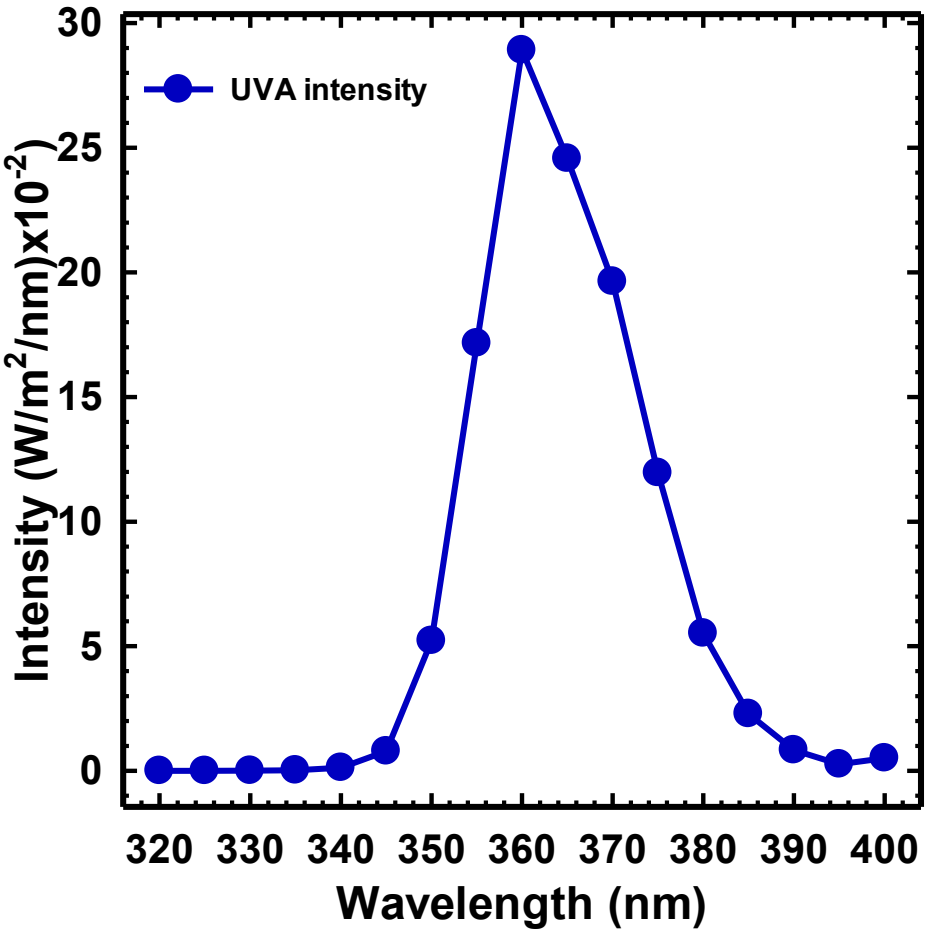


Figure 2. 14. Spectrum of the twin-tube UV lamp with a maximum emission in the UVA region (360 nm). The data represent a single measurement.

2.2.8.3 Irradiation with UVB

Irradiation with UVB was carred out using a twin-tube lamp (TL-20W/12RS; Philips, Guildford, UK), within similar circumstances. THP-1 cells were exposed to (0, 0.125, 0.25, 0.5 and 1 K J/m^2) for (0, 40, 78, 156 and 318 sec) of UVB radiation. The UVB source was positioned directly above the cell suspension with intensity (3.154 W/m^2) calculated by using integrated intensity between 280 - 340 nm (peak intensity of 310 nm) by a Macam spectroradiometer (Macam SR9910, Livingston, UK) (Figure. 2.15).

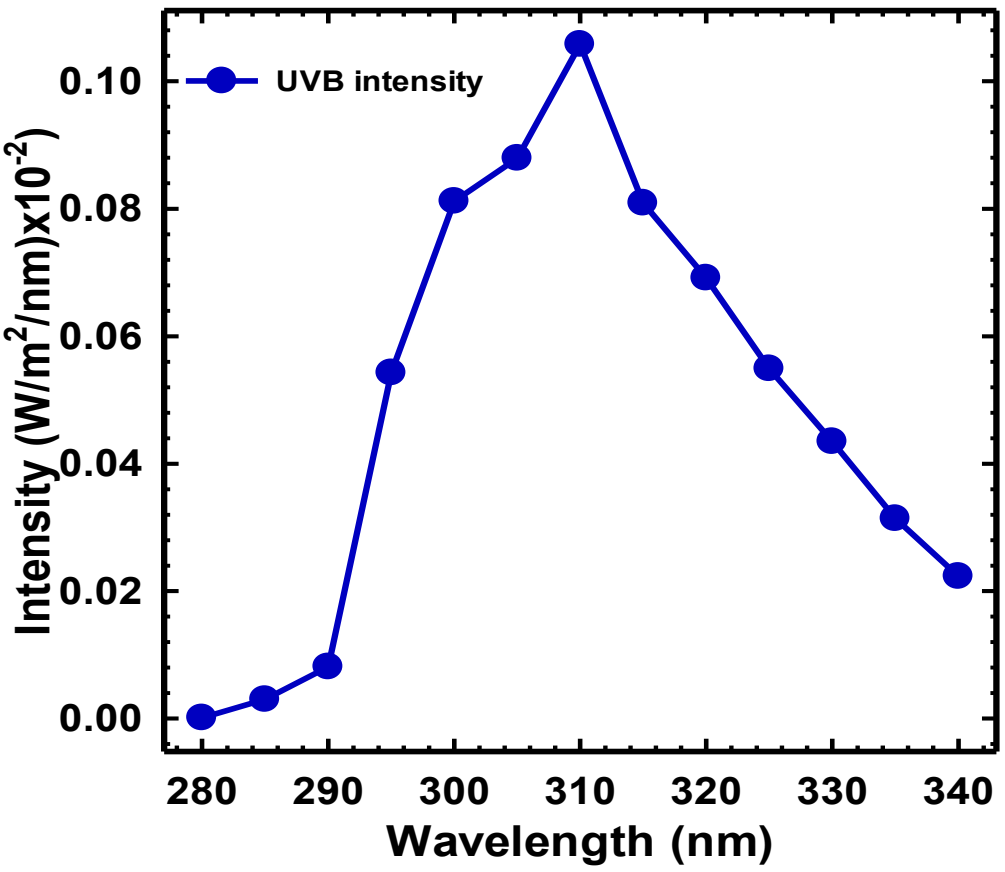


Figure 2. 15. Spectrum of the twin-tube UV lamp with a maximum emission in the UVB region (310 nm). The data represent a single measurement.

2.2.8.4 Irradiation with UVC

UVC radiation was delivered from (UVP UVLS-28 EL Series UVLamp 8Watt, 254/365 nm, P/N 95-0201-02, 0.16 Amps/ 230V ~50-60 Hz Upland, CA, USA). Cells, within similar cercomstances, were irradiated with (0, 12, 23.8 and 35.7 J/m^2) of UVC radiation for different durations of time (5, 10 and 15 sec) at maximum intensity of 2.38 W/m^2. The intensity has been calculated using integrated intensity between 245 - 252 nm (peak intensity of 248.25 nm) by a Macam spectroradiometer (Macam SR9910, Livingston, UK) (Figure. 2.16).

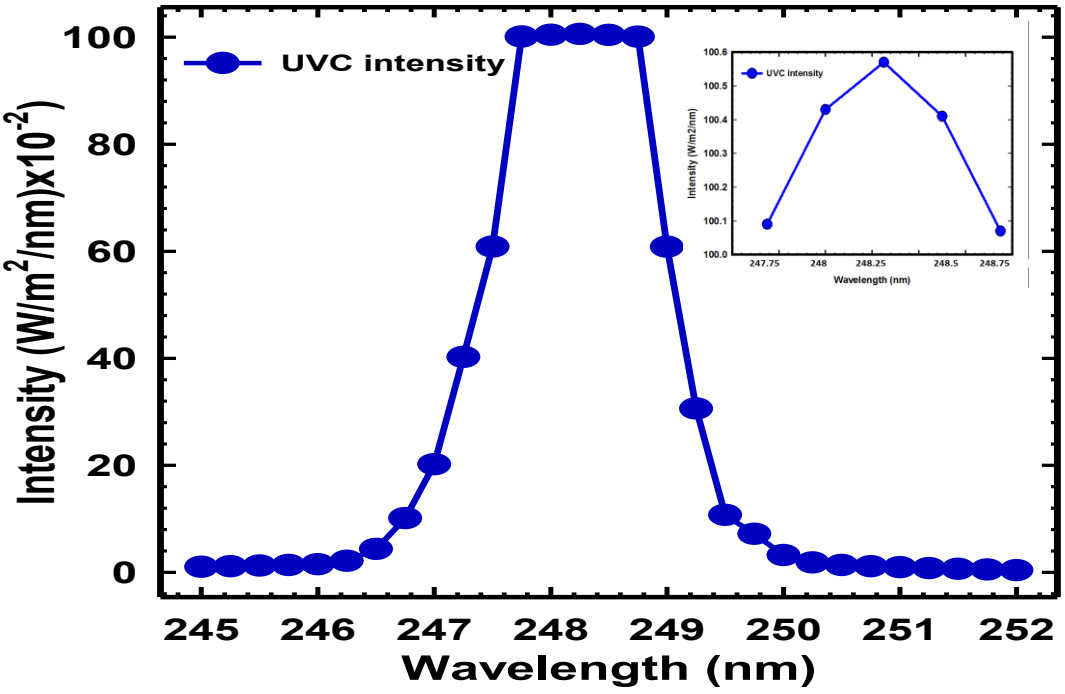


Figure 2. 16. Spectrum of the twin-tube UV lamp with a maximum emission in the UVC region (248.25 nm). The data represent a single measurement.

2.2.9 Photobiomodulation therapy (PBM)/Low Level Laser Therapy (LLLT)

2.2.9.1 Experimental techniques for the design and fabrication of BELT

In order to study the Biological Effect of Laser Therapy (BELT) at the cellular and molecular level, several processes are needed. The instrumental platform for BELT system was designed and built during the course of this work. Our system was schematically designed with Sketchup program, and different microbenchs designed to be suitable for the diameter of beam splitters and mirrors (Figure 2.17). The instrument was built on an optical bench using the designed and standard mounts (optical microbench) and fashioned mechanical components. The basic system shown in figure 2.18, consists of the optical components, a laser light source, two lenses with different focal length for focusing light, a polarizer, a detector, three pairs of beam splitter (40:60, 50:50and 30:70) and mirrors. The system is controlled by convenient data acquisition software built within the NI LabVIEW environment.

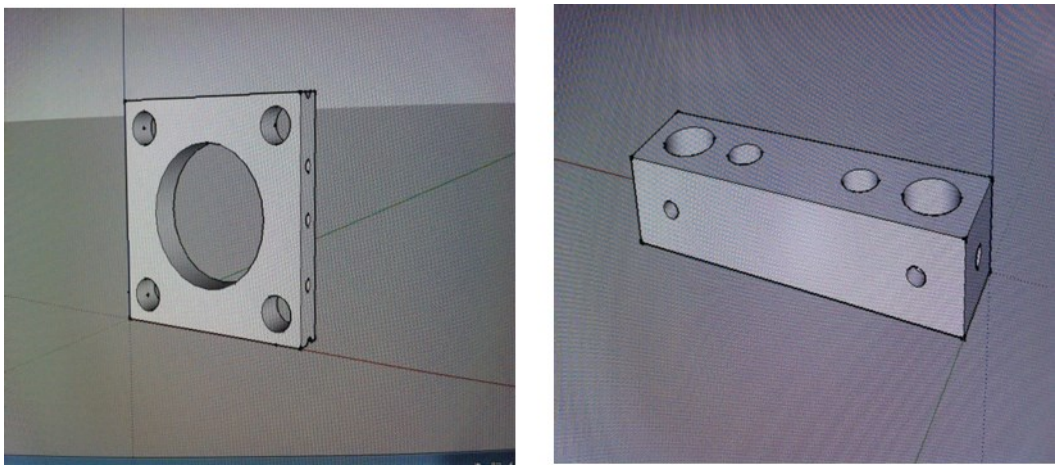


Figure 2. 17. Designing of optical microbenchs for the BELT system

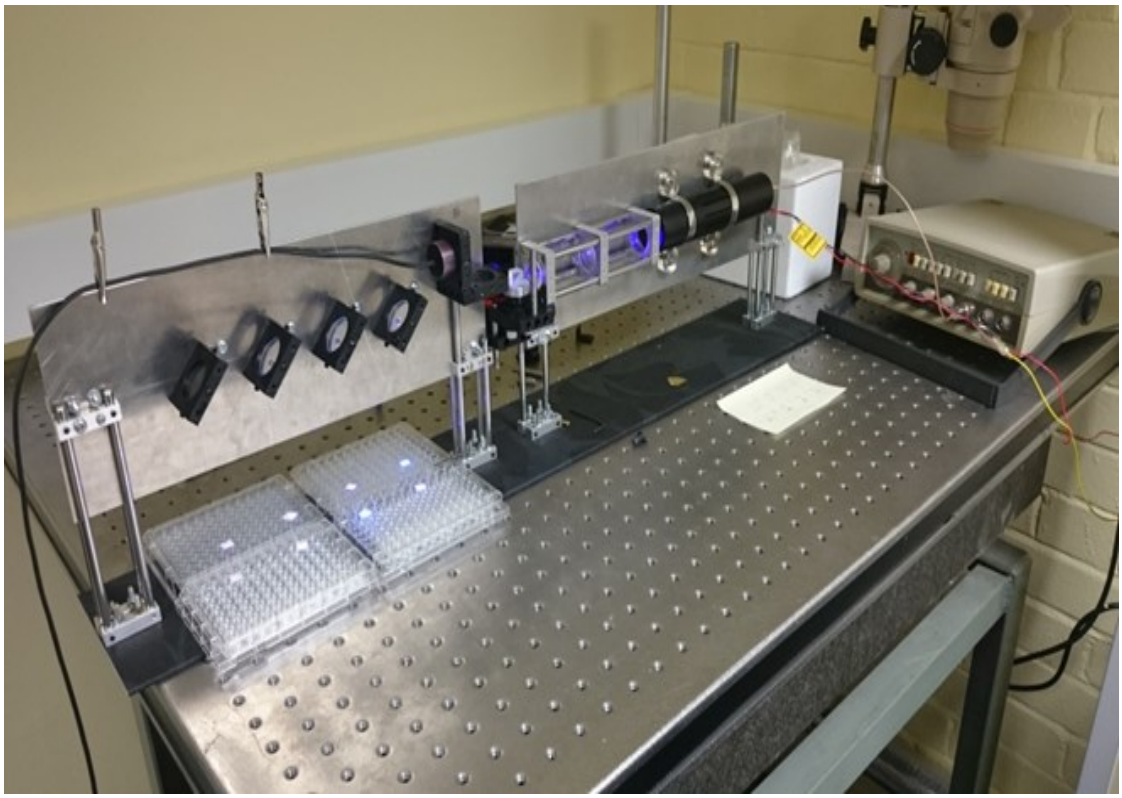


Figure 2. 18. The experimental BELT system

2.2.9.2 PBM/LLLT irradiation

The laser device used in this study was the diode laser model APMT25 (850-40)/5342 (Power Technology Incorporated, Alexander, AR USA) with a wavelength of 850 nm and power output of up to 27 mW. The light spot was delivered with diameter of 8.7mm in the continuous wave (CW) mode. The laser spot with respect to the cell well is shown in Figure 2.19a. It was assumed that the laser intensity was constant across the spot diameter. The laser power delivered was constant at 11.1 mW, giving a power density of 34.6 mW/cm^2. After accounting for the fact that 86 % of the power reaches the cells in the well, the laser power becomes 9.5 mW with a power density of 29.6 mW/cm^2. The laser intensity, and hence temperature, is not constant across the cells, and can be considered to be represented by the 'bands'

shown in Figure 2.19b, with T_0 representing the maximum temperature. In this work the mean delivered power was used, with the only variable being irradiation time, as given in Equation 2.3 below.

The dose was calculated as:

$$\text{Dose=Power Density . Irradiation Time} = \frac{\text{J/s}}{\text{cm}^2}\text{. s} = \text{Jcm}^{-2} \quad \ldots\ldots\ldots (2.3)$$

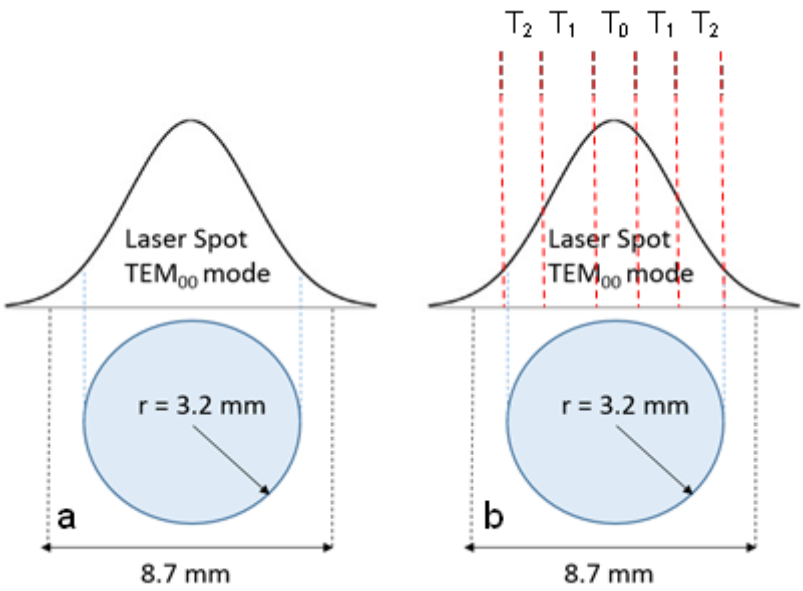


Figure 2. 19. (a) The diode laser beam is expanded to deliver stimulating light to the full well area. (b) Temperature bands arising from the Gaussian intensity distribution.

THP-1 cell line were transferred to a 96 well plate with cell density of (10,000 cells / 200 µl) to be irradiated by the diode laser for the durations of 20, 40, 120, 300, 600 and 900 sec for different fluences of 0.6, 1.2, 3.6, 8.9, 17.8 and 26.8 J/cm^2, respectively with one group of cells non-irradiated as control. Determination of cell proliferation, cell viability and comet assay were achieved immediately after irradiation and post 12 hr and 24 hr incubation following irradiation.

CHAPTER 3

GENOTOXIC AND CYTOTOXIC EFFECT OF ULTRAVIOLET (UV) RADIATION

Hypothesis: Ultraviolet radiation exposure induces genotoxic and cytotoxic effects detectable in human monocytic leukaemia THP-1 cell line.

3 Genotoxic and cytotoxic effect of ultraviolet (UV) radiation

3.1 Introduction

Ultraviolet (UV) radiation is part of the electromagnatic spectrum emitted by the Sun. UV rays are invisible to human eye, fall in the range of the electromagnetic spectrum between visible light and x-rays, (Figure 3.1), have frequencies of about 8×10^{14} to 3×10^{16} cycles per second, or hertz (Hz) 1, and energies more than visible rays energy and less than x-rays energy (Noonan and De Fabo, 1992; Zamanian and Hardiman, 2005; Schuch et al., 2013). The human eye can see in the range of 400 nm (Blue) to 700 nm (Red). UV radiation covers the range of 100 – 400 nm. The spectrum is often expresed in terms of the frequency of the electromagnetic radiation. The spectrum goes from γ ray with wavelength of the order pm (picometre) up to radiowaves of mm (millimeter) and larger.

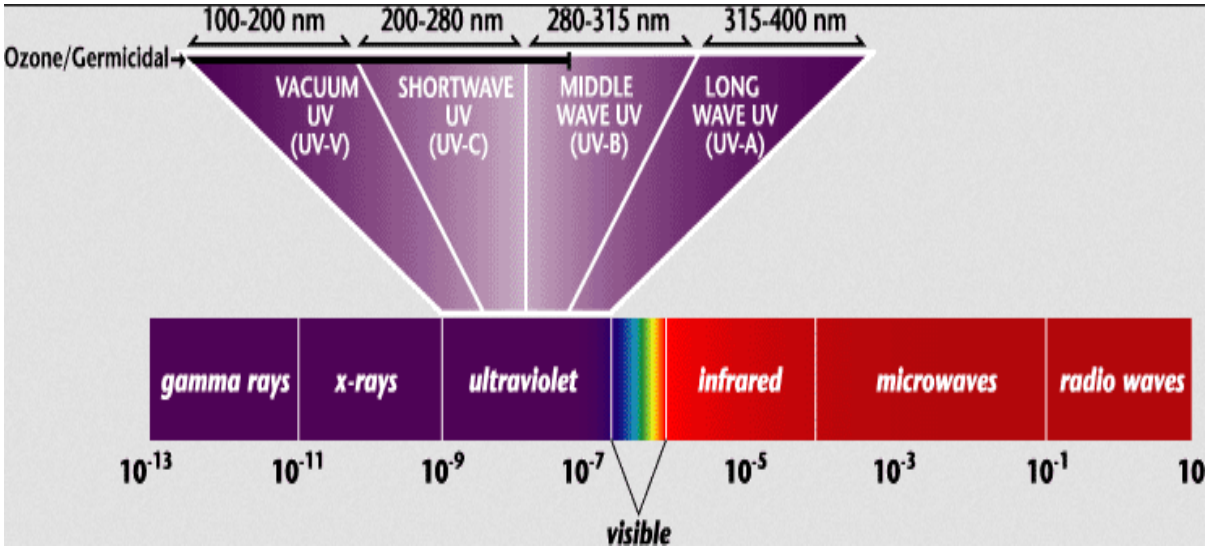


Figure 3. 1. Electromagnetic spectrum (Zamanian and Hardiman, 2005).

UV radiation levels are recorded and specified using a UV Index (UVI) by many organizations such as Word Health Orginisation (WHO) and United Nations Environment Programme (UNEP) (Gies et al., 2004). The amount of UV radiation reaching the surface of Earth varies during the day, the level of UV radiation is more intense between 10am- 3pm. Accordingly, these are the most dangerous periods throughout the day when skin damage occurs the fastest (Hoskin et al., 2008).

UV radiation has a low power of penetration, therefore its effects are limited to human skin, dermis and epidermis. UV with low energy is considered non-ionizing radiation, because its photons have low energy not enough to take of the atomic or moleular electron, but UV with high energy have photons energy capable to remove an electron from an atom of the substances to become an ionizing radiation (Ravanat et al., 2001).

The boundary of UV radiation within electromagnetic spectrum is ranged from 10 nm, on the X-rays side to 400 nm, on the visible light side. Although, in physics UV radiation is divided into four regions: near (400–300 nm), middle (300–200 nm) and far (200–100 nm), and extreme (below 100 nm). In biology, UV radiation is specified by three bands: UVA, UVB, and UVC. This is based upon the interaction between UV photons and biological materials. The three UV regions are: UVA (400–315 nm), UVB (315–280 nm) and UVC (280–100 nm). Due to the separation of ozone, wavelengths < 290 nm (100-280 nm), which represent UVC, are filtered by the stratosphere and doesn't reach the Earth (Alapetite, 1996) except at high altitudes (Henderson, 1977). The wavelengths 290-400 nm reach us as a solar UV radiation including (5%) UVB (280–315 nm) and (95%) UVA (315–400 nm) and is constant throughout the year (Svobodová et al., 2003). Accordingly, different biological effects are induced with different wavelengths of UV light (Schuch and Menck, 2010).

Increasing the importance of environmental UV radiation is related to its harmful effect on biological systems (Remenyik et al., 1999). It is counted one of the genotoxic and mutagenic factors (Douki et al., 2003; Ikehata et al., 2008) , and its contribution to skin carcinogenesis has been well investigated (Remenyik et al., 1999; Alexandrov et al., 2013). These issues have led the investigators to evaluate the deleterious effects of solar UV radiation on living cells and significantly its DNA. The wavelength dependence of this phenomena generally reflects the absorption spectrum of DNA (Clingen et al., 1995). It is well establitied that shorter wavelengths of sunlight in the UV are highly absorbed by DNA with a maximal effect around 250 nm. UVC radiation.UVB radiation constitutes a small proportion of solar radiation spectrum, but contains the most energetic component of terrestrial sunlight and are one thousand times more active than UVA radiation in prompting photo-carcenogenisis (Besaratinia et al., 2005; Pérez-Sánchez et al., 2014) . UVC radiation does not reach the Earth's surface, and so exposure for this radiation could happen by exposure to artificial sources emiting UVC radiation (Matsumura and Ananthaswamy, 2004). However, it is the most serious spectral region to humans as it is strongly absorbed by DNA molecule (Shi, 1992; Goodsell, 2001) .

UV radiation can damage DNA directly via absorption of UV photons by DNA molecules (DNA bases), and indirectly through absorption of UV rdiation by non-DNA cellular chromophores (photosensitisers) (Kielbassa et al., 1997). Formation of *cis-syn* Cyclobutane pyrimidine-dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4)PPs, due to dimerization of pyrimidines, are the most frequent generation by direct excitation by UVC and UVB radiation, and the main cause of cytotoxicity, mutagenisis and carcenogenisis development (Kielbassa et al., 1997, Debacq-Chainiaux et al., 2005, Jang et al., 2012, Kong et al., 2015). While oxidative

induced DNA damage such as 8-hydroxyguanine (8-oxoG), strand breaks, base loss sites and DNA-protein crosslinks are produced as a result of indirect DNA excitation due to the poor absorption of UVA radiation by DNA, which potentially triggers mutagenesis by photosensitiser reactions (Wölfle et al., 2014). Reactive oxygen species mediate the mechanism of oxidative DNA damage (Ito et al., 2006, Hoeijmakers, 2009, Poljšak and Fink, 2014, Chaisiriwong et al., 2016). An excited intracellular photoacceptor result in oxidative DNA modifications; either through react directly with DNA by single electron transfer or via free radicals, singlet oxygen ($^1O_2$) or hydroxyl radicals ($^{\cdot}OH$) formed by a superoxide (O_2^{-}) and a Fenton reaction (Kielbassa et al., 1997).

In human cells DNA damage is identified by sensor proteins, which transport the signal to start the mechanism of repair (Batista et al., 2009). However, defective in DNA damage repair process due to DNA lesions such as CPDs, 6-4 PPs and some oxidative bases such as 8oxoG block polymerases through replication, and this produces mutations, contributing to the development of carcinogenic process (Kozmin et al., 2005, Menck and Munford, 2014). Accumulation of unrepaired DNA damage leads to cell death by apoptosis. Furthermore, UV radiation has been demonstrated to be implicated in immunosuppression and has a significant role in the production of melanoma (Mouret et al., 2006). Human and animal studies have established that UV radiation has an essential role in the alteration of the immune function (Ichihashi et al., 2003). UV radiation exposure causes induction of immune suppressive pathways and modulate immune response (Duthie et al., 1999) by affecting different immune subsets, mainly to suppress T cells, a type of lymphocyte (a subtype of white blood cell) that plays a central role in cell-mediated immunity, to

proliferate and pro-inflammatory cytokine through upregulation of suppressive monocyte cells (Blanco et al., 2014).

3.2 Aims and objectives

(a) Evaluate the cytotoxic effects of UVA, UVB and UVC irradiation on human monocytic THP-1 cells.
(b) Using the comet assay to assess DNA damage in THP-1 cells and investigate if oxidative DNA damage is the key mechanism behind the genotoxicity of UV radiation.
(c) Elucidate in what way different wavelengths of UV radiation have different biological responses.
(d) Demonstrate that the biological responses of human monocytic THP-1 cells to UV irradiation is dose-dependent.

3.3 Material and methods

3.3.1 Chemicals, cell culture materials and cell culture technique.

All THP-1 cell culture materials and chemicals have been listed in Chapter 2, table 2.1. THP-1 cell culture technique has been described in Chapter 2, section 2.2.1.

3.3.2 UV exposure of THP-1 cells

THP-1cells exposure with UVA, UVB and UVC radiation has been described in Chapter 2, sections 2.2.8.2, 2.2.8.3 and 2.2.8.4, respectively. The overall UV treatment strategy for THP-1 cell line and each assay has been summarised in Figure 3.2.

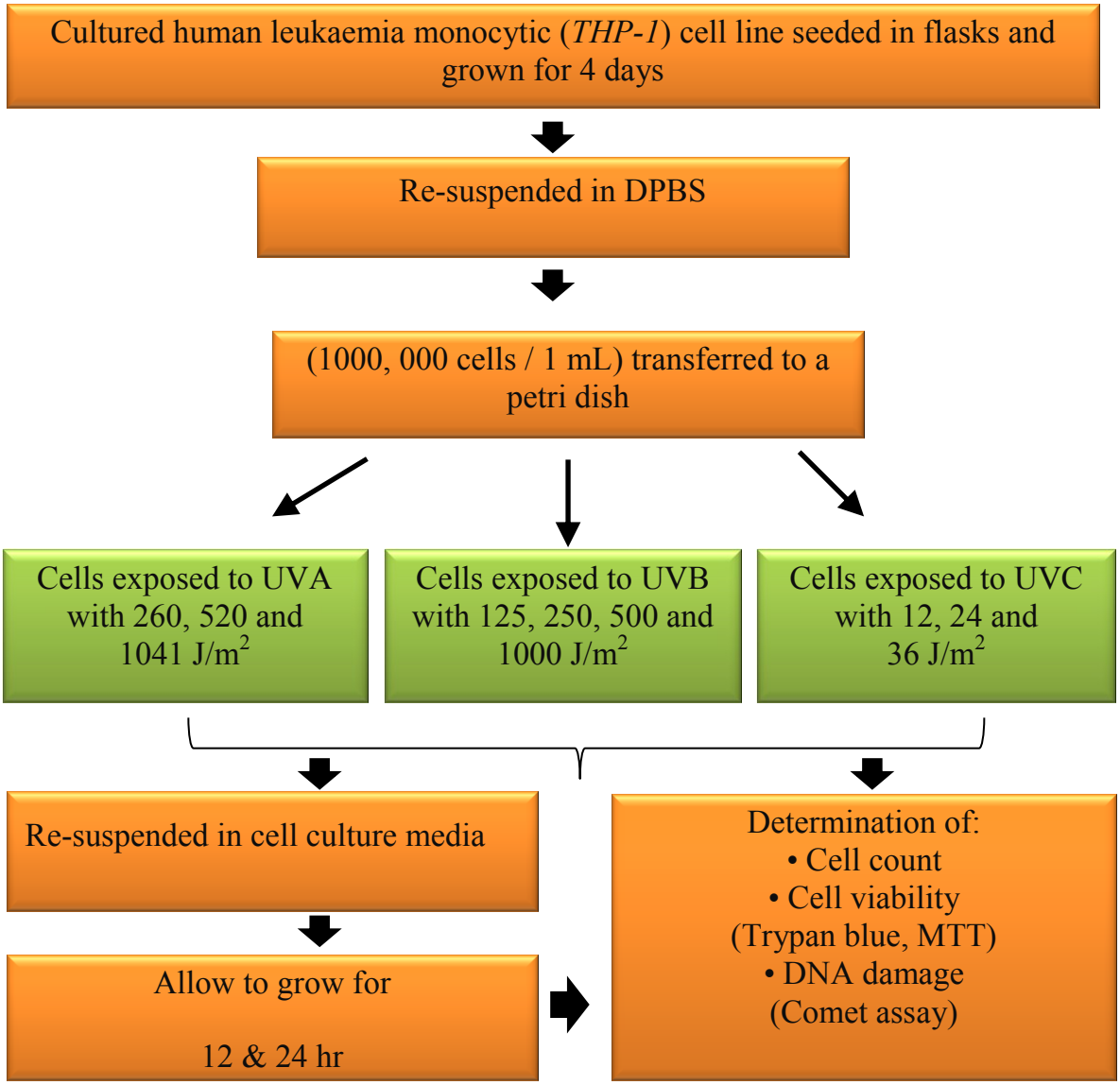


Figure 3. 2. Flow diagram showing overall experimental design to determine the impacts of UV radiation on THP-1 cells.

3.3.3 Proliferation rate of THP-1 cells

To study the impact of UV radiation on the THP-1 cell line after exposure for a range of doses of UVA, UVB and UVC radiation the process sequence in Figure 3.2 was used. The actual proliferation rate of THP-1 cells was determined by counting the number of irradiated cells microscopically in a Neuberger haemocytometer chamber, as described in Chapter 2, section 2.2.4.

3.3.4 Cell viability and cytotoxicity

Cell viability and vitality are highly important endpoints in regards to acute toxic effect that include reduced integrated cellular membrane, low mitochondrial effectiveness and impaired ability to proliferate. The viability of THP-1 cells has been determined to assess the cytotoxic effect of UV radiation. The protocols for investigating the integrity of cell membranes using the trypan blue exclusion test and mitochondrial activity by using MTT assay have been detailed in Chapter 2, section 2.2.5.1 and section 2.2.5.2 respectively.

3.3.5 Comet assay to determine DNA damage

THP-1 cells were processed for comet assay to determine DNA damage following UV irradiation as stated in the protocol in Chapter 2, section 2.2.6 to investigate the genotoxicity of UV radiation. All chemicals and buffers of comet assay have been listed in table 2.1. The main steps of comet assay have been elucidated in Figure 2.1. Viability for comet assay experiments was measured for each sample before starting the comet experiments as described in Chapter 2, section 2.2.5.1, and only 90%

viability was included in the experiment in accordance with the recommendation of Tice et al.(2000) .

3.3.6 Statistical analysis

All data were tested for normality using Anderson-Darling Normality test and Shapiro-Wilk. Appropriate parametric test was used with ANOVA General Linear Model for cells count and equal variance with (Brown-Forsythe) test used for cells viability, and for not equal variance used Holm-Sidak test for % tail DNA. Statistics were calculated using Minitab (version 15.1.0; Minitab Inc., State College, PA, USA) and SigmaPlot (version 13). The significance for all tests was set at $p \leq 0.05$.

3.4 Results

3.4.1 Evaluation of the cytotoxic effects of UVA, UVB and UVC irradiation on human monocytic THP-1 cells.

Human monocyte cells were used in these experiments because they represent one of the immune system cells, which is present in the blood and regularly exposed to UV radiation. To obtain comparable results for three types of UV radiation, different ranges of dose have been used for each type. We chose 260 – 1041 J/m^2 for UVA, 125 – 1000 J/m^2) for UVB, and 12 – 36 J/m^2 for UVC, that based on doses used in previos poplushed studies concerning DNA damage. The measurements for all tests have been conducted for immediate, 12 hr and 24 hr after irradiation.

To evaluate the cytotoxicity of UV radiation, first we determined microscopically the number of viable cells as the first indicator for the harmful effect of UV radiation exposure. Secondly, viability tests of irradiated THP-1 cells were achieved by testing THP-1 cell membrane integrity (trypan blue test), as discribed in section 2.2.5.1 and mitochondrial activity (MTT assay), section 2.2.5.2 Chapter 2.

3.4.1.1 Impact of UV radiation on cell number

UVA irradiated cells showed a significant decline in number with increasing doses compared to control at immediate exposure,12 hr and 24hr incubation post irradiation ($p < 0.001$; Figure 3.3). However, the number of irradiated cells increased after 12 hr incubation and increased more after 24 hr incubation post irradiation comparing to immediate irradiation, due to the proliferation of unaffected cells to radiation and cells of repaired DNA (Figure 3.3). Whereas, the number of control cells increased significantly ($p < 0.05$) with increasing incubation time, because of proliferation.

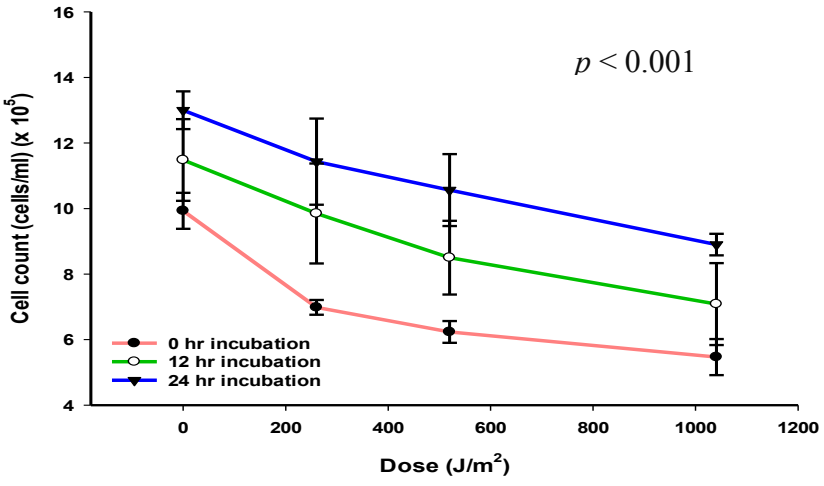


Figure 3. 3. Figure 3.3. Number of UVA irradiated cells: (•) represents number of cells immediately after exposure; (o) represents number of cells 12 hr after exposure; (▼) represents number of cells 24 hr after exposure. Doses – dependent cells number *(p* < 0.001), and comparison between each dose *vs* control is significant (*p*<0.05). Data shown are mean ± SEM for three separate independent experiments (*n* = 3).

On the other hand, UVB and UVC exposed cells needed longer time to respond for irradiation. THP-1 cels showed significantly maximum decrease in cell numbers ($p < 0.05$) at 12 hr of UVB and UVC radiation compared to immediate exposure (Figure 3.4 and Figure 3.5), where statistically, no significant changes in cells number were observed. Then, the number of cells returned to increase after 24 hr incubation post irradiation ($p < 0.05$) from these outpoints, we can reach to a consequence that THP-1 cells have time-dependent response to UV radiation. As for the responding for the applied doses, the irradiated cells showed a dose-dependent response. The number of THP-1 cells is significantly decreased ($p < 0.001$) after irradiation with different doses of UVA, UVB and UVC radiation compared to the control, which showed a persistent significant increase ($p < 0.05$) in number following the incubation due to the proliferation.

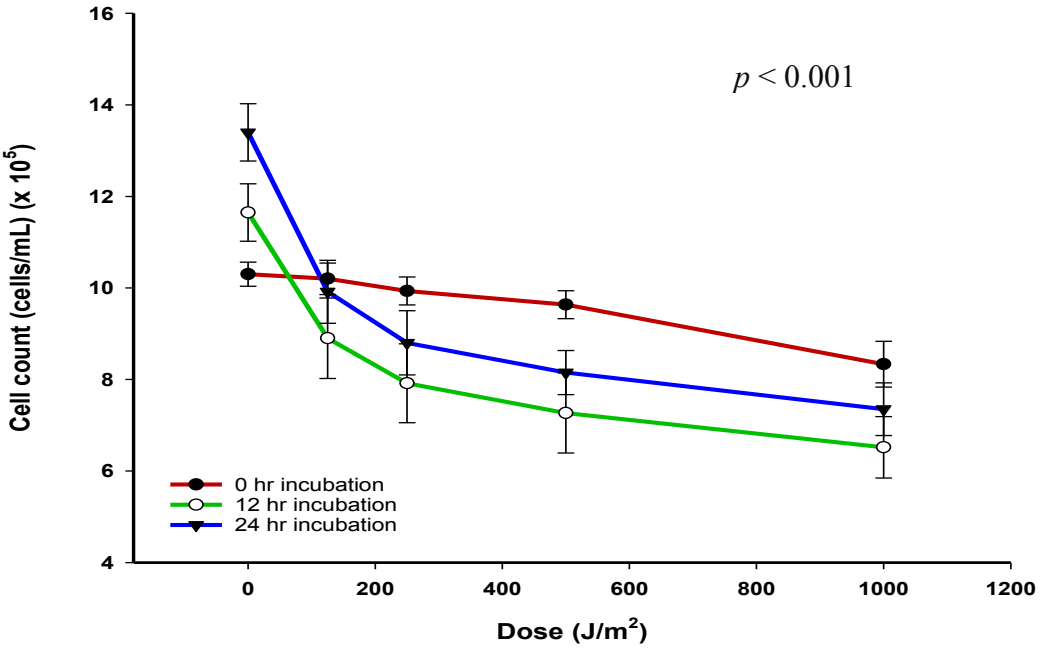


Figure 3. 4. Number of UVB irradiated cells: (•) represents number of cells immediately after exposure; (°) represents number of cells 12 hr after exposure; (▼) represents number of cells 24 hr after exposure. Doses – dependent cells number ($p < 0.001$), and comparison between each dose *vs* control is significant ($p < 0.001$). Data shown are mean ± SEM for three separate independent experiments ($n = 3$).

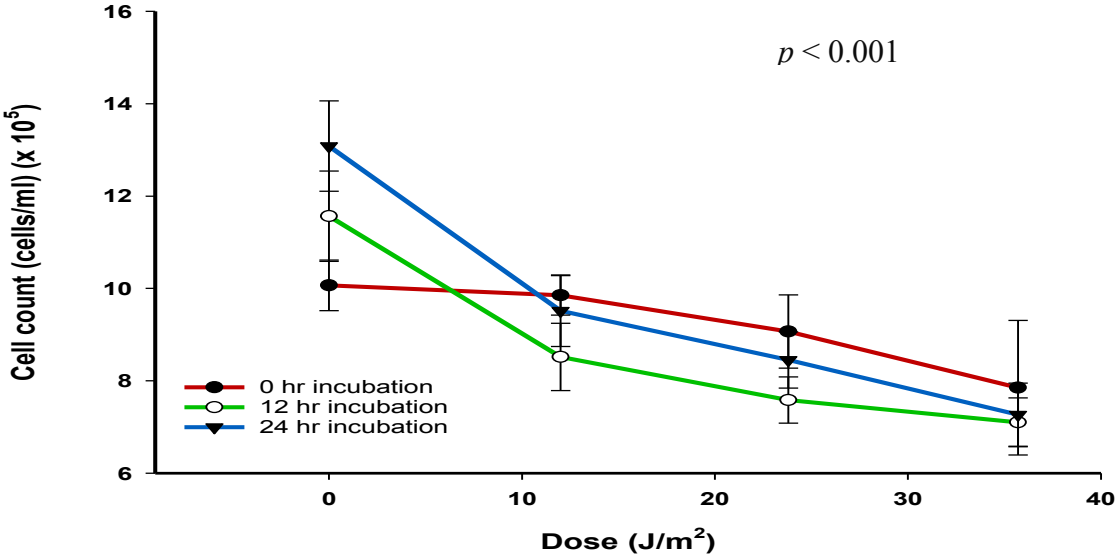


Figure 3. 5. Number of UVC irradiated cells: (•) represents number of cells immediately after exposure; (ᵒ) represents number of cells 12 hr after exposure; (▼) represents number of cells 24 hr after exposure. Doses – dependent cells number ($p < 0.001$), and comparison between each dose *vs* control is significant ($p < 0.001$). Data shown are mean ± SEM for three separate independent experiments ($n = 3$).

3.4.1.2 Cells viability

The viability of cells has been measured by trypan blue dye and MTT assay as discribed in section 2.2.5.1 and section 2.2.5.2 respectively in Chapter 2. The results in both showed that the response of cells to UV radiation is time and dose-dependent. The cells exhibited a significant decrease ($p < 0.05$) in viability immediately after being exposed to the doses range of UVA compared to the untreated cells at the three incubation periods. Nevertheless, the viability increased again significantly ($p < 0.05$) with increasing incubation times as presented in Figure 3.6 and Figure 3.7. Control cells (zero dose) showed slight consist increase in viability with inceasing the incubation times.

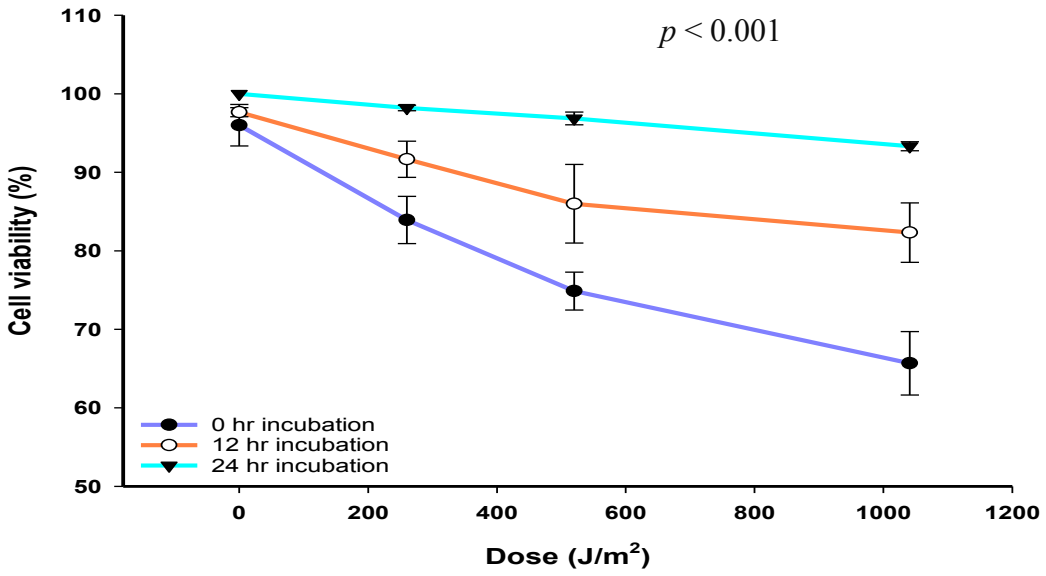


Figure 3. 6. Viability of UVA irradiated cells using trypan blue test: (•) represents cells viability immediately after exposure; (ᵒ) represents cells viability 12 hr after exposure; (▼) represents cells viability 24 hr after exposure. Doses – dependent cells viability (p <0.001), and comparison between each dose *vs* control is significant (p < 0.001). Data shown are mean ± SEM for three separate independent experiments (*n* = 3).

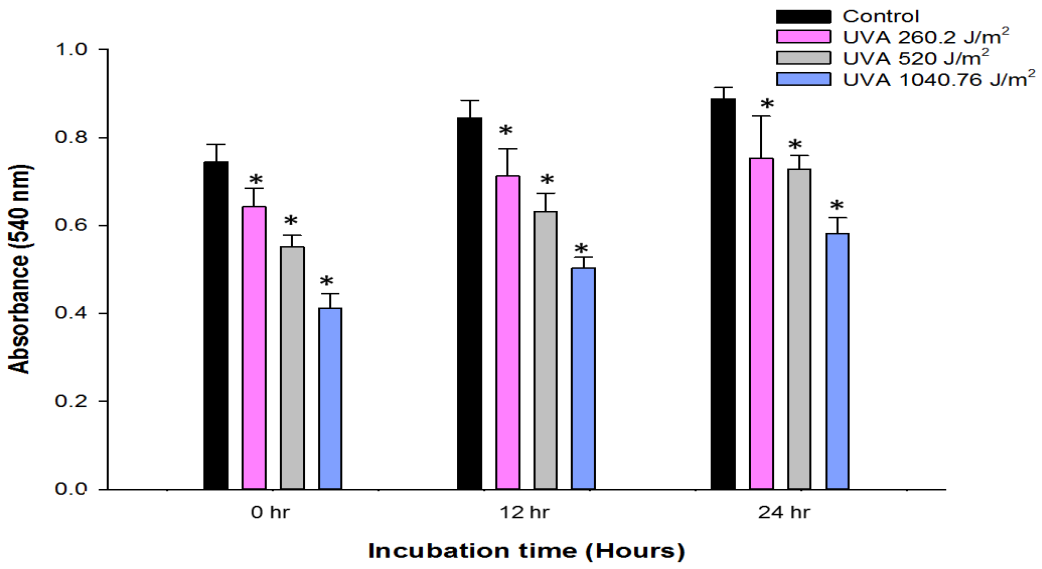


Figure 3. 7. THP-1 cell viability post UVA exposure was determined for different incubation time using MTT assay. Cell viability represented by absorbance, decreased significantly ($p < 0.001$), (*) represents significant differences between treated groups and control ($p < 0.001$). Data are mean ± SEM for three separated experiments, n=3.

While UVB and UVC irradiated cells did not exhibit an immediate significant change ($p > 0.05$). A maximum significant decrease ($p < 0.05$) was observed after 12 hr incubation post exposure, compared to the viability after immediate exposure to UVB and UVC radiation (Figure 3.8 and Figure 3.10). and returned to elevate significantly ($p < 0.05$) after 24 hr incubation. The viability of unirradiated cells is increased significantly ($p < 0.05$) with increasing incubation time. These results were also showed in Figure 3.9 and Figure 3.11 respectively, for investigating the mitochondrial activity by MTT assay to assess the viability of THP-1 cells post UVB and UVC exposure. All doses produced significant changes for cell viability ($p < 0.05$), which in the graph represented by absorbance (540 nm), in compared to the viability of untreated cells, except for UVC exposed cells at 12 hr post irradiation for 12 J/m^2, no statistically significant change was found (Figure 3.11; $p > 0.05$)

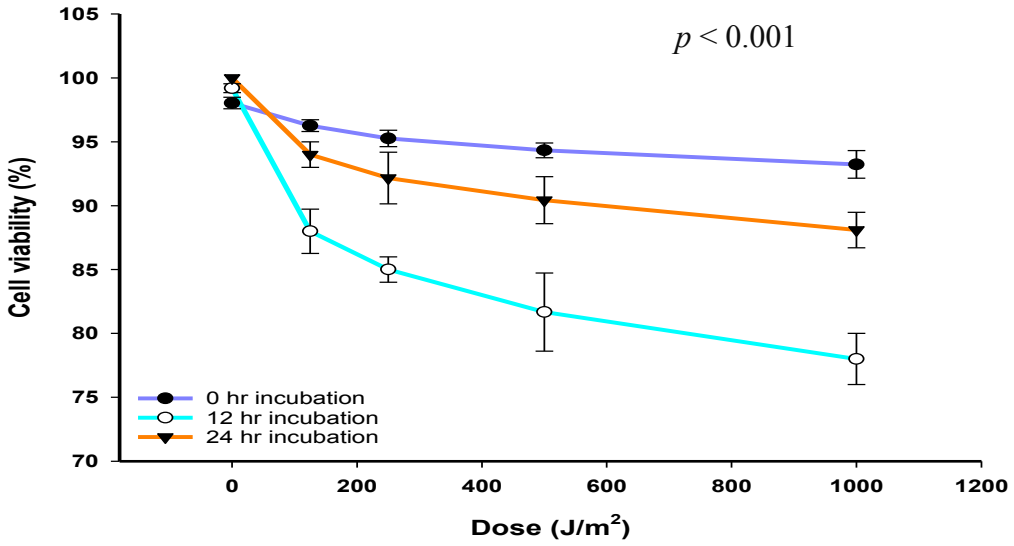


Figure 3. 8. Viability of UVB irradiated cells: (•) represents cells viability immediately after exposure; (o) represents cells viability 12 hr after exposure; (▼) represents cells viability 24 hr after exposure. Doses – dependent cells viability ($p < 0.001$), and comparison between each dose *vs* control is significant ($p < 0.05$). Data are mean ± SEM for three separated experiments, n=3.

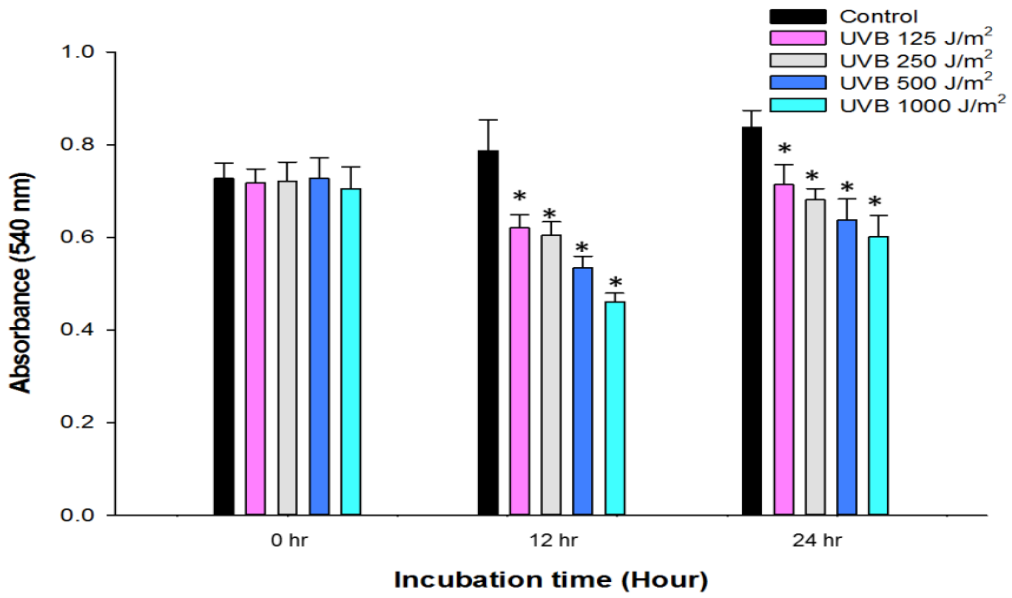


Figure 3. 9. THP-1 cell viability post UVB exposure was determined for different incubation time using MTT assay. Cell viability represented by absorbance, decreased significantly ($p < 0.001$), (*) represents significant differences between treated groups and control ($p < 0.001$). Data are mean ± SEM for three separated experiments, n=3.

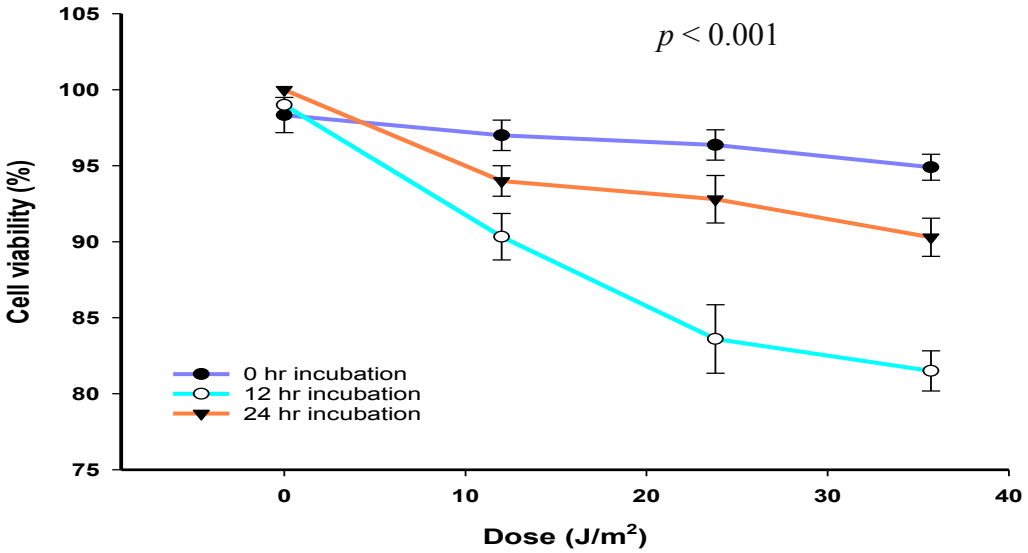


Figure 3. 10. Viability of UVC irradiated cells: (•) represents cells viability immediately after exposure; (ᵒ) represents cells viability 12 hr after exposure; (▼) represents cells viability 24 hr after exposure. Doses – dependent cells viability ($p < 0.001$), and comparison between each dose *vs* control is significant ($p < 0.005$). Data shown are mean ± SEM for three separate independent experiments ($n = 3$).

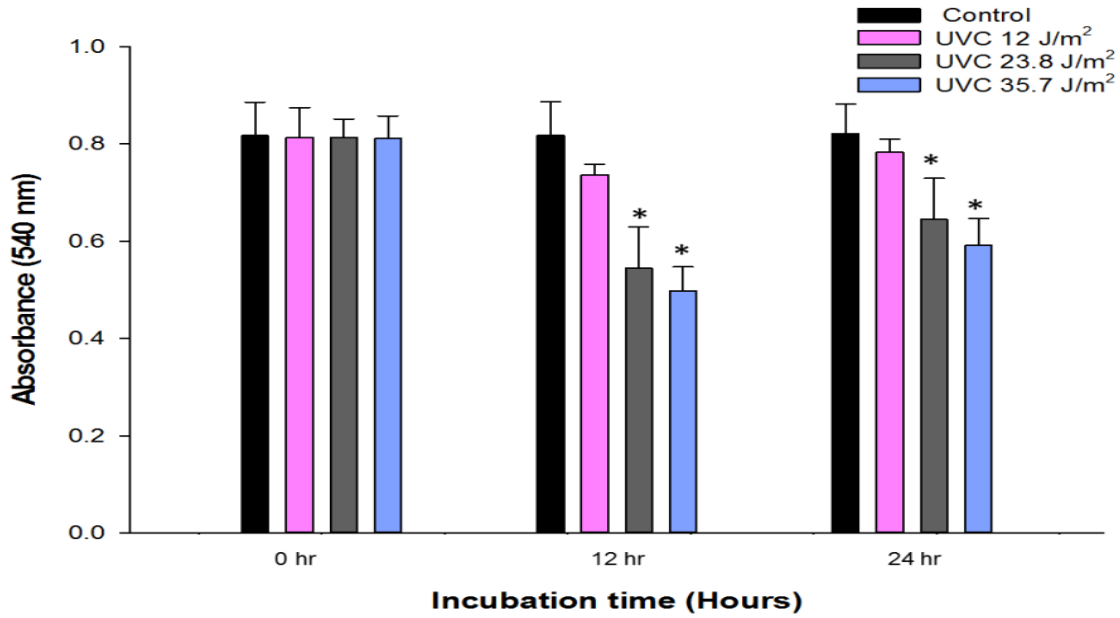


Figure 3. 11. THP-1 cell viability post UVC exposure was determined for different incubation time using MTT assay. (*) represents significant differences between treated groups and control ($p < 0.001$). Data are mean ± SEM for three separated experiments, (n=3).

3.4.2 Comet assay to determine DNA damage to assess the genotoxicity of UV radiation in THP-1 cells

Cells were treated with UV radiation of different wavelengths for different doses. The results indicated that DNA damage was significantly affected by exposur to UV radiation, and the effect was time and dose-dependent. UVA irradiated cells showed a significant elevation ($p < 0.001$) in oxidative DNA damage after exposure to range of doses 260 – 1041 J/m^2 compared to the control, that indicates the dose-dependent response of THP-1 cells post immediate irradiation, 12 hr ($p < 0.001$) and 24 hr ($p < 0.001$) incubation following UVA irradiation . A considerable increase in DNA damage ($p < 0.005$) was observed immediately after irradiation compared to the control group, but because of the repair processes that activated through incubation time , a significant decrease ($p < 0.005$) in DNA damage was found after 12 hr and 24 hr of incubation (Figure 3.12).

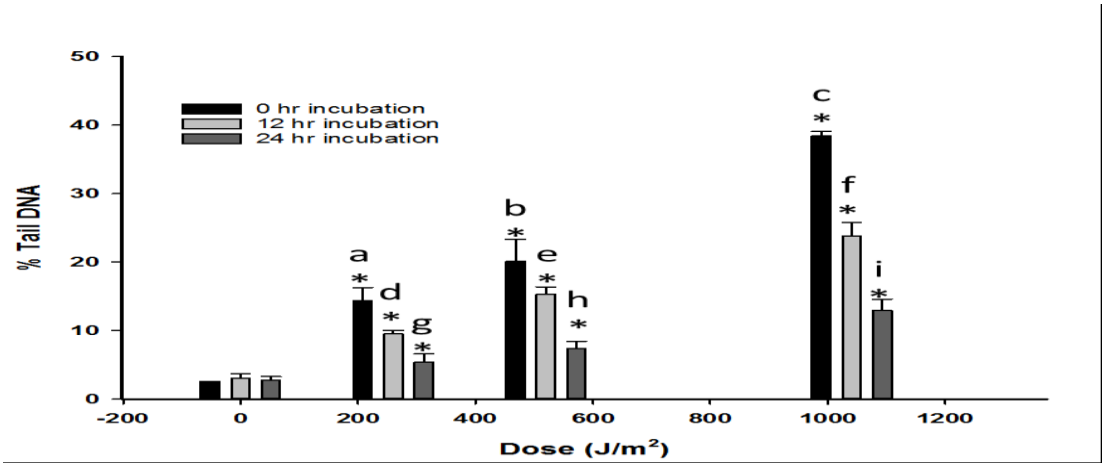


Figure 3. 12. Genotoxic effects in monocytic THP-1 after exposure to UVA radiation. (*) represents significant differences between treated groups and control (p < 0.001), and the letters represent significant differences between treated groups. Data shown are mean ± SEM for three separate independent experiments (n = 3).

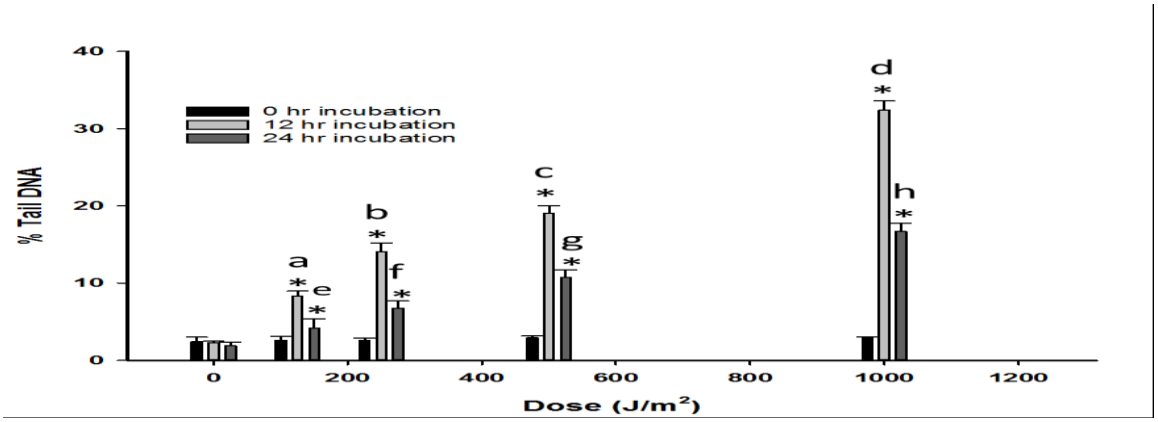


Figure 3. 13. Genotoxic effects in monocytic THP-1 after exposure to UVB radiation. (*) represents significant differences between treated groups and control (p <0.001), and the letters represent significant differences between treated groups (p < 0. 001). Data shown are mean ± SEM for three separate independent experiments (n = 3).

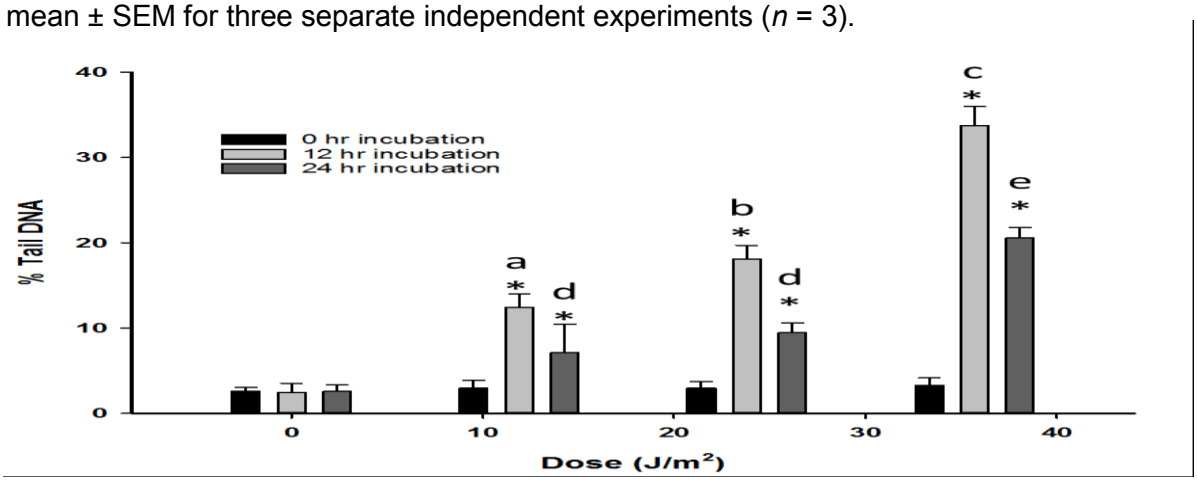


Figure 3. 14. Genotoxic effects in monocytic THP-1 after exposure to UVC radiation. (*) represents significant differences between treated groups and control (p < 0.001), and the letters represent significant differences between treated groups (p < 0. 001). Data shown are mean ± SEM for three separate independent experiments (n = 3).

While cells irradiation with UVB and UVC for doses ranged 125 – 1000 J/m^2 and 12 – 35.7 J/m^2 respectively, showed a higher increase in DNA damage ($p < 0.001$) after 12 hr incubation following treatment, which followed by a segnificant drope in DNA damage ($p < 0.001$) 24 hr post treatment due to DNA repair, as represented in Figure 3.13 and Figure 3.14 respectively. There was no significant differences ($p > 0.9$) between treated groups and control after immediate UVB and UVC irradiation.

3.4.3 Wavelength-dependent response of THP-1 cells for UV irradiation

The results in the present study proved that effects of UV radiation on biological systems are strongly dependence on wavelength (Diffey, 1991). In this study we used different bands of UV rays; UVC (100 – 280 nm) at peak of 248 nm, UVB (280 – 320 nm) at peak of 310 nm and UVA (320 – 400 nm) at peak of 360 nm to irradiate human monocytic THP-1 cell line, and revealing the biological impact of each band. The findings showed that UVA, UVB and UVC irradiation affected differently on various cellular processes included cell proliferation (Figure 3.15), viability and vitality (Figure 3.16), in addition to DNA damage and repair mechanism (Figure 3.17). Thus, this study demonstrated the wavelength –dependent response of THP-1 cells for UV irradiation as follows.

Figure 3.15 shows the changes in cell number ($p < 0.05$) according to change the wavelength of the applied UV rays. A clear significant reduction ($p < 0.05$)in No. of cells immediately following UVA band exposure at peak of 360 nm, while statistically insignificant reduction ($p > 0.05$) was observed following immediate UVC band and

UVB band exposure at peaks of 248 nm and 310 nm respectively. Whereas, a significant reduction in cell number ($p < 0.05$) appeared after 12 hr incubation following irradiation with UVC and UVB bands. The number of cells significantly increased again ($p < 0.05$) was after 24 hr incubation post irradiation with UV band (100 – 400 nm). More details are available in Table 3.1. Similar observations about cell viability were obtained after irradiation with UV band (100 – 400 nm) in Figure 3.16, and detailed in Table 3.2.

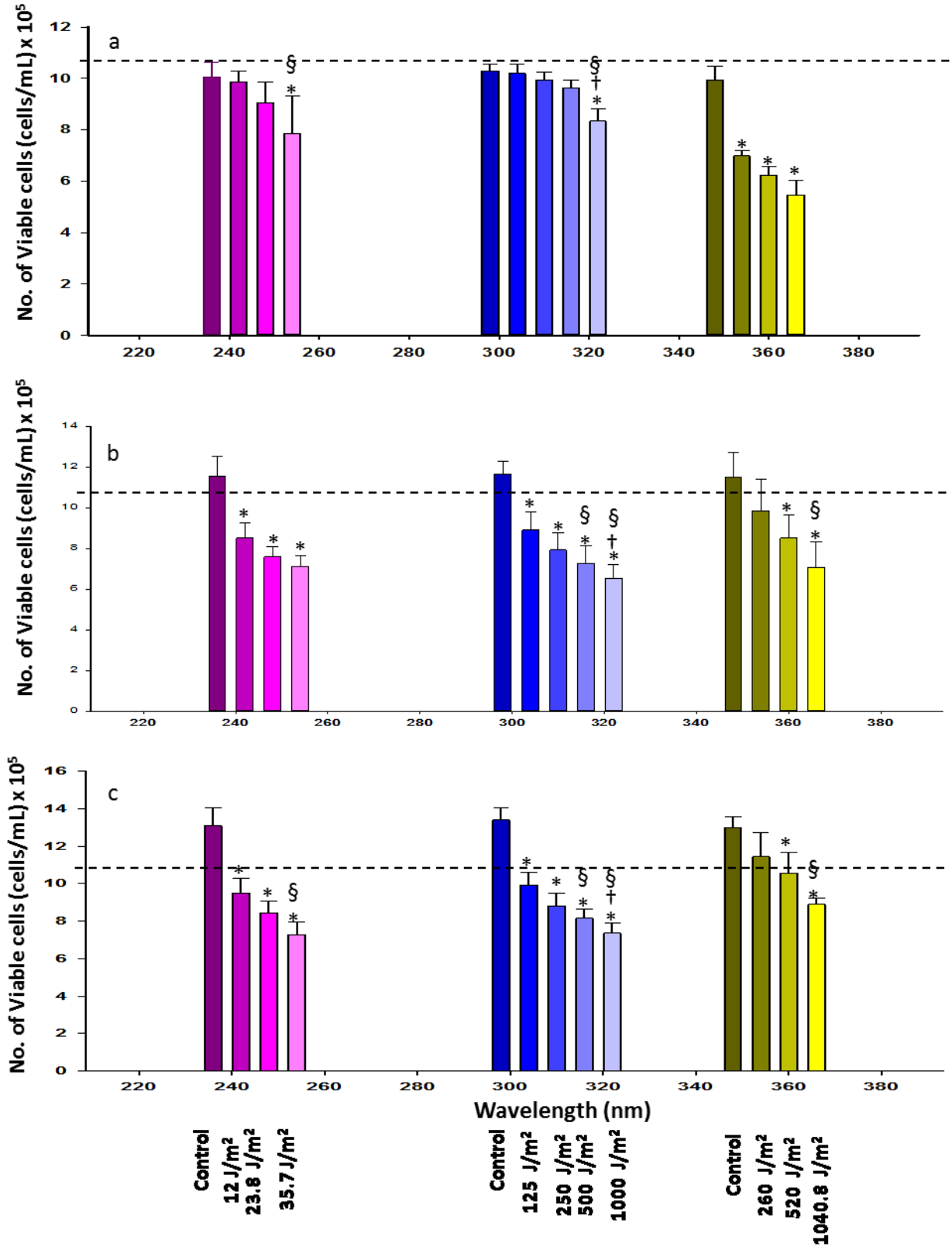


Figure 3. 15. The UV wavelength effect on the number of viable THP-1 cells irradiated with different wavelengths of UV radiation for different doses after: (a) 0 hr incubation; (b) 12 hr incubation and (c) 24 hr incubation following irradiation. (*) represent differences from control, (§) represent differences from first dose, (†) represent differences from second dose. . The dashed line refers to the maximum values of cell number at 0 hr incubation Each point is mean ± SEM for three separate independent experiments (*n* = 3).

Table3.1:The effect of UV irradiation on No. of THP-1 cells immediately, 12 hr incubation and 24 hr incubation after irradiation. Mean ± SEM. (*) represent differences from control, (§) represent differences from first dose, (†) represent differences from second dose.

UV band type	Dose (J/m^2)	No. of viable cells Mean ±SE Immediately post irradiation	No. of viable cells Mean ±SE 12hr post irradiation	No. of viable cells Mean ±SE 24 hr post irradiation
UVA	0	9.933 ± 0.317	11.483 ± 0.719	13.000 ± 0.333
	260.2	6.983 ± 0.130*	9.850 ± 0.881	11.433 ± 0.758
	520.4	6.233 ± 0.192*	8.500 ± 0.650*	10.567 ± 0.633 *
	1040.76	5.467 ± 0.318*	7.083 ± 0.722*§	8.900 ± 0.189*§
UVB	0	10.300 ± 0.153	11.650 ± 0.362	13.400 ± 0.362
	125	10.200 ± 0.200	8.900 ± 0.507*	9.917 ± 0.398*
	250	9.933 ± 0.176	7.917 ± 0.497*	8.800 ± 0.404*
	500	9.633 ± 0.176	7.267 ± 0.505*§	8.150 ± 0.278*§
	1000	8.333 ± 0.290*§†	6.517 ± 0.388*§†	7.350 ± 0.333*§†
UVC	0	10.067 ± 0.317	11.567 ± 0.564	13.083 ± 0.564
	12	9.850 ± 0.247	8.517 ± 0.420*	9.517 ± 0.447*
	23.8	9.067 ± 0.459	7.583 ± 0.289*	8.450 ± 0.351*
	35.7	7.850 ± 0.840*§	7.100 ± 0.306*	7.267 ± 0.394*§

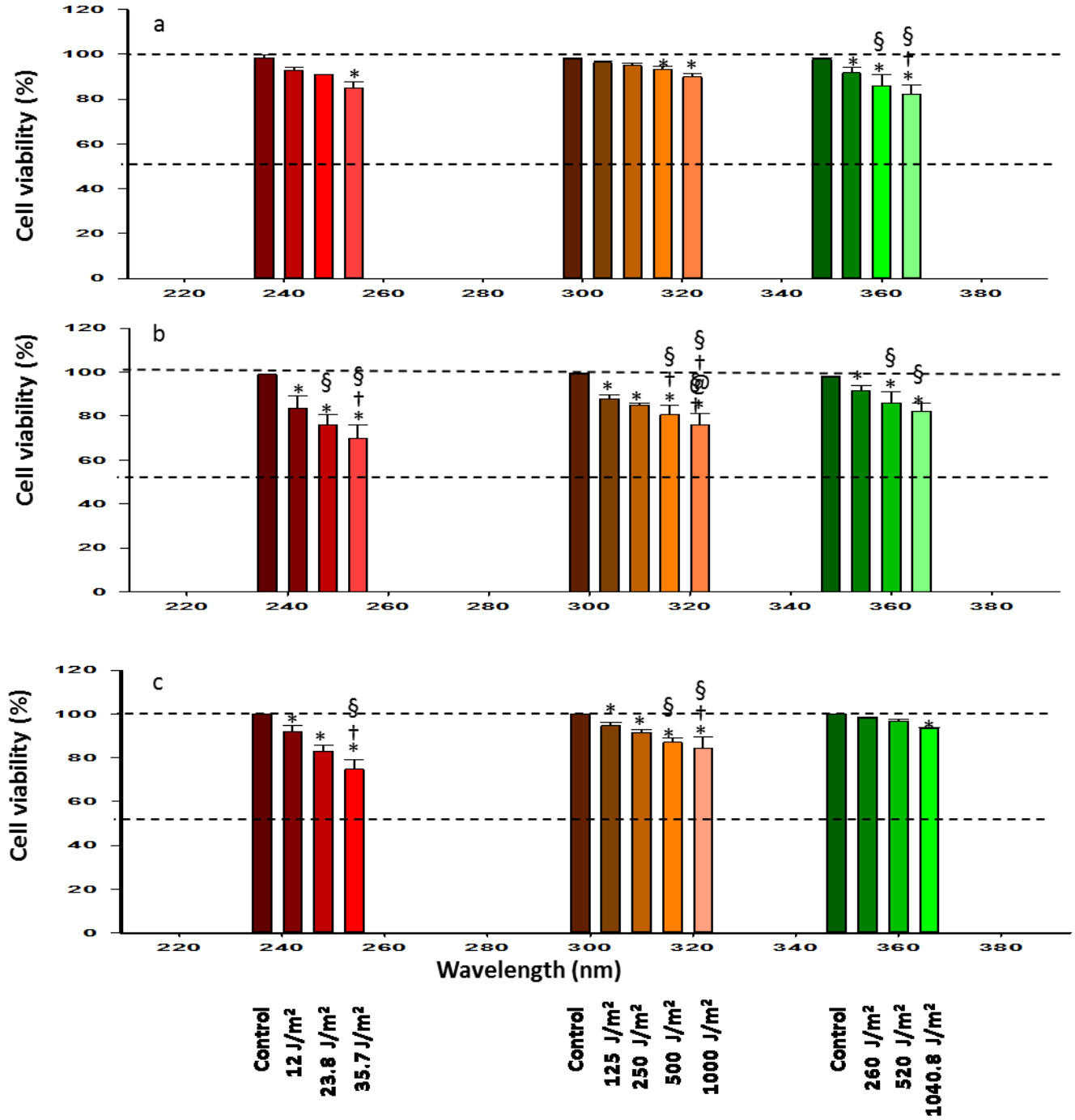


Figure 3. 16. The UV wavelength effect on the viability of THP-1 irradiated cells with different bands of UV radiation for different doses after (a) 0 hr incubation, (b) 12 hr incubation and (c) 24 hr incubation following irradiation. (*) represent differences from control, (§) represent differences from first dose, (†) represent differences from second dose. The dashed line refers to the maximum and mean levels of cell viability. Each point is mean ± SEM for three separate independent experiments (*n* = 3).

Table 3. 2: The effect of UV irradiation on viability of THP-1 cells immediately, 12 hr incubation and 24 hr incubation after irradiation. Mean ± SEM. (*) represent differences from control,(§) represent differences from first dose, (†) represent differences from second dose and (@) represent differences from third dose.

UV band type	Dose (J/m^2)	Cell viability % Mean ±SE Immediately post irradiation	Cell viability % Mean ±SE 12 hr post irradiation	Cell viability % Mean ±SE 24 hr post irradiation
UVA	0	96 ± 1.528	98 ± 0.333	100 ± 0.000
	260.2	84 ± 1.733*	92 ± 1.333*	98 ± 0.2
	520.4	75 ± 1.397*§	86 ± 2.887*§	97 ± 0.467
	1040.76	66 ± 2.333*§†	82 ± 2.186*§	93 ± 0.333*
UVB	0	98 ± 0.2	99 ± 0.362	100 ± 0.000
	125	96 ± 1	88 ± 0.507*	94 ± 0.577*
	250	95 ± 0.577	85 ± 0.497*	92 ± 1.167*
	500	94 ± 1.764*	82 ± 0.505*§†	90 ± 1.059*§
	1000	93 ± 1.155*	78 ± 0.388*§†@	88 ± 0.802*§†
UVC	0	98 ± 0.667	99 ± 0.000	100 ± 0.000
	12	97 ± 0.577	90 ± 0.882*	94 ± 0.577*
	23.8	96 ± 0.578	84 ± 1.301*§	93 ± 0.901*
	35.7	95 ± 0.493*	82 ± 0.764*§†	90 ± 0.723*§†

Figure 3.17 elucidates the response of DNA for irradiation with different UV bands (100 – 400 nm) of UVC, UVB and UVA. A significant increase ($p < 0.05$) in DNA damage (% tail DNA) was shown after irradiation with UVA band at peak of 360 nm, whilst no significant change in DNA damage following UVC and UVB bands exposure at peaks of 248 nm and 310 nm respectively. On the other hand, a significant increase ($p < 0.05$) in DNA damage was obtained after 12 hr incubation post irradiation with UVC and UVB radiation. 24 hr incubation following irradiation showed significant decrease ($p < 0.05$) in DNA damage in UVA and UVB irradiated cells and less in UVC irradiated cells. More details are inTable 3.3.

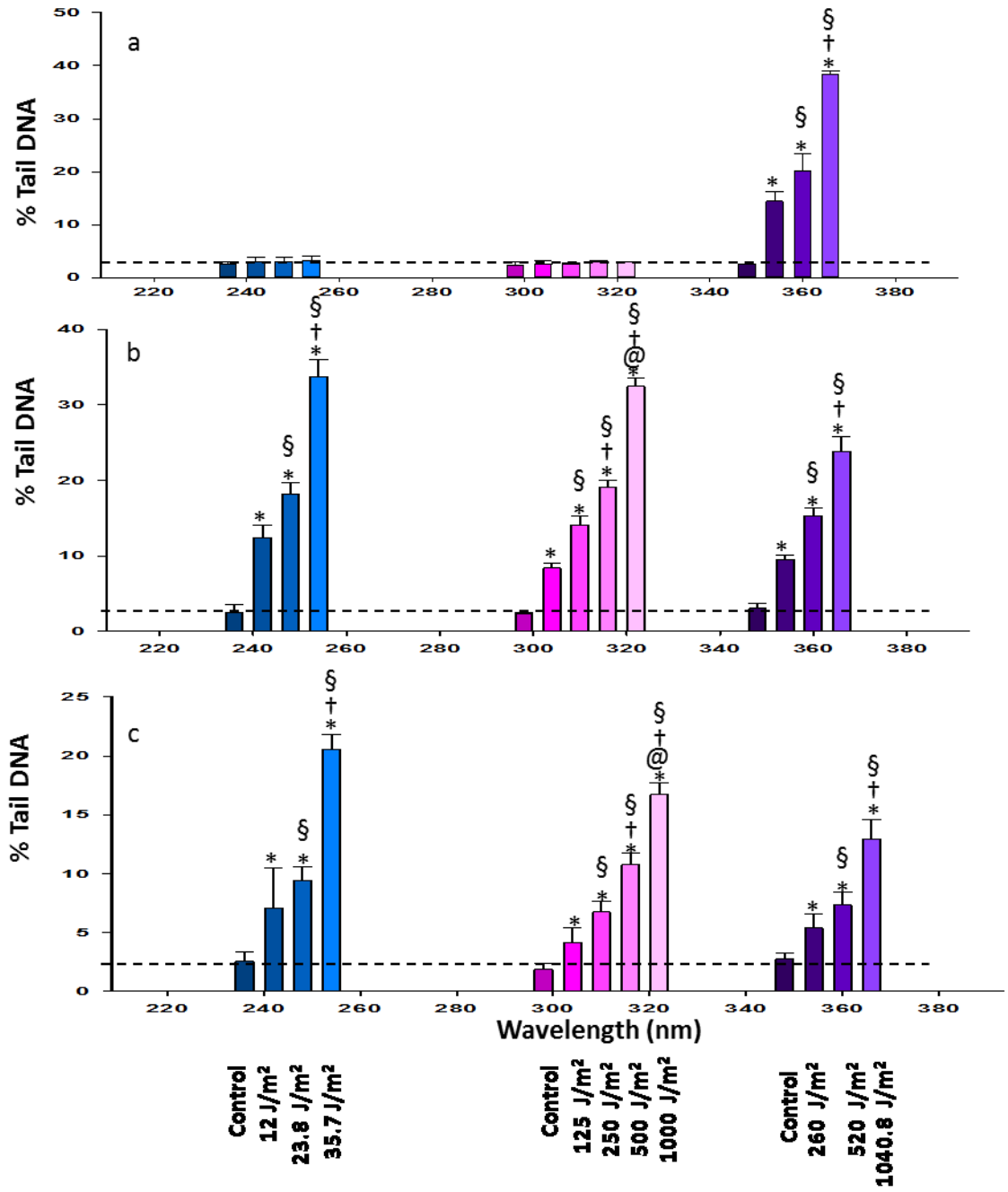


Figure 3. 17. The UV wavelength effect on DNA damage (percentage of tail DNA) in THP-1 irradiated cells with different bands of UV radiation for different doses after (a) 0 hr incubation, (b) 12 hr incubation and (c) 24 hr incubation following irradiation. the dashed line refers to level of DNA damage in control cells. Each point is mean ± SEM for three separate independent experiments (*n* = 3).

Table 3. 3: % Tail DNA in THP-1 cells immediately, 12 hr incubation and 24 hr incubation after irradiation with UVA & UVB. (*) represent differences from control, (§) represent differences from first dose, (†) represent differences from second dose and (@) represent differences from third dose. The dashed line refers to the DNA damage for control. Mean ± SE. for three separate independent experiments (*n* = 3).

UV band type	Dose (J/m^2)	% Tail DNA Mean of median ±SE Immediately post irradiation	% Tail DNA Mean of median ±SE 12 hr post irradiation	% Tail DNA Mean of median ±SE 24 hr post irradiation
UVA	0	2.560 ± 0.016	3.015 ± 0.380	2.744 ± 0.322
	260.2	14.383 ± 1.084*	9.504 ± 0.307*	5.372 ± 0.707*
	520.4	20.090 ± 1.878*§	15.296 ± 0.624*§	7.332 ± 0.633*§
	1040.76	38.386 ± 0.377*§†	23.843 ±1.129*§†	12.939±0.943*§†
UVB	0	2.430 ± 0.336	2.289 ± 0.126	1.856 ± 0.290
	125	2.575 ± 0.307	8.336 ± 0.359*	4.139 ± 0.702*
	250	2.583 ± 0.160	14.057 ± 0.653*§	6.731 ± 0.547*§
	500	2.917 ± 0.134	19.075 ± 0.547*§†	10.761 ± 0.549*§†
	1000	3.001 ± 0.290	32.396±0.689*§†@	16.697±0.593*§†@
UVC	0	2.609 ± 0.257	2.461 ± 0.608	2.554 ± 0.446
	12	2.947 ± 0.549	12.423 ± 0.924*	7.081 ± 1.947*
	23.8	2.963 ± 0.453	18.119 ± 0.915*§	9.433 ± 0.667*§
	35.7	3.299 ± 0.488	33.758 ± 1.283*§†	20.567 ± 0.724*§†

3.4.4 Relationship analyses between cell viability and % Tail DNA

Figure 3.18 displayed the correlations obtained between DNA damage (% tail DNA) and cell viability after *in vitro* exposure for UV radiation bands (100 – 400 nm). Significant and high positive correlations were apparent for three UV bands, UVC, UVB and UVA bands. These findings reflect the genotoxic and cytotoxic effect of UV radiation.

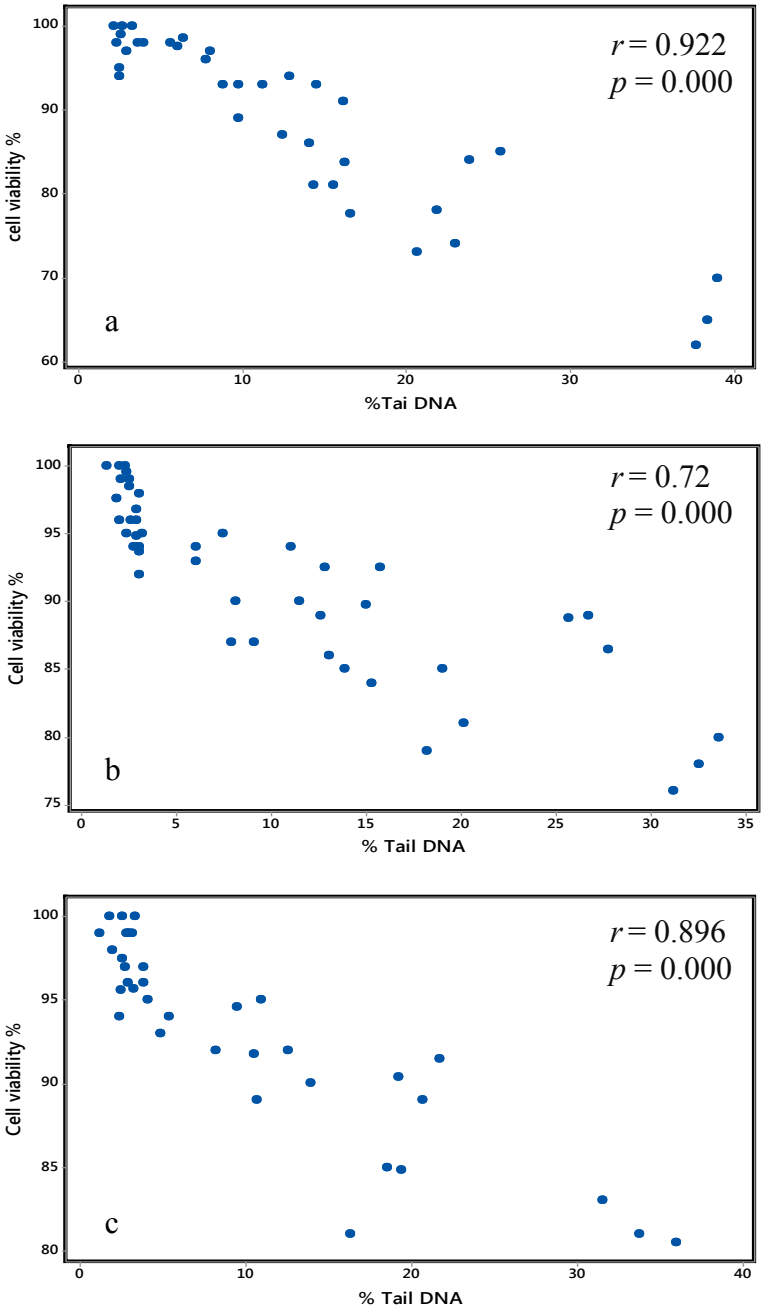


Figure 3. 18. Pearson's correlation analyses of % Tail DNA and Cell viability in: a. UVA; b. UVB; c. UVC irradiated cells.

3.5 Discussion

Because of increasing evidences for the carcenogenic, and apoptotic effects of UV radiation on cells, the cytotoxic and genotoxic effects of UV irradiation are investigated in this study. The irradiation of THP-1 cells has different influences on cell counts (Figures 3.3, 3.4, 3.5), the percentage of cells viability (Figures 3.6, 3.8, 3.10) and DNA damage for UVA, UVB and UVC. A clear reduction in cell number immediately after irradiation with UVA could be attributed to the stimulation of apoptosis due to the generation of ROS and superoxide radicals (Kulms et al., 1999), and may be related to the enhancement of decreasing the mitochondrial membrane potential that reflects mitochondrial membrane depolarization (Widel et al., 2014). This, however, did not seem to show irreversible damage, because the number of cells increase after 12 hr and the level of cell survival is close to control level after 24 hr post irradiation (Figure 3.3).While, a maximum decrease in cell numbers occurs at 12 hr after irradiation with UVB and UVC (Figures 3.4, 3.5),.

The main cause for UVB irradiation-induced cell death is the induction of cell apoptosis . However, the molecular mechanisms underlying UVB-induced apoptosis of human monocytic cells are poorly understood (Sollberger et al., 2015). Apoptosis induction of UVB irradiated cells is based on the activation of caspase-1, an evolutionarily conserved enzyme that proteolytically cleaves other proteins. Caspase-1 has a crucial protective role in innate immunity (Sollberger et al., 2015, Strowig et al., 2012) , as an inflammatory response initiator. Caspase-1 is involved in pyroptosis, a lytic form of cell death, that leads to inflamation enhancement *in vivo* via releasing intracellular components (Bergsbaken et al., 2009, Miao et al., 2011). Caspase-1 activates multi-protein complexes called inflammasomes, which

assemble and activate upon sensing of stress signals (Lamkanfi and Dixit, 2012).Caspase-1expression, caspase-1 activity and the activation of inflammasoe are essential for UVB-induced immun cells apoptosis (Miao et al., 2011, Sollberger et al., 2015). Decreasing the mitochondrial activity in irradiated cells (MTT) may lead to quantitative loss of mitochondrial membrane confirmed by the requirement of caspase-1 for UVB-induced apoptosis. Also, a growing body of evidence demonstrates that UVB-induced apoptosis is related to the activation of lincRNA-p21 (Hall et al., 2015), a long noncoding RNA and a trsnscriptional target of phosphoprotein (tumor protein) p53 (Tang et al., 2015). UVB irradiation increases potently the level of lincRNA-p21 in irradiated immun cells, and p53 regulating lincRNA-p21 transcript levels in response to UVB, causing apoptosis in UVB-treated cells may it could be mentioned that p53, a tumor suppressor, plays a role in UVB irradiated cells by triggering apoptotic death of irrepairable DNA-damaged cells ,and causing cell cycle arrest to give time for damaged DNA to repair, thus prevent UVB induced mutation and cancer (Hall et al., 2015, Tron et al., 1998).

Cell death and reduced cell proliferation after irradiation with UVB radiation (observed in Figure 3.4) could be attributed to the downregulation level of p21 protein in UVB treated cells. p21 is a protein and functions as a regulator of cell cycle progression at G1 and S phases. This p21 downregulation in UVB irradiated cells produces a reduction in cells proliferation, increase in cells apoptotic death and promoting of G2 phase arrest (Chen et al., 2015). Irradiation with UVC light showed a decline in the number of monocytic cells and viability with increasing doses after 12 h and 24 h post irradiation. The main reason for this diminution is the induction of apoptosis (Takasawa et al., 2005, Nawkar et al., 2013, Van Houten et al., 2016). UVC-induced apoptosis could be related to the activation of JNK (c-Jun N-terminal

Kinases); proteins belong to mitogen-activated protein kinase family, and participate in cellular responses to stress stimuli, such as UV irradiation. A sustained JNK activation in apoptosis is potently induced by UVC radiation (Chen et al., 1996), which is much more cytotoxic than both UVB and UVA (Uehara et al., 2014).

The biophysical and biochemical effects of UV radiation, including cell viability and DNA damage, are relatively well understood in mammelian cells (Schuch and Menck, 2010). However, kinetics of DNA damage induced by UV radiation is still a mater of debate. Assays for detecting DNA damage have already been used. In this respect, single cell gell electrophoresis or the alkaline comet assay is the preferred test to assess the damage and repair of DNA in cells irradiated with UVA, UVB and UVC radiation (Alapetite, 1996). Image analysis software reveals the importance of a comet assay by giving the quantification of DNA damage in individual cells (Clingen et al., 1995).

The results in the present study provide quantitative information on DNA damage in human monocyte cell lines after exposure for the three types of UV radiation in three spectral bands with varying doses. The wavelength dependence of the induction of DNA damage in THP-1 cells (Figure 3.17) shows good agreement with the previous studies for different cultured cells (Besaratinia et al., 2011, Pfeifer and Besaratinia, 2012, Pronin et al., 2017). This observation is also similar to that showed for the promoting of damage in isolated DNA (Matsunaga et al., 1991).

Comet assay assist in showing the response of THP-1 cells with damaged DNA immediately after exposure to UVA radiation through visualizing the initial formation of strand breaks. This is in accord with the results obtained on human fibroblast cells (Alapetite, 1996), and human B-lympoblast cell line (Bock et al., 1998), by alkaline

comet assay and human epithelioid cells by alkaline elution (Peak et al., 1991). The repair of damaged DNA has shown at 12 hr and more evidently after 24 hr incubation following the irradiation. These findings contradict the previous studies, which reported that the recovery time of induced DNA damage in humane cells occurs through 1 hr post irradiation with UVA.

While, the recovery time of our monocyte cells, as one of immun system cells, agree another studies which observed that cultured human immun cells take a longer period for DNA damage repair than other cultured human cells. Bock et al. 1998 indicated that B-lymphocytes take more than 150 min for DNA damage to repair (Bock et al., 1998). It is also reported that the repair of oxidative DNA damage by lymphocytes takes a longer period of time than for HeLa cells to be completed (Collins et al., 1995). Furthermore, in accordance with several studies, the results in the present study suggest that the high amount of DNA damage after immediate UVA treatment is a consequence of the formation of cyclobutane pyrimidine dimers CPDs in addition to the strand breaks, which may elongated the recovery time of DNA damage (Perdiz et al., 2000, Pfeifer et al., 2005, Courdavault et al., 2005)

The indirect genotoxic effect of UVA is commonly related to the interaction between UVA photons and endogenous photosynthesizers (Cadet et al., 2012). This stimulation triggers the generation of reactive oxygen species (ROS) mediated induction of UVA-oxidation DNA damage (Sage et al., 1996), which likely contribute to its toxicity and mutagenicity (Cadet et al., 2005). UVA radiation is more efficient than UVB and UVC in producing oxidative damage for DNA bases in cells. The generation of singlet oxygen causes selective guanine oxidation (8-oxo-7, 8-dihydroguanine). In addition, hydroxyl radical formed by superoxide anions reactions, that contribute to DNA degradation, in turn producing oxidized purine and pyrimidine

bases simultaneously with single-strand breaks (Cadet et al., 2015). However, recent studies have reported the ability of UVA radiation to produce several orders of magnitude fewer CPDs (but no 6:4 Py:Pys) in oxygen-independent reactions in mammalian cells (Karran and Brem, 2016).

In contrast to UVA, the percentage of DNA damage is observed remarkably 12 hr post irradiation with UVB and UVC. The direct interaction between UVB or UVC radiation and DNA enables the cells to analyze by alkaline single cell gell electrophoresis whenever they process the promoted adducts and dimers from cleavage to ligation (Speit and Hartmann, 1995).

The absence of comets in THP-1 cells immediately following irradiation with UVB and UVC indicates that no breaking in DNA strands are produced in cells in the applied doses range. The capacity of cells to form UV-induced photoproducts makes the comets a function of post-exposure time dependence. To evaluate acute UVB induced DNA damage, acheiving the cell lysis as soon as posible following exposure is essential, to minimize the photochemicaly generated strand breaks arising from the repair of damaged DNA by cells enzymes (Patton et al., 1999), in addition to the diversity of UV induced nucleobase photoproducts due to the excision repair (Alapetite, 1996; Patton et al., 1999).

Through applying comet assay to THP-1 cell line, it is found that the response of cells to UVC is relatively more than that of UVB in the applied doses range used in our study. The maximum intensity of UVC lamp used in the present study is at 248.25 nm, that is within the short-wave radiation (100-280 nm), and maximaly absorped by DNA as maintioned above. The bands of these wavelengthes coincide with the absorption spectra of DNA, RNA and proteins (Widel et al., 2014), therefore,

considered relatively to be the most dangerous environmental exposure for cells (Lee et al., 2016). As seen in the present study, the response of cells is dose-dependente. This may be due to the few transporting energy suficient to generate low level of excision and repair that the cells are undergoing (Patton et al., 1999).

Investigating the repair of DNA damage has been conducted under more physiologically relevent conditions by irradiating cells to UV radiation and incubating the irradiated cells in growth media for 12 and 24 hr, and then subjecting them for alkaline comet assay. The percentage tail of DNA showed a significant decrease for applied range of UVB and UVC after 24 hr incubation post irradiation (Figures 3.13, 3,14). While, the reduction in percentage tail of DNA observed clearly after 12 hr incubation and higher reduction demonstrated after 24 hr incubation following exposure to UVA radiation (Figure 3.12).

It has been demonstrated that the correction of major DNA damage representing most helix distortion, in particular the pyrimidine products, induced by UV light, from nucleotide excision repair (NER) (Lankinen et al., 1996, Lindahl, 1993). NER pathway is an important mechanism utilized for damaged repair in mammalian cells (Katiyar et al., 2017). The induction of NER is enhanced by the cytokine interleukin-12, secreted firstly by antigen presenting cells such as monocytes cells (Robertson and Ritz, 1996, Zou et al., 1995), also suppressing UVB-induced cell death (Kulms et al., 2002). NER is a key mechanism that preventing the potential mutation, either by completely repairing the damaged DNA before replecation, or using specific DNA polymerase for postreplication repair to synthesize DNA free from error. It is an extremely conserved strategy for repairing huge of DNA damage, such as CPDs and (6-4)pp (Wood, 1997). Although the tail percent of DNA (DNA damage) decreased with increasing the incubation time refer to the effective NER in human

monocytes to repair the majority of direct formation of CPDs and (6-4)pp. However, NER is limited and less efficient in repairing indirect UVA induced base damage and strand breaks. These lesions are most likely removed primarily by base excision repair (BER) pathway (Kryston et al., 2011). BER is a multistage process depending on succesive activity of many proteins, in particular, initiated by DNA glycosylase, that contribute in the repair of several oxidized bases such as 8-oxo-7,8-dihydro-2_-deoxyguanosine (8-oxodG) and 5,6-dihydroxy-5,6-dihydrothymine and single strand breaks (SSBs) (Aburatani et al., 1997, Gueranger et al., 2014, Wallace, 2002).

The cells exhibited a relatively long repair time and the resynthesis/ legation process tended to be slow following the damage they underwent for each type of UV light exposure. The delay of the recovery time is could also be attributed to resedual breaks of alkali-labile-sites ALS, which are slow and need longer period to repair than strand breaks. These results agree with Bock et al. (1998), who examined the repair periods of UVA induced DNA damage in B-lymphocyte cells, the damage is composed of single strand breaks in addition to alkali-labile-sites, that known to repair more slower than SSB. Bianchi et al. (1990) and Lankinen et al. (1996), who reported that the kinetics of repair in UV-damaged lymphocytes need 24 hr to process. Green et al. (1992) observed that granulocytes and lymphocytes take more than 4 hr to repair UV-induced DNA damage. Different types of repair mechanisms are envolved after UV irradiation (Churchill et al., 1991, Peak et al., 1991), however, under the experimental conditions of our study, it is likely, limited types of repair mechanisms or may be one type is activated, that represented through decreasing the level of DNA damage post 12 and 24 hr incubation after irradiation. The type of cell could also be important and have a vital role in this observations, as Henriksen et al. detected, they irradiated human lymphocytes with UV radiation and got single

phase kinetics for DNA damage repair (de With et al., 1994). While, Churchill et al 1991 has used chinese hamster overy cells and human epithelioid P3 cells respectively, and obtained biphasics repair kinetics (Churchill et al., 1991).

3.6 Conclusions

The effects of UV radiation on human immune monocytic THP-1 cells are extremely convoluted and illustrated the complications, which include the growing immune cells response. It is noteworthy that for different incident doses of the three wavelength bands of UV radiation caused a reduced survival and increased frequency of inducted apoptosis, and comets from irradiated THP-1 cells displayed a considerable mean percentage of tail DNA due to direct and indirect photosensitizer interaction. However, damage varied according to the spectral bands of UV radiation. The results in the present study confirmed that exposure to UV radiation generates damage in human immune THP-1 cells and has an adverse impact on their viability. In spite of that, the damage wasn't permanent and decreased in general after 24 hr post irradiation, which implying participation of varied mechanisms and effective pathways of cell repair. This study integrated multiple biomarker approach suggests that UV radiation carries a potential health risk due to its direct interaction with critical cellular molecules such as DNA, and indirectly by ROS induction.

The results have emphasised that the response of DNA damage and cells death was dependant on UV dose, wavelength and time post irradiation.

CHAPTER 4

THE EFFECT OF PBM/LLLT ON CELL AND DNA UNDER *IN VITRO* CONDITIONS

Hypothesis: Photobiomodulation (PBM) or Low level laser therapy (LLLT) has the ability to increase the rate of cell proliferation and accelerate DNA damage repair mechanism via photostimulation effect.

4 The effect of PBM/LLLT on cell and DNA under *in vitro* conditions

4.1 Introduction

Photobiomodulation therapy (PBM therapy) or Low-level laser (light) therapy (LLLT) is an important technique for many circumstances that require energizing of healing, relief of pain and inflammation, and restoration of function (de Abreu Chaves et al., 2014). Master was the first who introduced (LLLT) for potential clinical applications in the late 1960s, where low intensity, non-thermal irradiance does not generate heat (Wetter et al., 2009). This process is also referred to as photostimulation, phototherapy or photobiomodulation (Kneebone et al., 2006, Adamskaya et al., 2011).

The biomodulatory effects of LLLT have been disseminated in variety of *in vitro* studies using different cell types such as fibroblasts, lymphocytes, keratinocytes, macrophage, HeLa and Stem cells (Adamskaya et al., 2011). It has been demonstrated that LLLT has the ability to modulate the process of tissue repair by prompting of cellular reaction such as migration, proliferation, apoptosis and cellular differentiation (Giannelli et al., 2011).

Low power lasers have been shown to be safe, non-invasive and highly beneficial in various fields of medicine, including dentistry and orthopedics (Cobb, 2006) (Kneebone et al., 2006). However, the mechanism of photobiostimulation by LLLT is

poorly understood. It seems that LLLT can be influential at the molecular, cellular and tissue levels (Chaves et al., 2014). The basic biological action of lasers is the absorption of red and NIR light photons by specific photoacceptors, such as cytochrome c oxidase (CCO). This chromophore is the fourth of five proteins located in the respiratory chain within the mitochondria, and possibly also photoreceptors in the plasma membrane of cells; so, a series of events occurs in the mitochondria, producing biological alterations for various processes (AlGhamdi et al., 2012). Studies have been conducted to record the absorption spectra of CCO in different oxidation states, which is found to be identical to biological responses to LLLT (Karu and Kolyakov, 2005, Hamblin and Demidova, 2006a, Karu, 2010, Huang et al., 2011). Light absorption may lead to photodissociation of the restrained nitric oxide from CCO, leading to an increase of enzyme activity, increasing electron transport, oxygen consumption, mitochondrial respiration, and ATP production. Thus, altering the mitochondria or redox state of cell, LLLT can stimulate the activation of many intracellular signalling pathways and changes of cellular activities; including proliferation, regeneration and cell survival (Adamskaya et al., 2011, Lins et al., 2010) .

Many different types of laser light sources, including diode lasers, helium-neon (He-Ne), and ruby, these are diode lasers used to deliver LLLT in different medical applications. In LLLT, energy is delivered to a biological system at low levels, and therefore does not generate significant heat (AlGhamdi et al., 2012). Studies regarding the biostimulation effect of LLLT found no increase in heat of the target tissue, and this can be ignored if the induced temperature rise is < 1°C (Hrnjak et al., 1994). Many investigators have shown that in fibroblast suspensions there is no temperature change with LLLT irradiation (Boulton and Marshall, 1986, Quickenden

and Danniels, 1993) . Other researchers, Schneede et al. (1988), are in agreement with these findings, he found the increase in temperature to be < 0.065°C, during laser irradiation of 40 mW/cm^2, using a microthermal probe in a monolayer of cells(Schneede et al., 1988) . In contrast, the surgical lasers of high energy (e.g., carbon dioxide lasers and neodymium-YAG lasers, Nd: YAG) are able to elevate the tissue temperature high enough to cut , cauterise, vaporise and sterilise it (Pogrel, 1991).

4.2 Aims and objectives

Within the context of the above information, this chapter have the following objectives:

(a) To investigate the ability of NIR low level laser (850 nm diode laser) to enhance proliferation of THP-1 human monocyte cell line under *in vitro* conditions.
(b) To identify the doses and exposure times at which the stimulatory effect occurs.
(c) To evaluate potential cytotoxicity and the genotoxic effects of different diode laser doses on the target cells.
(d) To find whether there is a valuable change in ATP level in low level laser exposed cells by using luminometer.
(e) To use an integrated experimental approach to correlate ATP levels and cell proliferation rate after low level laser application.

4.3 Materials and methods

4.3.1 Experimental techniques for the design and fabrication of the Biological Effect of Laser Therapy (BELT).

In order to study the Biological Effect of Laser Therapy (BELT) at the cellular and molecular level, an instrumental platform for BELT was designed and built in Plymouth during the course of this work. Figure 4.1 shows the schematic designed. The 850 nm laser source has a linearly polarised circular beam, with variable output power (up to 25 mW). The beam expander increases the beam diameter to match the size of the well containing the cells. The polarising beam splitter enables ‘p’ or ‘s’ polarised light to be delivered to the cells, according to the orientation of the laser diode. In this work ’p’ polarised light was used. The ‘p’ polarised light passes through a series of beam splitters, delivering 40%, 30%, 20% and 10% of the input beam to four wells which contain the cells in suspension. In the work reported here, only 40% of the laser power was delivered to the cells. The laser power was fixed and the exposure time varied to expose the cells to the different doses. The 30%, 20% and 10% options are part of the system design and will be used for future experimental studies, which will also include ‘s’ polarised light.

The laser diode output was calibrated using a power meter (at 850 nm) in parallel with the in-built power monitoring capability of the laser diode. Subsequently power delivered was determined from in-built power monitor diode. A stopwatch controls the exposure time, which is the only variable. The uncertainty in exposure time was +/- 1 s.

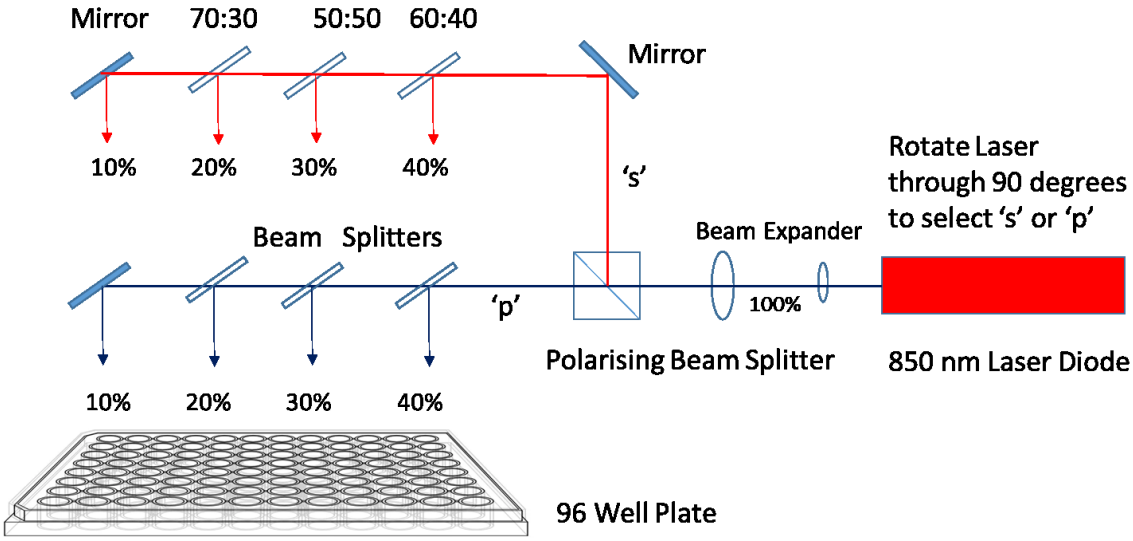


Figure 4. 1. Schematic representation of the biological effect of PBM/LLLT. A polarized beam of 850 nm laser diode is split by different beam splitter with angular separation of output beam at 45° to irradiate cells in well plate.

4.3.2 Experimental design and laser exposure conditions of THP-1 cells

The experimental design to study the effect of diode laser on THP-1 cells has been elucidated in Figure 4.2

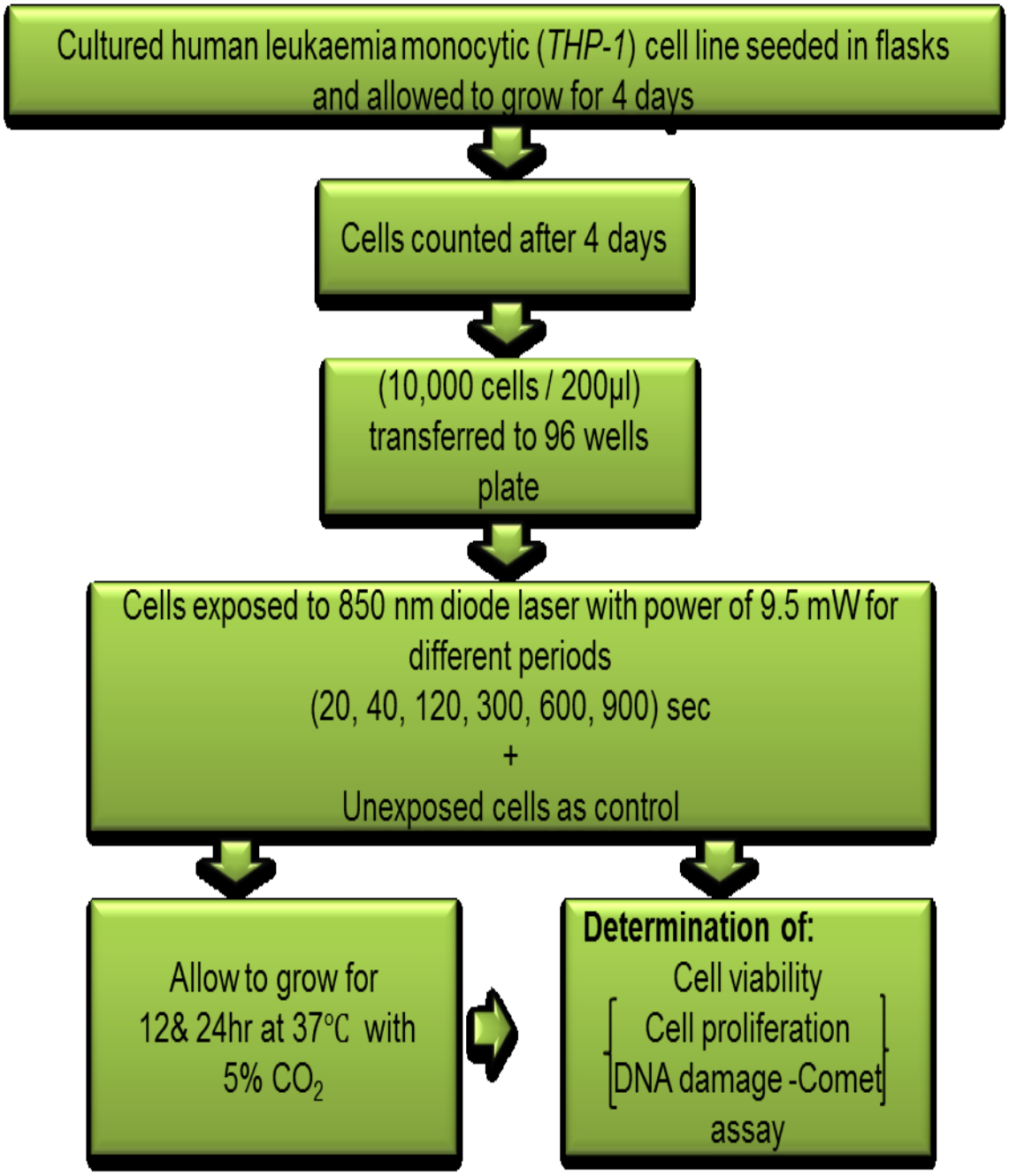


Figure 4. 2. Schematic diagram showing experimental overview for the *in vitro* studies to test the Impact of PBM/LLLT at cellular and molecular level.

4.3.3 Cell culture materials and cell culture technique

All THP-1 cell culture materials have been listed in Chapter 2, table 2.1. THP-1 cell culture technique has been described in section 2.2.1.

4.3.4 PBM/LLLT irradiation of THP-1 cells

The irradiation of THP-1 cells with diode laser and dose calculation to study the stimulatory effect of PBM/LLLT on cells have been described in Chapter 2, section 2.2.9.2.

4.3.5 Proliferation rate of THP-1 cells

Proliferation of cells was determined before and after exposure to various doses of diode laser. THP-1 cells were counted microscopically in a Neuberger haemocytometer chamber as described in Chapter 2, section 2.2.4.

4.3.6 Cell viability and cytotoxicity

Viability of THP-1 cells has been determined to test the cytotoxic effect of diode laser. The protocols for investigating the integrity of cell membrane by using trypan blue exclusion test and the mitochondrial activity by using MTT assay have been detailed in Chapter 2, section 2.2.5.1 and section 2.2.5.2 respectively.

4.3.7 Cell Viability Kit SL Assay for ATP measurement

A cell Viability Kit SL Assay obtained from BioThema, Sweden (Product no. 188-441; www. biothema.com) was used to measure ATP content in human monocytic THP-1 cell line after irradiation with different doses of 850 nm near infrared low level laser therapy NIR-LLLT light. The cell Viability Kit SL is intended for cell proliferation and cytotoxicity studies by determination of the total amount of ATP. This assay generates a “glow-type” luminescent signal produced by a luciferase reaction with cellular ATP. In all cases the cell Viability Kit SL assay is performed as described by Lundin (2000).

To be specific, 50 µl of extract ant B/S was added to 10 µl of each sample of cell suspension in the cuvette and mixed gently. 400 µl of ATP Reagent SL, (this reagent is diluted with Diluent B before using. Pour approx. half of the content of Diluent B into the ATP Reagent SL vial and swirl the content to mix. Thereafter pour the liquid back again into the Diluent vial and swirl to mix), was added into the cuvette and mixed to be read within 30 sec by using a reporter luminometer. Then, 10 µL of ATP standard was added, mixed and read within 1.30 min. All reagents were stored in the dark in a freezer, and assays are curried out at room temperature. The amount of ATP present in the THP-1 cells was quantified in relative luminescent

Units (RLUs) for each sample according to the following equation:

$$\text{ATP smp} = 100 \times \text{I samp} / (\text{I smp} + \text{std} - \text{I smp}) \ldots\ldots (4.1)$$

The factor 100 is the amount of ATP Standard added (100 pool).

4.3.8 Comet assay to determine DNA damage

In order to evaluate the genotoxic effect of PBM/LLLT in THP-1 cell line, the alkaline comet assay (single cell gel electrophoresis SCGE) has used to determine the damaged DNA in THP-1 cells post exposure to different fluences of 850 nm diode laser. All chemicals and buffers of comet assay have been listed in Chapter 2, table 2.1. The main steps of comet assay have been elucidated in Figure 2.1. THP-1 cells were processed for comet assay to determine DNA damage as described in section 2.2.6.

4.3.9 Statistical analysis

All data acquired were tested for normality using Anderson-Darling test and Shapiro-Wilk and for equal variance with Brown-Forsythe test, and appropriate parametric or non-parametric tests were used. For parametric data, appropriate regression and correlations were determined using Pearson's correlation coefficient. Analysis was made using a 2-way ANOVA with Holm-Sidak for pairwise comparisons and comparisons versus a control group, and Tukey's pairwise comparisons as post hoc tests. For non-parametric, Kruskal-Wallis test for pairwise comparisons and Mann-Whitney for comparisons versus a control group.

For the comet assay data, the median was calculated for % tail DNA values for each slide. The resulting data set based on medians was not normally distributed and was analysed by Kruskal-Wallis test for pairwise comparisons and Mann-Whitney for comparisons versus a control group. Statistics were calculated using SigmaPlot Statistics (version 13.0.0) and Minitab (version 17.1.0; Minitab Inc., State College, PA, USA). Significance for all tests was set at $p \leq 0.05$.

4.4 Results

4.4.1 Effect of PBM/LLLT in enhancing cell proliferation

Cell counting was used to elucidate how PBM/LLLT affects the proliferation rate of human monocytic cells (Figure 4.3). The doses of the PBM/LLLT (diode laser) at 850 nm were chosen according to the literature (Chow and Barnsley, 2005) and primary optimization (Hashmi et al., 2010a, Leal Junior et al., 2008, Faria Amorim et al., 2006, Aver Vanin et al., 2016). Proliferation of THP-1 cell line has been determined by using a microscope and a haemocytometer. The monocytic cells irradiated with 850 nm diode laser at fluences of (0.6, 1.2, 3.6, 8.9, 17.8 and 26.8 J/cm^2) showed a remarkable change in the number of viable cells, and hence proliferation, compared to non-irradiated cells (Figure 4.3).

Comparing the effect of different diode laser fluences statistically showed no significant change in cell proliferation at the time of irradiation (0 hr incubation) with low doses (0.6, 1.2 and 3.6 J/cm^2) in comparing to non-irradiated cells, while a significant drop was clear immediately post irradiation with high level doses (8.9, 17.8 and 26.8 J/cm^2). On the other hand, a noteworthy significant increase in proliferation ($p < 0.001$) at low doses (0.6- 3.6 J/cm^2), and significantly decreased proliferation ($p < 0.001$) at higher doses (8.9- 26.8 J/cm^2) after 12 and 24 hr incubation post irradiation as compared to the immediate irradiated cells. A 2-way ANOVA with Tukey's pairwise comparisons as post hoc tests showed a significant change in cell count for different laser doses ($p < 0.05$).

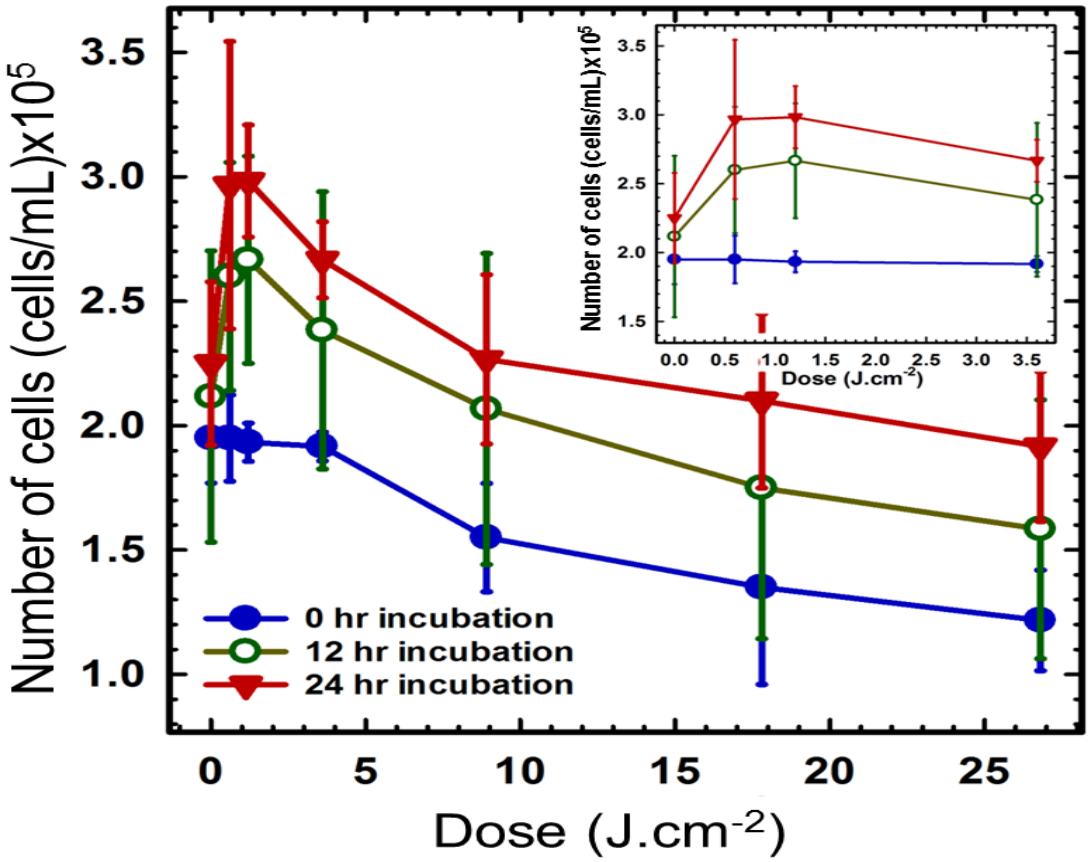


Figure 4.3. Number of THP-1 cells after irradiation with diode laser: (•) Immediately post irradiation; (o) After 12 hr incubation post irradiation; (▼) After 24 hr incubation post irradiation. Each point represents the mean of three separately performed experiments. *Error bars*, SEM. The inset shows in detail the data at very low doses.

Table 4. 1: The proliferation of THP-1 cells after PBM/LLLT.

NIR diode laser energy density J/cm^2	Number of THP-1 cells immediately post irradiation x (10^5 cells/mL) Mean ± SE	Number of THP-1 cells 12 hr post irradiation x (10^5 cells/mL) Mean ± SE	Number of THP-1 cells 24 hr post irradiation x (10^5 cells/mL) Mean ± SE
control	1.95 ± 0.10	2.12 ± 0.34	2.25 ± 0.19
0.6	1.95 ± 0.10	2.60 ± 0.26*	2.97 ± 033*
1.6	1.93 ± 0.04	2.67 ± 0.24*	2.98 ± 0.13*
3.6	1.92 ± 0.03	2.38 ± 0.32*†	2.67 ± 0.09*†
8.9	1.55 ± o.13*	2.07 ± 0.36*$	2.27 ± 0.19$
17.8	1.35 ± 0.23*§	1.75 ± 0.35*§	2.10 ± 0.20*$
26.8	1.22 ± 0.12*&	1.58 ± 0.30*&	1.92 ± 0.17*&

* refer to significant changes between the irradiated and the unirradiated cells (control), the symbols refer to significant changes between the irradiated groups.

4.4.2 Evaluating the cytotoxic effect of PBM/LLLT in THP-1 cells

4.4.2.1 Effect of PBM/LLLT on cell viability

Cell viability was determined in order to assess the cytotoxic events taking place in the cells following 850 nm diode laser irradiation to investigate the photobiomodulation PBM effect. The viability of irradiated cells was determined immediately after irradiation, twelve hours and twenty four hours incubation after irradiation with the diode laser. Cell membrane integrity (Figure 4.4) and

mitochondrial activity (Figure 4. 5) were tested by trypan blue exclusion test and MTT assay respectively to evaluate the viability of irradiated THP-1 cells. A 2-way ANOVA with Holm-Sidak test for pairwise comparisons and comparisons versus a control group showed a significant change in cell viability ($P < 0.001$) and mitochondrial activity (absorbance 540 nm) ($P < 0.05$) for different laser doses. THP-1 cells show a time and dose-dependent manner response, as show in Figures 4.4 and Figure 4.6 ($p < 0.05$).

4.4.2.2 Cell viability using trypan blue exclusion test

In respect of investigate that if NIR diode laser has a cytotoxic influence in THP-1 cells, we tested the membrane integrity of the cells using trypan blue exclusion test following irradiation with NIR diode laser over various fluences. Figure 4.4 showed no significant changes in THP-1 cell viability ($p > 0.05$) immediately after exposure to low doses of NIR diode laser irradiation (< 5 J/cm^2), while a remarkable decrease was shown after exposure to high doses (> 5 J/cm^2) ($p < 0.05$) compared to unirradiated cells. However, due to the proliferation, the viability of irradiated cells significantly increased after12 hr and 24 hr post irradiation over the applied low and high doses of diode laser compared to 0 hr incubation, as show in Figure 4.4 ($p < 0.05$). There were no significant changes ($p > 0.05$) in viability at low doses after 12 hr and 24 hr following irradiation, because approaching the viability from the control (Figure 4.4; $p > 0.05$).

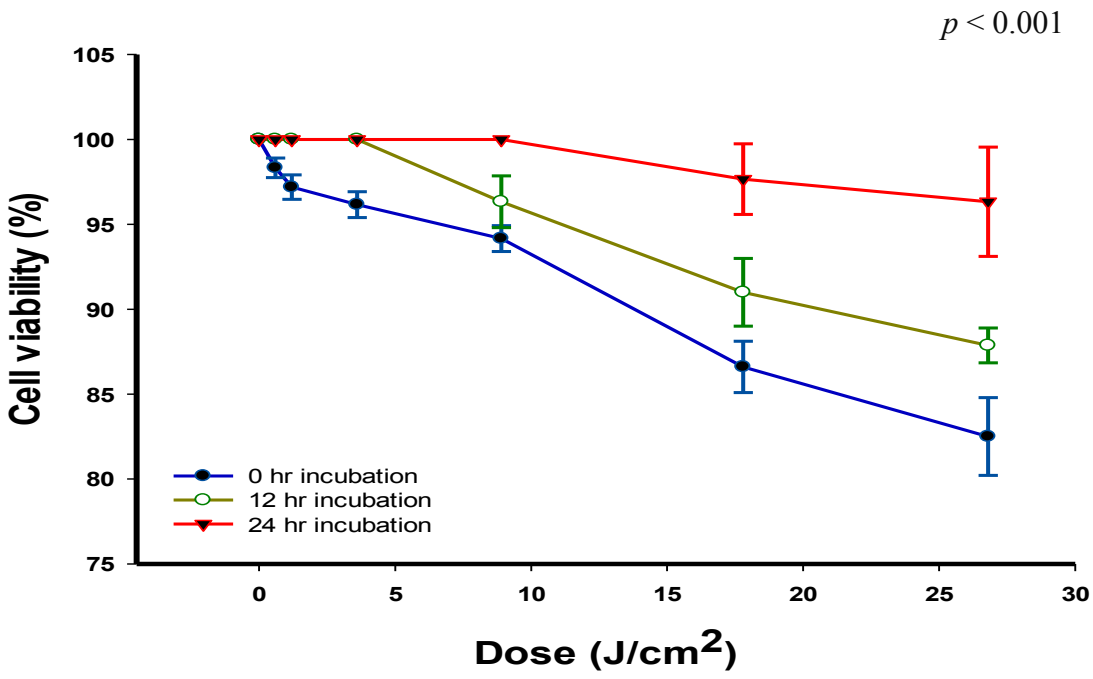


Figure 4. 4. Viability of THP-1 cells after irradiation with diode laser using trypan blue exclusion test: (•) Immediately post irradiation; (ᵒ) After12 hr incubation post irradiation; (▼) After 24 hr incubation post irradiation. Each point represents the mean ± SEM of three separately performed assays.

4.4.2.3 Cell viability by MTT assay

The viability of THP-1 cells were determined to estimate the cytotoxic effect of NIR diode laser through investigating the mitochondrial activity in irradiated cells using MTT assay. Similarly, the activity of the mitochondria at the time of irradiation of THP-1 cells exhibited no change at low doses (Figure 4.5; $p > 0.05$), and again a significant decrease was found after irradiation with high doses of NIR diode laser (Figure 4.5; $p < 0.05$) compared to unirradiated cells. Also, because of the proliferation, the viability of THP-1 cells increased significantly for all doses

irradiation after 12 hr incubation, and increased further after 24 hr incubation following irradiation compared to immediate exposure ($p < 0.05$). Were there was no significant difference between the most doses > 5 J/cm^2 and the low doses irradiation and the control after 24 hr incubation post irradiation ($p > 0.05$).

Interestingly, the results in Figure 4.5 revealed that a slight change in mitochondrial activity and the viability at the low doses after 12 hr and 24 hr incubation post irradiation compared to unirradiated cells (control). These results indicate that NIR diode laser has a stimulatory effect at doses < 5 J/cm^2 increased the activity of mitochondria of irradiated THP-1 cells.

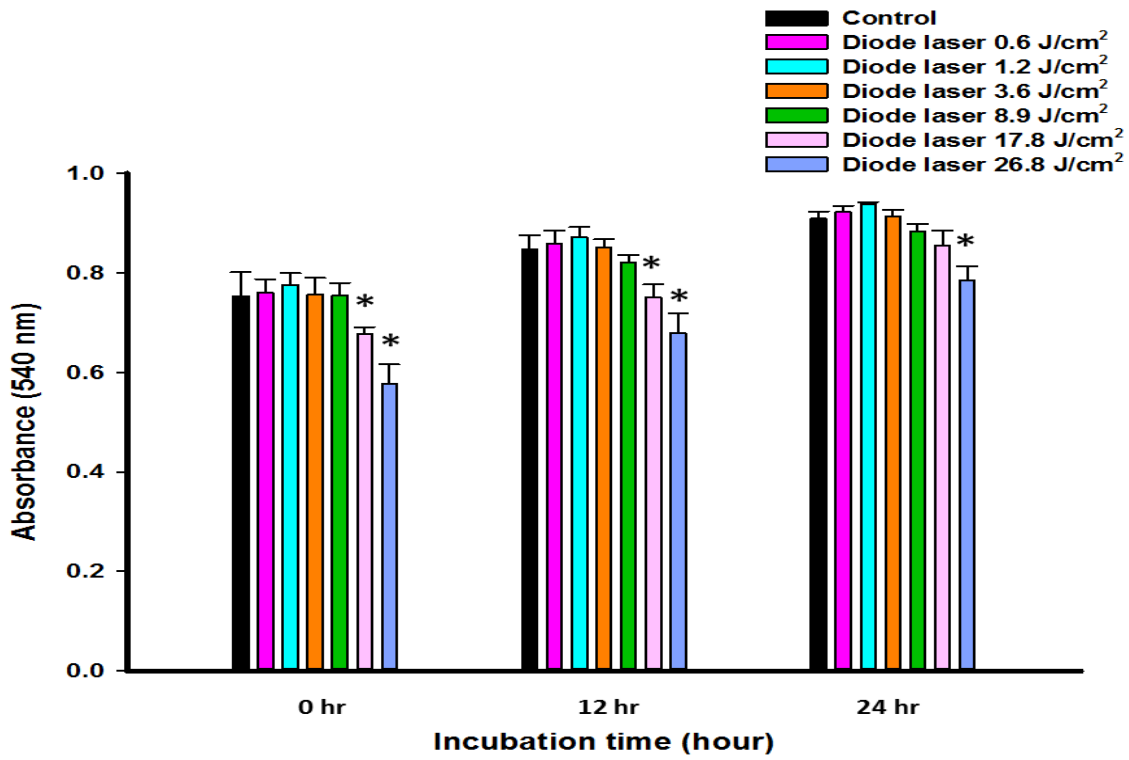


Figure 4. 5. Viability of THP-1 cells after diode laser exposure using the MTT assay. Cells were irradiated with 850 nm diode laser at different doses for the incubation times, 0 hr (immediate irradiation), and 12 hr and 24 hr post irradiation. Data are mean ± SEM for three separated experiments, results were analysed by two ways ANOVA. Significant difference between irradiated and unirradiated (control) cells. *$p < 0.001$.

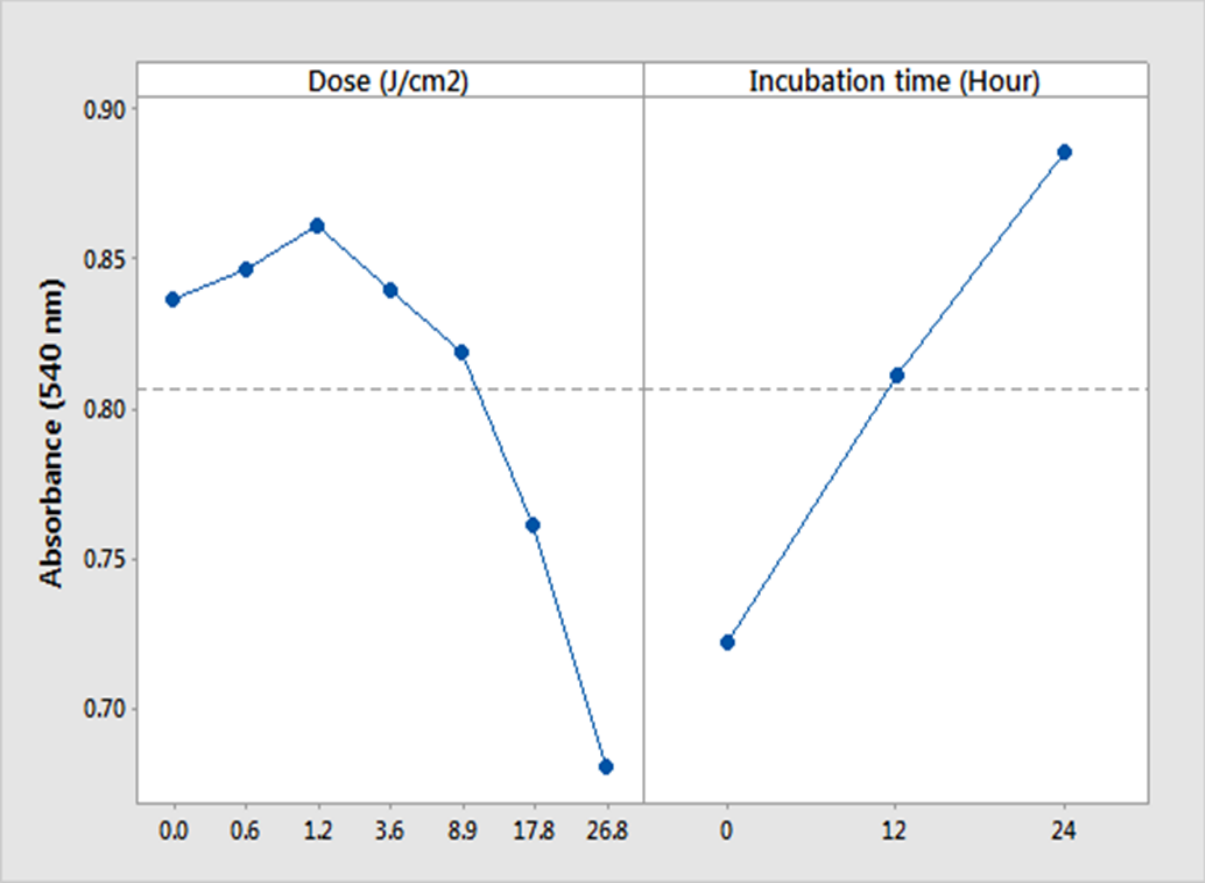


Figure 4. 6. Mitochondrial activity (MTT assay) change in: (a) dose and (b) time -dependant manors in cultured THP-1 cells after 850 nm PBM irradiation.

4.4.3 ATP measurement

To determine the effect of PBM/LLLT on cellular ATP, a cell Viability Kit SL assay performed on cultured THP-1 cells after exposure to a range of doses 0.6, 1.2, 3.6, 8.9, 17.8 and 26.8 J/cm^2 with 850 nm NIR at intervals of 20 min, 12 h, and 24 h. The results illustrated the photobiomodulation effect of NIR radiation through modulating the level of ATP content in monocytic THP-1 cells. The results indicated that the level of ATP synthesis represented by ATP Luminescence (RLU) is changed in a dose-dependent manor, (Figure. 4.7a, $p < 0.001$).

ATP synthesis significantly increased ($p < 0.001$) after irradiation with low doses 0.6, 1.2 and 3.6 J/cm^2, and decreased significantly ($p < 0.001$) after irradiation with high doses 8.9, 17.8 and 26.8 J/cm^2 compared to control, (Figure 4.7a). In addition to the effect of diode laser doses, the incubation time after irradiation showed significant changes in ATP synthesis (Figure 4.7b). Figure 4.8 shows a statistically significant increase in ATP synthesis with increased incubation time ($p < 0.001$), reaching maximum significant increase at 12 hr post irradiation (Figures. 4.9; $p < 0.001$), and decreased significantly at 24 hr (Figures. 4.10; $p < 0.001$), (12 hr > 24 hr > 20 min; Figure 4.11**)**.

Figure 4.12 showed an association between increasing ATP synthesis and mitochondrial activity, represented by absorbance (540 nm), after exposure for various doses of NIR diode laser. ATP synthesis and mitochondrial activity displayed significant increase ($p < 0.005$) at doses < 5 J/cm^2 and both showed considerable reduction at doses > 5 J/cm^2. THP-1 cells showed a time-dependent response to NIR diode laser through increased ATP synthesis (Figure 4.7b) and mitochondrial efficacy (Figure 4.6b). Elevating ATP synthesis and mitochondrial activity (absorbance) at 20 min and maximum at 12 hr ($p < 0.001$), while decreased ATP synthesis ($p < 0.001$), in spite of the persistent increase in absorbance, at 24 hr ($p < 0.001$) post irradiation. This indicated that increasing mitochondrial activity induced ATP synthesis in irradiated THP-1 cells maximally at 12 hr following irradiation.

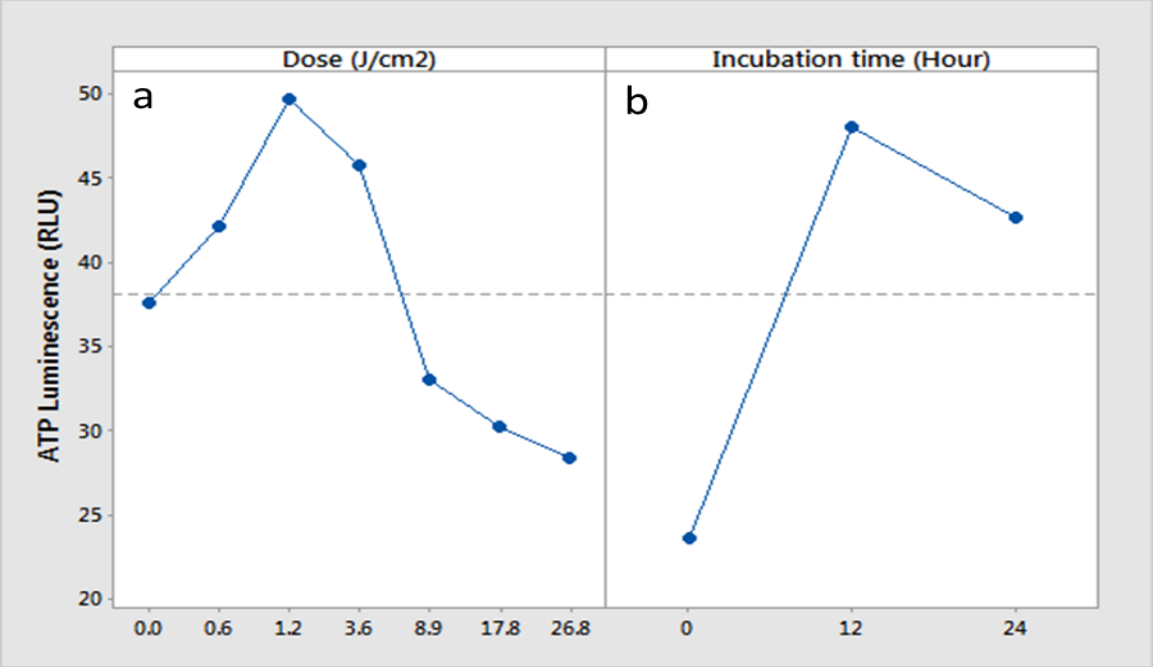


Figure 4. 7. ATP synthesis change in: (a) dose and (b) time-dependant manner in THP-1 cells after 850 nm PBM irradiation.

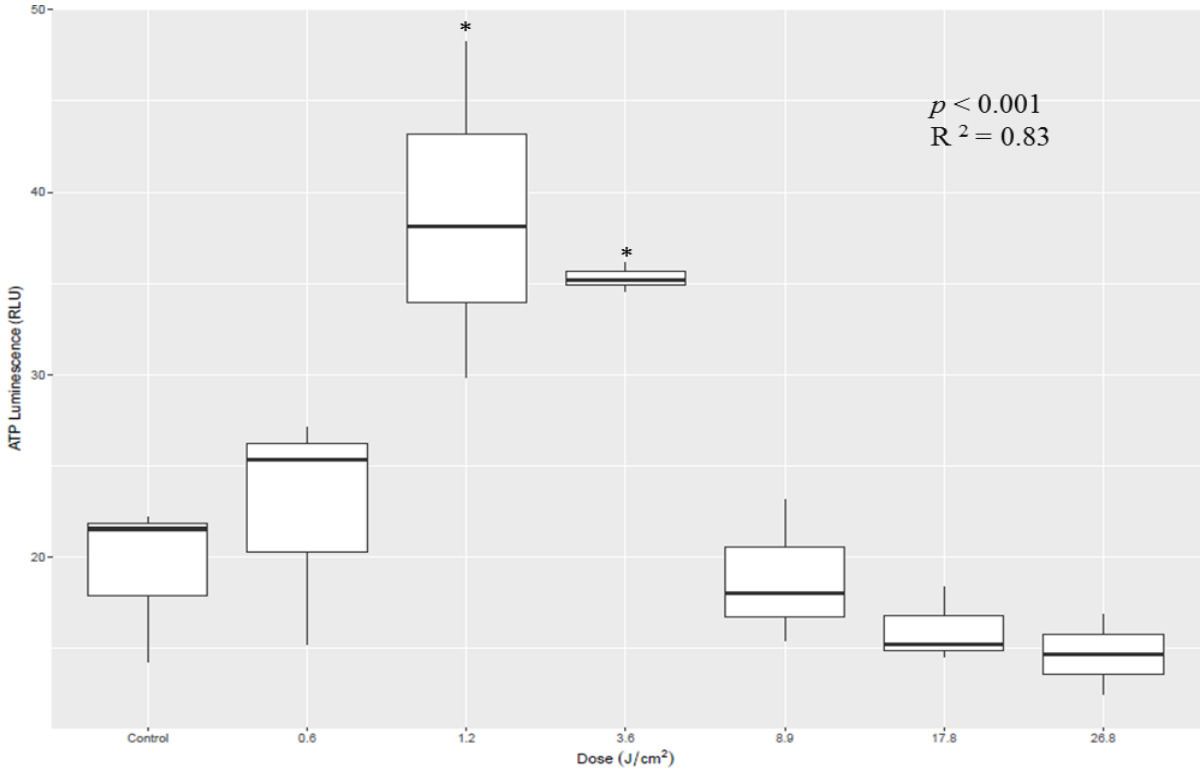


Figure 4. 8. Effect of PBM on intracellular ATP in the cultured THP-1 cell line. Diode laser irradiation at 1.2 J/cm^2 and 3.6 J/cm^2 produced significant change in ATP synthesis ($p <$ 0.005) and ($p <$ 0.01), respectively and insignificant at 0.6, 8.9, 17.8 and 26.8 J/cm^2 irradiation ($p >$ 0.05) after 20 min post irradiation compared to unirradiated cells. Data shown are mean ± SEM for three separate independent experiments (n = 3).

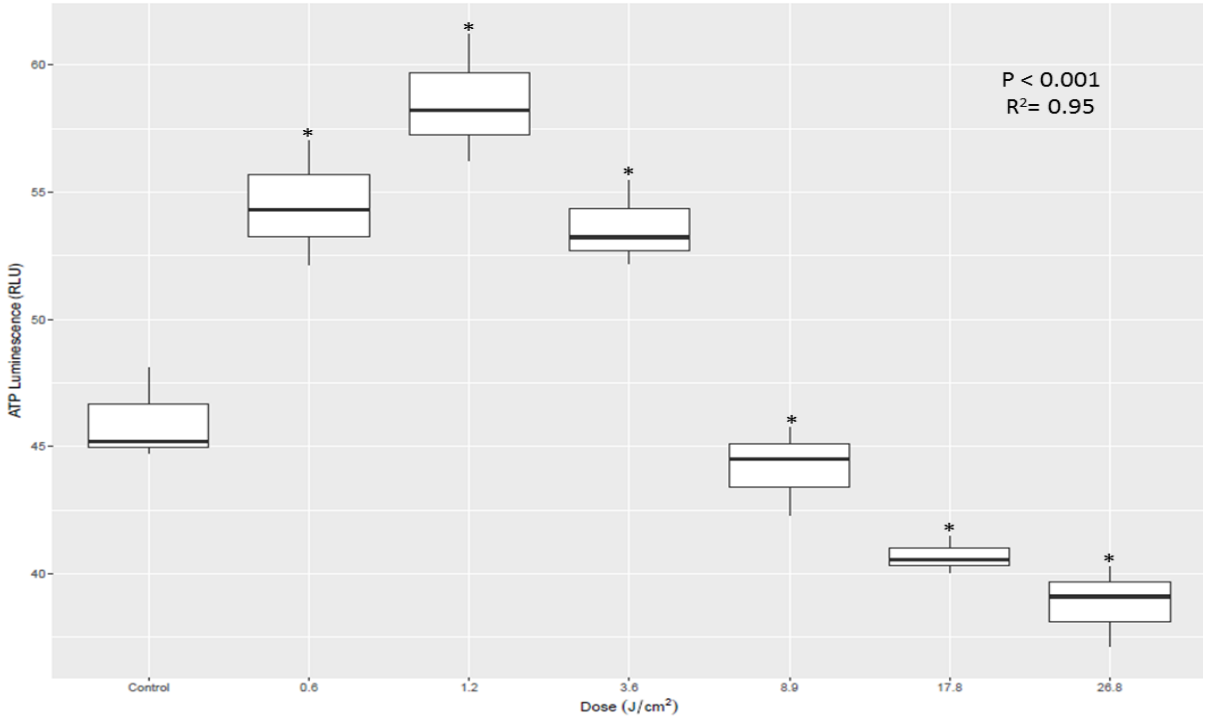


Figure 4. 9. Effect of PBM laser on intracellular ATP in the cultured THP-1 cell line. Diode laser irradiation produced a significant change in ATP synthesis ($p < 0.001$) after 12 h incubation post irradiation compared to unirradiated cells. Data shown are mean ± SEM for three separate independent experiments (n = 3).

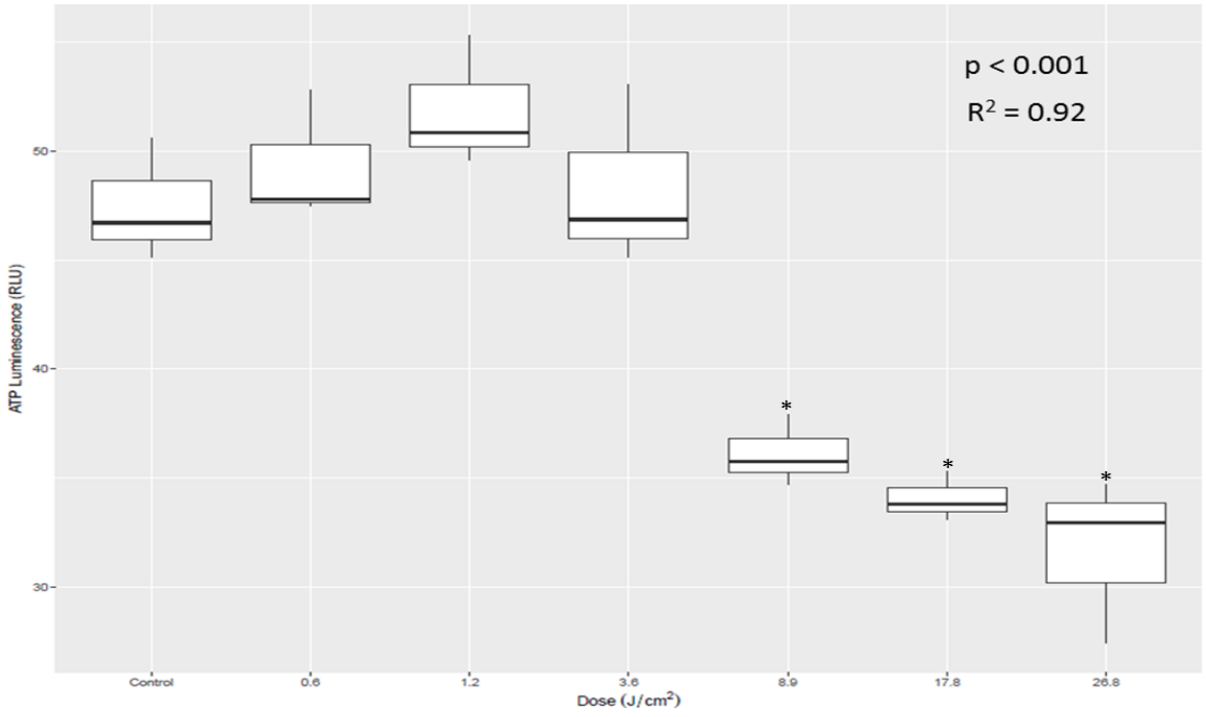


Figure 4. 10. Effect of PBM on intracellular ATP in the cultured THP-1 cells. Diode laser irradiation at 8.9, 17.8 and 26.8 J/cm^2 produced a significant change in ATP synthesis ($p < 0.001$), and insignificant at 0.6, 1.2 J/cm^2 and 3.6 irradiation ($p = 0.694$), ($p = 0.241$), ($p = 0.722$), respectively after 24 hr post irradiation compared to unirradiated cells. Data shown are mean ± SEM for three separate independent experiments (n = 3).

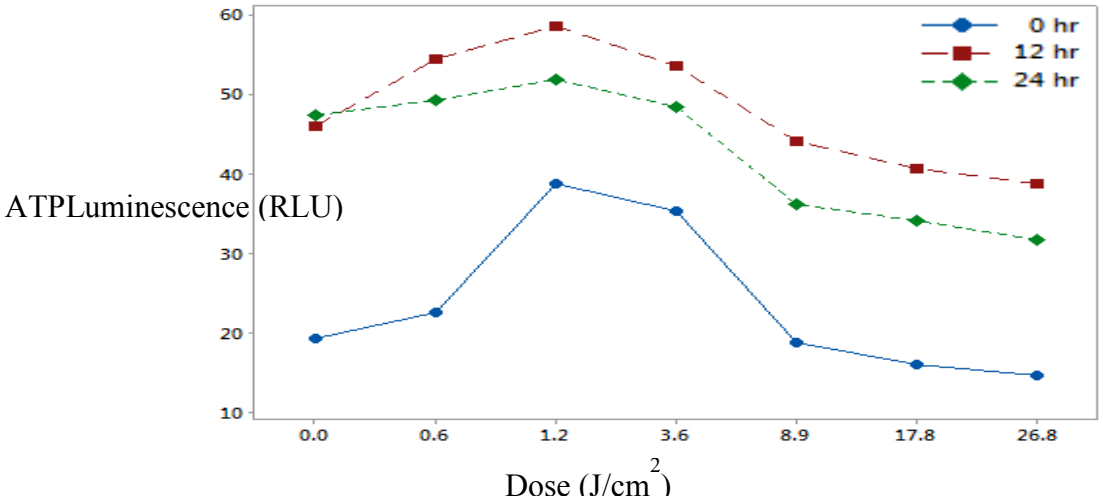


Figure 4. 11. Effect of PBM irradiation on intracellular ATP in the cultured THP-1 cell line after 20 min, 12 hr and 24 hr incubation post irradiation.

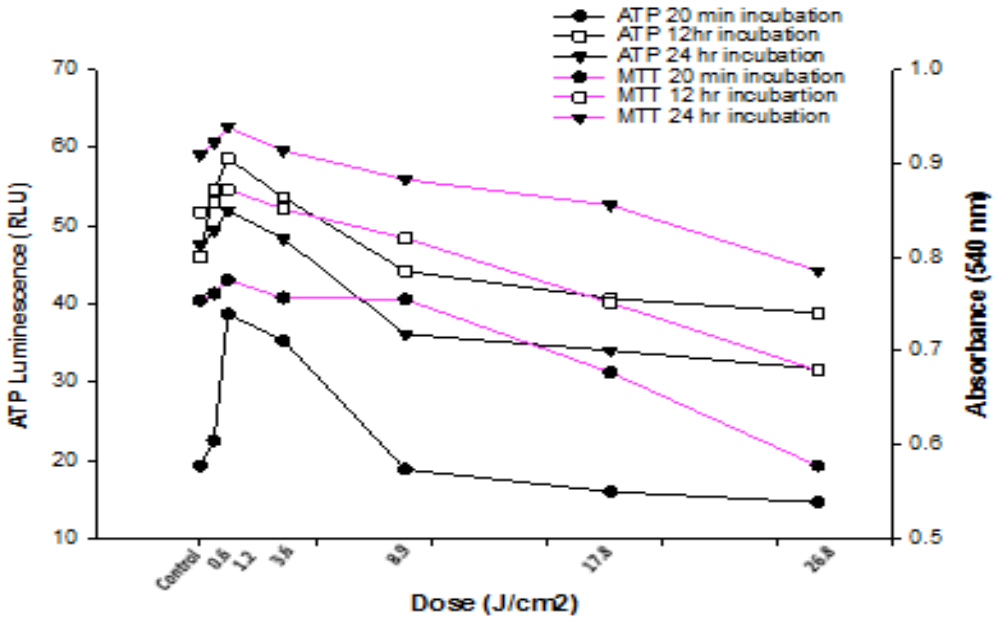


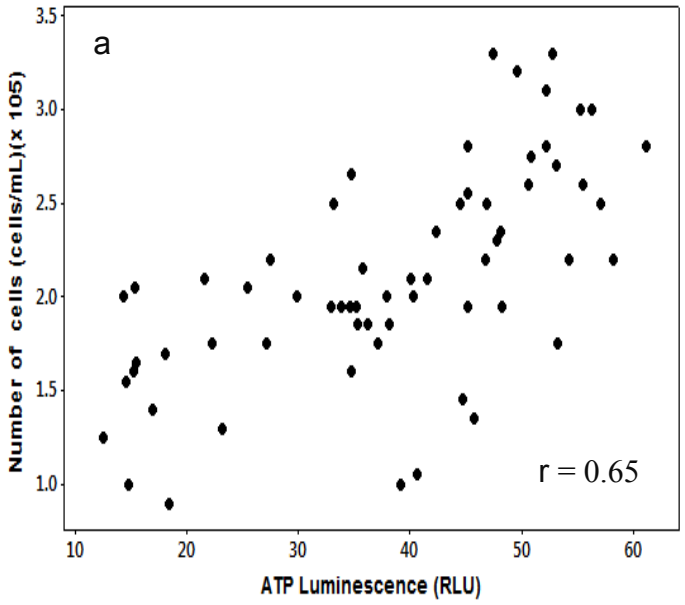
Figure 4. 12. Intracellular ATP and mitochondrial activity (MTT assay) in the cultured THP-1 cell line. The parameters existed time and dose-response to PBM at 20 min (•), 12 hr (□) and 24 hr (▼) after irradiation. Data shown are mean for three separate independent experiments (n = 3).

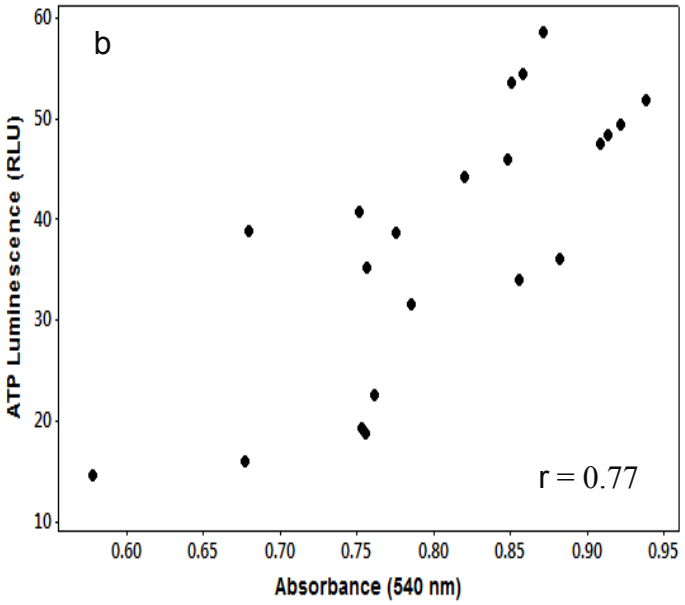
4.4.4 Relationship analysis

Figure 4.13 displays the correlations obtained for ATP synthesis and number of cells, and ATP synthesis and mitochondrial activity (absorbance) in THP-1 cells after irradiation with diode laser. There were positive correlations between ATP induction and the proliferation of THP-1 cells (r = 0.67), and apparent relationship between ATP synthesis and the mitochondrial activity (r = 0.765).

These correlations indicated that the NIR diode laser at low fluences (< 5 J/cm^2) increased the activity of mitochondria, which is well known as the home of energy (ATP) support the cell to achieve its most functions such as proliferation, so increased the level of ATP synthesis as explored in Figure 4.13b. This relationship, in turn, produced a significant increase in the number of THP-1 cells or proliferation, as clarified in Figure 4.13a.

Figure 4. 13. Pearson's correlation analyses of (a) No. of viable cells (cells / mL) and ATP luminescence (RLU) (b) absorbance (540 nm) and ATP luminescence (RLU) in PBM laser irradiated cells.





4.4.5 Estimation of the genotoxic effect of PBM/LLLT in THP-1 cell line

Alkaline comet assay was performed to determine DNA damage, for assessing the genotoxicity of NIR diode laser in irradiated THP-1 cells. The effect of diode laser on DNA damage in THP-1 cells was observed to be a dose-dependent manner (Figure 4.15). The results in this study showed a significant changes in percentage tail DNA (DNA damage) for all incubation periods following exposure to different doses of diode laser (Figure 4.14a; p = 0.008), 12 hr (Figure 4.14b; p = 0.007) and 24 hr (Figure 4.14c; p = 0.013) incubation following irradiation (Figure 4.14; Kruskal-Wallis test, $p < 0.05$).

At immediate irradiation a significant increase was indicated ($p < 0.05$) for high doses (> 5 J/cm^2), while no significant changes ($p > 0.05$) was displayed for low doses (< 5 J/cm^2) compared to unirradiated cells (Figure 4.14a). Similar observations were found after 12 hr and 24 hr incubation post exposure to NIR diode laser as referred by Figure 4.14b and Figure 4.14c respectively. A significant dose-dependent increase was observed between the high doses (> 5 J/cm^2) and the control and the low doses (< 5 J/cm^2), while insignificant increase between the low doses and control (Figure. 4.14; Mann-Whitney U tests, $p < 0.05$). However, the percentage of tail DNA (DNA damage) decreased significantly for all doses after 12 hr and 24 hr incubation post exposure to NIR diode laser compared to immediate exposure (Figure 4.15; $p < 0.05$), due to repair processes of DNA damage.

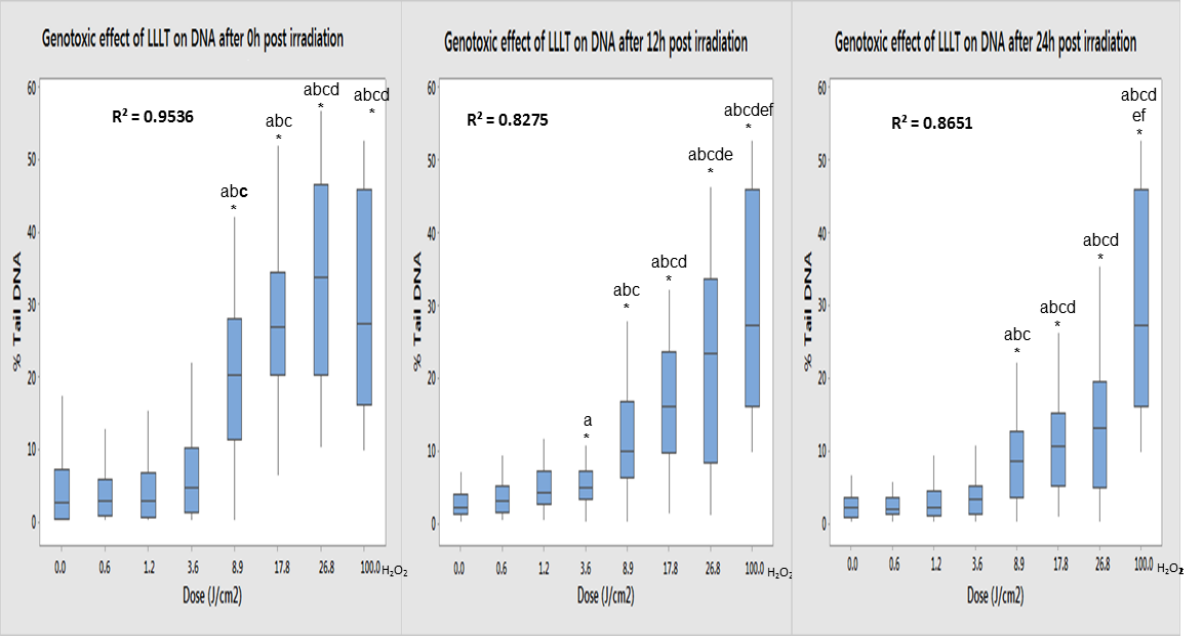


Figure 4. 14. The genotoxic effects in THP-1 cells after *in vitro* exposure to PBM, unirradiated (control) samples used as negative control and 100µm H_2O_2 as positive control: (a) Immediately post irradiation; (b) 12 hr incubation after irradiation; (c) 24 hr incubation after irradiation. Asterisks refer a significant difference from the control (p < 0.05); mismatched lower case letters refer significant differences between treatment groups (p < 0.05). Kruskal Wallis tests for the three time points gave p values of 0.008 (immediately post irradiation), 0.007 (12 hr) and 0.013 (24 hr). Pearson's correlation statistics are shown on each graph.

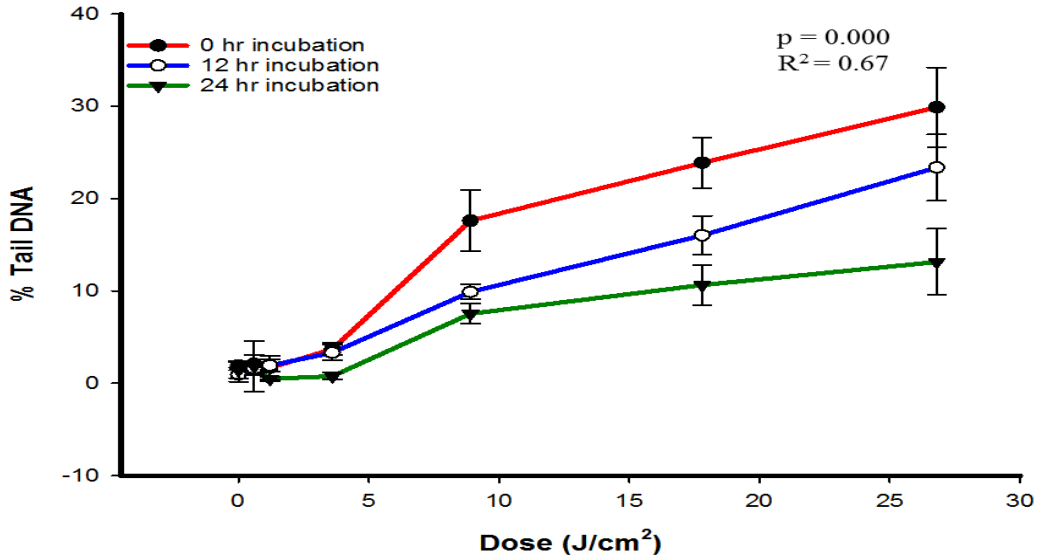


Figure 4. 15. . Effect of PBM on DNA damage in THP-1 cells immediately, 12 hr and 24 hr incubation following irradiation with 850 nm diode laser. Each point represents the mean of three separately performed assays. Error bars, SEM. Kruskal Wallis test gave p = 0.000. Pearson's correlation analyses was R^2 = 0.67.

4.5 Discussion

This study identified a well-defined dose and time-response of cells, such as proliferation, viability, vitality and cellular DNA, for the PBM/LLLT mediated by change in ATP synthesis and mitochondrial activity in human monocyte THP-1 cells cultivated *in vitro*. The wide range of the doses used was based on previous studies that reported usefulness of PBM/LLLT on human cells and DNA (Greco et al., 1989, Karu, 1989a, Masoumipoor et al., 2014, Migliario et al., 2014). Cellular responses such as proliferation, viability as well as DNA and RNA synthesis are considerable biotechniques widely utilised to explore the influences of PBM/LLLT irradiation on biological systems (Gao and Xing, 2009a, Karu, 2008) . The findings in the present study, obviously confirm for the first time that the NIR-diode laser has a photobiomodulation effect that significantly promotes the proliferation rate of the THP-1 cell line. There was a remarkable consistent increase in the proliferation through 24 hours after irradiating cells with low doses (< 5 J/cm^2; Figure 4.3). The results of the present study support those of previous studies, which emphasised the role of therapeutic lasers with low doses in enhancement of proliferation of different human cell types *in vitro* through 24hr post laser application (Hou et al., 2008, Karu, 2008, Irvine and Schell, 2001b, Santana-Blank et al., 2005, de Freitas and Hamblin, 2016).

The basic action of PBM/LLLT therapy or LLLT on biological system is electron transport. Mitochondria contains a respiratory electron transport chain (ETC), five proteins complexes *(*I,II, III, IV, V) located in the inner surface of mitochondrial membrane (Schagger et al., 1994, Rouslin, 1983, Kühlbrandt, 2015). The respiratory ETC is able to transport electrons through these complexes (Perry et al., 2011).

The photon energy absorbed by intracellular chromophores (Conlan et al., 1996, Eells et al., 2004, Karu, 2014), in particular, cytochrome c oxidase in mammalian (Karu, 1999, Karu, 2014) IV (CCO) and converted to metabolic energy, generates photochemical changes that produce an elevation in the CCO enzymatic activity (Yadav et al., 2016), and various redox reactions lead to an electronically excited state able to transfer electrons in the ETC, and ultimately increase synthesis of ATP (Yadav et al., 2016, Xu et al., 2017). Cellular ATP levels increase almost twofold after irradiation by red to near infrared laser light (Karu et al., 1995). ATP works by multiple nucleotide receptor subtypes to increase intracellular calcium concentration (Ca^{2+}) and cell proliferation (Corr and Burnstock, 1994, Kalthof et al., 1993, Kitajima et al., 1994) . It has been demonstrated that ATP induces activation of the extracellular signal-regulated kinase (ERK) cascade, ERK1/ERK2 in a phosphatidylinositol 3-kinase (PI3K)-independent manner, where both ERK1/ERK2 and PI3K activity (pathways) play an important role in cell proliferation (Sonis et al., 2016, Shefer et al., 2001). Therefore, increasing the proliferation of THP-1 cells (No. of viable cells) due to increasing ATP synthesis following irradiation with diode laser at doses (< 5 J/cm^2) is well demonstrated through the positive correlation between cell proliferation and ATP content measured for each doses after 20 min (immediate effect) to 24 h (long effect), that found in the present study. The results obtained in this study are in agreement with results of many experiments on PBM/LLLT-induced ATP increase in irradiated cells and extrasynthesis in isolated mitochondria (Passarella et al., 1984). Whereas, decreasing the number of viable THP-1 cells that appeared at high irradiation doses is likely related to generation of large amount of reactive oxygen species ROS, which induce cell apoptosis through motivated mitochondrial signalling pathways, mainly, induction of Caspase-3 (Wu et al., 2007,

Wu et al., 2009), Caspase-3 is one of caspases family, and is required for some typical hallmarks of apoptosis, and is important for DNA fragmentation in all cell types (Porter and Jänicke, 1999).

ATP synthesis and mitochondrial activity levels were increased observably at low doses (< 5 J/cm^2) of applied 850 nm diode laser. Our results are in agreement with studies reported previously that increasing ATP synthesis post low doses of NIR irradiation (Karu, 1999, Wang et al., 2015). However, there were differences in minimum and maximum doses and time-response of rising ATP synthesis among various cell types after PBM/LLLT application (Quirk et al., 2016). In this study, THP-1 cells showed a persistent elevation in ATP synthesis up to 12 hr, but decreasing at 24 hr, while mitochondrial activity was increased for 24 hr following the irradiation. However, the response of cells to different doses was not the same, a significant decline in ATP synthesis and mitochondrial activity were observed at high doses (> 5 J/cm^2). This decline is seemingly attributed to the increasing generation of reactive oxygen species ROS (Rai, 2016). One of the biological effects of PBM/LLLT is stimulation of ROS production and modulation of cellular redox activity (Therapy), although, these species have a heterogeneous impact on cells according to the applied fluences and hence ROS concentration (Sharma et al., 2011). The generation of ROS depends on laser intensity and total dose delivered to the single cell, and that can affect the characteristics of ROS generation such as amount of ROS (Pal et al., 2007). At low fluences, PBM/LLLT (red and NIR) generates a beneficial low concentration of ROS act as signalling molecules activate the redox-sensitive transcription factor NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells. This protein complex has a crucial role in controlling many cellular activities like DNA transcription, cytokine production cell survival, anti-apoptosis and

cell proliferation (Gilmore, 2006a, Brasier, 2006) . Whereas, at high fluences, LLLT generates a deleterious high level of ROS, which is sufficient to produce actual cellular damage and initiating apoptosis (Wu et al., 2009, Chen et al., 2011). This causes imbalance between the production of ROS and reduction in the antioxidant defence, leading to oxidative stress (Huang et al., 2013), in addition, high fluences of PBM/LLLT decrease mitochondrial membrane potential (Sharma et al., 2011). Mitochondria perform essential functions in the cell such as ATP synthesis, to maintain Ca^{+2} concentration, protein import and plays an important role in cellular apoptosis process (Richter and Kass, 1991, Hirose et al., 1999, Rizzuto et al., 2008). Losing mitochondrial membrane potential ($\Delta\Psi m$) causes disabling these functions, and ultimately apoptosis (Cooper and Hausman, 2000, Brenner and Kroemer, 2000). High dose of PBM/LLLT able to induce mitochondrial permeability transition (MPT), that leads to immoderate release of Ca^{+2}, which exhaust the reserves of cellular ATP (Hawkins and Abrahamse, 2005). Moreover, excessive ROS produce reduction in respiration, due to damaged mitochondria, and decreased mitochondrial membrane potential ($\Delta\Psi m$) as a preamble for cell apoptosis (Wu et al., 2009, Sharma et al., 2011). High dose of PBM/LLLT has a harmful effect on irradiated cells. Two signalling pathways are involved through irradiation with high fluences, primary and secondary pathways. The primary signalling pathway is production high level of ROS, which plays a key role in the induction of (MPT); occurs in the early phase of apoptosis. The secondary signalling pathway is translocation of *Bax* protein, a regulating molecule for apoptosis that occurs in the last phase of cellular apoptosis (Wu et al., 2009).

The damaging effect of LLLT is controversial. In the present study the genotoxic effect of the NIR diode laser is measured by single cell gel electrophoresis (comet

assay) method. No genotoxic effect or detectable DNA damage were observed after irradiation with a low fluence laser (< 5 J/cm^2), while a remarkable increase in % DNA damage post irradiation with higher fluences (> 5 J/cm^2) was measured at 0 hr incubation time (Figure 4.14a). However, a significant decrease in % DNA damage at 12 hr and 24 hr (Figures 4.14b,c) respectively, compared to 0 hr incubation after irradiation have been shown due to the activation of DNA repair mechanisms. Exposure to laser therapy at high fluences leads , as mentioned above, to the formation of reactive oxygen and nitrogen species which trigger the initiation of lipid peroxidation, protein damage or DNA modification (Huang et al., 2011, Gagnon et al., 2016).

Electrons that pass through the respiratory chain of the mitochondria, interact with oxygen and hydrogen to produce H_2O and ATP energy in addition to toxic by-products, such as oxygen free radicals, which are released (Karu, 2010, Pieczenik and Neustadt, 2007). Appling PBM/LLLT with high intensity increases electron transport, and this impedes the flow of electrons through the respiratory chain. This enables an elevated transformation to oxygen, which induces the generation of free radicals. These free radical as known, are highly reactive and can attack the proteins of mitochondrial respiratory chain, DNA and many cell components (Pieczenik and Neustadt, 2007, Wallace, 1997). Few studies have evaluated effects of low-level lasers on DNA at different doses or powers (Hawkins and Abrahamse, 2006b, Kohli and Gupta, 2003). However, there is consensus that irradiation with PBM/LLLT (red and NIR) at high fluences present lethal effects (Fonseca et al., 2012). They capable of promoting a superoxide dismutase SOD response in E.coli cells, (Fonseca et al., 2011, da Silva Sergio et al., 2013b) , and to induce DNA lesions in mammalian cells (Houreld and Abrahamse, 2007, Mbene et al., 2009). Also, these lasers trigger time

and dose-response DNA damage in cultivated cells (Hawkins and Abrahamse, 2006b). Moreover, an intensive study investigated the effect of high fluence red and near infrared laser therapy on DNA in blood cells, and emphasised that oxidative mechanisms induced by red and near infrared laser light at high doses caused DNA damage through differently stimulated chromophores. These lesions are targeted by formamidopyrimidine DNA glycosylase, such as 8-oxiguanine, and endonuclease III (Sergio et al., 2015), an enzyme that cleave the phosphodiester bond within a polynucleotide chain such as DNA molecule (Venter et al., 2006, Kong et al., 2017).

4.6 Conclusions

Results in the present study clarified that human monocytic THP-1 cells were affected by PBM laser, and the applied doses of diode laser produced measurable changes in THP-1 cells. A laser diode at 850 nm is capable of modulating cellular processes after irradiation in a dose-dependent manner. PBM therapy/LLLT at this wavelength has the ability to promote the proliferation rate of THP-1 cell line at doses < 5 J/cm^2, on the contrary, a cytotoxic and genotoxic effect were occurred after irradiation at doses > 5 J/cm^2. The alterations of mitochondrial complexes are illustrated by changing the activity of mitochondria, which increased ATP synthesis. This investigation supports employing the beneficial effect of PBM therapy/LLLT to treat the lack of energy and metabolic deficit associated by mitochondrial dysfunctions. On the other hand, this study also demonstrated the deleterious effect of LLLT applied at high fluences (> 5 J/cm^2), which manifests as a reduction in mitochondrial activity associated by decreasing ATP biosynthesis. This investigation however, could be a therapeutic treatment for cancer. As, there is clearly an optimal

range of doses for stimulation of tissue and cell lines. Therefore, further investigation into optimal conditions for cell stimulation is needed. Identification of the proper treatment conditions for the particular cell lines or tissue is crucial for achieving optimal photobiostimulation.

CHAPTER 5

EFFECT OF PBM/LLLT ON UV IRRADIATED CELLS

Hypothesis: Photobiomodulation (PBM) therapy or Low level laser therapy (LLLT) has the ability to prevent or repair the detrimental outcomes resulting from UV exposure.

5 Effect of PBM/LLLT on UV irradiated cells

5.1 Introduction

It is widely accepted that exposure to UV radiation is highly responsible for the cytotoxic and genotoxic impact on mammals (Yaar and Gilchrest, 2007, Karol, 2009, Tsatsou et al., 2012). In spite that UV radiation is the best natural source supporting the body with vitamin D (Holick, 2008, Kockott et al., 2016), it counts as the most influential factor causing skin damage (Sawhney and Hamblin, 2014). The pathways occurring UV-induced damage to the biological system could be related to the breakdown of collagen, the generation of free radicals, DNA damage and inhibition of the immune system (Agrawal et al., 2014). Adequate protection and prevention of UV risk is essential, and can be setup by minimizing UV exposure, which is achieved by either avoiding UV exposure, or through using sunscreens (Agrawal et al., 2014, Helger Stege and Krutmann, 2000). However, avoiding sun exposure is not suitable solution for many people, such as those outdoor occupations. Moreover, the limited photoprotective efficacy of sunscreens after water exposure or perspiration and the potential toxicity of T_1O_2 nanoparticles (Jeon et al., 2016) that exist in most sunscreens, reduced its role of skin protection from UV radiation (Agrawal et al., 2014, Avci et al., 2013b). Therefore, it has become more important for scientists to constantly search for a new mechanism with minimal side effects to reduce the malignant effects of UV radiation. It has recently been suggested that exposure to

infrared radiation (IR) with spectrum range of 780 -1000, 000 nm, in particular infrared A (IRA) portion (near infrared) (780-1400 nm), might have protective effect against UV radiation (Akhalaya et al., 2014, Schieke et al., 2003, O'Leary et al., 2014). Previous studies demonstrated that photobiomodulation PBM or LLLT (400-1400 nm), which exerts non-thermal biological effects has a photoprotective effects for UV radiation (Fekrazad and Chiniforush, 2014, Avci et al., 2013b). LLLT is defined as "Treatment using irradiation with light of low power intensity so that the effects are a response to light only and not due to heat. " (Anders et al., 2015). LLLT using light at red and near-infrared wavelengths (630-904 nm), has high penetration into tissues and has the ability to enhance the remodelling and repair processes (Tumilty et al., 2010, Brosseau et al., 2005a, Akgul et al., 2014). The action of LLLT based on exciting endogenous photoacceptors leading to catalysing photophysical and photochemical events at various biological levels, that produce unlimited beneficial therapeutic outcomes such as pain and inflammation relief, immunomodulation and wound healing (Weber et al., 2006, Nascimento et al., 2010, Fronza et al., 2013, Tsai and Hamblin, 2017). Thus, the term photobiomodulation (PBM) is more accurate for the therapeutic application that encompasses the inhibitory and stimulatory biological responses of low power laser therapy (Tang and Arany, 2013). Illustration the molecular mechanism of interaction between light and tissue is come by identifying the chromophores molecules (photoacceptors) through light irradiation. Cytochrome c oxidase and intracellular water are the main photoacceptors induced by LLLT during exposure (Bashkatov et al., 2005, Passarella and Karu, 2014). Absorption of laser photons by the photoacceptors converts the light energy into signals that can stimulate various cellular processes

such as production of ATP, DNA and RNA synthesis, Ca^{2+} activation (Cotler et al., 2015, Passarella and Karu, 2014).

Previous laboratory and clinical studies have demonstrated the wide range of beneficial impacts of LLLT or PBM therapy on various cells and different medical indications and conditions without any adverse effects (Huang et al., 2011). Therefore, recently it has become important to understand the therapeutic effects and the mechanisms of LLLT action (Gkogkos et al., 2015). The present study has used an integrated experimental approach with different conditions to illustrate the photobiomodulation influence of LLLT.

5.2 Aims and objectives

a) To evaluate the survival of UV irradiated human monocytic THP-1 cell line after exposure to 850 nm PBM diode laser by:
    1. Examining the cytotoxicity of UV radiation in THP-1 cells before and after exposure to diode laser.
    2. Estimating the motivated mitochondrial activity of UV radiated THP-1 cells after exposing to diode laser.
b) To investigate the role of PBM/diode laser in accelerating the repair mechanism of UV-induced DNA damage by using comet assay.

5.3 Materials and methods

5.3.1 Cell culture materials and cell culture technique.

All THP-1 cell culture materials have been listed in Chapter 2, table 2.1, and the technique of THP-1 cell culture has been described in section 2.2.1.

5.3.2 UV irradiation

We chose using UV radiation with doses that produced maximum response of THP-1 cells and DNA damage according to the data obtained in Chapter 3, to produce cells with high cytotoxicity and genotoxicity before applying 850 nm diode laser were selected to assess the role of LLLT effect on the viability of UV irradiated cells and DNA damage. Table 5.1 show the values of intensities, time of exposure, doses and wavelength of each radiation used.

Table 5. 1: The characteristics of the used radiation.

UV Radiation	Wavelength (nm)	Intensity (W/m^2)	Exposure Time (sec)	Dose (J/m^2)
UVA	360	0.29	3600	1041
UVB	310	3.15	318	1000
UVC	248	2.38	15	36

5.3.3 PBM/LLLT irradiation

According to the results in Chapter 4 in the present study, we selected low doses of 850 nm diode laser that produced no significant damage for DNA, no change in cell viability but instead increased the proliferation rate of cells, to be applied after UV irradiation. Table 5.2 illustrates the characteristics of LLLT used to evaluate its influences on UV irradiated THP-1 cells.

Table 5. 2: The characteristics of PBM/LLLT.

LLLT	Wavelength (nm)	Power (mW)	Intensity (mW/cm^2)	Exposure Time (sec)	Dose (J/cm^2)
Diode laser	850	11.1	29.6	20	0.6
				40	1.2

Figure 5.1 summaries the overall THP-1 cells treatment for each radiation and examination for each test. 5 ml of cell suspension in 50 ml test tube was centrifuged at 1000 rpm for 5 min and re-suspended in 5 ml DPBS solution after removing the supernatant. 1000,000 cells per 1 ml DPBS was added into two petri dishes and kept on ice before and after exposure to UV radiation. Following UV irradiation, the cells of one petri dish were re-suspended in cell culture media and 200 µl of cell suspension of density (10, 000 cells / 200 µl) was transferred to 96 well plate to be exposed for 20 and 40 sec of 850 nm diode laser light. The percentage of cell viability and percentage of tail DNA were measured immediately by trypan blue and MTT assay and by comet assay respectively after UV irradiation alone and after diode laser exposure following UV irradiation, to assess the impact of PBM therapy on viability and DNA damage of UV irradiated cells. To study the effect of diode laser radiation on repair process, UV irradiated cells were incubate before and after laser

exposure for 12 and 24 hr in 37℃ with 5% CO_2 in a humidified incubator, then determining the viability assays of irradiated THP-1 cells and comet assay for DNA damage of THP-1 cells after the incubation periods.

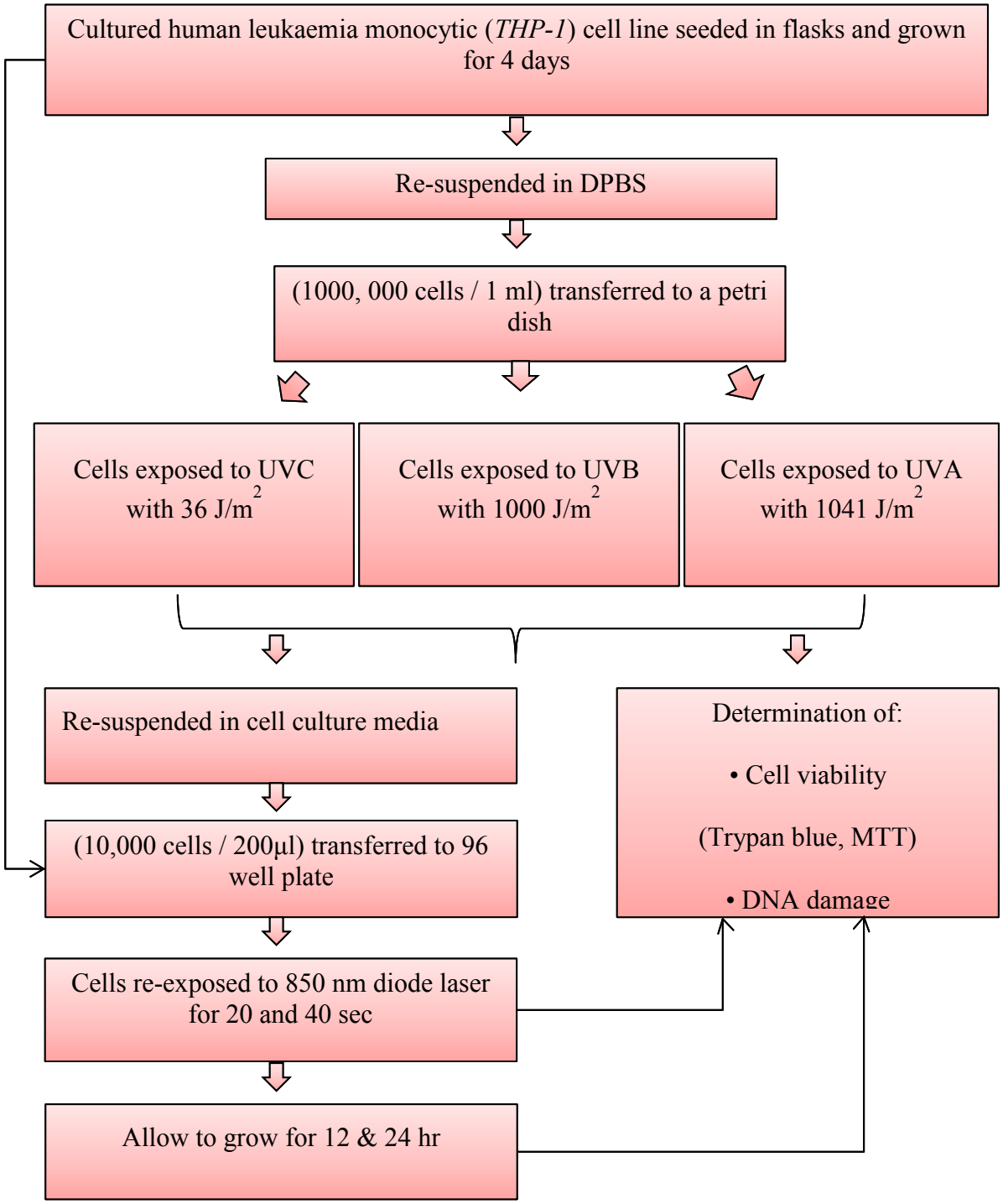


Figure 5. 1. Schematic diagram for UV radiation and LLLT exposure strategy used for different assays.

5.3.4 Cell viability and cytoxicity

Viability of THP-1 cells has been determined to test the cytotoxic effect of the laser exposure and to evaluate the cytotoxicity of UV irradiated cells before and after exposure for 20 sec and 40 sec at 850 nm. The protocols for investigating the integrity of cell membranes using the trypan blue exclusion test and mitochondrial activity by using the MTT assay have been detailed in Chapter 2, section 2.2.5.1 and section 2.2.5.2 respectively.

5.3.5 Comet assay to determine DNA damage

Determination of DNA damage and repair were assessed using comet assay to evaluate the genotoxicity of UV irradiated cells, before and after laser exposure. All chemicals and buffers of comet assay have been listed in Chapter 2, Table 2.1. THP-1 cells were processed for comet assay to determine DNA damage was described in Chapter 2, section 2.2.6, and the main steps of comet assay have been elucidated in Figure 2.1.

5.3.6 Statistical analyses

The mean and standard deviation (SD) were calculated to express the data in the present study. Data were tested for normality using Anderson-Darling tests. The comparison of cell viability between groups was performed by using two-tailed student's t-test. For the comet assay data, the median of % tail DNA of 100 cells per one slide (pair slides for each sample) was calculated, and in order to compare the results in the groups, one-way ANOVA was used and followed by the post hoc Tukey test. Statistics were calculated using Minitab (version 17.1.0; Minitab Inc., State College, PA, USA), and SigmaPlot 13.0. Significance for all tests was set at $p \leqslant 0.05$.

5.4 Results

The present study was conducted to achieve an investigational strategy to assure that photobiomodulation (PBM) or low level-near infrared laser radiation is capable of reducing the deleterious effect of UV radiation. The results obtained were analysed by the comparing between the UV or LLLT irradiated cells and the unirradiated groups as negative control and between UV irradiated cells, as positive control and the UV irradiated cells following LLLT exposure for 20 sec or 40 sec.

5.4.1 Evaluating the survival of UV irradiated human monocytic THP-1 cell line after exposure to 850 nm PBM /diode laser

5.4.1.1 Examining the cytotoxicity of UV irradiated cells following diode laser exposure

To assess the ability of PBM therapy or LLLT to reduce the cytotoxic effect and cell death induced by UV radiation, trypan blue exclusion test for membrane integrity was used to determine the percentage of cell viability before and after exposure to 850 nm diode laser at 0, 12 and 24 hr incubation periods after laser irradiation.

For UVA irradiated cells, a significant decrease in cell viability percentage was observed immediately (Figure 5.2a; $p < 0.005$), and less after 12 hr and 24 hr incubation after irradiation (Figure 5.2b; $p < 0.05$ and Figure 5.2c; $p < 0.05$), whilst no significant effect was indicated for laser exposure alone on THP-1 cells for the three incubation periods compared with control or untreated cells, (Figure 5.2abc; $p > 0.05$).

Interestingly, the data in the present study showed that at 850 nm with 0.6 J/cm^2 and 1.2 J/cm^2 significantly increased ($p < 0.05$) the percentage of mean viability of THP-1

cells, which decreased after UVA irradiation. However, laser exposure at 1.2 J/cm^2 was more effective in producing a significant elevation in the mean of viability percent of UVA irradiated cells following (0 hr) irradiation and at 12 hr incubation following irradiation than the change produced after diode laser exposure with 0.6 J/cm^2 (Table 5.3). While no significant change in viability of UVA irradiated cells at 24 hr incubation post exposure for both LLLT doses (0.6 J/cm^2 and 1.2 J/cm^2) was shown, this indicates that cell viability is close to the control.

Table 5. 3: Percentage of viability of THP-1 cells using trypan blue exclusion test, after UVA and LLLT post UVA irradiation at 0, 12 and 24 hr incubation following irradiation. The results are assessed by mean ± SD. (*) represents significant difference from control group ($p < 0.05$), (§) represents significant difference between UVA and UVA+Laser irradiated (L) groups ($p < 0.05$).

Type of radiation	Viability after 0 hr Mean ± SD	Viability after 12 hr Mean ± SD	Viability after 24 hr Mean ± SD
Control	99.3% ± 0.6	99.6% ± 0.5	99.8 ± 0.3
UVA	72.7% ± 4.5 *	81.7% ± 4.9 *	91.3 ± 1.5 *
UVA + L20	86 % ± 1.7 * §	86% ± 3.6 *	92.3% ± 2.5 *
UVA + L40	89% ± 3.5 * §	88% ± 1.0 *§	94% ± 1.0 *
L20	99% ± 1.0	99.3% ± 0.6	99.5% ± 0.5
L40	98.8% ± 0.8	99% ± 1.0	99.5% ± 0.5

Abbreviation as in tables 5.3, 5.4, 5. 5:

L20: laser exposure for 20 sec.

L40: laser exposure for 40 sec

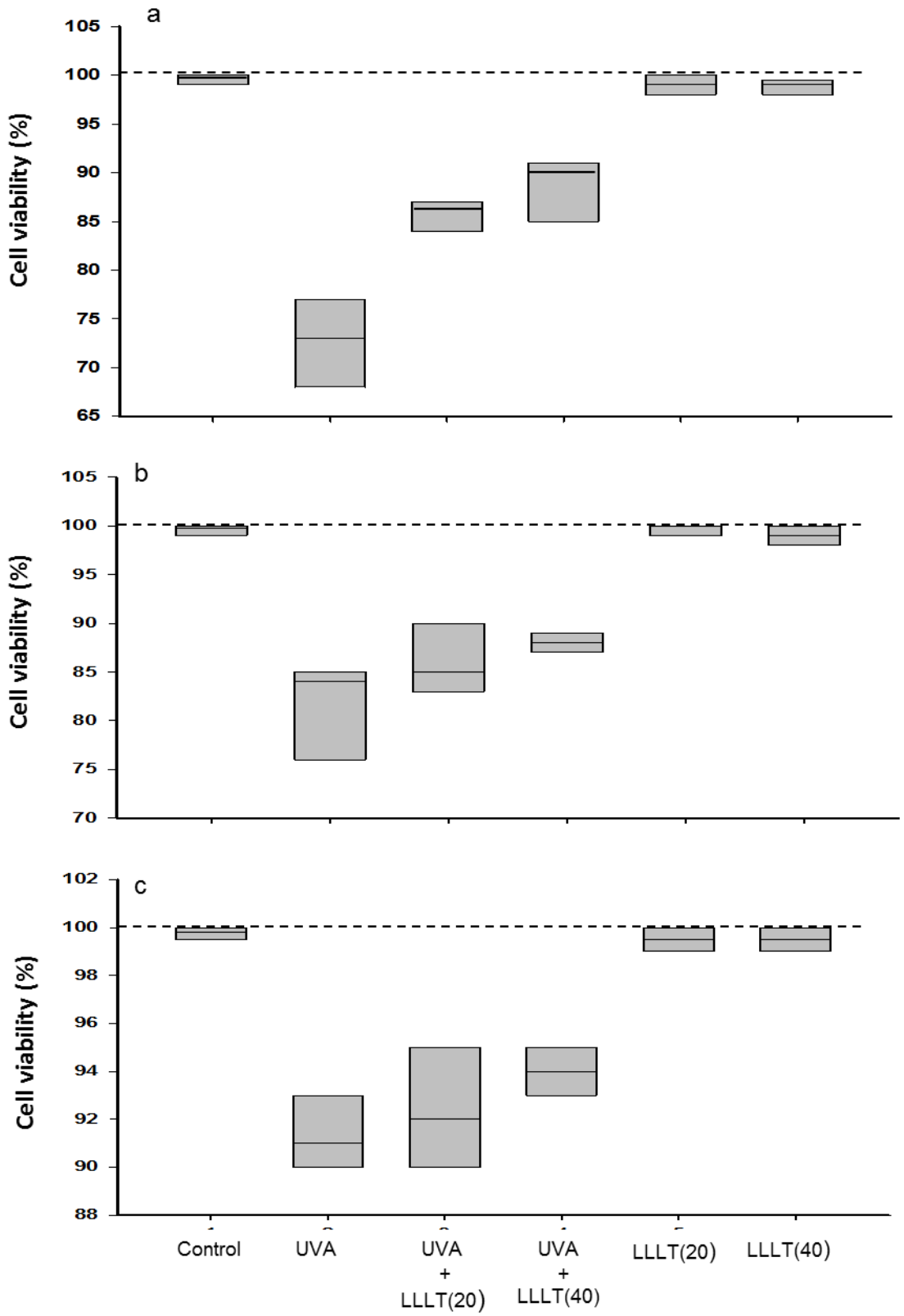


Figure 5. 2. Effect of LLLT exposure on the percentage of THP-1 cell viability using trypan blue exclusion test before and after irradiation with 1041J/m^2 UVA radiation at: (a) 0 hr incubation (immediate irradiation); (b) 12 hr incubation; (c) 24 hr incubation. The dotted line refers to the presence of live cells in full. Each box of the graph represents the mean ± SEM of three independent experiments (*n* =3).

Figure 5.3 and Figure 5.4 showed that exposure for UVB and UVC radiation alone produced no significant effect on THP-1 cells after immediate exposure ($p > 0.05$), but showed a maximum reduction in cell viability at 12 hr and 24 hr after exposure ($p < 0.001$). Whereas, laser exposure after UVB and UVC irradiation produced a significant increase in cell viability appeared after 12hr and 24 hr incubation ($p < 0.005$), but insignificant change was observed at 0 hr incubation post laser exposure ($p > 0.05$). Data obtained in the present study revealed that laser exposure with 1.2 J/cm^2 after UVB and UVC irradiation respectively produced a significant increase in the mean of cell viability percent compared with UVB exposure alone at 12 hr and 24 hr and with UVC exposure alone at 24 hr incubation ($p < 0.005$). However, exposure with 0.6 J/cm^2 after UVB and UVC irradiation produced a small increase in mean of cell viability percent ($p = 0.09$) and ($p = 0.1$) compared to the viability post UVB and UVC irradiation respectively, (Table 5.3 and Table 5.4). There was no significant change in mean of cell viability percent after laser exposure alone at both doses ($p > 0.05$) compared to non-exposed cells. In contrast a significant change in cell viability was observed after exposure to UVB and UVC radiation alone ($p < 0.005$) compared to control group (not exposed cells).

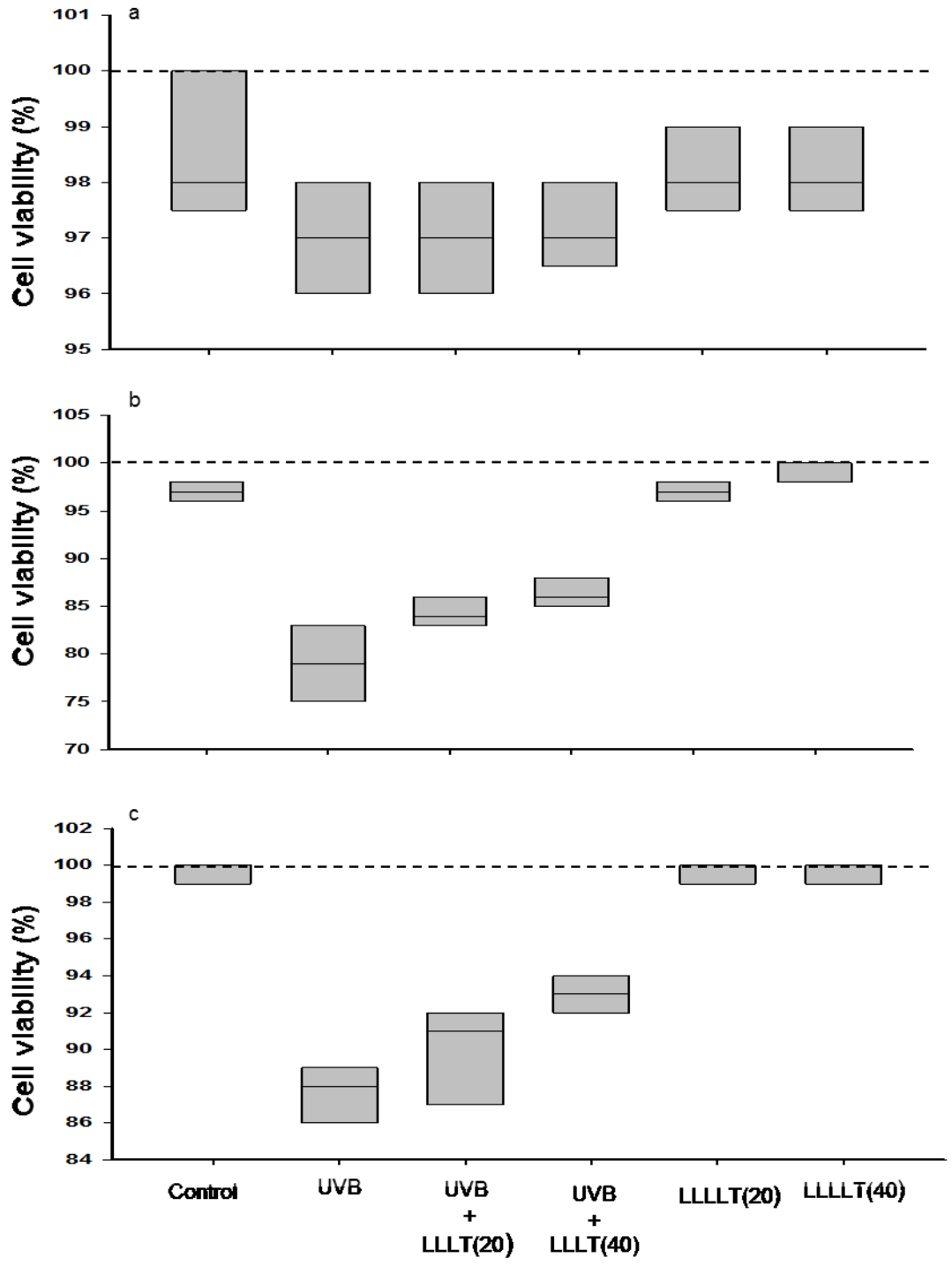


Figure 5. 3. Effect of LLLT exposure on the percentage of THP-1 cell viability using trypan blue exclusion test before and after irradiation with 1000 J/m^2 UVB radiation at: (a) 0hr incubation (immediate irradiation); (b) 12 hr incubation; (c) 24 hr incubation. The dotted line refers to the presence of live cells in full. Each box of the graph represents the mean ± SEM of three independent experiments (n=3).

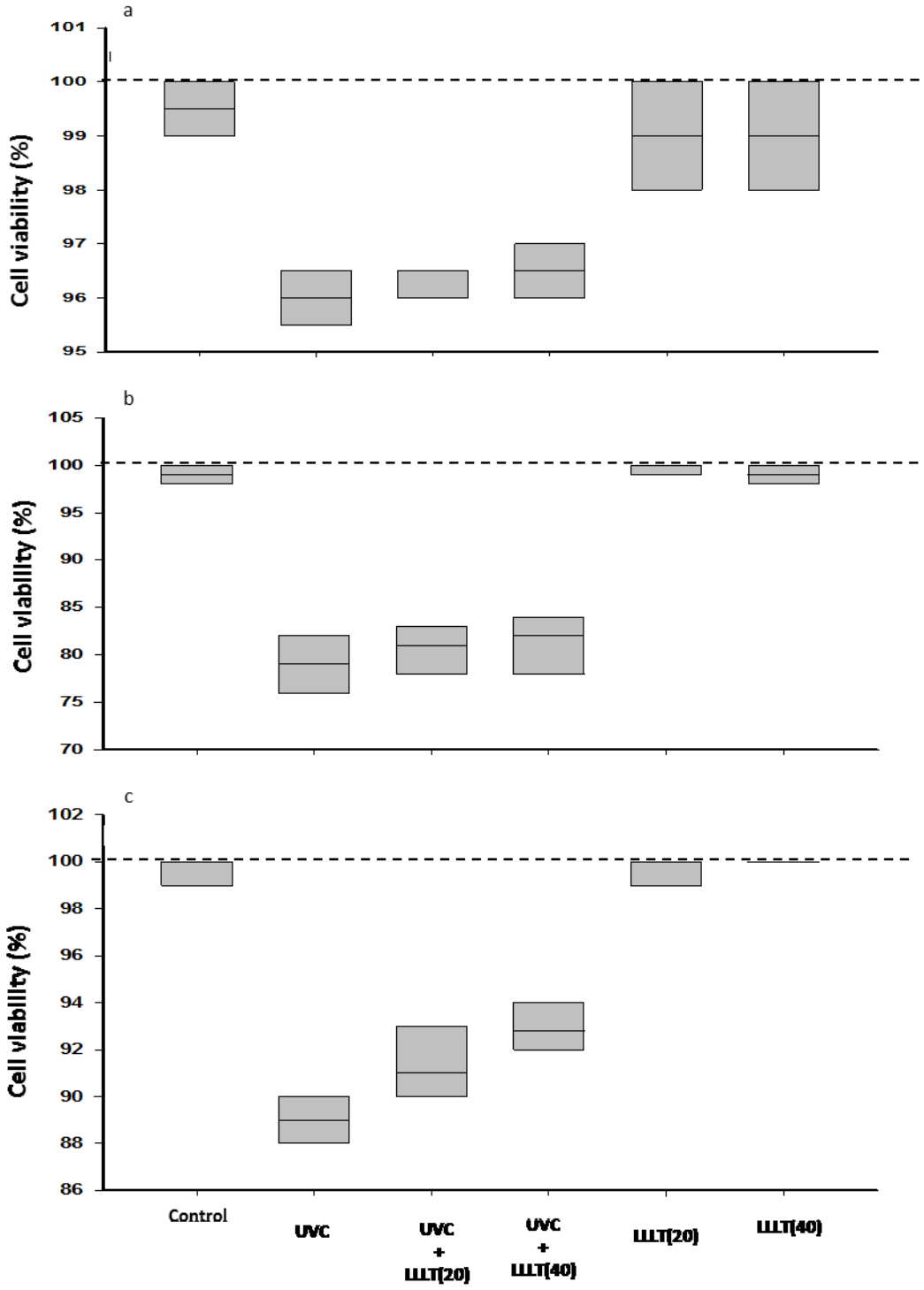


Figure 5. 4. Effect of LLLT exposure on the percentage of THP-1 cell viability using trypan blue exclusion test before and after irradiation with 36 J/m^2 UVC radiation at: (a) 0hr incubation (immediate irradiation); (b) 12 hr incubation; (c) 24 hr incubation. The dotted line refers to the presence of live cells in full. Each box of the graph represents the mean ± SEM of three independent experiments (n=3).

Table 5. 4: Percentage of viability of THP-1 cells using trypan blue exclusion test, after UVB and LLLT post UVB irradiation at 0, 12 and 24 hr incubation following irradiation. The results are assessed by mean ± SD. (*) represents significant difference from control group ($p < 0.05$), (§) represents significant difference between UVB and UVB+L irradiated groups ($p < 0.05$).

Type of radiation	Viability after 0 hr Mean ± SD	Viability after 12 hr Mean ± SD	Viability after 24 hr Mean ± SD
Control	99% ± 1.0	97% ± 1.0	99.3% ± 0.6
UVB	97% ± 1.0	79% ± 4.0 *	87.7% ± 1.5 *
UVB + L20	97.2% ± 1.0	84.3% ± 1.5 *§	90% ± 2.6 *
UVB+ L40	97.2% ± 1.0	86.3% ± 1.5*§	93% ± 1.0 *§
L20	99.5% ± 0.5	97.3% ± 1.5	99.3% ± 0.6
L40	99.6% ± 0.5	98.7% ± 1.2	99.3% ± 0.6

Table 5. 5: Percentage of viability of THP-1 cells using trypan blue exclusion test, after UVC and LLLT post UVC irradiation at 0, 12 and 24 hr incubation following irradiation. The results are assessed by mean ± SD. (*) represents significant difference from control group ($p < 0.05$), (§) represents significant difference between UVC and UVC+L irradiated groups ($p < 0.05$).

Type of radiation	Viability after 0 hr Mean ± SD	Viability after 12 hr Mean ± SD	Viability after 24 hr Mean ± SD
Control	96% ± 1.0	99% ± 1.0	99.3% ± 0.6
UVC	95% ± 1.0	79% ± 3.0 *	89% ± 1.0 *
UVC + L20	96.2% ± 1.2	80.7% ± 2.5 *	91.3% ± 1.5 *
UVC+ L40	96.3% ± 0.6	81.3% ± 3.0 *	93% ± 1.0 *§
L20	97% ± 1.0	99.7% ± 0.6	100% ± 0.0
L40	98.3% ± 0.6	99% ± 1.0	99.7% ± 0.6

5.4.1.2 Investigating the mitochondrial activity of UV irradiated THP-1 cells after exposing to diode laser.

The mitochondrial activity of the human monocytic THP-1 cell line was assessed for UV irradiated group before and after exposure for 850 nm laser radiation using MTT assay, to confirm that photobiomodulation laser therapy (PBM) at low doses (0.6 and 1.2 J/cm^2) has the ability to motivate the activity of mitochondria that infected due to UV radiation exposure. MTT assay is estimating the metabolic and mitochondrial activity, which reflects cell vitality and survival.

The ability of PBM diode laser in enhancing monocytic THP-1 cell survival through modulating the metabolic and mitochondrial activity, which suffered from disorders occurred by UV irradiation is well demonstrated in the present study. Our data showed that the mitochondrial activity, which presented as Absorbance (540 nm), in UVA irradiated cells is significantly increased after exposure to 0.6 J/cm^2 and 1.2 J/cm^2 of 850 nm diode laser (Figure 5.5; $p < 0.001$), immediately, 12 hr and 24 hr incubation following exposure compared to groups irradiated with UVA radiation alone. Also, a significance between these groups, laser irradiated alone and unirradiated cells for the three incubation periods was obviously shown, (Figure 5.5; $p < 0.001$),

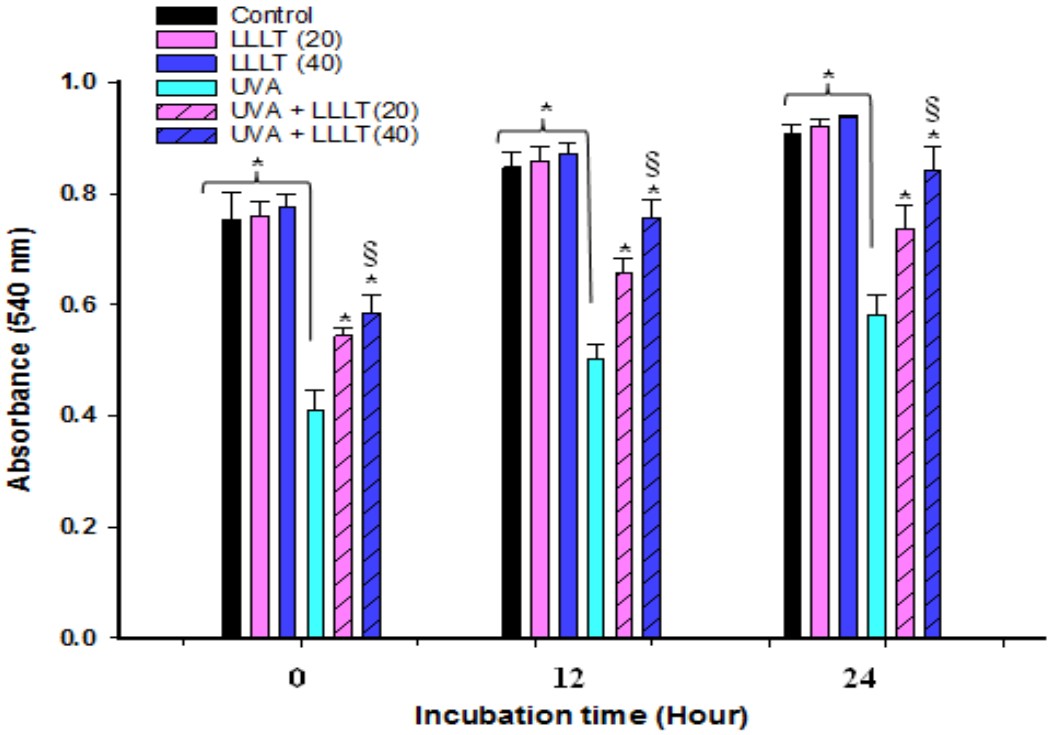


Figure 5. 5. PMB influence on viability of UVA irradiated cells at 360 nm with 1040.76 J/m^2 before and after exposure for 20 sec and 40 sec 850 diode laser using MTT assay. Data are presented as absorbance (540 nm) for control and irradiated groups for each incubation time. Asterisks indicate significant differences from UVA irradiated groups alone, while (§) indicate significant differences between UVA irradiated cell after 40 sec laser exposure and UVA irradiated cell after 20 sec laser exposure. A significant difference indicates $p < 0.001$. Data shown are mean ± SEM of three separated experiments ($n = 3$).

Whereas, the role of PBM (diode laser) to modulate the mitochondrial activity in UVB and UVC irradiated cells was evidently significant after 12 hr and 24 hr incubation post diode laser exposure. The absorbance increased significantly following exposure for 20 sec and 40 sec ($p < 0.005$; Figure 5.6 and Figure 5.7) respectively. Figure 5.6 showed insignificant increases ($p = 0.143$) in the absorbance in UVB irradiated cells after 24 hr incubation post exposure for 20 sec diode laser. While insignificant increases in the absorbance in UVC irradiated cells after 12 hr ($p = 0.092$) and 24 hr ($p = 0.054$) incubation following diode exposure for 20 sec, (Figure 5.7). In both UVB and UVC irradiated cells, there were insignificant increases in the

absorbance (mitochondrial activity) immediately after laser exposure for 20 sec and for 40 sec respectively ($p > 0.05$).

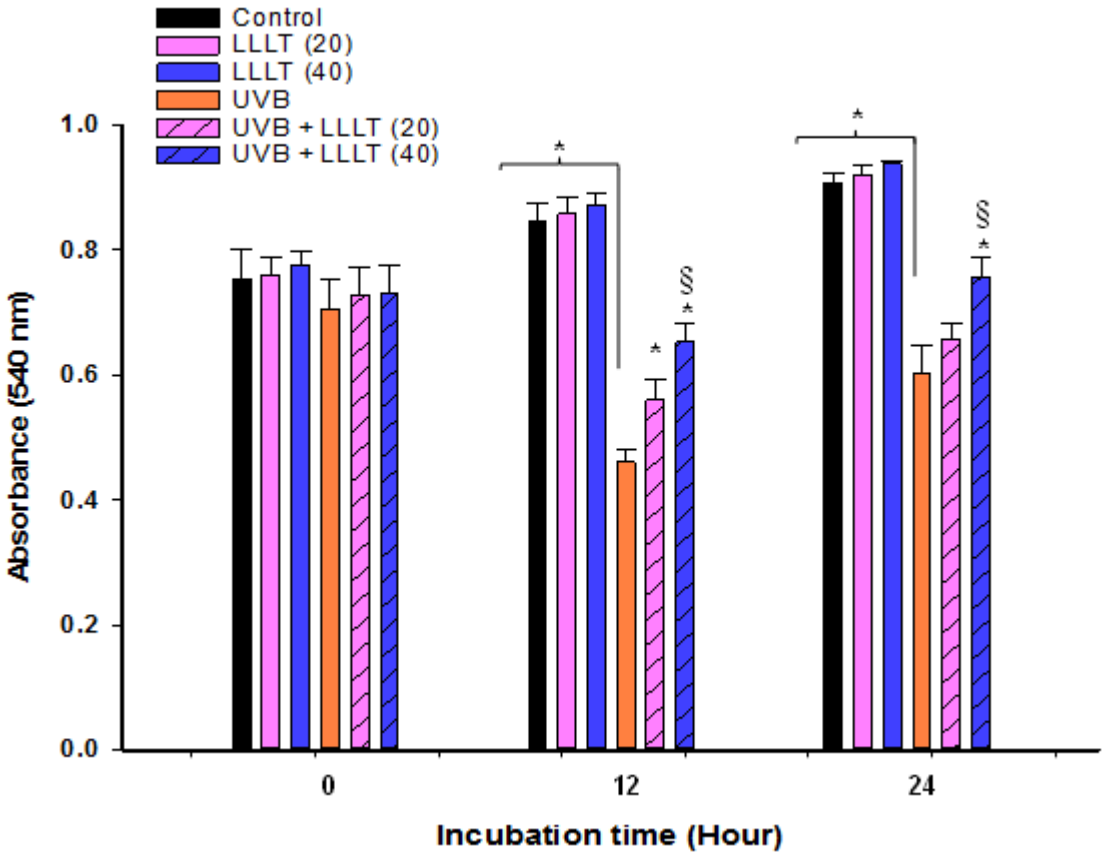


Figure 5. 6. PMB influence on viability of UVB irradiated cells at 310 nm with 1000 J/m^2 before and after exposure for 20 sec and 40 sec 850 diode laser using MTT assay. Data are presented as absorbance (540 nm) for control and irradiated groups for each incubation time. Asterisks indicate significant differences from UVB irradiated groups alone, while § indicate significant differences between UVB irradiated cell after 40 sec laser exposure and UVB irradiated cell after 20 sec laser exposure. A significant difference indicates $p < 0.001$. Data shown are mean ± SEM of three separated experiments ($n = 3$).

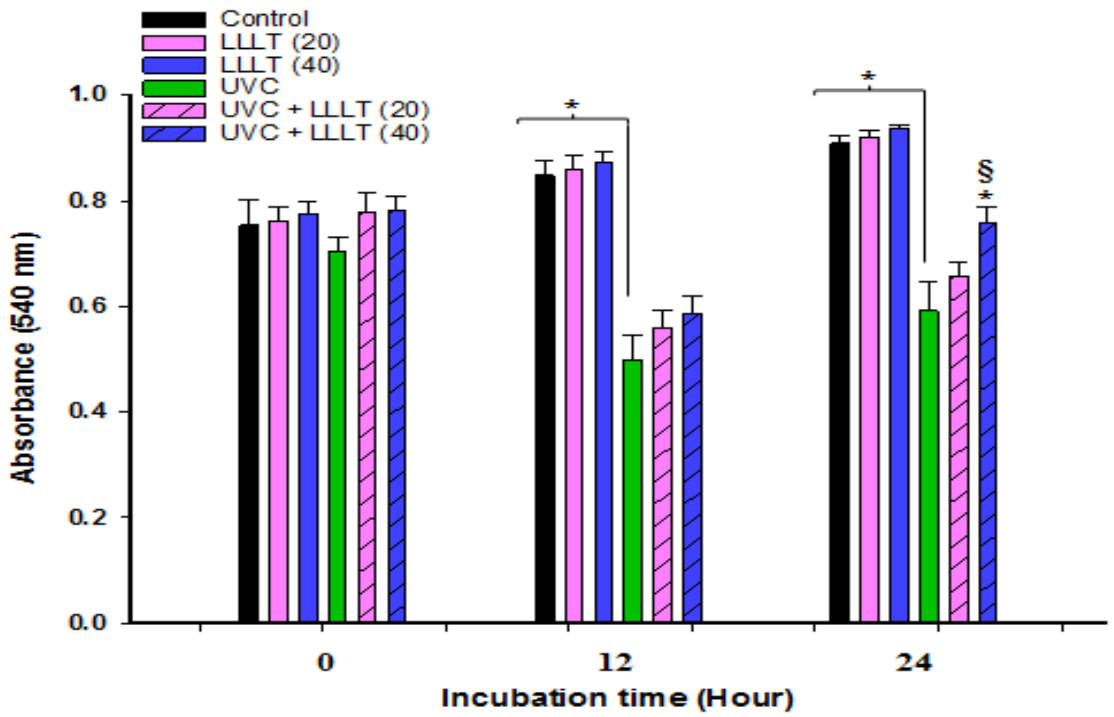


Figure 5.7. PMB influence on viability of UVC irradiated cells at 248.25 nm with 35.7 J/m^2 before and after exposure for 20 sec and 40 sec 850 diode laser using MTT assay. Data are presented as absorbance (540 nm) for control and irradiated groups for each incubation time. Asterisks indicate significant differences from UVC irradiated groups alone, while § indicate significant differences between UVC irradiated cell after 40 sec laser exposure and UVC irradiated cell after 20 sec laser exposure. A significant difference indicates $p < 0.001$. Data shown are mean ± SEM of three separated experiments ($n = 3$).

5.4.2 Investigating the role of PBM/diode laser in accelerating the repair mechanism of UV-induced DNA damage

The alkaline comet assay or (single cell gel electrophoresis) is used as a rapid and sensitive technique to detect and quantify DNA damage in any cell population. As a major trigger of UV-reduced survival (viability), is that of DNA damage, this study conducted to investigate the effect of UV radiation followed by laser exposure on DNA damage in THP -1 cell line. The data obtained by comet assay showed a

considerable influence of UV radiation on DNA damage in irradiated THP-1 cells. A significant increase in the percentage of tail DNA was detected after immediate UVA exposure (Figure 5.7; p <0.05), and at 12 hr following UVB and UVC exposure (Figure 5.8 & Figure 5.9; $p < 0.05$). Whereas, no significant effect on DNA of THP-1 cells after exposure for 40 sec and 20 sec diode laser after 0, 12 and 24 hr incubation periods post irradiation ($p > 0.05$).

Comet assay was also used to evaluate the impacts of laser exposure on DNA damage induced via UV irradiation and DNA repair. The results in the present study revealed that DNA damage induced by UV radiation is significantly decreased ($p < 0.05$) following diode laser exposure for 40 sec and 20 sec, and more significant reduction ($p < 0.005$) was appeared after 40 sec laser exposure at 0 hr incubation, for UVA irradiated cells (Figure 5.7; $p < 0.001$) and at 12 hr and 24 hr for UVB and UVC irradiated cells (Figure 5.8 and Figure 5.9; $p < 0.001$) respectively, post laser exposure.

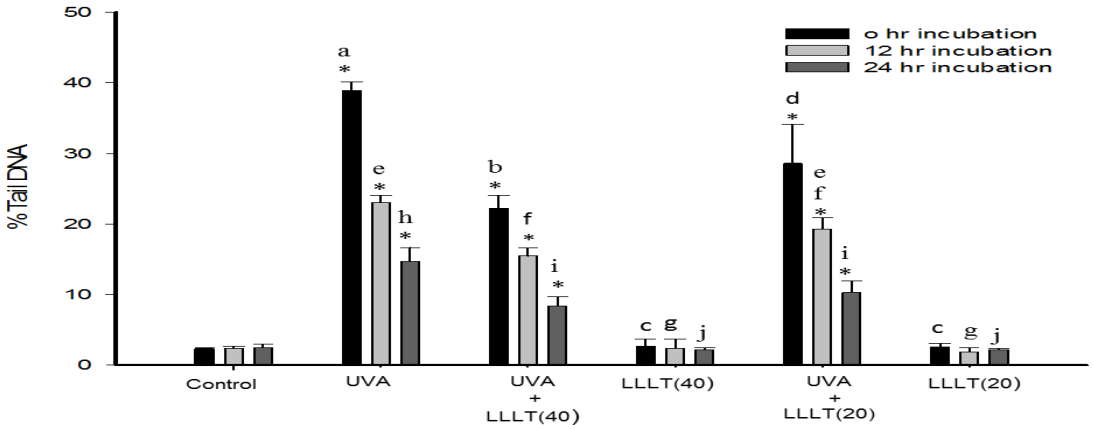


Figure 5. 8. Effect of LLLT exposure on the percentage of tail DNA (DNA damage) in THP-1 cells before and after irradiation with 1041 J/m^2 UVA after 0, 12 and 24 hr incubation post irradiation. Asterisks (*) indicate significant differences from the corresponding control. Mismatching lower case letters indicate significant differences between irradiated groups. No significant differences exist between the same letter ($p > 0.05$) Data are mean ± SEM for separated experiments, ($n = 6$).

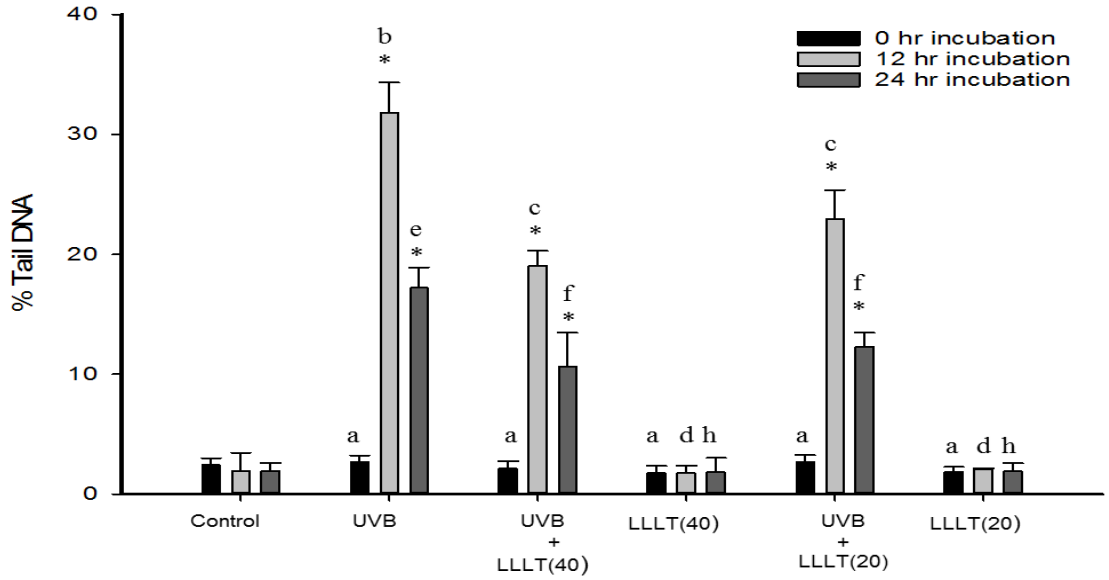


Figure 5. 9. Effect of LLLT exposure on the percentage of tail DNA (DNA damage) in THP-1 cells before and after irradiation with 1000 J/m^2 UVB after 0, 12 and 24 hr incubation post irradiation. Asterisks (*) indicate significant differences from the corresponding control. Mismatching lower case letters indicate significant differences between irradiated groups. No significant differences exist between the same letter (p > 0.05) Data are mean ± SEM for separated experiments, (n = 6)

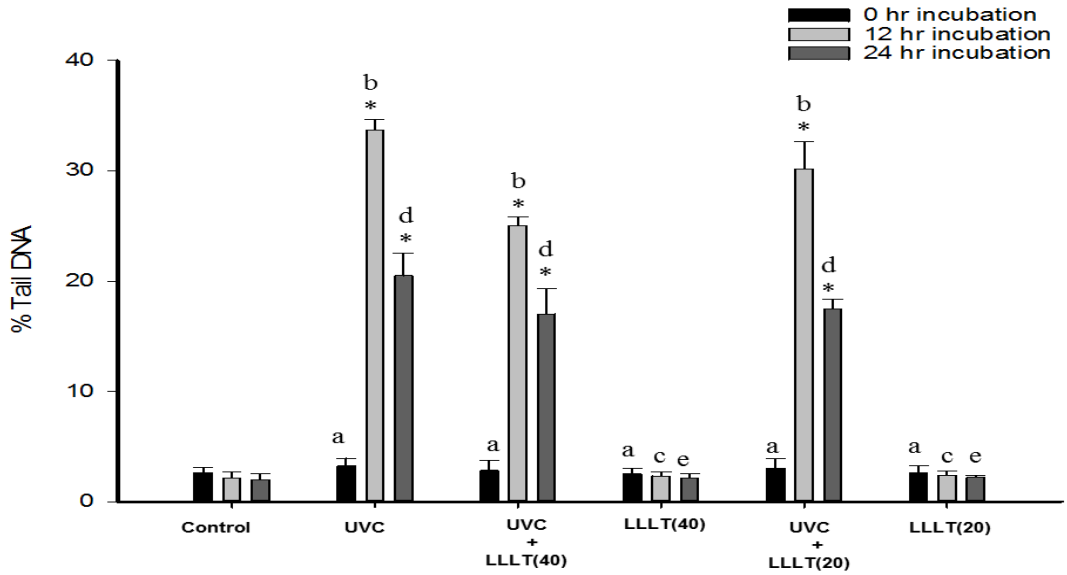


Figure 5. 10. Effect of LLLT exposure on the percentage of tail DNA (DNA damage) in THP-1 cells before and after irradiation with 36 J/m^2 UVC after 0, 12 and 24 hr incubation post irradiation. Asterisks (*) indicate significant differences from the corresponding control. Mismatching lower case letters indicate significant differences between irradiated groups. No significant differences exist between the same letter (p > 0.05). Data are mean ± SEM for separated experiments, (n = 6).

5.5 Discussion

Initially, the effect of UV irradiation and 850 nm diode laser at 0.6 J/cm^2 and 1.2 J/cm^2 severally on viability and DNA damage in THP-1 cells was investigated. Then, the effect of PBM / LLLT (diode laser) on the viability and DNA damage in human monocytic THP-1 cells before and after exposure to UV radiation was explored. The mechanisms of actions of the two radiations are completely different. PBM / LLLT action on the biological system is a photostimulation effect, and is non-thermal. While, the action of UV radiation is a photochemical reaction effect, with a high probability for temperature rise. The data in the present study revealed that near infrared wavelength (850 nm) and low level intensity (<5 J/cm^2) have the ability to decrease DNA damage and increase the viability of UV irradiated THP-1 cells. Increasing cell viability is associated with decreasing DNA damage and is related to the biostimulation effect of a laser. The data in the present study indicated that the near infrared (NIR) diode laser used in this work significantly increased the viability of pre-irradiated cells for UV radiation, which decreased cell viability mainly due to apoptosis induction. Several pathways mediated the reduction of apoptosis of UV exposed cells after NIR laser exposure including reduction of DNA damage and modulation of antiapoptotic proteins (Tsai and Hamblin, 2017). It was observed that NIR light significantly reduces apoptosis induced by UV radiation through inducing anti-apoptotic proteins $FLIP_L$ (FLICE-Like Inhibitory Protein Long form) a major endogenous anti-apoptotic protein that inhibits the death receptor-induced apoptosis through the inactivation of caspase-8 pathway (Safa, 2013, Quintavalle et al., 2010) and activation of BCL-XI (B-cell lymphoma-extra-large) is a transmembrane molecule in the mitochondria (Korsmeyer, 1995), and through downregulating the

proapoptotic protein *BAX*, also known as Bcl-2 protein (B-cell lymphoma 2), is a protein that in humans is encoded by the *BAX* gene that regulate cell death or apoptosis (Westphal et al., 2011), which is upregulated by UV radiation (Biasibetti et al., 2014, Jantschitsch et al., 2009, Lettnin et al., 2016). Moreover, staurosporine, a natural antibiotic normally induces apoptosis, but it is also inactivated by LLLT through inhibition of the Bax pathway (Zhang et al., 2010). Recently, evidence confirmed the stimulation effect of NIR on extrinsic apoptotic pathways (Kimeswenger et al., 2016). NIR or infrared A (750 nm- 1400 nm) effectively induces the extrinsic apoptotic pathways via inhibition of UVB-induced rising of Caspase-8 activity, which is inactivated by the anti-apoptotic molecule $FLIP_L$ (Subramaniam et al., 2013), and by blocking UVB- induced upregulation of pro-apoptotic death receptor Fas ligand (Fasl or CD95L), is a type of transmembrane protein and its binding to its receptor induces apoptosis or cell death (Kulms and Schwarz, 2002, Bang et al., 2002, Lettnin et al., 2016).

However, UV-induced DNA damage is one of the most molecules triggers UV-induced apoptosis where lessening of DNA damage is associated with a reduction of apoptosis (Kulms et al., 1999). A significant decrease in DNA damage in THP-1 cells induced by UVA radiation after LLLT exposure for 0, 12 and 24 hr incubation times is attributed to a reduction in oxidative stress induced by UVA-induced ROS generation. The evidence indicates that LLLT is capable of increasing antioxidant enzymes in UV irradiated cells (Arakelyan, 2005). In addition, LLLT can reduce DCFH (2, 7-Dihydrodichlorofluorescein) oxidation levels and superoxide dismutase (SOD) activity, which caused a significant reduction in DNA damage (Biasibetti et al., 2014). Moreover, antioxidative properties of LLLT are related to its ability to induce ferritin, a protective protein produced by most living organisms controlling the concentration of

cellular iron (Casiday and Frey, 2000). LLLT-induced ferritin functions as scavenger of Fe^{2+} (Avci et al., 2013b, Parrow et al., 2013), which increases with UV radiation and participates in redox reactions. Removing this species is very important because it reacts rapidly with hydrogen peroxide (H_2O_2), producing highly toxic hydroxyl radical ($OH^{\cdot}$), which can damage biological molecules including DNA (Halliwell, 2006). UVB and UVC irradiated cells, also exhibited a significant reduction in DNA damage at 12 hr and 24 hr incubation after exposure to LLLT. The decreased DNA damage could be attributed to the activation of the DNA-repair enzyme photolyase (Anacystis nidulans), a light sensitive enzyme that adheres directly to the dimers in damaged DNA, induced by UVB radiation exposure, converting cyclobutane dimers CPDs into their original structure of DNA after exposure the enzyme-DNA complex to photoreactivating light (Stege et al., 2000).

Many studies have investigated the cellular response to LLLT at the molecular level, and demonstrated the influence of LLLT on genes. All genes from the antioxidant related category and cell proliferation were upregulated (Song et al., 2003). Some genes such as JAKs, a family of Janus protein tyrosine kinases (JAKs), which regulate cellular processes (Yasukawa et al., 1999), were upregulated (Song et al., 2003). In the present study, decreasing DNA damage after LLLT may be related to upregulating the expression of specific genes due to DNA synthesis and repair, which caused indirect stimulation for cell growth (Song et al., 2003, Hamblin and Demidova, 2006a). In further studies, Frank et al. (Frank et al., 2004), investigated the role of the p53 cell signalling pathway to prevent the cytotoxic effect of UV radiation post LLLT irradiation, suggesting that LLLT prepares cells to repair DNA damage and to resist further damage induced by UV radiation (Frank et al., 2004). P53 protein is thought to play an important role in DNA repair. The expression of

mutant forms of p53 might change cellular resistance to the DNA damage caused by UV radiation (Lee and Bernstein, 1993).

5.6 Conclusions

The results obtained in the present study revealed the considerable role of LLLT in the inhibition of deleterious effects of UV radiation on human monocytic THP-1 cells. These results suggest that the use of LLLT (850 nm) with 0.6 J/cm^2 or 1.2 J/cm^2 is able to stimulate the repair mechanism pathways and reduce DNA damage, that evidenced by increasing the survival of cells. The data showed that LLLT was effectively achieving a significant response in the reduction of UV induced apoptosis. The important role for LLLT is concentrated in stimulating the p53 signalling pathway to change cell behaviour and resist further the harmful effect of UV radiation. Thus, LLLT has become a safe photoprotector tool against UV radiation, which may enhance the suggestion that LLLT stimulates the endogenous radio protector, and so provide some protection.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE WORK

6 General discussion and Future work

6.1 General discussion

The treatment of infected tissues still remains quite a challenge in medicine. The care and management of acute health conditions and chronic diseases require long time to get significant consequences. So, finding an effective treatment without harmful side effects (Macedo et al., 2015), has become concern of researchers in both veterinary and human medicine (Saltmarche, 2008), and outstanding researchers have supported new therapy options in order to boost and accelerate the repair processes. Among the treatment approaches which have been presented in recent years, such as; ultrasound therapy and electrical therapy, low level laser/light therapy (LLLT) is highlighted (Brassolatti et al., 2016a, da Silva et al., 2010). Low-level laser therapy (LLLT), or as more recently termed photobiomodulation (PBM) has supported by a large body of evidence of its efficacy and effectiveness in treatment (Zecha et al., 2016).

PBM therapy/LLLT is a rapidly expanded therapeutic technique, showing tantalizing promise and encouraging consequences for treatment of a wide range of health conditions (Mignon et al., 2017). This approach, however, has encountered difficulties and complications in interpretation due to the inconsistent published experimental designs. In this perspective, the project at hand included preparing a well experimental design to study the effect of different parameters of PBM therapy or LLLT on cells and DNA under *in vitro* conditions. The primary aim is to demonstrate the photobiostimulation action of near infrared laser therapy in

enhancing cell survival and accelerate the repair processes at cellular and molecular levels. Whilst, diode laser has used widely in recent years for therapeutic purposes.

Diode laser of near infrared (850 nm) used is effectively enhanced the proliferation of THP-1 cells, at wide range of doses < 5J/cm^2, which supported by recent study for Gagnon and Co-workers (Gagnon et al., 2016), and corresponded with findings of previous studies (Frozanfar et al., 2013, Tuby et al., 2007, Sroka et al., 1999, Pereira et al., 2002, Hou et al., 2008). This is confirmed by the changes in cellular energy as cellular ATP level is largely increased in diode laser exposed cells at doses < 5 J/cm^2, in addition to the induced mitochondrial activity of exposed cells reflected by upregulation in viability using MTT assay, both are known as marker for cellular metabolic activity (Riss et al., 2016); this is supported by Brendan(2016) (Quirk et al., 2016) indicating that applying NIR laser therapy lead immediately to promot the generation of cellular ATP, which act as signalling molecule stimulates cell proliferation via signalling pathways, it is likely affecting the progression of cell cycle (Schwiebert and Zsembery, 2003, Buckley et al., 2003). Although, the increase in cellular ATP in our study was dose-dependent as agree with results of many published reports (Sharma et al., 2011). Nevertheless, it appeared to be temporal increase that the cellular ATP gradually decreased within 24 hr after exposure. Whiles, the increase in activity of mitochondria was persistent with increasing the period post the exposure to PBM NIR laser therapy, as supported by de Olivera (2017), demonstrating that the influence of NIR laser therapy on increasing mitochondrial activity was dose-dependent, and constantly with increasing period following exposure (de Oliveira et al., 2017, Souza et al., 2014). It has been reported that the energy of NIR laser therapy inducing electrons transition and ATP synthesis

in mitochondria of exposed cells (Fernandes et al., 2016, Desmet et al., 2006, Gao and Xing, 2009b).

Estimating DNA damage in diode laser exposed cells at doses < 5 J/cm^2 revealed that near infrared (NIR) diode laser has no genotoxic impact (Chapter 4); these findings agreed by Karu (1987), Kujawa (2004) and Michle (2014), they unanimously confirmed that the exposure for LLLT with a low level doses does not cause DNA damage, but on the contrary LLLT exposure increases DNA synthesis at irradiation with low doses (Kujawa et al., 2004, Karu, 1987, Biasibetti et al., 2014). While this was not the case with diode laser exposed cells at doses > 5 J/cm^2 (Chapter 4). It has been shown that proliferation of diode laser exposed cells is downregulated, synchronous with downregulation of cellular ATP level; similar results have been obtained for cells exposed for NIR laser therapy at energy densities > 5 J/cm^2 (Byrnes et al., 2005, Lukowicz et al., 2013). In addition, there was an oxidative state generated after exposure for diode laser at energy densities > 5 J/cm^2 led to increase DNA damage in a dose-dependent manor. There were differences in the energy density of LLLT that increases DNA damage and occurring cytotoxic effect of the applied LLLT, according to the type of laser, maximum intensity and the wavelength used, in addition to the sort of the target cells. However, scientists in this area unanimously found that energy density greater than (5-8) J/cm^2 inhibit cellular activity (Tuner and Hode, 2002), and increase DNA damage (Hawkins and Abrahamse, 2006b). It has been reported that NIR laser therapy at high energy density can have a cytotoxic impact on the integrity of DNA (Rai, 2016), causing cell photodamage and larger DNA damage (Callaghan et al., 1996) related to formation of oxidative stress induced by generation of ROS (Sharma et al., 2011, Kolarova et

al., 2008), such as singlet oxygen, superoxide anion radical and hydrogen peroxide (Lavi et al., 2003).

The PBM therapy or LLLT has been used in various medical conditions applications because of its beneficial effects, at balanced normal redox state. Of these applications, which in particular occurred by external factors, wound healing, burns and cutaneous infections. However, some tissues could be affected through exposure to this radiation like blood. Therefore, it is important to study the impact of this radiation on blood cells, which have a crucial role in organism's functions. Monocyte cells are important defence cells of the body, as one of immune system cells and its existence in blood vessels, in particular within the skin, that more likely affected by therapeutic laser during treatment. In addition, skin and blood cells encounter one of the most harmful exogenous factors including UV radiation (Schuch et al., 2017). In order all of that, in the present study we chose human monocytic THP-1 cell line as a target for UV irradiation and PBM/LLLT exposure in order to investigate the photothermal and photostimulation effects of UV radiation and PBM/LLLT respectively, under *in vitro* conditions.

In spite of the positive effect of UV radiation, in particular UVB, in supporting the body with vitamin D which has a crucial role in calcium maintenance and in other important processes (Holick, 2007), many medical and dermatology associations denounced sun exposure, because of its risk impact associated with skin cancer (Lucas et al., 2015, Holick, 2016). In mammalian system, genotoxic agents (e.g. UV radiation) that cause DNA damage and initiate an important event in carcinogens, are of the greatest relevance (Møller, 2005). In this investigation, we estimated the direct and indirect impacts of UV radiation on DNA damage in irradiated THP-1 cells, ae well as, cell proliferation and viability.

THP-1 cells have effectively demonstrated the toxic effect of UV rays through their different responses to the range of doses to which they have been exposed (Chapter 3). In addition, they are remarkably displayed various biological reactions affected by different wavelengths bands, which is confirmed the reason behind dividing UV band as described in Chapter 3, section 3.1. Increased the cytotoxic impact of UV radiation, which caused killing cells and reduces the survival, is strongly correlated with elevated DNA damage after irradiation with increased doses of UV radiation, in addition to photo damaging of other macromolecules including protein oxidation and extracellular degradation of collagen (Kammeyer and Luiten, 2015).

Certainly, the direct absorption of UV radiation triggers DNA damage owing to, as cleared by literature, vast majority of lesions that induce cell cancer (Markovitsi, 2016). Lesions formed by UV irradiation such as cross linking, oxidative DNA bases and the most common products of UVB, cyclo-butane pyrimidine dimers and 6,4-pryimidine-pyrimidones are attack and alter the structure of DNA and consequently inhibit DNA polymerases and arrest cell replication (McKenzie et al., 2011, McKenzie et al., 2007, Dipple, 1995), that give rise to tumour progression (Miller and Miller, 1981, Hathway, 2013). Assessment of DNA lesions induced in THP-1 cells irradiated with UVA, UVB and UVC radiation and caused DNA damage in the present study has been carried out by using alkaline comet assay. There were clear evidences for the damaging effect of UV radiation through scoring the irradiated THP-1 cells, and determining the increased DNA damage with increasing the applied doses of UV radiation.

To minimize genetic disorders produced, cells are equipped with DNA damage response pathways and DNA repair proteins to remove these lesions (Hoeijmakers, 2001), primarily by nucleotide excision repair NER or by photoreactivation (Menck

and Munford, 2014, Katiyar, 2016, Essen and Klar, 2006, Vink and Roza, 2001). The repair mechanisms were effectively processed to reform the damaged DNA in UV irradiated THP-1 cells, generally following a day of the irradiation with three bands of UV radiation, and they also were appraised by comet assay (Chapter 3). Nevertheless, there were considerable dead cells due to UV-induced apoptosis, which may owing to accumulated unrepaired damaged DNA in UV irradiated THP-1 cells (Menck and Munford, 2014, Cortat et al., 2013, Perdiz et al., 2000). Authors have been suggested that unrepaired DNA damage can be toxic, and produces apoptotic and necrotic death via promoting cell elimination pathways (Roos and Kaina, 2006, Lee and Choi, 2008), at the same time, these pathways are function as tumour suppressor and prevent cancer (Lowe and Lin, 2000). Moreover, unrepaired DNA damage exists in a survived cell, and re-enter cell cycle, causes the cell to be cancerous (Jackson and Bartek, 2009).

To find a way that enhances and accelerates the repair mechanisms of DNA damage induced by UV radiation, a method was adopted as more efficient than sunblock to prevent and protect the human body, in specifically skin from the risk of carcinogenesis triggered by the often unavoidable exposure to solar UV radiation (Schuch et al., 2017). In this regard, we have decided to use the results we have obtained through investigating the effects of PBM diode laser of NIR at the cellular and molecular level (Chapter 4), that produced most satisfied positive outcomes (0.6 J/cm^2 and 1.2 J/cm^2 of 850 nm diode laser), and applied on UV irradiated THP-1 cells (see Chapter 5). Where these doses were able to stimulate the mechanisms of reform and accelerate the repair of DNA damage catalysed by UV radiation, and enhanced cell survival (Figure 6.1) (Fallahnezhad et al., 2018).

It is well known that DNA is UV preferential cellular target (Gomez-Mendoza et al., 2016). Also, it become prevalent that NIR radiation can exhibits genoprotector capacity for UV-induced DNA damage (Rostand, 1997, Bell and Rostand, 1998). Although, the sun is the main source for NIR (infrared A or IRA) (Kochevar et al., 2008), but to identify the specific wavelength and the energy density that effectively produce a protective effects against UV radiation and the induced damage, artificial sources have been developed and utilized for therapeutic purposes. The present study showed that the employed doses of the NIR diode laser, which identified with fixed intensity for different exposure times, in addition to the wavelength of NIR radiation (850 nm) were able to modulate and rearranged the metabolic processes and the repair pathways in UV irradiated THP-1 cells. Lanzafame and co-workers (2007), clarified that evaluating the effect of NIR radiation requires considering that determining the influence of radiation exposure in biological model is occur by dose value, since the response of cells can be affected by various dose frequency or period of exposure. They added that photostimulatory impact can be observed at does as low as 1-10 J/cm^2, while the photoinhibitory effects are shown at high doses exposure (Lanzafame et al., 2007).

There is an evolutionary standpoint confirm that NIR pre-exposure protect cells from the hazard impacts of UV exposure, and the re-exposure for NIR radiation could be important for protection maintenance (Continenza et al., 1993, Lettnin et al., 2016). However, according to point of view of Karu (1989 & 2003), who suggested that the main action of PBM/LLLT involves the injured cells or which has defect in some cellular functions more than normal cells (Karu, 2003, Karu, 1989c). Therefore, we investigated the protective effect of PBM diode laser on THP-1 cell line following UV irradiation, because the response of cells to NIR LLLT, as reported recently,

depends on cellular conditions and irradiation parameters (i.e. energy density and wavelength) (Almeida‐Lopes et al., 2001a, Volpato et al., 2011, Chung et al., 2012).

The photobiomodulation effect of controlled NIR diode laser (LLLT) exposure up-regulated anti-apoptotic proteins, and inhibited activity of some pro-apoptotic genes (*Bax*) induced by UV radiation. Furthermore, the limitation of cytotoxic effect of UV radiation by PBM or LLLT is mediated by induced antioxidant defence system and reduced lipid peroxidation, which increased by UV radiation, specifically UVB (Ramachandran and Prasad, 2008, Terra et al., 2012). The excited mitochondrial cytochrome c oxidase after absorbing NIR radiation photon generates ROS that causes changing the oxidation state of the mitochondrial membrane, which in turn, activates the production of transcription nuclear factor NF-kB, inactive protein that controls transcription of DNA, cytokine production and cell survival, exist in a complex with IkB inhibitory protein (Gilmore, 2006a, Perkins, 2007). The generated ROS induces IkB-kinase (IkK), which stimulates the phosphorylation of IkB, and consequently decay of IkB complex occur accompanied by liberate of NF-kB protein. This protein is transported into the nucleus, where 150 genes being involved in the defence reactions are activated (Akhalaya et al., 2014). Thus, PBM/LLLT can stimulate the cells to resist further UV exposure.

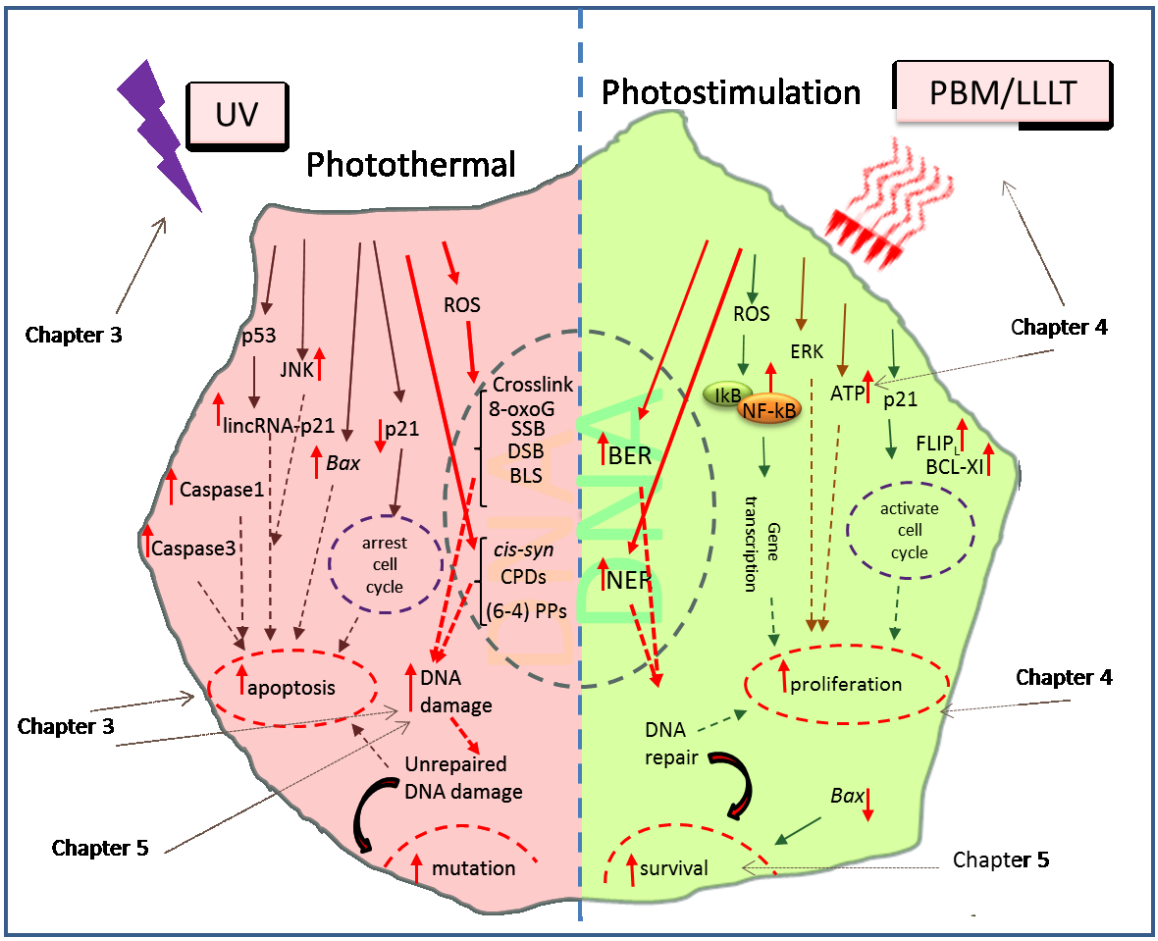


Figure 6. 1. Photo-action profiles differ between UV radiation and photobiomodulation therapy/LLLT in THP-1 cell line. At UV irradiation, the thermal action induced ROS generation formed DNA lesions led to DNA damage. Unrepaired DNA damage may produce mutation or causes apoptosis. Moreover, increased lincRNA-p21 by inducing p53, increased JNK and Bax, decreased p21, which arresting cell cycle, and produced cell apoptosis. While, at PBM/LLLT- NIR diode laser, the photobiostimulation action enhanced ATP synthesis, ERK cascade and induced cell cycle progression due to increased p21 resulted in increase in cell proliferation. Furthermore, induced ROS in excited mitochondrial c oxidase drives to release NF-kB, which activated gene transcription and in turn promoted cell proliferation. PBM/LLLT exposure, induced the anti-oxidant proteins FLIPL and BCL-XI, the down-regulation in Bax gene, and enhanced activity of BER and NER, which catalysed DNA damage repair created up-regulation in the survival. (→) refer to direct effect of PBM/LLLT, (- - -►) refers to pathway modulation by PBM/LLLT and (→) refers to upregulation or downregulation effect of PBM/LLLT.

6.2 Future work

PBM/LLLT is widely regarded as one of the most successful public therapeutic radiation (Torres-Silva et al., 2015). In this study, we attempted to develop a safe, therapeutic design for PBM/LLLT irradiation with considered laser therapy parameters, amongst energy density or dose and wavelength. This study has revealed, as many published researches, although wavelength of PBM/LLLT used have different biological effects at cellular and molecular levels, the varying levels of applied doses (energy density) within the same wavelength has great association with the variety of biological responses involved cell proliferation and cell viability in addition to DNA damage. The PBM/LLLT of NIR has phototherapeutic and photopathologic effects, at low level doses the PBM/LLLT can causes therapeutic stimulating impacts such as increasing proliferation, ATP synthesis and cell survival, which is established in the present study the key role of increased ATP synthesis in a remarkable increase in the proliferation of human monocytic cells following exposure for NIR diode laser. This *in vitro* system for human cells may offer a suitable tool for further interpretation of the mechanism involved in PBM/LLLT effects on the immune system. While, PBM/LLLT generates pathological effects at high level doses. Therefore, the factors that determine the specific biological outcomes elicited by PBM/LLLT of NIR exposure must be characterized.

The photostimulation effects induced by NIR laser therapy should provide valuable information. This study may reveals novel therapeutic and pathological applications of PBM/diode laser in clinical medicine. The comparative evaluation that conducted for the beneficial effects of NIR diode laser and deleterious effect of UV radiation regard cell survival and DNA repair showed that it is practically important to estimate

the relevance of NIR (IRA) in comparison to UV for solar radiation-promoted effects on extracellular matrix turnover, immune function and the stress response *in vivo*. Moreover, the findings of the present study should support the development of photoprotective strategies versus undesired NIR results, and help to reduce the development of pathological manifestations.

IRA radiation accounts for more than one third of the solar energy that reaches human skin (Schroeder et al., 2010), and it is considered essential environmental factor that is effectively able to regulate gene expression in cutaneous cells (Schieke et al., 2003, Schroeder et al., 2008). Furthermore, it is reported that solar NIR a potent factor in increasing the concentration of antioxidant in skin cells, which is always correlated with the increased defense status against UV-induced ROS (Akhalaya et al., 2014). In order to understand the photoprotection effect of NIR laser therapy against UV radiation and unwanted NIR radiation, understanding the action of NIR radiation modes is paramount (Schroeder et al., 2008). In addition to understanding the photobiological mechanisms of NIR laser therapy, it is important to design the parameters and irradiation of NIR laser therapy with regard to clinical experience and the goal of the desired therapy to obtain optimum medical and biological impacts. In clinical practice, It is important to considerate the biphasic dose response effect to get optimal clinical outcomes (Huang et al., 2011).

According to our results obtained in Chapter 4, the NIR diode lasers at high energy densities can upregulate DNA damage and downregulate the cell viability. It would therefore be tempting to investigate the mechanism of this inhibitory effect as a future study. It has previously been observed that NIR laser therapy was able to decrease cell proliferation in tumor cells (Ramos Silva et al., 2016), and increasing apoptosis and tumor cell death through induce cytotoxic effect in cancer cells at 9

J/cm^2 (Lettnin et al., 2016) by generation of ROS causing acute inflammation and thus it has been suggested that PBM/LLLT at energy densities > 20 J/cm^2 may be employed as the best energy dose associated with Photodynamic Therapy (Petrellis et al., 2017). This can give importance to our results and encourage employing the high energy densities of 850 nm diode laser to be used confidently as a therapeutic radiation for cancer cells, so counted part of the therapeutic spectrum in cancer treatment (König et al., 2018).

Unlimited studies involved the deleterious effect of UV radiation and causing DNA damage and aging. Effectively, our results in Chapter 5 confirmed the ability of NIR diode laser with experienced energy densities to downregulate the DNA damage and accelerate the repair mechanism by inducing the adequate cellular responses to DNA damage. However, unrepaired DNA damage is seen as an enabling factor of cancer formation (Jackson and Bartek, 2009, Bartek et al., 2007) and the enhanced genomic instability in spontaneously arising (Vollebergh et al., 2012). On the other hand cancer treatment commonly relies on DNA damage induction by irradiation (Helleday et al., 2008). It has been reported that the integrity of the DNA is threatened by many of endogenous and exogenous agents, It can produce instability in the genes due to failure in response to DNA damage (Jackson and Bartek, 2009). Genomic instability is a crucial agent in promoting the process of cancer formation. At the same time, inducing DNA damage by irradiation is a critical driver leads the cancer cells to death (Stechow and Olsen, 2017). Accordingly, and depending on the results in Chapter 4, irradiation of cancer cells with the high level doses of NIR diode laser used in current study constitutes a key therapeutic strategy to kill fast-dividing cancer cells. Thus, more studies are needed to investigate the ability of the applied NIR diode laser to verify such a strategy may be of therapeutic value.

It is commonly known that PBM/LLLT is non-invasive radiation and counted non-thermal phototherapy has been recognized worldwide for its expansive use in medicine (O'Kane et al., 1994, Al-Watban and Andres, 2012, AlGhamdi et al., 2012, Barolet et al., 2016). Nevertheless, researches published recently revealed the other side of the coin that a potent inhibitory impact has been generated after exposure to PBM/LLLT with high energy density, which is likely due to increased temperatures. Studies maintained that in addition to the beneficial effect, PBM/LLLT without doubt has deleterious effects (Salehpour et al., 2017). In view of study to Logan (1994), suggested that the possibility of an LLLT mediated intracellular thermal effect should be considered (Logan et al., 1994). Another study emphasized by evidence that exposure to LLLT with high energy density elevated the temperature and caused significant increase in DNA damage, inhibition in cellular functions and induced apoptotic cell death. Therefore, further studies are still required to assess if the PBM/LLLT has a role in inducing the temperature of the target cells or tissues through and after exposure to varying energy densities of PBM/LLLT. Which was supposed to accomplish such an experiment and analyze these measurements in the current study, but unfortunately time constraints prevented us from completing this work.

Through the review we found that the PBM/LLLT affects different cellular functions, one of them is cell cycle phases, but there are very little published researches, most involved using PBM/LLLT of visible red and very few used NIR, despite their importance in conserving the progression in other functions such as proliferation. The cell cycle is a series of crucial repeated events allowing the cell to grow and duplicate correctly (Massagué, 2004, Nurse, 1994). A research study for Ramos and Co-workers 2016 revealed that PBM/LLLT was capable of increasing proliferation of

cells at S phase (Ramos Silva et al., 2016). It has been emphasized that PBM/LLLT irradiation can induce progression of cell cycle and allows the cells to pass through G1 phase and enter S phase through redistribution and degradation of checkpoint regulator protein (PML protein) (Gavish et al., 2004), and by affecting early cell-cycle regulatory genes, that enabling cell proliferation at last (Ramos Silva et al., 2016, Shefer et al., 2003). Therefore, we need a broader study to investigate the effect of PBM/LLLT on the cell cycle and its role in promoting cell proliferation.

Finally, it would be interesting to investigate the role of NIR laser therapy in activation the mechanism of heat and anticancer drug in the nanocomposite. Where, the NIR laser-induced targeted cancer thermo-chemotherapy is a therapeutic mean with practical efficacy, and demonstrates a novel anti-cancer targeting strategy (Zhang et al., 2014). Thus, future studies might target a cancer cells through manipulating the laser therapy used very precisely and flexibly.

APPENDICES

Appendix 1 has been removed due to copyright restrictions.

Mussttaf *R. A., Jenkins D.F.L., Jha A. N. 2017,* Photo-stimulatory effect of LLLT on the proliferation rate of human monocytic leukaemia cells*. IET Nanobiotechnology*, 12(2), 175-181. DOI: 10.1049/iet-nbt.2017.0035

RESEARCH WITH PLYMOUTH UNIVERSITY

Assessing the impact of low level laser therapy on biological system

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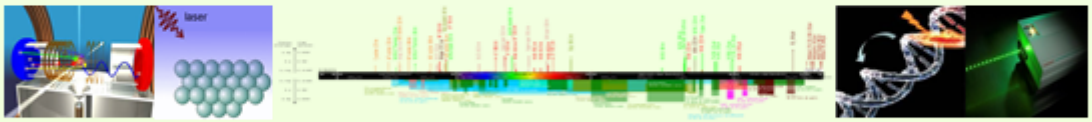


BACKGROUND

- Laser light has a high power beam energy, because it is highly coherent (waves in phase), polarized, focused and monochromatic (a single wavelength)
- Low level laser therapy (LLLT) has been used mainly for medical applications such as pain relief, inflammation and wound healing, however its biological action is not well understood.

Aims:

- Identify any knowledge gaps in the obtainable literature.
- Identify parameters (e.g. coherence and polarization) for LLLT whose therapeutic impacts are not fully characterized, to enable further studies.

METHODS

Literature Search included (LLLT, cells, DNA)

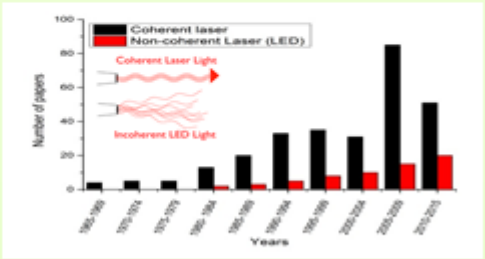
↓

Determining source, coherence and target (cells or DNA) for each reference and counting number of papers in each category

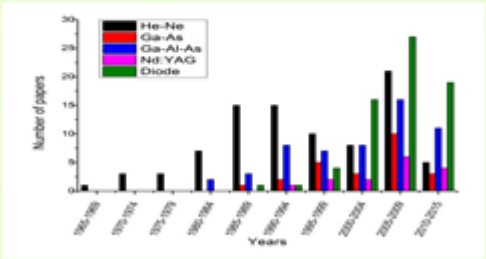
↓

Tabulated data (using Origin program) | Graphical analyses (using Origin program)

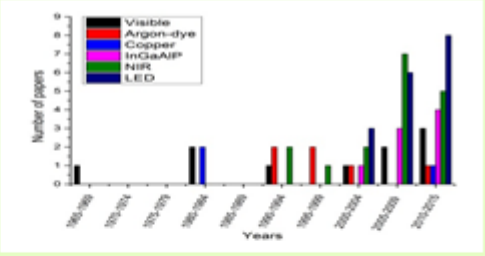
RESULTS



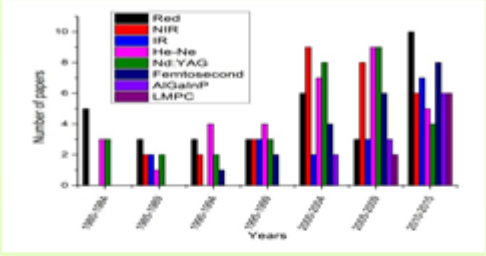
Coherent sources and non-coherent (LED) of LLLT in clinical and laboratory studies on the effect of LLLT on cell and DNA from 1965-2015



Most used sources in clinical and laboratory studies on the effect of LLLT on cell functions from 1965-2015



Least used sources in clinical and laboratory studies on the effect of LLLT on cell functions from 1965-2015



Sources used in clinical and laboratory studies on the effect of LLLT on DNA from 1980-2015

CONCLUSIONS

- Most studies concern dose and wavelength of LLLT.
- There is only a limited number of studies using other physical parameters of LLLT such as coherence and polarization of light.
- There were conflicted opinions about whether coherent laser or non-coherent LED has the most beneficial therapeutic impacts on biological systems.
- Studies using the same parameters of LLLT to assess cell survival or effects on DNA are almost non-existent.

RESEARCH WITH PLYMOUTH UNIVERSITY

The Photo-stimulatory Effect of Low Level Laser Therapy on the Proliferation Rate of Cell Cultures

ukems

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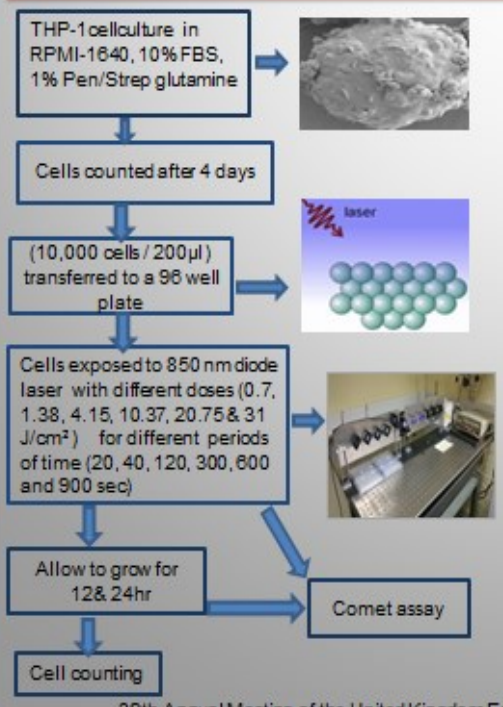
Background

- Low level laser therapy (LLLT) has been used mainly for medical applications, which involves visible red and near infrared (NIR) electromagnetic waves, and broadly termed as laser therapy and photobiomodulation.
- LLLT irradiation promotes proliferation of multiple cells, which is mainly through the activation of mitochondrial respiratory chain and the initiation of cellular signalling.
- Effects of LLLT on proliferation of cell cultures depend on energy density, total power, exposure time, wavelength, irradiation mode as well as the cell type.

Aims

- Highlight the ability of NIR low level laser to enhance proliferation of THP-1 cell line *in vitro*.
- Identify the doses and exposure time at which the stimulatory effect is performed to enhance cell proliferation rate.
- To evaluate potential cytotoxicity and genotoxic effect of different doses of LLLT on the cells.

Experimental



Results

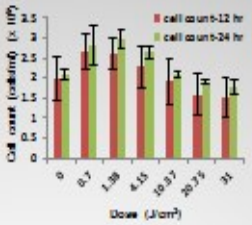


Fig. 1 The effect of LLLT doses on cell proliferation 12 hr& 24 hr after irradiation.

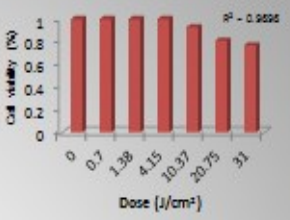


Fig. 2 The cytotoxic effect of LLLT doses on cell viability after irradiation

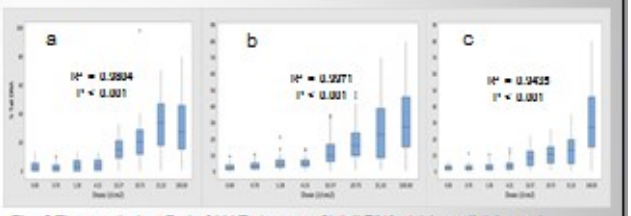


Fig. 3 The genotoxic effect of LLLT doses on % tail DNA (a) immediately post irradiation, (b) 12 hr after irradiation, (c) 24 hr after irradiation.

Conclusions

- LLLT with near-infrared (850 nm) diode laser has the ability to promote the proliferation rate of THP-1 cell line.
- The results confirm the stimulatory effect of LLLT is dose dependent.
- There is range of doses may exist for tissue and cell lines which is necessary in order to perform the stimulation and /or a unique dose at which maximal stimulation of cellular metabolism and proliferation.
- Below the threshold of 0.1J/cm² the cells are not stimulated to proliferate, therefore further investigation into optimal conditions for cell stimulation is needed.
- Identification of the proper treatment conditions for the particular cell lines or tissue is crucial for achieving optimal photobiostimulation.

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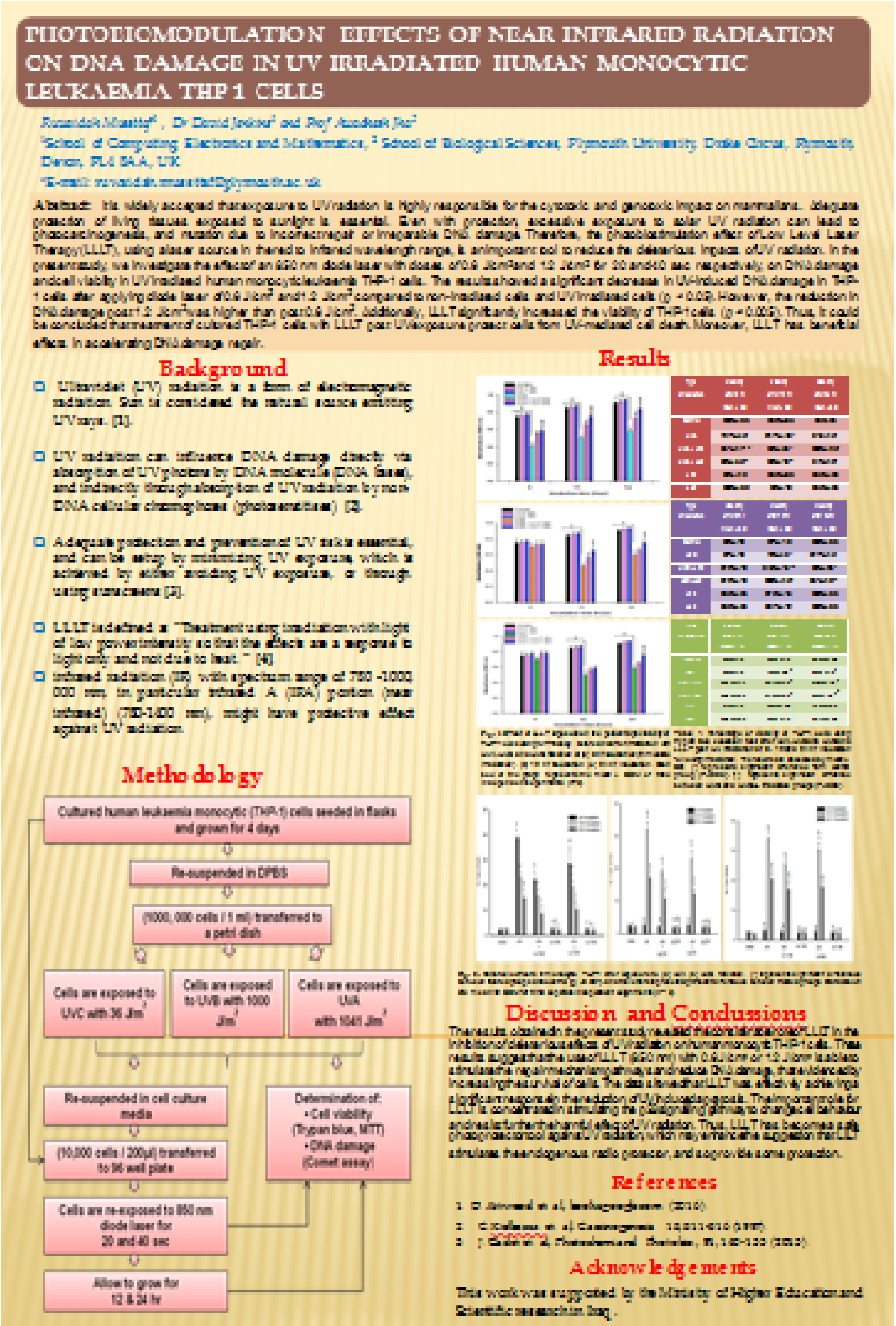
PHOTOBIOMODULATION EFFECTS OF NEAR INFRARED RADIATION ON DNA DAMAGE IN UV IRRADIATED HUMAN MONOCYTIC LEUKAEMIA THP 1 CELLS
Results
Background
Methodology
Cultured human leukaemia monocytic (THP-1) cells seeded in flasks and grown for 4 days
Re-suspended in DPBS
(1000, 000 cells / 1 ml) transferred to a petri dish
Cells are exposed to UVC with 36 J/m²
Cells are exposed to UVB with 1000 J/m²
Cells are exposed to UVA with 1041 J/m²
Re-suspended in cell culture media
(10,000 cells / 200µl) transferred to 96 well plate
Cells are re-exposed to 850 nm diode laser for 20 and 40 sec
Allow to grow for 12 & 24 hr
Determination of:
• Cell viability (Trypan blue, MTT)
• DNA damage (Comet assay)
Discussion and Conclussions
References
Acknowledgements

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