In vitro cytotoxic and apoptotic action of zinc oxide nanoparticle, diode laser and their combination against colorectal cancer

A Thesis

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DECLARATION

I declare that the M.Sc. thesis entitled: **(*In vitro* cytotoxic and apoptotic action of zinc oxide nanoparticle, diode laser and their combination against colorectal cancer),** my original work, and hereby I certify that unless stated, all work contained within this thesis is my pendent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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We certify that we have read this thesis: **(*In vitro* cytotoxic and apoptotic action of zinc oxide nanoparticle, diode laser and their combination against colorectal cancer),** and as an examining committee examined the student(**Chrakhan Salih Rahman**) in its content and what related to it. We approve that it meets the standard of a thesis for the degree of MSc. in MLT.

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SUMMARY

Colorectal cancer (CRC) is the third most diagnosed tumor worldwide, with a very high mortality rate. The treatment strategies, such as surgery, chemotherapy, or radiotherapy, are not effective enough and show several limitations. Therefore, emerging strategies, such as nanomedicine offers a very powerful tools for cancer treatment. Recently, the combination of nanoparticle antitumor effect with a triggering external stimulation was formulated to boost up the cytotoxic activity. In the present study, the synergistic action of Zinc oxide nanoparticles and Diode laser was assessed in the treatment of Colorectal cancer cell line (Caco2) *in vitro*.

Different concentrations of ZnO2-NP (0.5, 1, 5, 10, 20, 40, 75, 100, 200, 1000) µg/ml were used for cytotoxic action. In addition, targeted laser power of (5 j/cm^2). The combination of ZnO2-NP and Diode laser for the cell was evaded properly. IC50 for CaCo2 was 20 µg/ml. The cytotoxic action was measured via MTT Assay for (24,48 and 72 hrs), while Flowcytometery (Propidium Iodide, Annexin VAssay) was applied for detection of apoptosis, while, analysis of cell cycle arrest was measured by flowcytometry. Moreover, the relative expression level of (P53, Bax, and Bcl2) was measured using quantitative real-time PCR (qPCR). Reactive Oxygen Species-level was determined by dichlorofluorescein diacetate (DCFDA) Assay method.

The percentage of cells that decreased in the G2 phase of the cell cycle relative to the S and G1 phases was significantly different when laser and zinc nanoparticles were combined (p-value<0.05). Significantly substantial variation in the use of a diode laser and zinc nanoparticles to stop the spread of cancer by boosting the expression of the p53 gene (p-value<0000). Bax, a key pro-apoptotic component of the B-cell lymphoma 2, is an essential gateway to mitochondrial dysfunction and was significantly expressed in the study (p-value<0.05) after cell treatment. Utilizing the laser and zinc nanoparticles together did not reveal any substantial differences in

the Bcl2 gene (p-value>0.05). Results of different techniques used in the study supported that combination therapy of ZnO2 and Diode laser is cytotoxic to Colorectal cancer *in vitro* and there is a possibility of developing an effective therapeutic agent against Colorectal cancer. The finding of cytotoxicity is a significant difference in combination therapy on the number of cells experiencing early apoptosis compared to live, late apoptosis, and necrosis (p-value<0.05). Highly noticed modifications and significant differences have been reported when the proportion of cell lines in DCFH- compared to DCFH+ has been reduced using laser and zinc nanoparticles alone or together (*p*-value<0.05), the percent of cells in the control remains at high rates and decreased in all treated cases with different ratios.

This study demonstrated that the combination of zinc oxide nanoparticles and diode laser therapy on colorectal cancer cells (Caco-2) *in vitro*, showed a synergistic anticancer impact.

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List of Abbreviations

CRC	Colo-Rectal Cancer
ZnO	Zinc Oxide
NPs	Nanoparticles
ZnO NPs	Zinc Oxide Nanoparticle
P53	Protein 53 kilos Dalton molecular mass
Bcl2	B-cell lymphoma 2
Bax	Bcl2 Associated X-protein
cDNA	Complementary deoxyribonucleic acid
ELISA	Enzyme-Linked immunosorbent assay
IC50	The half maximal Inhibitory Concentration
PMS 1, 2	Polymeric micelles 1, 2
MLH 1, 6	DNA mismatch repair protein (MutL homolog1, 6)
MSH2	DNA mismatch repair protein (MutS homolog2)
HNPCC	Hereditary nonpolyposis colorectal cancer
CIN	Cervical intra-epithelial neoplasia
TNBC	Triple Negative Breast Cancer
ER+	Estrogen Receptor positive
DCFH	Dichloro-dihydro-fluorescein diacetate
J	Joules
ROS	Reactive Oxygen Species
qPCR	quantitative real-time PCR

CHAPTER ONE
Introduction

1. Introduction

Colorectal cancer (CRC) is the second most common cause of cancer-related death. accounting for 9.2 % globally, and is the third most prevalent adult cancer in males and the second most prevalent adult cancer in women (Gupta et al., 2020, Dekker et al., 2019). Around 900,000 fatal CRC cases were reported in 2018, and by 2035, it's believed that there would be approximately 2.5 million additional cases (Dekker et al., 2019, Bray et al., 2018). The proximal colon is affected by around 41% of all colorectal cancers, whereas the rectum and the distal colon are each affected by 22% and 28%, respectively (Cheng et al., 2011). Men and women with colon cancer are, on average, 68 and 72 years old at diagnosis, whereas both sexes with rectal cancer are, on average, 63 years old (Islami et al., 2018). Age, environment, and hereditary factors all have a big impact on how colon cancer develops. Lynch Syndrome (Hereditary Nonpolyposis Colorectal Cancer), Familial Adenomatous Polyposis (FAP), and MUTYH-Associated Polyposis are hereditary colorectal cancer syndromes (MAP) (Lakkis et al., 2021).

Chemotherapy, immunotherapy, radiation, therapy, surgery, targeted therapy, and hormone therapy are some of the traditional therapeutic modalities utilized during cancer therapy (Park et al., 2018, Jovčevska and Muyldermans, 2020).

In the past twenty years, The characteristics of NPs have facilitated the development of several NPs-based therapy techniques (Bi et al., 2016). Many different NP platforms, including those based on lipids, polymers, inorganic materials, viruses, and drug conjugates, are now being researched as nanocarriers for cancer treatment. In contrast, some of these NP systems have been authorized for usage in clinical settings. One of the most innovative and promising methods of development of

future cancer treatments is nanomedicine (Singh et al., 2017). The majority of scientific literature demonstrates that cancer may be successfully treated using nanomedicine treatments both in vivo and vitro. the capability of specifically delivering anti-cancer drugs to tumors, the ability to image tumors, the capacity to store 1,000 drug molecules, and the capacity to overcome problems with soluble, stable, and resistible materials are the key benefits of using NPs as anticancer agent carriers (Hua et al., 2018).

In the field of biomedicine, nanoparticles of metal oxide have demonstrated promise for a variety of uses, involving the transport of anticancer drugs and genes, biosensing, and cell imaging (Maaza et al., 2015, Verma et al., 2018). Brain, muscle, bone, and skin are just a few of the human components that contain zinc, which is often recognized as an important trace element. In addition to its involvement in metabolic processes, Zinc is a crucial cofactor for many enzyme systems and is necessary for the synthesis of nucleic acids and proteins, neurogenesis, and hematopoiesis (Ruszkiewicz et al., 2017). Due to their high exciton binding energy and wide bandgap, zinc oxide nanoparticles (ZnO-NPs) exhibit unique chemical and physical characteristics. This metal oxide nanoparticle is one of the most significant ones and is utilized extensively across many fields (Ruszkiewicz et al., 2017). Numerous investigations were required to control the consequence of ZnO NPs on cancer cells' ability to proliferate less quickly, however it has been demonstrated that they may very specifically trigger cacancerell apoptosis. The p53 pathway is most likely what is causing this, as opposed to reactive oxygen species.(Ahamed et al., 2021).

Photothermal therapy (PTT) and Photodynamic therapy (PDT) are two types of uses of nanomedicine in laser-assisted therapy that have been studied. PTT and PDT are

two laser-based therapy methods that have gained a lot of interest in the last ten years (Jiang et al., 2017b).

A chemical known as a photosensitizer (PS) is given to a patient as part of photodynamic therapy (PDT), which then activates the drug in cancerous tissue using light of a certain wavelength. Cancerous tissues are destroyed as a result of the photochemical reaction's generation of reactive oxygen species and oxygen free radicals (Matoba et al., 2018). However, variables involving inflammation, antitumor immunity, and vascular shutdown effects all have an impact on enhancing the anticancer effects (Oinuma et al., 2016). PDT, compared to other prevalent cancer therapies like radiation and chemotherapy, induces acute inflammation in addition to directly killing cancer cells, which stimulates the immune system and enhances the T-cell exposure to tumor-derived antigens (Railkar and Agarwal, 2018). PDT is a well-known and effective method for the treatment of several cancer types, including gastrointestinal (GI) cancer (Yu et al., 2018, Kaneko et al., 2018, Hosokawa et al., 2018).

The aim of study is to find out the anticancer action of ZnO NP and Diode laser and to look into the possibility of synergistic anticancer effect of combining zinc oxide nanoparticles and diode laser therapy on colorectal cancer cells (Caco-2). Also to find the mechanisms of therapeutic action involving apoptosis, cell cycle arrest, and increases the levels of reactive oxygen species (ROS) in the cancer cells.

CHAPTER TWO

Literatures Review

2.1. Cancer

Cancer is a difficult disease that has the potential to spread to the body's other organs as well as spread locally. In the next 20 to 40 years, it is expected that the number of cancer cases would more than quadruple globally, and the primary cause of death to surpass heart disease (Jemal et al., 2010). Additionally, a growing issue in an aging population is the treatment of cancer which is crucial in emerging nations (Siegle et al., 2012). By the year 2030, the International Agency for Research on Cancer (IARC) estimates that there will be 26 million new instances of cancer and 17 million cancer-related deaths (Ferlay et al., 2008). As a result of population growth, aging, an increase in the frequency of unhealthy behaviors, and particular risk factors like smoking, cancer incidence rates have been rising in the majority of countries since 1990. This constitutes an increasing threat to public health on a global scale (Fitzmaurice et al., 2015, Badwe et al., 2014). 17.5 million new cases of cancer have been reported globally since 2005, a 33 percent rise (Fitzmaurice et al., 2017, Hussain and Lafta, 2021).

2.1.1 Risk factors for cancer

In Iraq, the frequency of cancer risk factors, including diabetes, obesity, smoking, poor diet, and obesity has recently increased without enough preventative measures being implemented. Iraq has high incidence rates for bladder, lung, and breast cancer in addition to the rising frequency of many other cancers (Jemal et al., 2010). With the aid of accurate and quantitative assessments of the burden of cancer, policymakers and health management may more effectively prioritize diseases and distribute resources. If the time of the deployment of a control measure is indicated, they can also establish what factors contributed to a drop or rise in the incidence of cancer and how this affected the illness rate (Funk et al., 2013).

2.2. Colon cancer

As a heterogeneous illness, colorectal cancer progresses to the formation of polyp-like malignant tumors in the rectum and colon's inner walls (Fanelli et al., 2020). The reports of WHO (2020) indicated that the second-leading cause of mortality worldwide was colorectal cancer leading to almost 1 million deaths per year and the third-most frequent cancer, in 2020, almost 2 million cases were diagnosed, respectively, based on the International Agency for Research on Cancer (IARC), 2022. In western nations, colorectal cancer has surged recently and now accounts for 10% of all deaths of cancer-related (Schreuders et al., 2017). The colorectal cancer prevalence in Iraq was low but has recently grown, according to many descriptive studies (Al Dahhan and Al Lami, 2018). However, colorectal cancer may be malignant, non-cancerous, or benign depending on the severity of the condition (Angell et al., 2020). The improvement of polyps in the inner lining of the

rectum and colon causes localized colorectal cancer (Bolat et al., 2020a). As time goes on, colorectal cancer develops a malignant state owing to an increase in the size of the polyps, which causes them to often metastasize to the liver and less frequently to the spinal cord, bones, brain, and lungs(Guo et al., 2020).

2.2.1. Related Causes and CRC Risk Factors

Old age, obesity, environmental pollutants, heredity, the use of foods high in animal protein, saturated fats, alcohol, and diets poor in fiber are other factors that contribute to colorectal cancer, albeit the specific process is yet unclear (Koliarakis et al., 2019). Age-related changes in the population, dietary habits, smoking, insufficient physical activity, and obesity have all been linked to an increase in colorectal cancer mortality(Schreuders et al., 2017). For colorectal cancer, age is the main risk factor; after the age of fifty, a substantially increased risk of CRC development, and colorectal cancer rarely develops prior to the fifth decade of life (aside from cancers inherited) (Levin et al., 2008). Age is not the only innate risk factor that could not be altered. Personal history of inflammatory bowel disease (IBD) or colorectal cancer—the risk is increased in persons with ulcerative colitis by up to 3.7 percent (Eaden et al., 2001), while among patients the risk rises by 2.5 percent that have a disease of Crohn (Canavan et al., 2006)—are substantial hazards for development of colorectal cancer. Chronic inflammation associated with IBD frequently leads to dysplasia, an abnormal cell growth. Dysplastic cells are more prone to develop into anaplastic cells and form tumors even though they are not yet malignant. The existence of relatives with a positive CRC family history, particularly those who were under fifty at the time of diagnosis, is another risk factor that could be added

to this category. Increased risk due to family history may be caused by inherited mutations or environmental factors (Johns and Houlston, 2001). Small changes to one's eating and exercise habits can help lessen numerous additional risk variables that are related to one's way of life. For instance, there is the notion that, despite the fact that the relationship between a sedentary lifestyle and colorectal cancer is not fully understood, it may increase the risk of the condition. Light exercise, however, has been proven to increase metabolic rates, gastrointestinal motility, and with time, metabolic efficiency, as well as lower blood pressure (Mármol et al., 2017). For colorectal cancer, another significant risk factor is obesity, which is associated with a sedentary lifestyle. Surprisingly, this elevated risk is associated with increased calorie intake as well as A hormonally active component of total body fat known as visceral adipose tissue (VAT) has been linked to an increased risk of colorectal cancer through secreting cytokines which leads to inflammation in the rectum and colon, causing insulin resistance, and altering the activity of certain metabolic enzymes, such as lectin or adiponectin (Martinez-Useros and Garcia-Foncillas, 2016). A recent meta-analysis and comprehensive assessment of the literature on the relationship between dietary factors and the risk of CRC, eating more red and processed meat was linked to an enhanced risk of the disease, whereas eating more fish, dairy products, vegetables, and whole grains had the opposite effect (Vieira et al., 2017). Dietary habits have also been linked to mortality among CRC survivors and CRC risk, according to previous meta-analyses (Jemal et al., 2010). A "healthy" diet, defined as one with an appropriate intake of low-fat dairy, poultry, soy, fish, olive oil, whole grains, vegetables, and fruit, and is associated with a decreased risk for colorectal cancer (CRC), whereas a "western" diet, defined as one with an extensive intake of high-fat gravy, potatoes, butter, high-fat dairy products, Red and/or processed meat, sugary foods, and refined carbohydrates are all associated with an increased risk of cancer (Feng et al., 2017).

2.2.2. Colorectal Cancer Genetics

Similar to other malignancies, colorectal cancer can be caused by gene alterations. Genes engaged in pathways of DNA repair, tumor suppressor genes, and oncogenes may all exhibit such alterations (Fearon and Vogelstein, 1990). Colorectal cancers may be divided into three categories according to the mutation source: family, irrational, and hereditary. Point mutations, which can affect just one cell and its offspring throughout life, are not connected to inherited diseases. Point mutations create sporadic tumors, which account for roughly 70% of all colorectal malignancies. Because several genes may be impacted by mutations, the molecular etiology of sporadic cancer is diverse (Fearon and Vogelstein, 1990). But in around 70% of CRC instances, a specific morphological sequence is produced that starts with the onset of an adenoma and ends with the carcinoma stage. As a tumor suppressor gene, Adenomatous polyposis coli (APC) experiences its first mutation, resulting in the development of non-cancerous adenomas, often referred to as polyps. Around 15% of such adenomas in a ten-year period, are anticipated to develop to the carcinoma stage. The KRAS, TP53, and DCC mutations come after this APC mutation (Fearon and Vogelstein, 1990). Only 5% of instances of CRC are brought on by inherited cancers. These malignancies have resulted from hereditary mutations impacting one of the changed alleles of a gene, This denotes that the cancer will eventually develop as a result of a point mutation in the other allele. To enable a more accurate categorization of hereditary cancers, two categories—non-polyposis and polyposis forms—have been created. The most common type of polyposis, familial adenomatous polyposis (FAP), is characterized by the growth of many colon polyps with the potential to become malignant (Balmana et al., 2013). On the other hand, HNPCC, or Hereditary Non-Polyposis Colorectal Cancer, is connected to changes in DNA repair mechanisms. Lynch syndrome, the main factor causing

HNPCC,is brought on by genetic changes in one allele that codes for DNA repair proteins including PMS2, PMS1, MLH6, MLH1, and MSH2. In the HNPCC category, The most prevalent syndrome, Lynch syndrome, is present in 2% to 3% of all cases of colorectal cancer (Balmana et al., 2013, Umar et al., 2004). About 25% of instances of colorectal cancer are familial, and they are likewise brought on by mutations of inherited; nevertheless, because they could not be part of any inherited cancer variation, they are not specifically classified as hereditary malignancies (Stoffel et al., 2014).

Genomic instability is a key element of the underlying structure of colorectal cancer. The pathogenic mechanisms that result in this syndrome may be explained by one of three different pathways: microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and chromosomal instability (CIN). The conventional pathway, also known as the CIN route, is characterized by chromosomal aberrations that cause aneuploydic malignancies and loss of heterozygosity. It is the origin of up to 80% to 85% of all CRC cases (Carethers, 2014). (LOH) DNA damage response, telomere malfunction, and chromosome segregation Some of the procedures supporting CIN involve modifications. Important genes including TP53, APC, KRAS, and PI3K, among others, that are necessary for maintaining healthy cell function are impacted by these modifications. While PI3K and KRAS mutations constantly activate MAP kinase, APC mutations enhance -catenin translocation to the nucleus and speed up the transcription of genes involved in invasion and cancer. increasing cell proliferation. Last but not least, mutations that induce loss-of-function in TP53, the primary cell-cycle checkpoint, and the gene that encodes p53, result in an uncontrolled entrance into the cell cycle (Pino and Chung, 2010).

2.2.3. Colorectal Cancer Diagnosis

A number of methods for diagnosing colorectal cancer have been developed, including MRI scans, CT colonography, barium enema X-rays, colonoscopies, flexible sigmoidoscopy, and blood tests in the stool. Due to the significant gaps that accompany over-testing, over-diagnosis, over-treatment, non-specificity, and heterogeneity of colorectal cancer, identification of the disease is difficult (Zhou et al., 2020). The area of medicine has undergone a revolution thanks to nanomedicine, which has also considerably improved the pharmacokinetic and pharmacological profile of the unsteady anticancer medications (Buabeid et al., 2020, Bai et al., 2019). Anticancer medications used to treat colorectal cancer shown decreased entrapment/encapsulation effectiveness (Rampado et al., 2019, Youn and Bae, 2018).

2.2.4. Colorectal Cancer Prevention and Treatment

Fortunately, a number of preventative interventions are very successful in lowering colorectal cancer risk (Rawla et al., 2019). Regular screening, consuming foods with high fiber content, maintaining an acceptable body mass index (BMI), and exercising are the most often used preventative methods (Thanikachalam and Khan, 2019). While colorectal cancer is being treated according to established procedures, benign-stage cancer does not need to be treated. Surgery is an option in cases of metastatic invasion to remove lymph nodes and malignant tumors (Hashiguchi et al., 2020). When treating colorectal cancer, bevacizumab and ramucirumab are used as targeted therapies to block the actions of certain particular proteins implicated in the process of the disease (Bennouna et al., 2019, Kanat and Ertas, 2019, Modest et al., 2019). Additionally, radiation therapy is often used to treat colorectal cancer since it

helps reduce tumor size by using high-intensity beams of radiation (Klement et al., 2019). There are two techniques to provide radiation treatment for colorectal cancer: external beam radiation and internal radiation (brachytherapy). Small radioactive seeds are injected into the body using an ultrasound-image-guided needle to give a very low, optimal dosage of radiation over a reasonably extended period of time (Chan et al., 2019). To the disappointment of everybody, however, colorectal cancer treatment procedures are usually connected to significant non-compliance and toxicities of the patient (Lew et al., 2019). Also, over time, cancer cells have become resistant to current therapies and medications (Yu et al., 2019).

2.3. Nanoparticles and nanotechnology

In the last ten years, the discipline of nanotechnology has grown tremendously, and numerous goods incorporating nanoparticles are now employed in servals fields, such as food science, cosmetics, and medicines (Kumar et al., 2012). Particles having one dimension between 1 and 100 nm are known as nanoparticles (NPs). NPs have various characteristics based on their size and surface functions (Gwinn and Vallyathan, 2006). The broad usage of NPs in a variety of fields, including both therapeutic and diagnostic medical applications, electronics, and cosmetics, is because they are small and have a large surface area (Missaoui et al., 2018b).

2.3.1. Nanoparticles

Nanoparticles may be created using a variety of techniques and substances. For instance, Sayed et al. synthesized nanometric delafossite using the flash process and

evaluated it using the diffusion assay method against different Candida species, fungus, and bacteria (Sayed et al., 2020b). Silver nanoparticles (Ag-NPs) were produced using wet chemical flash and combustion procedures, according to ElBassuony et al (El-Bassuony and Abdelsalam, 2020a, El-Bassuony and Abdelsalam, 2020b). Sayed et al. synthesized spinel nanoferrites using several techniques in a separate research and tested them against four harmful fungus (Sayed et al., 2020a). The results showed that the antibacterial efficacy is influenced by both the microbial species and the nanoparticle manufacturing process. As a result, nanoparticles seemed to be promising candidates for stabilizing the encapsulation of anticancer medications, increasing their safety, therapeutic index, half-life, solubility, and effectiveness (Salvioni et al., 2019). Through direct internalization into tumor cells by micropinocytosis or endocytosis, multi-drug resistance may be passed on by using nanoparticle site-specific drug delivery (Manzanares and Ceña, 2020). Active targeting and passive targeting are two balancing tactics used in the manufacture and application of nanoparticles to the treatment of fatal diseases(Xu et al., 2020, Bort et al., 2020, Sindhwani et al., 2020). Selecting tiny natural compounds (like folic acid), peptides (against the receptor of integrin), and specific ligand receptors and antibodies (VEGFR and EGFR) that are overexpressed in colorectal carcinoma cells is necessary for active targeting of these tumors (Zhi et al., 2020). The capacity of a drug-loaded nanocarrier (200 nm diameter) to extravasate from the circulation of systemic drugs, however, can be improved to achieve passive targeting of the faulty solid tumor's leaky vasculature and remain there through dysfunctional lymphatic drainage (Narum et al., 2020). Therefore, a variety of nanomedicines have been successfully developed in relation to colorectal cancer, such as plant-derived lipid nanoparticles (curcumin, ginger), lipid nanodiscs, gold metallic nanoparticles, polymer-drug conjugates, nanomicelles, and solid lipid nanoparticles, core-shell nanoparticles, pro-drugs, dendrimers, and liposomes (stimuli-sensitive, active-

targeting, long-circulating, cationic, and conventional liposomes) (Bolat et al., 2020b, Carvalho et al., 2020, Ansari et al., 2020, Chen et al., 2020, Weng and Goel, 2020).

2.3.2. Nanoparticles use in medicine

Due to their controlled medication release and tumor-selective features, nanoparticles are desirable delivery systems for anticancer medicines (Vinod et al., 2011). Inorganic particles, synthetic and natural polymers, and liposomes/lipids have all been used to create nanoparticles (Wang et al., 2016). Pharmaceutical drug carriers called NPs are used in both therapeutic and diagnostic procedures. These NPs, which include solid NPs, liposomes, polymeric NPs, and nanoemulsions, are thought to have potential therapeutic uses. Their therapeutic relevance is affected by a number of factors, like their drug release, drug loading effectiveness, chemical, and physical characteristics, and, more significantly, minimal or no carrier toxicity (Puri et al., 2009). The delivery method and level of exposure determine the toxicity of NPs, just as with any medicine or chemical (Missaoui et al., 2018a). Skin contact, ingestion, injection, inhalation, and other routes of exposure to NPs are all possible. Following exposure to NPs, the organ-specific toxicity is dependent on the mode of administration and systemic dispersion. Unintentional exposures may occur, such as when people breathe in NPs from the environment or factories. Lung tissue fibrosis, necrosis, and inflammatory responses are all possible outcomes of exposure via the lungs (Shi et al., 2013).

2.3.3. Nanoparticles Toxicity

The size, surface charge, and surface area of NPs are among their biophysical characteristics and aggregation state, which affect how hazardous they are (Wu and Tang, 2018). The membrane integrity disruption, changing protein structures and activities, DNA damage, and Reactive oxygen species (ROS) generation are one of the processes by which NPs are hazardous to their target organs. Areas with a large surface that at the target regions promote molecular interaction are among the NP characteristics that seem to favor these pathways. Solid NPs including metal oxide or metal-containing NPs have been found to produce DNA damage, inflammation, and oxidative stress after acute systemic exposure (Nemmar et al., 2016). These NPs have been shown to suppress the actions of antioxidants while inducing oxidative stress in the kidneys, liver, and spleen (Couto et al., 2016, Teodoro et al., 2016). DNA damage, mitochondrial malfunction, and activating stress-related cell signaling pathways all contribute to NP-induced ROS, which in turn causes death and cell cycle arrest (Teodoro et al., 2016, Khanna et al., 2015b).

2.4. Types of Nanoparticle

2.4.1. Polymeric NPs

Polymeric NPs may have a wide range of conceivable shapes and features since they could be produced using monomers or readymade polymers, as well as natural or synthetic components. They may be made to give fine control of a range of NP features, they have simple formulation requirements, and they are often efficient delivery systems. Emulsification (solvent displacement or diffusion), among other techniques,(Brown et al., 2020), nanoprecipitation (Zhang et al., 2019, Le et al.,

2018), ionic gelation (He et al., 2020), and microfluidics, are used to create polymeric NPs, each of which produces a distinct end product (Zhang et al., 2020a).

2.4.2. Carbon-based NPs

Different biological applications, such as medication administration, gene therapy, and imaging, carbon-based NPs are being employed more and more. A significant class of these NPs are carbon nanotubes (CNTs), which come in multi-walled (MWCNTs) and single-walled (SWCNTs) varieties. Due to their distinct physiochemical characteristics, CNTs are excellent candidates for a variety of biological uses, such as tissue engineering, biosensors, and gene and drug delivery (Serpell et al., 2016, Klumpp et al., 2006, Chaudhari et al., 2016, Alshehri et al., 2016b). Additionally, they exhibit unique surface chemistry and great stability that increases the capacity of drug loading. CNTs have been demonstrated to be harmful to tissues of healthy after continuous exposure, hence their safety is still under doubt (Alshehri et al., 2016a, Zhang et al., 2014, Kolosnjaj et al., 2007, Lam et al., 2006). Carbon-based nanomaterials with their distinctive electrical, optical, mechanical, and thermal characteristics, including Graphene, graphene quantum dots (GQDs), carbon nanotubes (CNTs), and carbon quantum dots (CQDs), may be easily functionalized (Augustine et al., 2017). The fields of environmental applications, energy, nano-medicine, and biomedicine have all received a lot of interest from these nanomaterials (Patel et al., 2019, Maiti et al., 2019). Because carbonaceous nanoparticles are naturally hydrophobic, they may load the desired medication through p-p stacking or hydrophobic interactions and could be used as effective nano-platforms of drug delivery (Saleem et al., 2018). Since they may be conjugated with different imaging agents or targeted moieties at high densities, such nanostructured materials have the ability for multimodality for cancer theranostics,

hence increasing the sensitivity or detection limit against cancer cells(Augustine et al., 2017).

2.4.3. Solid NPs

Silver, gold, iron oxide, and other NPs of metal-based are examples of solid NPs. Iron oxide nanoparticles are created by joining a biocompatible polymer to a magnetite (Fe3O4) or maghemite (Fe2O3) organic core. Since the previous decade, iron oxide NPs have garnered a lot of attention, particularly as a result of their superparamagnetic properties (Xie et al., 2010, Wu et al., 2015). Nanoparticles of iron oxide (iron oxide NPs) are combined with magnetic fluid hyperthermia, targeted medication and gene delivery, and MRI in biosensors (Wu et al., 2016). Additionally, due to their distinct optical characteristics that allow them to function as biosensors in live cells, Several applications for iron oxide nanoparticles (NPs) include imaging and diagnostic procedures (Kumar et al., 2013). Gold nanoparticles (NPs) have been proposed for applying in radiation, treatment, and cancer diagnostics, based on their surface, shape, and size characteristics (Calavia et al., 2018). Even theranostic systems, which integrate diagnostics, imaging, and medicines for better treatment, may be created using gold nanoparticles (Ashraf et al., 2016). Although it has been shown that gold NPs are less harmful than other solid NPs, complete knowledge of their toxicity feature is not available (Connor et al., 2005). Another group of solid NPs that has drawn attentions is silver NPs. Silver nanoparticles are one example of how they might be used as biosensors because of their optical properties and ability to both absorb and scatter light. Electronics, fabrics, biomedical equipment, antimicrobial coatings, and wound dressings all make extensive use of silver nanoparticles (NPs) (Li et al., 2010, Deshmukh et al., 2019).

2.4.4. Lipid-based nanoparticles (LBNPs)

Lipid-based nanoparticles (LBNPs), including liposomes, nanostructured lipid carriers (NLC), and solid lipid nanoparticles (SLN), have been the subject of numerous studies. Because of their long half-lives and controlled drug release, these nanoparticles prolong the duration of pharmacological activity. They may move both hydrophobic and hydrophilic molecules and pose very little risk (Ozpolat et al., 2014). Lipid nanosystems may include chemical alterations (such as polyethylene or gangliosides glycol (PEG)) to thwart immune system detection or to increase a drug's solubility. Additionally, they may be manufactured in pH-sensitive formulations in an acidic environment to facilitate drug release, and they may be combined using antibodies that bind to the receptors on tumor cells (for example, folic acid (FoA)) (R Rama et al., 2016).

2.5. Nanoparticles of Zinc Oxide

ZnO nanoparticles are utilized often in numerous customer service applications. ZnO nanoparticle Worldwide production is thought to range between 0.1 and 1.2 million tons annually (Swain et al., 2016). White, naturally occurring ZnO nanoparticles have a broad variety of uses and are thermally stable. Widespread applications for ZnO nanoparticles include biosensors, cement, rubber, and plastic. They are also used in coatings and pigments (fungicides in paints, UV protection), culinary additives, electrical devices, and catalysts (Jiang et al., 2018c). Because of their shown anti-inflammatory, wound-healing, antibacterial, anti-tumor, and antidiabetic characteristics, zinc oxide nanoparticles have been investigated for biomedical uses. Additionally, they are included in biosensors and imaging agents (Mishra et al., 2017, Jiang et al., 2018b). ZnO NPs and aggregates with various three-

dimensional structures alter the surface properties of hierarchically porous frameworks, which in turn affects biological processes (Jiang et al., 2018b, Jin et al., 2019). Due to the micro-/macrochannels in the nanomaterials, These hierarchically porous designs increase light scattering and multiple reflections, which enhance mass transfer (Chen et al., 2017, Falgenhauer et al., 2017). A typical wide band-gap semiconductor is ZnO. Due to its special characteristics, it is suitable for a variety of biomedical purposes, such as those that are antibacterial, antifungal, and anticancer (Rasmussen et al., 2010b).

2.5.1. Mechanism of action ZnO2 NPs

The majority of ZnO NPs' biological uses derive from their capability of a cell's capacity to generate ROS, which results in cell death to fight oxidative stress is reduced (Ryter et al., 2007). Their capacity to produce ROS is influenced by ZnO's semiconductor characteristics. The band gap, a void that is approximately 3.3 eV wide for crystalline ZnO and extends from the top of the complete valence band to the base of the unoccupied conduction band, is where the energy of the electrons (e) in semiconductors is contained. UV photons possess sufficient energy to advance e to the conduction band and leave holes (h+) behind. The NPs' surfaces are invaded by e and h ions, which then interact with oxygen and hydroxyl ions, respectively. As a result, hydroxyl and superoxide radicals are produced (Rasmussen et al., 2010b, Sharma et al., 2015). Due to the structural defects of nanoscale materials, a significant amount of conduction-band e and/ or valence-band h+ are still exist in ZnO NPs even in the absence of UV radiation. The main mechanism by which ZnO NPs are ROS and which cause cytotoxicity in cancer cells(Wang et al., 2017, Lin et al., 2019, Tanino et al., 2020). The various ROS generated then cause irreversible oxidative damage to cells by initiating redox-cycling cascades (Sharma et al., 2016).

Additional mechanisms for ZnO NPs' biological activity include necrosis and apoptosis (Wilhelmi et al., 2013, Yang et al., 2015). ROS-induced DNA damage triggers releasing apoptogenic factors from the space of mitochondrial intermembrane, which in turn triggers apoptotic pathways (Saud Alarifi et al., 2013). As a result, apoptosomes are created, which in turn trigger executioner enzymes (Shi, 2002), whose specific substrates are then broken down to cause apoptosis and cell death. Another theory is that ZnO causes apoptosis by rapidly dissolving after being ingested in its particle-agglomerated form, in the acidic lysosomes of macrophages (Wilhelmi et al., 2013).

2.5.3. Mechanism of ZnO Nanoparticle Toxicity

2.5.3.1 Zinc ions (Zn2+) released by ZnO nanoparticles

One notable factor contributing to ZnO nanoparticle toxicity is their potential for dissolving into free Zn2+. Cellular hydrated zinc ions in the acidic lysosomal environment combine with unharmed ZnO nanoparticles, resulting in mitochondrial injury and disturbance of cellular zinc homeostasis, both of which cause cell death (Aydin Sevinç and Hanley, 2010). The nanotoxicity of ZnO nanoparticles is caused by coordinated internalization or cell uptake of leaking Zn2+ from the environment, and such toxicity depends on the solubilized Zn2+ concentration in the medium. DNA damage and catalytic framework disruption are also caused (Premanathan et al., 2011).

2.5.3.2 Reactive oxygen species (ROS) production

The primary factor responsible for the toxicity of ingested ZnO nanoparticles is the formation of ROS (Tang et al., 2018). Cell defense mechanisms are activated and start to produce ROS, if ZnO nanoparticles penetrate the cell (Khanna et al., 2015a). If such a ROS generation exceeds the cell's limit of antioxidant protective, it leads to the generation of potent and incendiary cytokines that cause inflammation (Preedia Babu et al., 2017). Due to damage to the membrane, cellular components, DNA, and increased lactate dehydrogenase production brought on by necrosis or apoptosis and cell death, this inflammation causes mitochondrial disturbance (Ghosh et al., 2016). Cell death is also brought on by extracellular ROS aging as a result of the interaction between ZnO nanoparticles and cell membranes (Teh et al., 2016). Fundamentally, extracellularly generated ROS react to the electrical movement chain in the mitochondrial inner membrane and can cause cell lysis and offer toxicity (Wahab et al., 2010). Additionally, it has been demonstrated that oral administration of ZnO nanoparticles drastically lowered the levels of the antioxidants CAT, SOD, and GSH, demonstrating how oxidative stress caused by ZnO nanoparticles weakens the brain's antioxidant system (Attia et al., 2018).

2.5.3.3 Mechanical harm of ZnO nanoparticles' direct contacts with cells

Numerous studies have suggested that ZnO nanoparticles could be connected to the divider of cell and cause mechanical damage, such as spillage or complication of intracellular structures, membrane distortion, cell morphology changes(Babele et al., 2018, Zhang et al., 2016), mitochondrial damage, and outflow of specific organelles (Peng et al., 2011). In some cells, ZnO nanoparticles increase their ability to penetrate by destroying the lipid and proteins that make up the cell membrane. They

also pierce the cell wall, allowing them to enter the cells and release ZnO nanoparticles (Chen et al., 2012).

ZnO is categorized by the Food and Drug Administration (FDA) as a medication that is "generally recognized as safe."(Rasmussen et al., 2010a). As a result, ZnO has gained popularity as a substance in nanomedicine (Rasmussen et al., 2010a). ZnO NPs demonstrate specific cytotoxicity for cancer cells, according to recent research (Yuan et al., 2014). Because ZnO has previously been proven to cause oxidative stress in cells, we looked at how the ZnO-NPs S4, S3, S2, and S1 affected the oxidative environment of ovarian cancer cells (Raghupathi et al., 2011, Liu et al., 2017). With the use of the RealThiol (RT) reagent, they are evaluated the effect of ZnO-NPs on the cellular redox environment using a method that precisely measures the free levels of glutathione in living cells (Jiang et al., 2017c). Lower levels of glutathione in cells are a sign of increased oxidative stress. We noticed that in the ALST cells, treatment of ZnO-NP resulted in a sharp decline in free levels of glutathione, indicating that treatment of ZnO NP causes oxidative stress (Liu et al., 2017).

Bare ZnONPs released Zn2+ much faster than MSN-ZnO-AuNSs, indicating a delaying effect for the MSN coating. These findings show that the ZnONPs of the MSN-ZnO-AuNSs will dissolve in the acidic intracellular environment of breast cancer cells and release Zn2+ ions locally, which may then cause apoptosis (Ruenraroengsak et al., 2019).

Human colorectal adenocarcinoma (Caco-2) cell lines were used to assess the cytotoxicity of ZnO-NPs and Ag-NPs. The Caco-2 cells were exposed to a range of dosages (10, 25, 50, 100, and 200 g/mL) of Ag-NPs and ZnO-NPs in order to study the nanoparticle toxicity mechanism on cancer cells. The results showed that Caco-2 growth was significantly impacted by both kinds of nanoparticles. But at the same

concentration, ZnO-NPs outperformed Ag-NPs in terms of dose-dependent cytotoxicity (Kim et al., 2017).

2.6. Diode Laser

Two categories of nanomedicine applications for laser-assisted therapy have been studied: photothermal therapy (PTT) and photodynamic therapy (PDT). PDT and PTT are two laser-based therapeutic methods that have attracted much notice over the past ten years (Jiang et al., 2017a).

2.6.1. PhotoDynamic Therapy (PDT)

In PhotoDynamic Therapy (PDT), malignant tissue is given a photosensitizer (PS) drug before being triggered by light of a certain wavelength. Reactive oxygen species and oxygen free radicals are produced by the photochemical process, which causes the cancerous tissues to be destroyed (Dolmans et al., 2003b). In order to directly destroying cancer cells, PDT also causes acute inflammation, which stimulates the immunesystem and increases the tumor-derived antigens presentation to T cells (Castano et al., 2006). PDT is a recognized and effective technique to treat several cancer types (Yu et al., 2018, Hosokawa et al., 2018).

2.6.2. PhotoThermal Therapy (PTT)

PTT is a technique to treat cancer in which NIR (650-950 nm) light deeply penetrates the tissue. Consequently, it can destroy tumor cells with minimal damage to surrounding normal tissues (Jiang et al., 2016). Using PTT, cancer cells are selected locally; hence, the adverse side effects are minimized. In PTT method, radiation can cause intercellular interventions by affecting DNA and protein denaturation.

CHAPTER THREE

Materials and Methods

3.1. Materials

3.1.1. Chemicals

Table (3-1) shows a list of chemicals and their supplier which are used in the study.

Table (3-1) Chemicals and their supplier list

No	Chemical	Supplier
1	Dulbecco's Modified Eagle Media	Gibco, Usa
2	Fetus Bovine Serum	Gibco, USA
3	Penicillin	Gibco, USA
4	Streptomycin	Gibco, USA
5	Amphotericin B	Gibco, USA
6	Trypsin	Gibco, USA
7	MTT Kit	Merck, Germany
8	Sodium Chloride	Merck, Germany
9	Potassium Chloride	Merck, Germany
10	Di-Sodium Hydrogen Phosphate	Merck, Germany
11	Sodium Dihydrogen Phosphate Dehydrate	Merck, Germany
12	Sodium Bicarbonate	Merck, Germany

13	Dimethyl Sulfoxide Solution (Dmso)	Merck, Germany
14	Ethanol 70%	Merck, Germany
15	Propidium Iodide	Sigma-Aldrich, USA
16	Triton X-100)	Sigma-Aldrich, USA
17	Annexin-V-FLUOS Staining Kit	Roche Diagnostics, Mannheim Germany
18	RNX-Plus	Qiagen, USA
19	Chlorophorum	Sigma-Aldrich, USA
20	Isopropanol	Sigma-Aldrich, USA
21	Alcohol-70%	Sigma-Aldrich, USA
22	Primers	Pishgam (Tehran, Iran)
23	Master Mix	Takara, Korea
24	Methanol	Merck, Germany

3.1.2. Instruments

Table (3-2) shows a list of instruments and their manufacturers which are used in the study

Table (3-2) Instruments and their manufacturer list

No	Instruments	Manufacturer
1	Laminar Air Flow Hood	Jal Tajhiz, Iran
2	Centrifuge	KAVUSHAZMA, Iran
3	Inverted Microscope	LW scientific, China
4	CO, Incubator	Memmert, Germany
5	Plate Reader Spectrophotometer	BioTek ELx808,
6	Liquid nitrogen container	YDS. China
7	Cell culturing flask T25	Iwaki, Japan
8	Microcentrifuge	Sigma, Osterode, Germany
9	Flow Cytometer (FACSCanto™ II BD)	Amersham Biosciences Corp. USA
10	Autoclave	Rahimi, Iran
11	Refrigerator	Zanussi, Italy
12	Syringe Filter	JET BIOFIL, China
13	Distilled Water Apparatus	Aqatron, UK
14	Neubauer slide	Assistant, Germany
15	96 well plate for culturing	Iwaki Japan
16	6 well plate for culturing	Iwaki, Japan.
17	Micropipette	Dragon, China

18	Pipette	Dragon, China
19	Disposable Centrifuge tubes	Nunc, Denmark
20	Micro-centrifuge tube	Brandtech, USA
21	Biosystems Step OneTM thermal cycler	Biosystems, USA
22	P1 LASER PIOON	Pioon China

3.1.3. Solutions and reagents for Tissue Culture

1. PBS buffer (pH 7.4)

Phosphate buffered saline (PBS) without Ca+2 and Mg+2 was prepared by mixing the following components and the volume was completed to one liter distilled water. D.W

8 g NaCl

0.2 g KCl

1.44 g Na2HPO4

0.24 g NaH2PO4

The final pH of this buffer was adjusted to be (7.4). Sterilization was performed by autoclave at 120C° at 1.5 bars for 15 min. This buffer was prepared without Ca+2 and Mg+2 for use in trypsinization. After preparation, it was stored at 4 C °. Prior to any usage, PBS was warmed to 37 C ° before use.

2. Fetal Bovine Serum (FBS)

Provided by (Gibco, USA), Stored at -20C°.

3.Antibiotic solutions

Benzylpenicillin sodium salt (1000000IU) and Streptomycin (1 g) (Gibco, USA) store at -20C°. Ten ml was added to one liter of the culture medium under preparation.

4.Trypsin

Provided by (Gibco, USA), Stored at -20C°.

5. Sodium bicarbonate solution

Dissolve 4.4gm sodium bicarbonate in 100ml D.W. Then autoclaved at 121C° for 15 min, then stored at 4C° (Freshney, 1994).

6. Cell culture media

Dulbecco‘s Modified Eagle Media (DMEM), (Gibco, USA) Medium powder high glucose (with L-glutamine, pyridoxine hydrochloride, 110mg/L sodium pyruvate), 13.48g was dissolved in approximately 1000ml D.W, and then the other components were added:

- 3.7 grams of sodium bicarbonate, to give a final pH of 7.4
- 100ml Fetal Bovine Serum (10% of total medium)
- Antibiotic solution 1%

- Antifungal (Amphotericin B) 0.1%

The volume was completed to one liter with D.W then sterilized using a 0.22μm filter unit. FBS and antibiotic and antifungal added to the medium before filtration.

7. Cell lines

Caco2 cell lines were purchased from the National Cell Bank of Iran (NCBI).

8. DCFH-DA solution preparation for ROS Assay

1. To create a 10 mM stock solution, combine 4.85 mg of DCFH-DA with 1 mL of dimethyl sulfoxide (DMSO).
2. Just before adding the stock solution to the wells, dilute it with pre-warmed DMEM to create a 10 μM working solution.
3. For 10 seconds, vortex the effective solution.

3.2. Methods

3.2.1. Research Design

The research was designed as two parts; the first part is to study the cytotoxic effect of ZnO2, Diode laser and Combination therapy (ZnO2+Diode laser), to evaluate the half-maximal inhibitory concentration for cell and detection of Reactive Oxygen Species (ROS). After the evaluation test cell cycle arrest and induction of apoptosis studied by using flow cytometry (figure 3-1). The second part was molecular study (figure 3-2) for some apoptotic genes (Bax, Bcl-2, P53) whether the nanoparticle and diode laser activates the apoptotic pathway or not. The study was performed in a private research center in Tehran, Iran in Iran High-Tech laboratory Network.

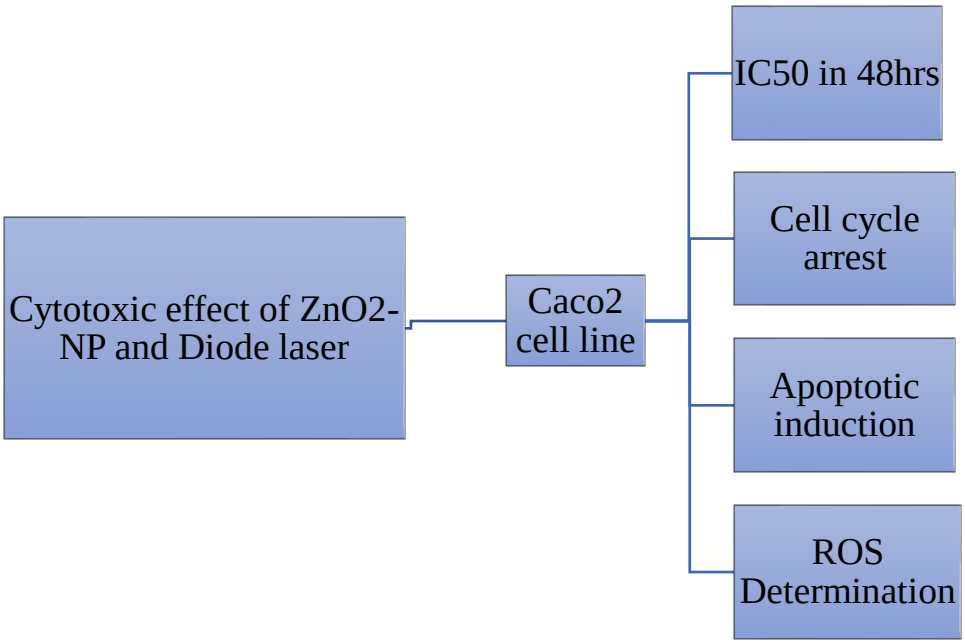


Figure (3-1): Cytotoxic Experimental Design for ZnO2-NP and Diode laser.

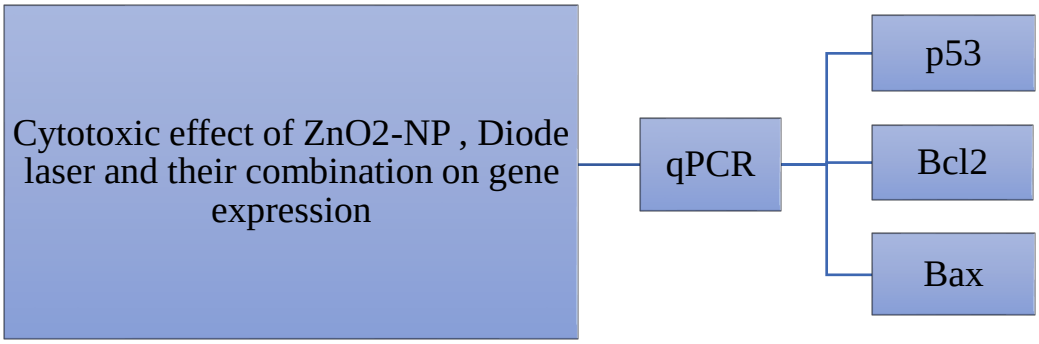


Figure (3-2): Gene expression study for apoptosis related genes in Caco2 cell line treated by ZnO2-NP , Diode laser and their combination.

3.2.2. Synthesis of Zinc Oxide nanoparticles

Zinc nitrate (0.5 M) is progressively added to a sodium hydroxide solution (1 M), and the mixture is continuously stirred for 15 minutes. Centrifuging and repeatedly washing the resulting white precipitate with distilled water. Drying occurs in a hot air oven at 60°C with the white powder (Zn(OH)2) that has been washed. Zn(OH)2 totally transforms into ZnO during the drying process.

3.2.3. Thawing of Frozen Cells

To thaw the cells, take one vial out from the LN2 tank or freezer and thaw vial immediately in a 37C° water bath. Thaw content with slight shake until only small ice is left in the vial. It usually takes 1 min. Spray vial with 70% ethanol all over and wipe its surface with clean tissue in the hood. Open the vial and transfer the content to a 15 ml tube already containing 5 ml of fresh medium. Spin down at 1000 rpm or 200g for 35 mins at 4C°. Aspirate supernatant. Re-suspend cells in fresh medium and transfer to 150mm x 25mm tissue culture dish. Check the cells under the microscope. Cells are cultured in a CO2 incubator and the medium is changed about every 3 days. It usually takes 3 days or more for cells to recover from freezing. After cell culture reaches 80-85% confluence, a subculture is conducted.

3.2.4. Cell Culturing

Cells were grown in DMEM media supplemented with 10% FBS, 1% penicillin and streptomycin 0.1% amphotericin-B. High glucose media contain L-glutamine and sodium bicarbonate solution. The cells were maintained as monolayers in 25 cm^2 plastic tissue culture flasks at 37C° in a humidified atmosphere containing 5% CO2 in air. Exponentially growing cells were used in all the experiments.

After cell culture reaches 80% confluence, a subculture is conducted. Remove media from the dish. Wash 1x with 10 ml of PBS. Add 5 ml of Trypsin and trypsinize for 5 mins at 37C°. Whack hard you should see the cells coming down. (It is important to remember that you should: never over trypsinize the cells, so work quickly). Add 5 ml of media and use it to rinse the dish to detach the cells off (4-5 times). The serum in the media will neutralize the trypsin. Spin down at 1000rpm for 3-5 mins at room temperature. Aspirate supernatant. Add 15 ml of media to 15ml tube

containing cell pellet, and pipette up and down to mix. Then, count the cell number by adding a drop on improved neubauer slide. Add 15 ml of media to each new 150mm x 25mm tissue culture dish(we split 1 dish into 3-4 dishes) add 5mls of cell culture containing media to each tissue culture dish. Make sure the media covers the entire area of the dish. Put the cells into 37C° with 5% CO2.

3.2.5. Laser Therapy and Zinc Oxide nanoparticle

P1 LASER,PIOON Medical Diode Laser

Different nano particle concentrations were used to treat the cells (0.5-1000 µg/ml)

Then three concentration (10,20 and 40 µg/ml) choose for treat with laser

Three laser power were test with three concentration

3 j/cm^2 (0.2W and 15 s)

5 j/cm^2 (0.5W and 10 s)

6.4 j/cm^2 (0.8W and 8 s)

And for another test choose 5 j/cm^2 and 20 µg/ml concentration of nanoparticle, for each experiment we have three different times (24, 48 and 72hrs).

3.2.6. Diphenyl Tetrazolium Bromide (MTT) Assay

Cell suspensions were seeded into a 96-well-plate and incubated for 24 hr (5- $6x10^3$ per well). Cells were allowed to reach exponential growth by resting at least 24 hours which is equal to one cell cycle duration of selected cells. After removing the medium, 100 µL medium was added to each well. The cells were incubated with 10 µL of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide (MTT), which was dissolved in 90 µL of medium for 4hr after each treatment (0.5, 1, 5, 10, 20, 40, 75, 100, 200, 1000) µg/mL. Cells that were alive, cleaved the yellow tetrazolium salt to an insoluble precipitate (formazan). The decrease in the percentage of living cells is correlated with the amount of formazan precipitate crystals (Fotakis and Timbrell, 2006). Then discarded the supernatant, kept the formazan precipitate and added 100 µL DMSO into the wells. The absorbance of the specimen was measured at 540 nm with a BioTek ELx808 microplate reader .The percentage (%) of cell viability was calculated by the following formula:

$$\% \text{ of cell viability} = \frac{Absorbance\ of\ treated\ cell}{Mean\ absorbance\ od\ control\ cell} \times 100$$

Cell viability results were shown as percentages in comparison with the control group to quantify the sensitivity of selected cell types the half- maximal inhibitory concentration (IC50) is the L.carduchorum concentration required for a 50% inhibition of cell growth was also measured.

3.2.7. Cell Cycle Analysis by Propidium Iodide Staining

Cells were seeded at 5x105 cells per well in six-well plates, treated with L.carduchorum, and harvested as described above. Cells were washed twice with DPBS and resuspended in 1.2 mL of ice-cold DPBS in polypropylene flow cytometry tubes. Next, 2.8 mL of 100% ice-cold ethanol was added dropwise with gentle vortexing, to achieve a final concentration of 70% ethanol. The fixed cells were stored at -20C° overnight, washed twice by centrifuging at 200x g for 10 mins at 4 C° and aspirating the supernatant. Cells were resuspended in freshly prepared propidium iodide (PI) staining solution consisting of 200 µg/mL PI (Sigma-Aldrich), 200 µg/mL RNase A (Sigma-Aldrich), and 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in DPBS, incubated at 37 C° for 15 mins, and then placed on ice protected from light. Stained cells were analyzed using a FACSCanto II (BD Biosciences) flow cytometer, acquiring at least 50,000 single-cell events per sample. Quantification of the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle was performed using the Watson (Pragmatic) model in FlowJo v10.4.1 (FlowJo, LLC, Ashland, OR, USA).

3.2.8. Apoptosis Assay by Annexin V and Propidium Iodide Staining

Cells were seeded at 5 x105 cells per well in six-well plates, treated with L.carduchorum, and harvested as described above. Cells were washed twice with DPBS and stained with the Annexin-V-FLUOS staining kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer‘s instructions. To compensate for the overlapping spectra of annexin V and PI, additional unlabeled and single-labeled samples, which contained dead cells, were prepared. Necrotic cells were prepared by heating a cell suspension in DPBS at 63 C° for 30 mins. Cells were analyzed

using a FACSCanto II (BD Biosciences), gating out debris and doublets, and acquiring at least 10,000 single-cell events per sample. Quantification of viable (double-negative), early apoptotic (annexin V-positive), late apoptotic (annexin V and PI double-positive) and necrotic cells (PI-positive) was performed using FlowJo v10.4.1 (FlowJo, LLC).

3.2.9. Quantitative Analysis of Gene Expression

According to the manufacturer's recommendations, the total amount of RNA was extracted from the Caco2 cells treated with ZnO2 and Diode laser in each step (0, 24, 48, 72, and 96 hr) by using RNeasy Mini Kit (Qiagen, USA). Concentrations of RNA were determined by UV spectrophotometry (Eppendorf, Germany). Add 500 µl ice-cold RNXTM – PLUS solution to 2ml tube containing trypsinated cell, Vortex 5-10 secs. and incubate at room temperature for 5 min. Add 200 µl of Chloroform. Then, mix well for 15 secs. by shaking (Do not vortex). Incubate on ice or 4C° for 5 mins. then centrifuge at 12000 rpm at 4 C° for 15 mins. Transfer the Aqueous phase to a new RNase-free 1.5 ml tube, (do not disturb the mid-phase) and add an equal volume of Isopropanol. Gently mix and incubate on ice for 15 mins. Centrifuge the mixture at 12000 rpm at 4 C° for 15 mins, then discard the supernatant and add 1 ml of 75% Ethanol, shortly vortex to dislodge the pellet and then centrifuge at 4 C° for 10 min. at 7500 rpm. Discard the supernatant and let the pellet dry at room temperature for a few minutes (do not let dry completely, it will decrease the solubility of the pellet). The cDNAs were synthesized from 500 ng of DNAase-treated RNA samples with a Quantitect Reverse Transcription Kit by using oligo (dT) primers. Thaw template RNA on ice. Thaw gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water at room temperature (15–25 C °). Mix each solution by flicking

the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then keep on ice, prepare the genomic DNA elimination reaction on ice 2µl gDNA, 4µl RNA and 8µl RNase-free water, total reaction volume is 14µl incubate for 2 mins at 42 C°, then place immediately on ice. Prepare the reverse-transcription master mix on ice: 1µl Quantiscript Reverse Transcriptase, 4µl Quantiscript RT Buffer, and 1µl RT Primer Mix, with 14µl entire genomic DNA elimination reaction, total reaction volume is 20µl mix and then store on ice. Incubate for 15 mins at 42C°. Place the reverse-transcription reactions on ice and proceed directly with real-time PCR. For long-term storage, store reverse-transcription reactions at –20C°. The specific primers used in PCR reactions are listed in Table and were created by the program Oligo7 for designing primers (Tehran, Iran). PCRs were performed by using Master Mix and Cyber Green in an Applied Biosystems Step One™ thermal cycler (Applied Biosystems, USA). The PCR program started with an initial melting cycle to activate the polymerase for 5 mins at 95 C°, followed by 40 cycles of melting 30s at 95 C°), annealing (30 s at 58 C°), and extension (30 s at 72C°). The quality of PCR reactions was confirmed by melting curve analysis. Efficiency was determined by using a standard curve for each gene (logarithmic dilution series of cDNA from the tests). The results were analyzed by $2^{-\Delta\Delta CT}$ method to calculate the relative changes in gene expression specified from RT-PCR.

Table (3-3): Apoptotic gene primers

Genes	Sequences	Temperature
BAX	F: CGAGAGGTCTTTTTCCGAGTG R: GTGGGCGTCCCAAAGTAGG	61 61
BCL2	F: AGCATCACGGAGGAGGTAGAC R: CTGGATGAGGGGGTGTCTTC	63 62.5
P53	F:CGTGTGGAGTATTTGGATGAC R:TTGTAGTGGATGGTGGTACAGTC	59.4 62
B-Actin	F: TGGAATCCTGT0GGCATCCATGAAAC R: TAAAACGCAGCTCAGTAACAGTCCG	60 60

3.2.10. Reactive Oxygen Species Determination

Colorectal cell lines were treated with various nZnO doses and Diode laser then incubated at 37°C for 30 mins with 10 µM dichlorofluorescein diacetate (DCF-DA, Sigma, USA). On a microplate reader, the fluorescence was observed at 525 nm with excitation at 488 nm after three rounds of cell washing (Thermo Fisher Scientific, USA).

-Summary of the DCFDA assay and ROS assay protocols (flow cytometry):

1. Collect cells in tubes
2. A stain for 30 minutes with DCFDA (without washing)
3. Use a flow cytometer to examine

3.2.11. Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA), with the Brown-Forsythe test and GraphPad Prism 8.0.2 (San Diego, USA) being expressed as mean ± SD. Data with P values lower than 0.05 were considered statistically significant.

CHAPTER FOUR

Results

4.1. Cytotoxic effect of ZnO2-NP on Caco2 cell line *in vitro*

Results showed a highly significant ($P < 0.001$) effect of Zinc oxide nanoparticle on Colorectal cancer cell line on different concentrations (10, 20, and 40) µg/ ml.The percentage of cell viability of Caco2 varied as cells treated with different concentrations of Zinc oxide nanoparticle. After treatment with different concentrations (0.5, 1, 5, 10, 20, 40, 75, 100, 200, 1000) µg/ ml, and 5 different readings for each concentration, the high dose was greatly effective in decreasing the proliferation of Caco2 as shown in Figure1. Thus, the highest dose (1000 µg/ ml) decreased the proliferation of Caco2 to mean optical density (M.O.D) (0.165) as compared to the control M.O.D. was (0.5431). As the concentration of treatment had increased gradually and respectively (0.5, 1, 5, 10) µg/ ml in low doses there were no significant effect of treatment on cancer cells. M.O.D. of Caco2 were (0.4834, 0.4342,0.4228 and 0.362), and the viability was similar with a slight inhibition in cells.

After increasing dose of treatment to (20, 40, 75) µg/ ml, the viability with M.O.D. decreased to (0.3456, 0.1752, 0.1568), were highly significant that reduced the growth of cancer cell. The dose of treatment with Zinc oxide nanoparticle increased to (100, 200) µg/ ml, the nanoparticle decreased the viability of Caco2, and the M.O.D. was (0.1514 and 0.155). Zinc oxide nanoparticle was highly effective as cells were treated with (10, 20, 40) µg/ ml.

The concentration of ZnO2-NP required for a 50% inhibition of cell growth (IC50) was obtained by extrapolation from an inhibition curve. The result showed that the IC50 for Caco2 cell was (20µg/ml) (Figure 4-2).

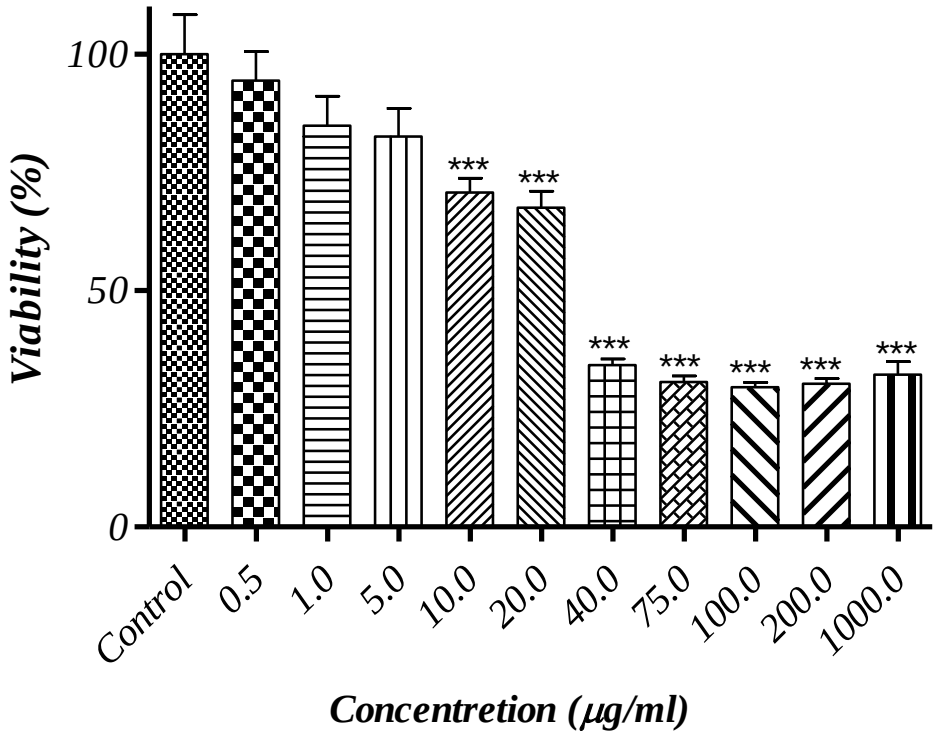


Figure (4-1). *In vitro* cytotoxicity of ZnO2-NP on Caco2 cell line with different concentrations (µg/ ml).

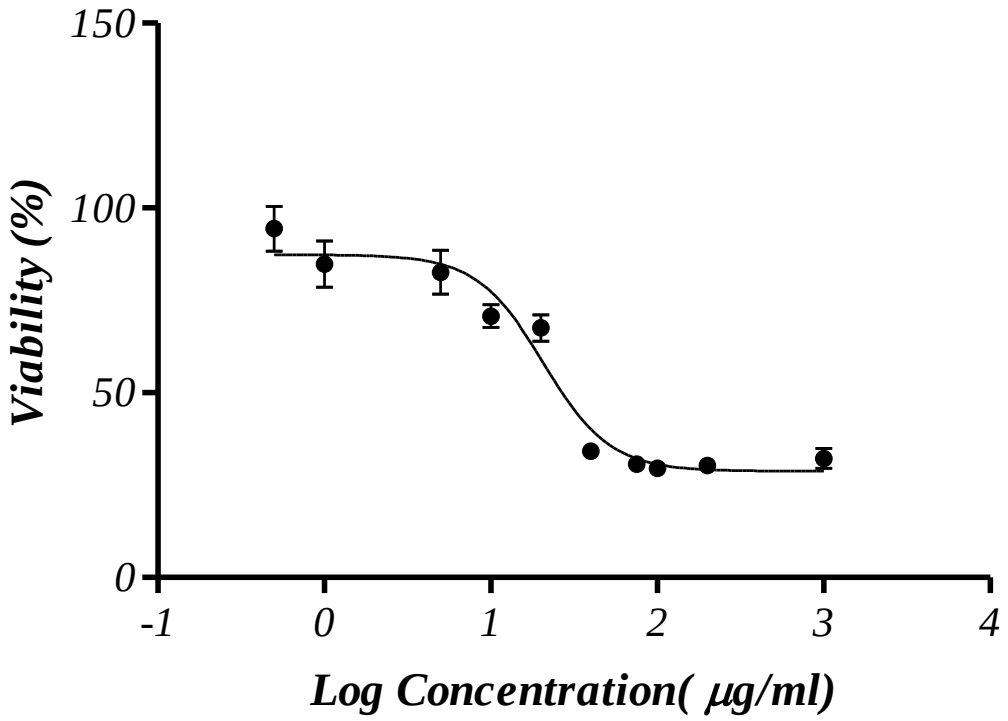


Figure (4-2). The half-maximal inhibitory concentration (IC50) of ZnO2-NP in Caco2 cells the (IC50 was 20 μg/ ml).

4.2. Analysis of Apoptosis in Caco2 cells treated with ZnO2-NP using Flow Cytometry

In this work, cells from a colorectal cancer cell line were employed, and they were stained using an Annexin-V-FLUOS labeling kit. By triggering apoptosis, laser and zinc alone and in combination were tested to see how they affected cell growth.

As shown in Figure (4-3), the combination of laser and zinc nanoparticles had a substantial impact on the proportion of cells undergoing early apoptosis compared to living, late apoptosis, and necrosis. The nano-laser combination therapy has an effect on late apoptosis and is more effective because the percentage of cells in late

apoptosis decreased significantly compared to early apoptosis and necrosis (p-value<0.05). In contrast, the use of zinc nanoparticles and laser to reduce the percentage of cell lines had clear effects on the early apoptosis (p-value<0.05), as evidenced by the decrease in the number of cell lines in the early apoptosis, which was not evident in the late apoptosis and necrosis Figure 4. Necrosis is a distinct phase that is more resistant to all forms of treatments, and when compared to live cases, the percentage of cell lines declines by a small amount.

Using a fluorescently tagged annexin V that binds to phosphatidylserine, flow cytometry may detect the early-stage apoptotic event known as phosphatidylserine exposure to the outer leaflet of the plasma membrane (Q3). While necrotic cells (Q1) stain only with PI, late apoptotic cells (Q2) have lost the ability to maintain the integrity of their cell membrane, allowing PI to penetrate (annexin V and PI double-positive). Early apoptotic cells exhibit increased staining only with annexin-V (annexin-V positive, PI negative). Annexin V and propidium iodide do not label viable cells (Q4) (annexin V negative, PI positive).

The results of the control and three times repeated experiments of treated colorectal cancer cell line (Caco2), 95.7 % of the Caco2 control cells in the experiment were alive and functional., and 96.9% of untreated were live as demonstrated in Figure 4. After cells received treatment with specified concentrations (IC50) and the process was repeated three times, An increase in annexin V staining, a sign of apoptosis, is caused by laser and zinc-oxide nanoparticle treatment in colorectal cancer cell lines (Figure 4-4). Zno and Laser significantly increased Caco2 cells that are necrotic and early apoptotic are more prevalent than control cells, this marked increase occurred but not significant. The mean of live cells for treated Caco2 cell with laser were 69.1% , Zno2 were 45.9% , Zno2 and Laser were 45.0% . The mean percent of necrosis were 20.5% for Laser treatment ,48.4% for Zno2 treatment and for

Zno2+Laser 31.3%, (Figure 4-3) shows the difference and mean of treated cells with Zno2 and Laser. There was a substantial rise in early and late apoptosis in Caco2 after cells treated with Zno2+Laser, as the mean percent of late apoptosis was 10.5%, and early apoptosis was 12.3% for Caco2 cells.

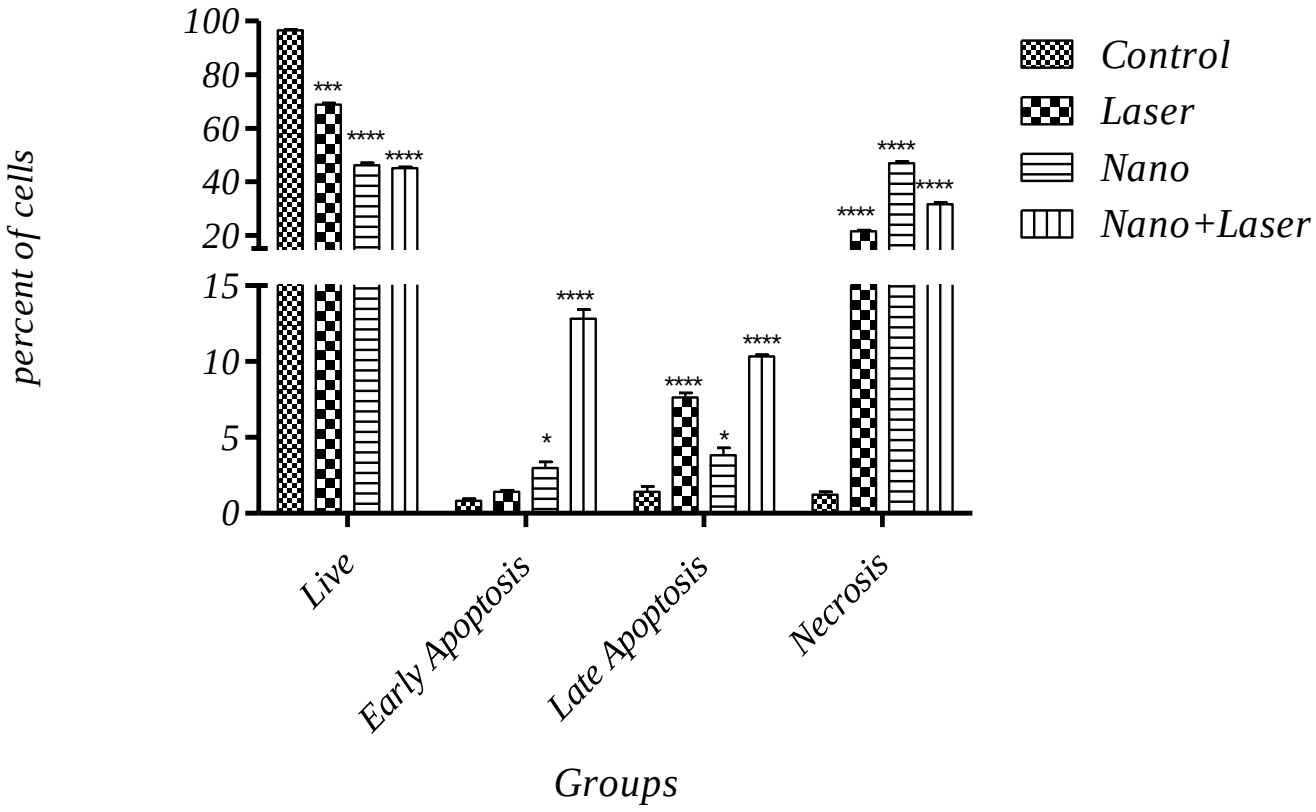
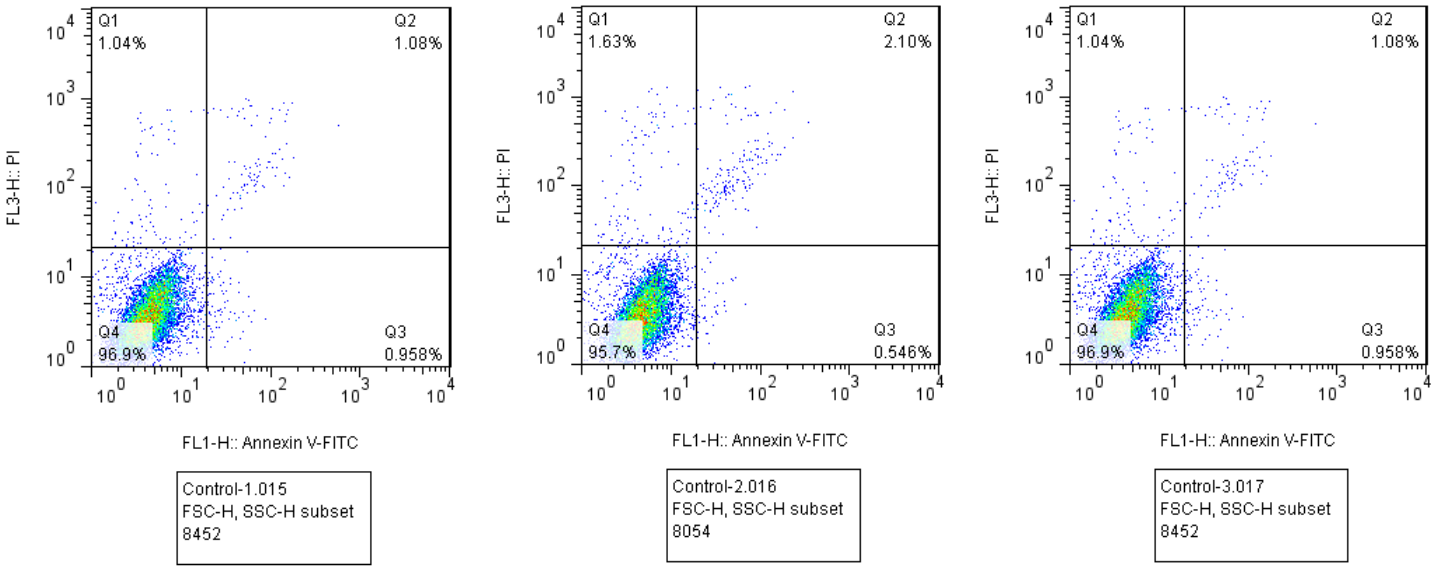
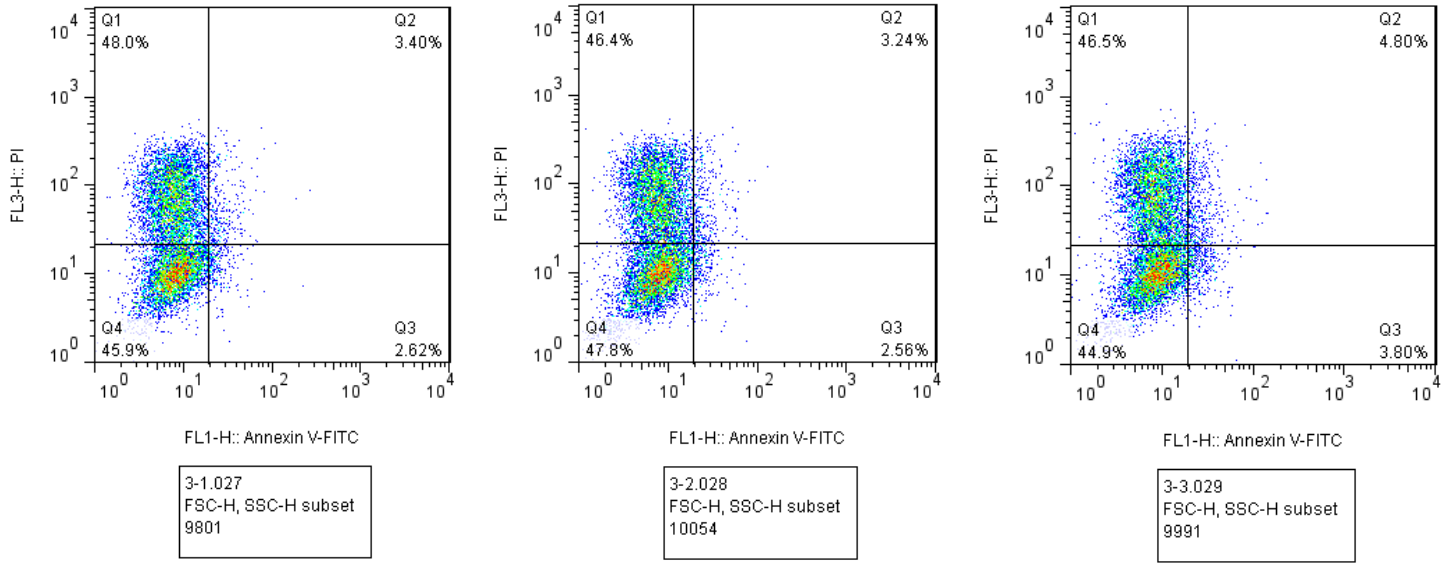


Figure (4-3). The impact of ZnO2-NP, Diode laser, and nano-laser combinations on the acceleration of apoptosis in cancer cell lines.

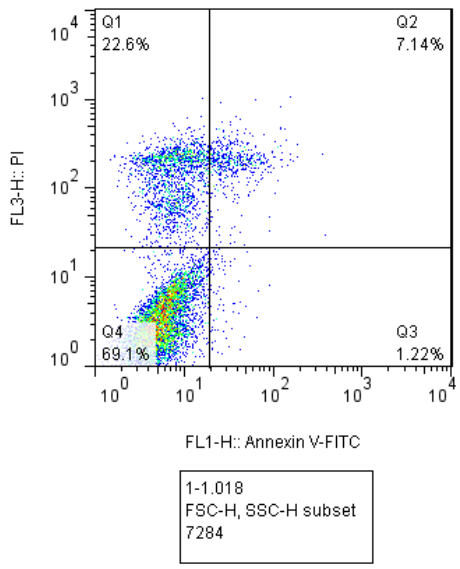


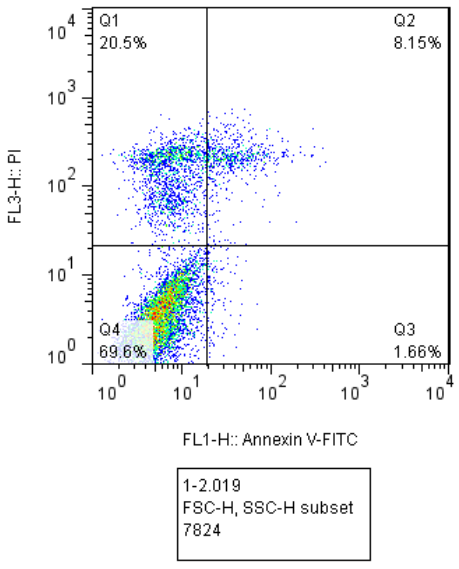
A (Control): 24hrs,48hrs,72hrs

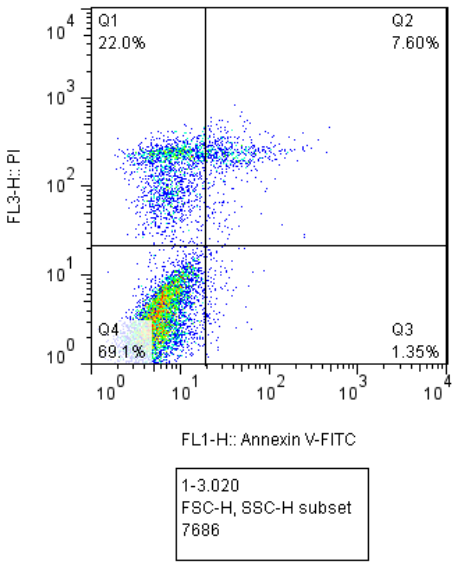


B (ZnO2-NP) : 24hrs,48hrs,72hrs

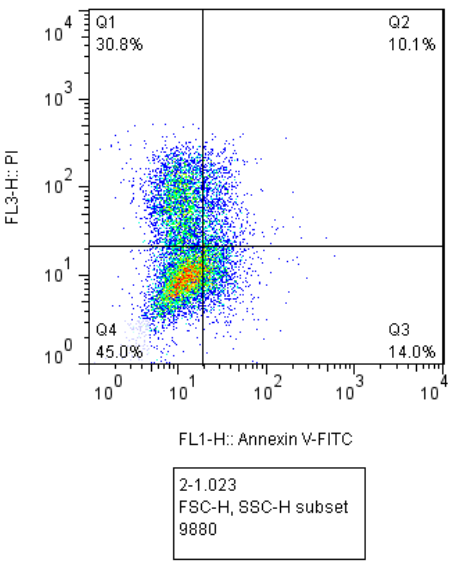
Figure (4-4). Cell line percent distribution of Caco2 cell line treated with (B) ZnO2-NP, (C) Diode laser, (D) nano+laser combination and (A) is Control. *Q1: Necrosis, Q2: Late Apoptosis, Q3: Early Apoptosis, Q4: Live Cells .

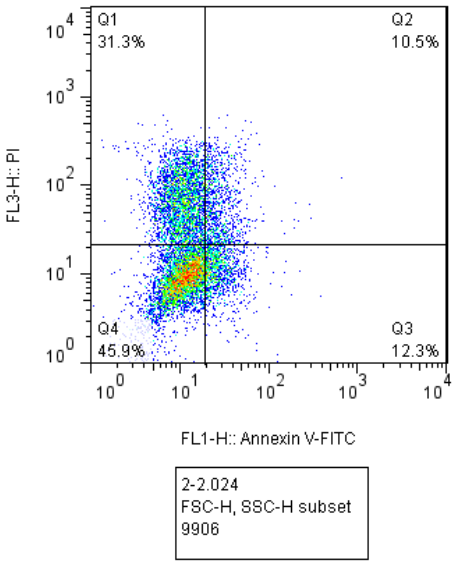


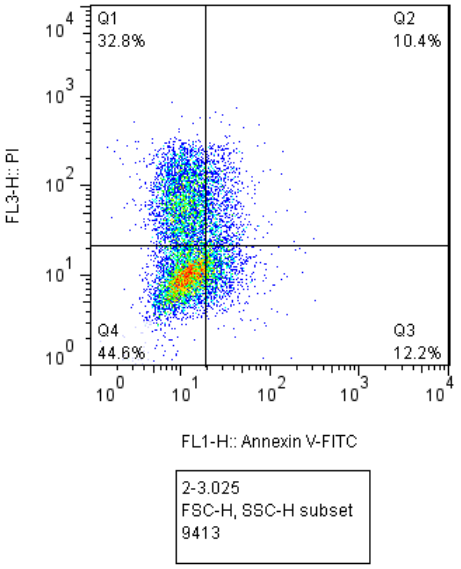




C (Laser) : 24hrs,48hrs,72hrs







D (ZnO2-NP+Laser) : 24hrs,48hrs,72hrs

4.3. Cell Cycle Analysis in CaCo2 cells treated with ZnO2 and Diode laser using Flow Cytometry

To investigate whether Zinc nanoparticles Laser and Zinc+Laser had an impact on the control of the cell cycle, according to the study's cell cycle analysis. The distribution of the cell cycle phase was examined using flow cytometry and PI labeling. Using Multicycle software, the percentage of cells in the G_1, S, and G_2/M phases were determined, respectively. All case of treatments has the same effects on the percent of cell lines in the G_2 phase of cell cycles when compared to the effects on another phase of cell cycles. Figure (4-5), shows that when laser and zinc nanoparticles were used together, there was a significant variation in the proportion of cells that are decreasing in the G_2 phase of the cell cycle compared to S and G_1 phases (p-value<0.05). On the other hand, using zinc nanoparticles and laser alone to reduce the number of cell lines in the phases of cell cycles had clear effects in different proportions (p-value<0.05), which shows that the number of cell lines decreased during G_2 during using laser in alone. The mean frequency G_1 in nanoparticle alone is (52.87), with laser is (72.34) and with combination therapy is (56.28). However mean frequency for G_2 phase with nanoparticle (8.57), with laser (5.38) and with combination therapy is (5.16). And the mean frequency for S phase with nanoparticle (15.9), with laser is (19.05), and with combination therapy is (12.2). Using of zinc nanoparticles in the declining of the percent of cell lines revealed the low proportion that have a low significant difference in the decreasing compared to the alternative treatment case Figure (4-6).

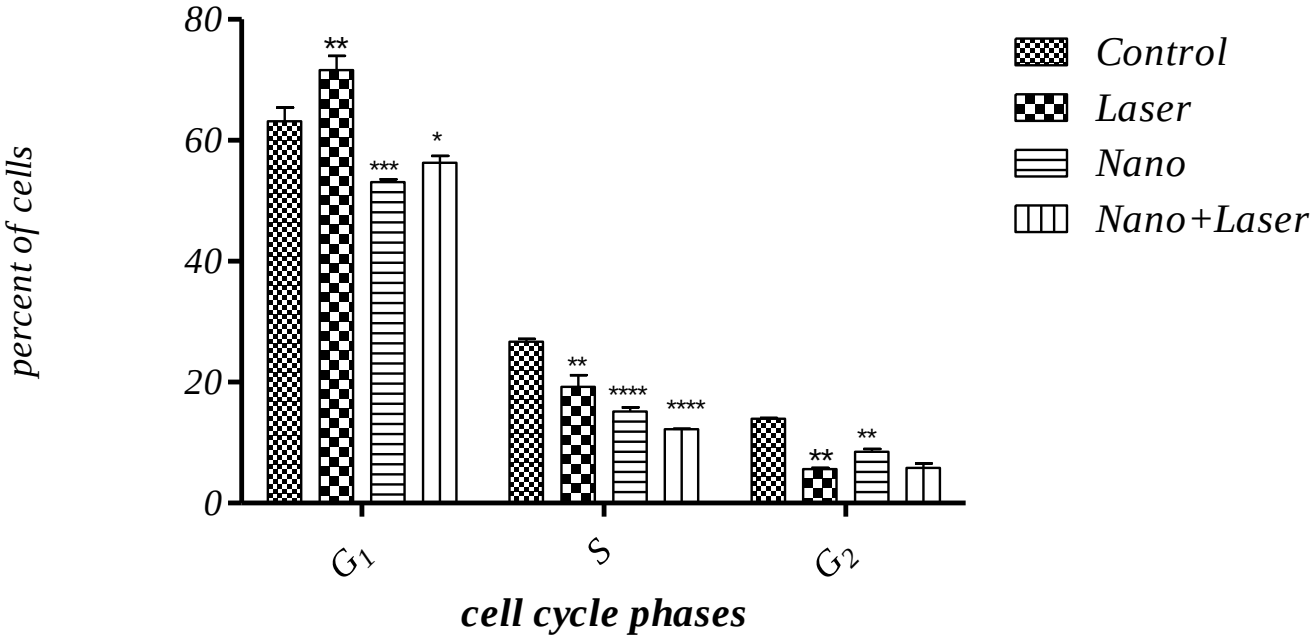
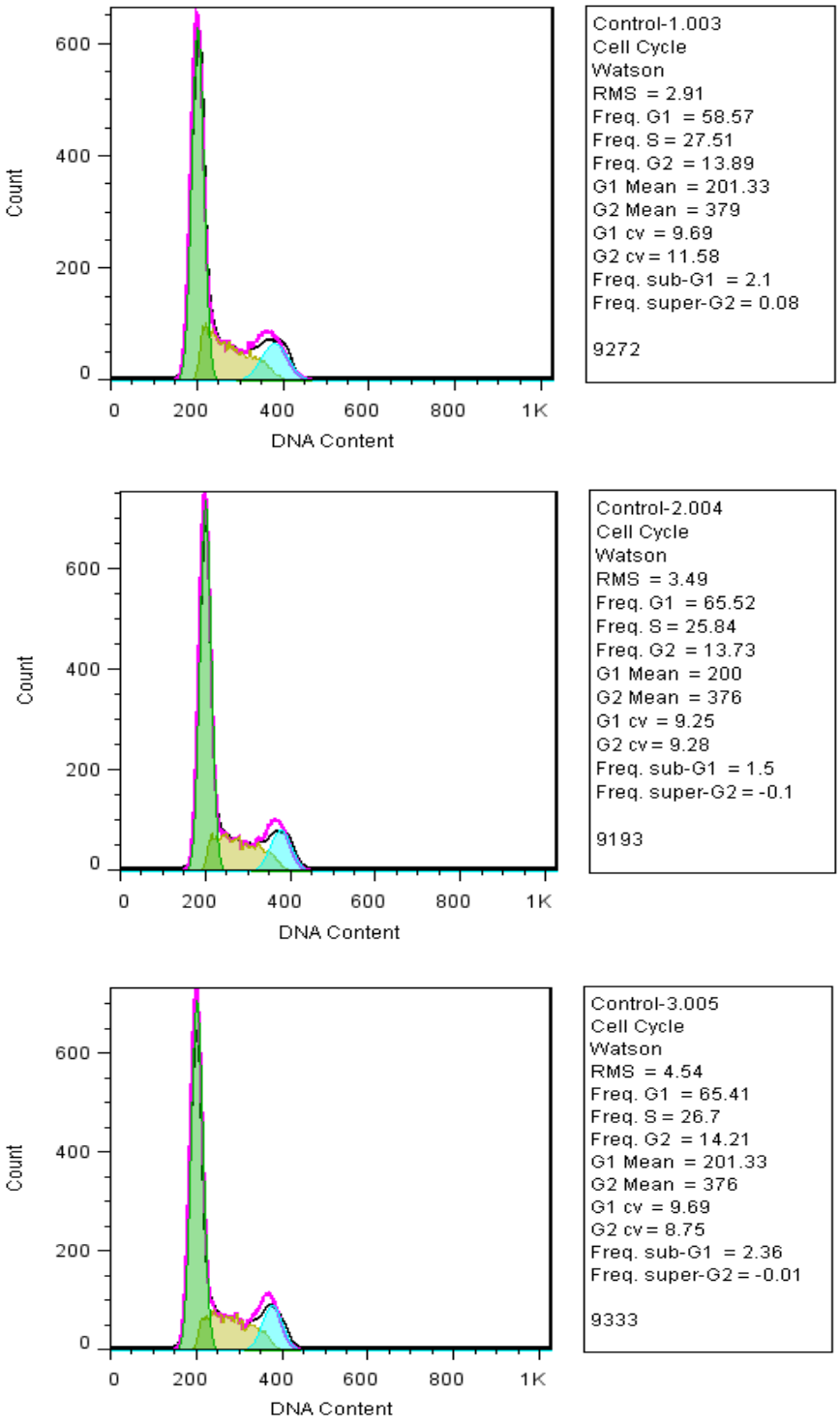
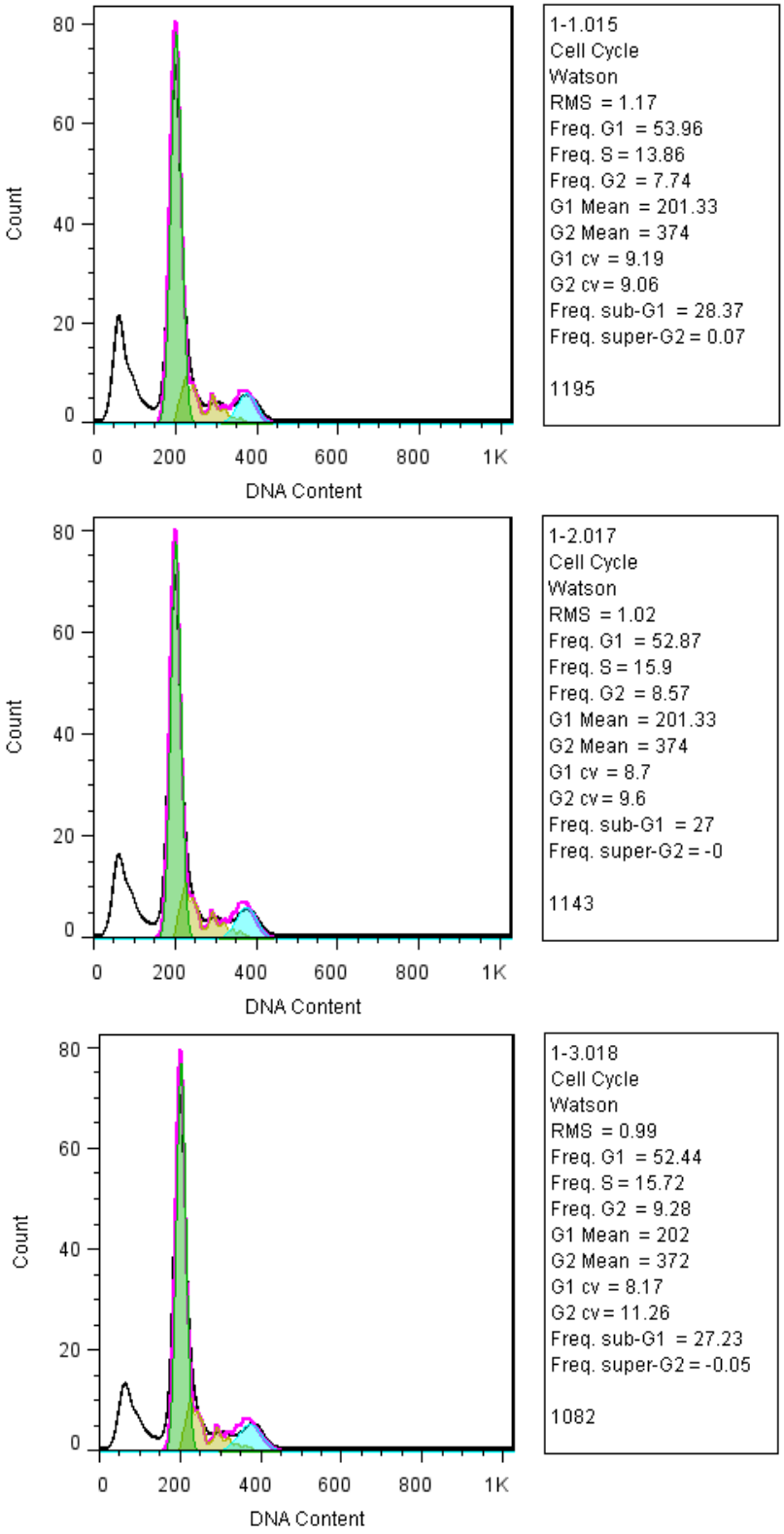


Figure (4-5). The influence of ZnO2-NP, Diode laser, and nano-laser combinations on the percentage of cells in various stages of the cell cycle.

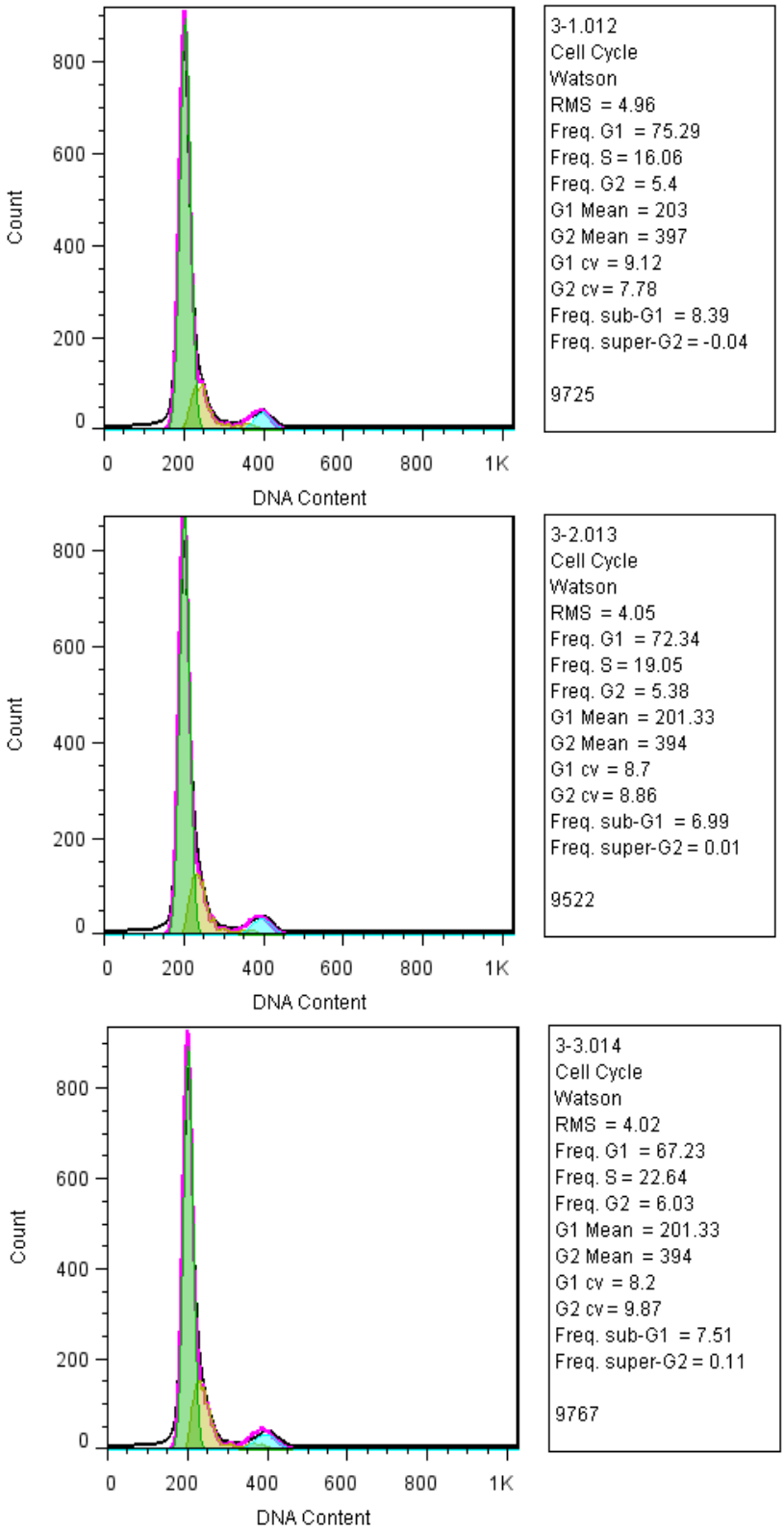


A(control) : 24hrs,48hrs,72hrs

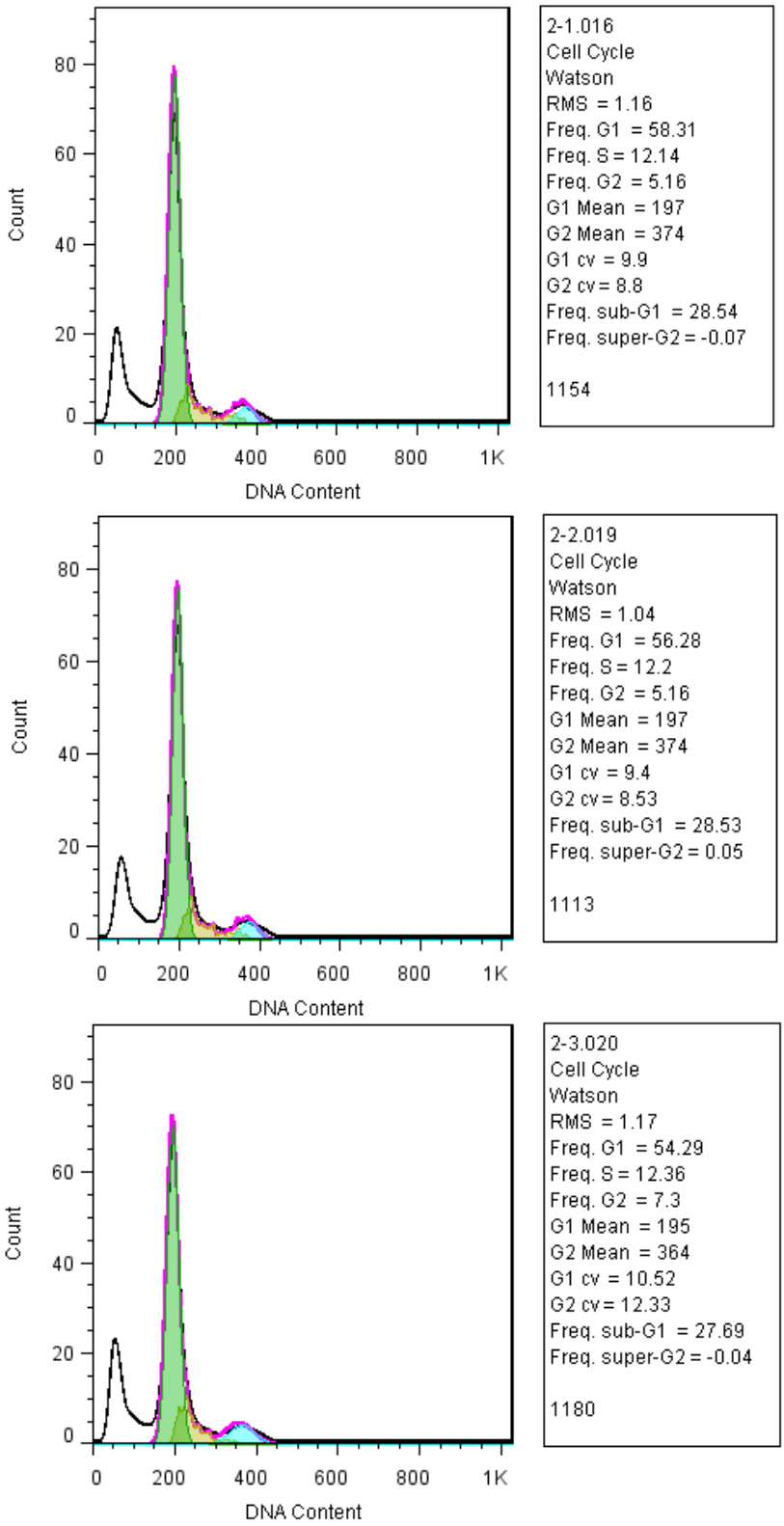
Figure (4-6). Cell cycle analysis of Caco2 cell line treated with (B) ZnO2-NP, (C) Diode laser, (D) nano+laser combination and (A) is Control.



B (ZnO2-NP) : 24hrs,48hrs,72hrs



C (Laser) : 24hrs,48hrs,72hrs



D (ZnO2-NP+Laser) : 24hrs,48hrs,72hrs

4.4. Detection of Reactive Oxygen Species by using Flow Cytometery

Under both physiological and pathological circumstances, oxidative stress is a significant occurrence. In this study, we show how to measure total reactive oxygen species (ROS) in colorectal cancer cell lines using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) labeling. ROS in cells can be found using the quick and affordable DCFH-DA staining method. It can be used to quantify ROS production following chemical or genetic alterations.

Based on the gating of living cells to check the amount of ROS. Based on the average staining ability of living cells (MFI) with fluorescent, about 90% of living cells have staining ability, which are reported with +. But for more accuracy, we report this amount with - because 10% of living cells may not have been dyed. When DCFH is exposed to oxygen, it turns into the fluorescent molecule 2′7′-dichlorofluorescein (DCF). Dichlorodihydrofluorescein diacetate (DCFH-DA). DCFDA is good to quantify ROS and nitric oxide. But DCFH-DA is an indicator of peroxynitrite formation.Both are cell permeable and once inside the cell, they undergo enzymatic cleavage of the diacetate groups by cytoplasmic esterase. After the removal of acetate, they readily get oxidized to form highly fluorescent product dichlorofluorescein (DHF). DCFDA can be oxidized by H2O2, NO or Superoxide, but DCFH-DA is oxidized by Peroxynitrite only and neither NO, superoxide, nor hydrogen peroxide alone appear to oxidize DCFH .

The percent DCFH- for control is (0.411%), and DCFH+ is (99.6%), the mean is (352). As showen in Figure (4-7). And abut nanoparticle therapy the DCFH- is (16.3%), and DCFH+ is (83.7%), the mean is (46.4). With laser therapy the percent of DCFH- is(16.2%), and DCFH+ is (83.6%), the mean is (32.7). However for the combination therapy the percent of DCFH- is (9.44%), and DCFH+ is (90.6%), the mean is (125).

The use of zinc nanoparticles and laser alone with zinc nanoparticles and laser in combination to lower the percentage of cell lines in DCFH- compared to DCFH+ (Figure 4-7), highly observed changes and strong significant differences have been recorded (p-value<0.05). the percent of cells in the control remains as high rates and decreased in all treats cases with different ratio (Figure 4-8).

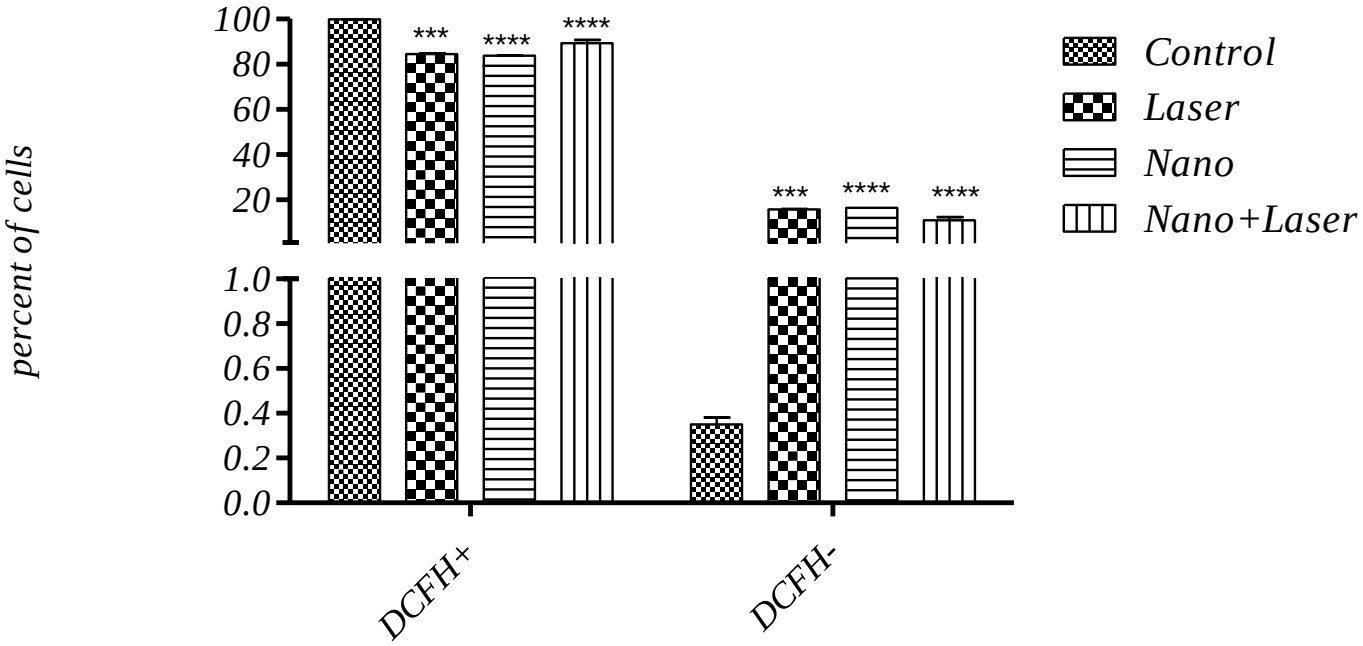
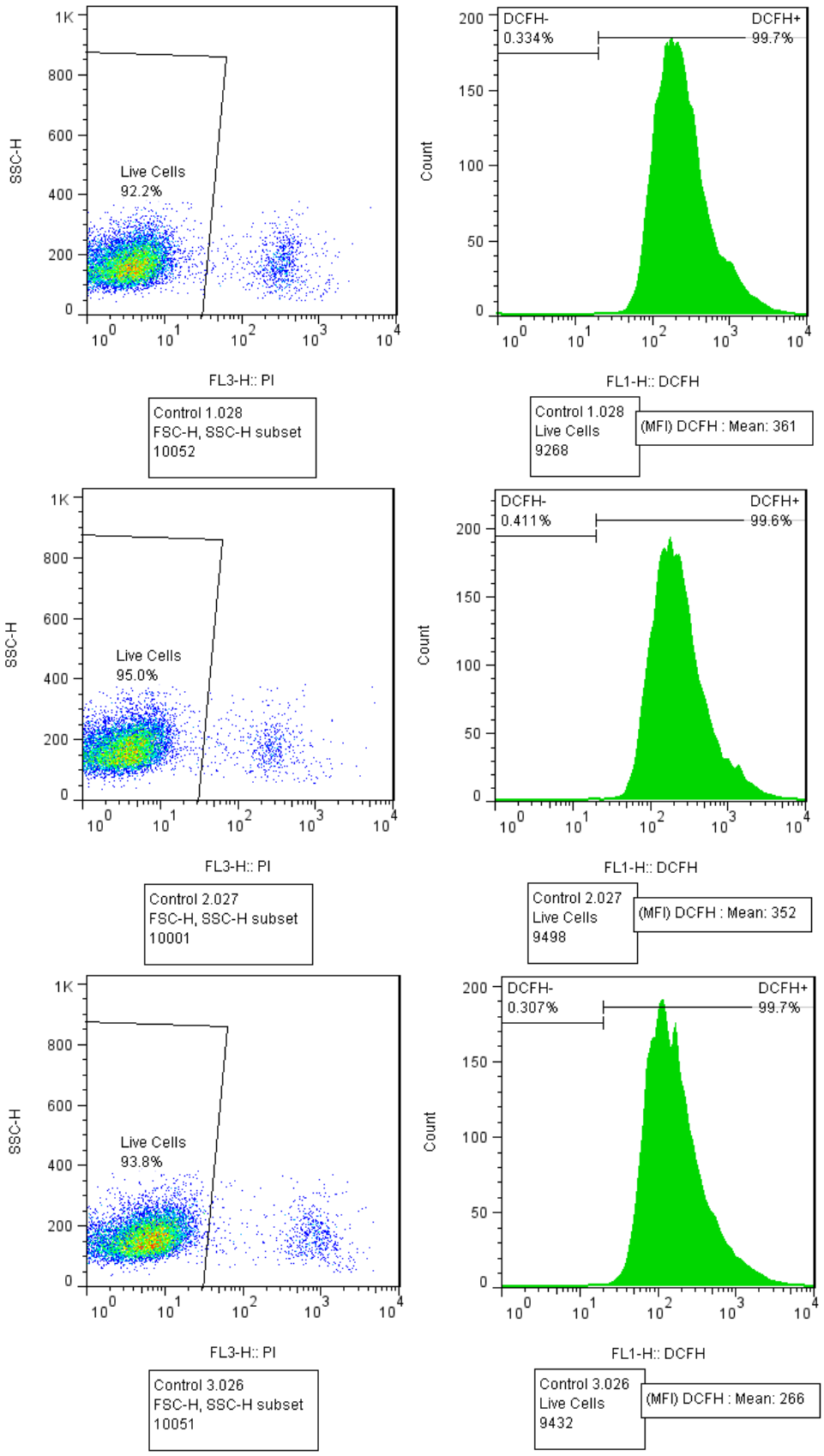
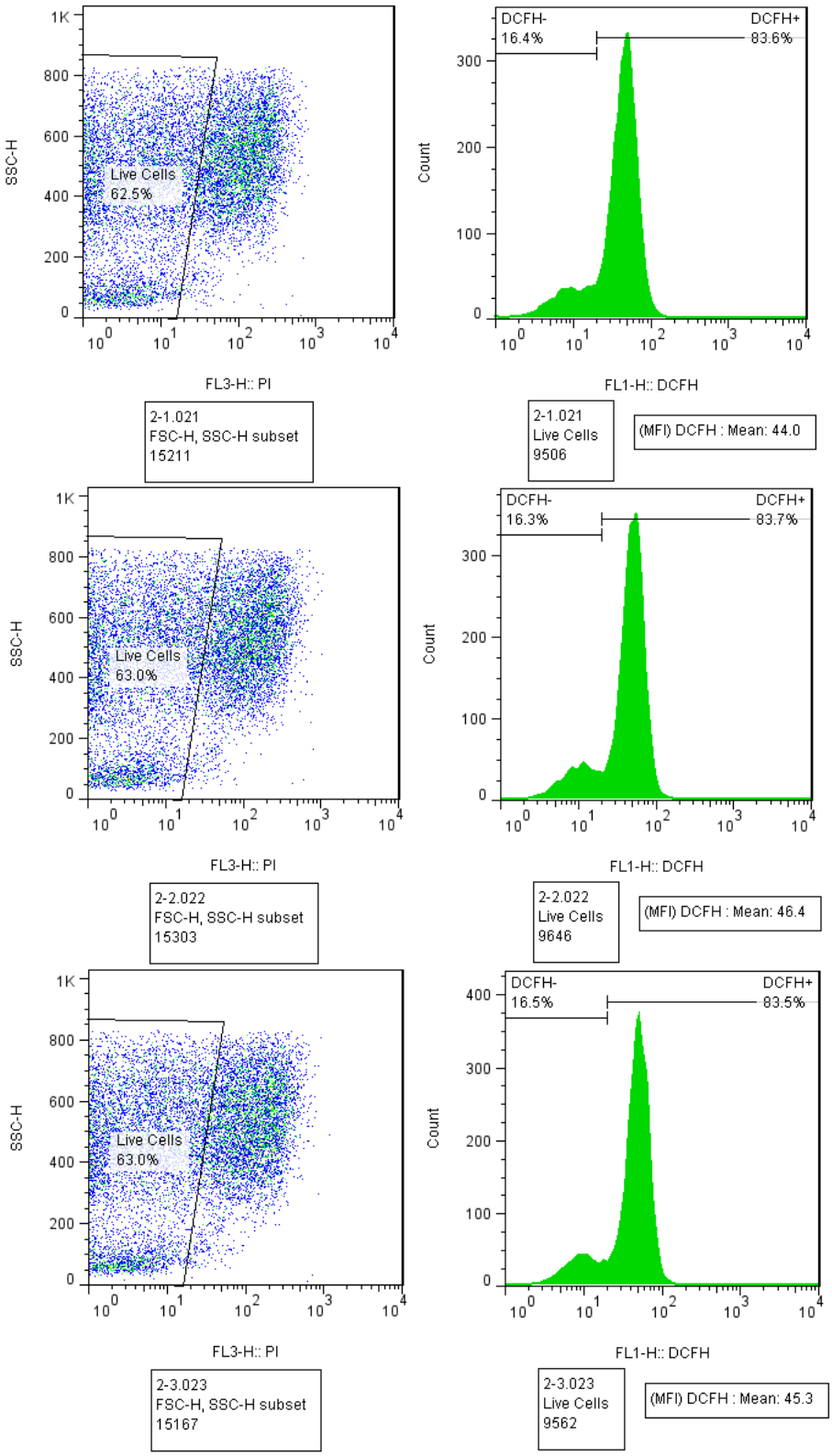


Figure (4-7). The impact of nano, laser, and nano-laser combinations on reactive oxygen species (ROS) in DCFH+ and DCFH-.

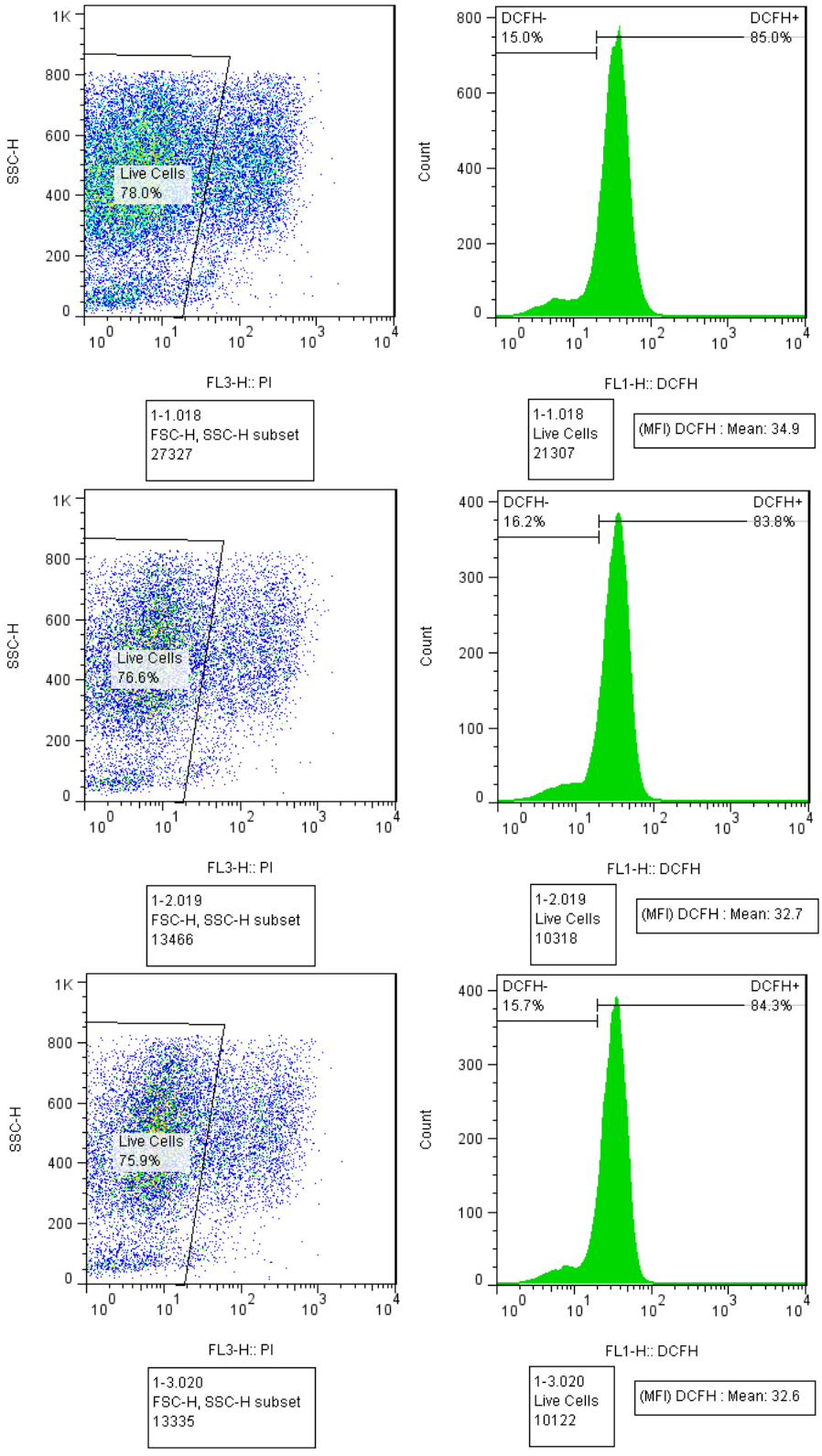


A(Control) : 24hrs,48hrs,72hrs

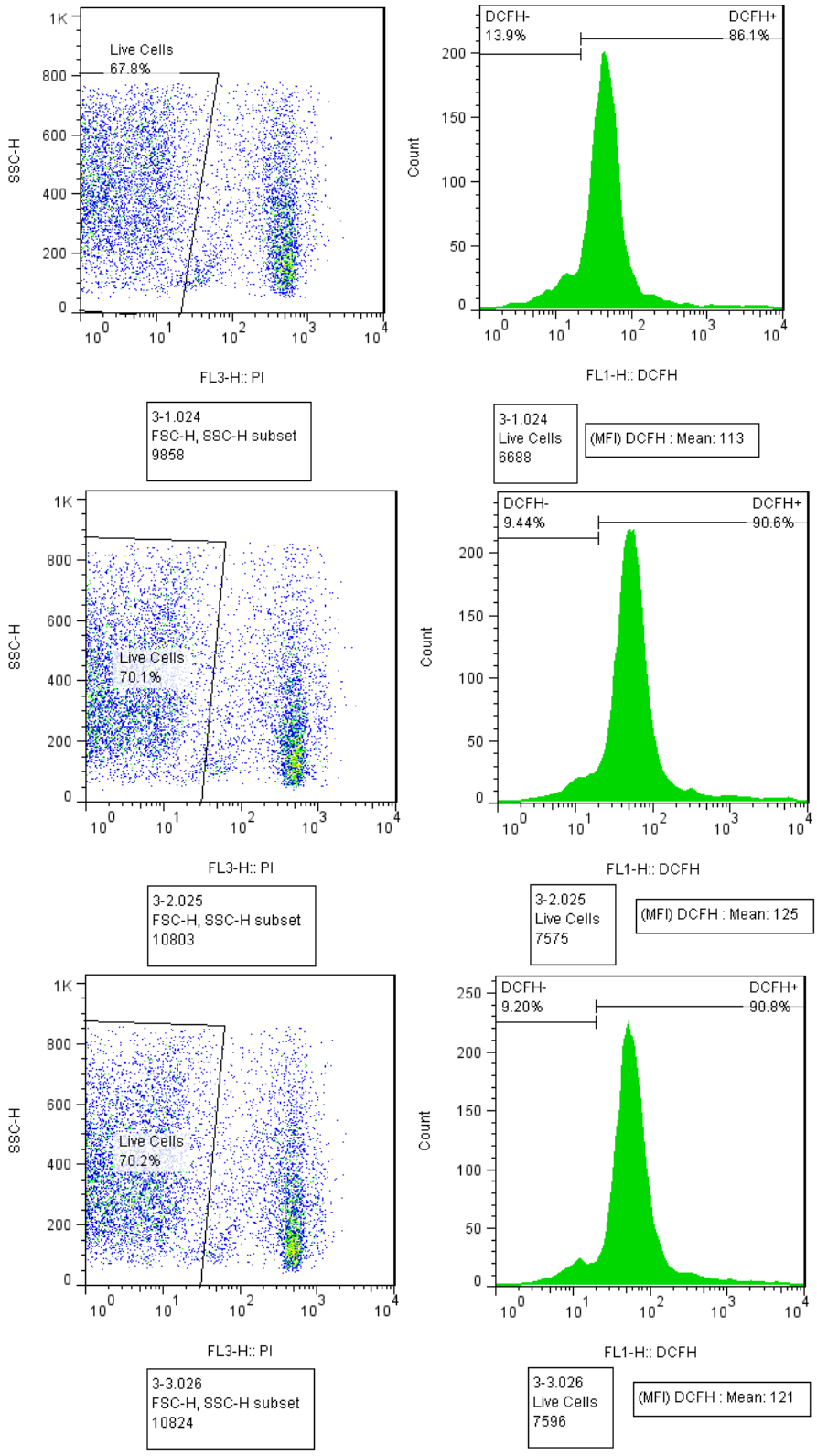
Figure (4-8). ROS determination in both DCFH+ and DCFH- Caco2 cell line treated with (B) ZnO2-NP, (C) Diode laser, (D) combination and (A) is Control.



B (ZnO2-NP) : 24hrs,48hrs,72hrs



C (Laser) : 24hrs,48hrs,72hrs



D (ZnO2-NP +Laser) : 24hrs,48hrs,72hrs

4.5. Expression of Apoptotic Genes in CaCo2 Cells Treated with ZnO2 and Diode laser

Results which is investigated Real-time PCR were used to examine the expression of certain apoptotic genes (P53, BAX, and Bcl2), and the results revealed a significantly significant difference in the using of diode laser with zinc nanoparticles for block the cancer progression by increasing the expression of *p53* gene (p-value<0000). In addition, in the treating of cancer cell lines via *BAX* gene expression (apoptosis regulator BAX), using both nano+laser affect the expression of BAX gene and accelerate and increase the rate of the gene and finally cause the death of cancer cell, which have significant difference when compared with each of control, treating with nano and laser in alone (p-value<0.05). However, there is no revealed any significant difference in case of Bcl2 gene during using combination treatment of laser with zinc nanoparticles (p-value>0.05).

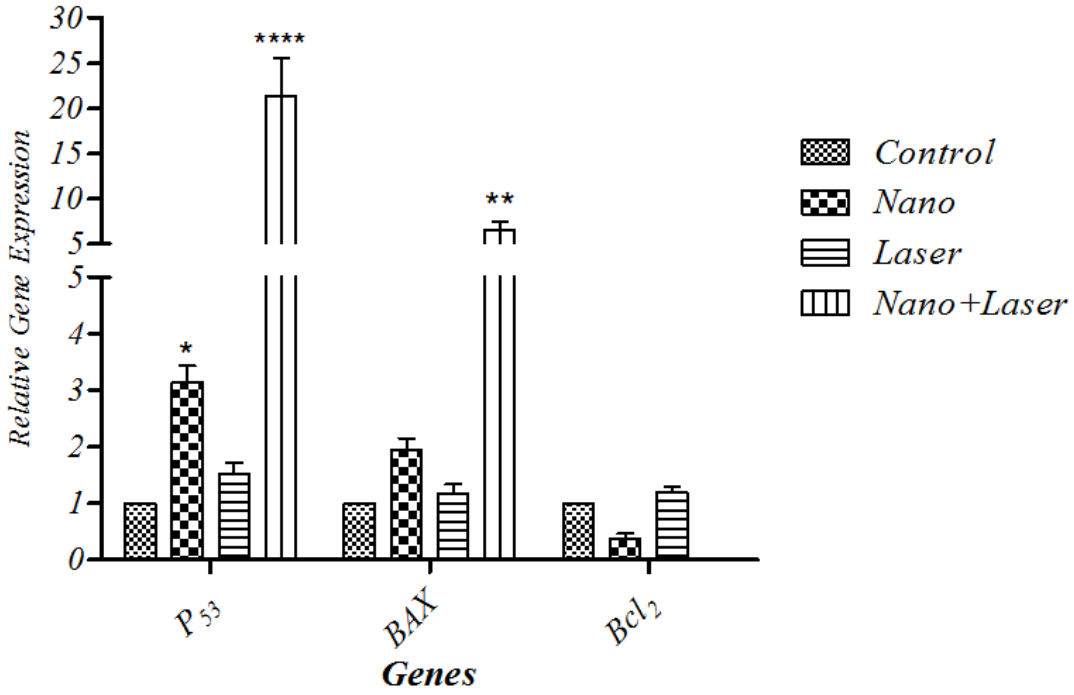


Figure (4-9). The effects of nano, laser and nano-laser combination in the cancer cell lines' gene expression.

CHAPTER FIVE

Discussion

5. Discussion

As a heterogeneous illness, colorectal cancer progresses to the formation of polyp-like malignant tumors in the rectum and colon's inner walls (Fanelli et al., 2020). Similar to other cancer treatments, (ZnO NPs) zinc-oxide nanoparticles are effective in treating colon cancer(Selim et al., 2020). In a recent study, Caco-2 cells were exposed to silver and ZnO nanoparticles to compare their cytotoxicity. It was discovered that by increasing the levels of ROS, ZnO nanoparticles are more harmful than silver NPs(Song et al., 2014). PDT is a recognized and effective technique to treat several cancer types (Yu et al., 2018, Hosokawa et al., 2018).

This study, which is work on colorectal cancer cell lines through 3 different types treating that are divided into 3 groups (Nanoparticle, Nanoparticle+Laser, Laser) Therapy. These therapies are used to assess the relative expression level of three different targetgenes including P53, Bcl2, and Bax. There is a different concentration of ZnOP and laser wavelength for Cell cycle, ROS, and qRT-PCR. According to recent research that used laser and gold nanoparticles on colon cancer in 2020, which is indicated based NPs exhibited the most efficient cytotoxicity and markedly enhanced inhibition effect on cells proliferation to SW480 cells under laser exposure when compared to the NPs merely with PTT or chemotherapy(Zhang et al., 2020b). In 2021 this study decide that Several (although small) *in vitro* and in vivo clinical experiments have examined the efficacy of PDT in the elimination of CRC, and the results have shown the treatment's exceptional potency with very few side effects(Ma et al., 2021). Despite the positive results, typical PSs have limitations that prevent PDT from reaching its full potential, including inadequate tumor

targeting, insufficient quantum yield, limited cellular absorption, and insufficient penetration depth (Montaseri et al., 2021). In this regard, NPs have been highlighted as favorable platforms to enhance the delivery of the PSs into the targeted CRC tumors, in 2021 (Nkune et al., 2021, Kruger and Abrahamse, 2019). NPs are prospective PDT options because they might help overcome some of the challenges: they can improve the PSs' bioavailability and solubility, their tumor selectivity and specificity with minimal side effects, and their overall increased PDT efficacy (Montaseri et al., 2021).

Furthermore, this study's results show that the combination of laser and zinc nanoparticles had a significant influence on the percentage of cells undergoing early apoptosis compared to living, late apoptosis, and necrosis. ZnO-NPs produced biologically had anticancer properties in HCT-116 cell lines, according to some research (Majeed et al., 2019). Numerous cancer cells were shown to undergo cell cycle arrest and death after being exposed to ZnO nanoparticles, according to studies(Boroumand Moghaddam et al., 2017, Rahman et al., 2016). Data analysis by (Sanaeimehr Z, Javadi I, and Namvar FJCn) demonstrated that the vitality of the treated cell lines was dose-dependent; as the concentration of ZnO-NPs rose, the viability fell (Sanaeimehr et al., 2018).

Growth inhibition is frequently caused by cell cycle arrest. Propidium iodide was employed as a probe in cell cycle distribution analysis flow cytometry to examine the effects of this substance on cell cycle progression and determine whether the action of extracts involves changes in cell cycle progression. Propidium iodide, a water-soluble dye that intercalates DNA, binds to a certain amount of DNA in a cell, and the amount of DNA in a population of cells determines where they are distributed during the cell cycle (Heidari et al., 2014).

This study discovered that when laser and zinc nanoparticles were used together, Compared to the S and G1 phases of the cell cycle, the percentage of cells dividing less rapidly in the G2 phase was significantly lower. According to research, ZnO nanoparticles can induce cell cycle arrest and death in a variety of cancer cells, which can inhibit the proliferation of cancer cells (Boroumand Moghaddam et al., 2017). Cell proliferation is characterized by the progression of the cell cycle. Cancer development and progression have been connected to dysregulation of the cell cycle (Drexler, 1998). As a result, one of the alluring therapeutic targets for the treatment of cancer is the cell cycle. Nevertheless, cell cycle-targeting siRNA or small molecule inhibitors have been created (Wang et al., 2011). Cell cycle arrest at the S-phase has only occasionally occurred, with the majority of chemotherapeutic drugs causing cell cycle arrest either at the G0/G1 or the G2/M stage. Cyclin-dependent kinase (CDK) enzymes are a class of proteins that regulate the cell cycle (Wohlbold et al., 2006). Through their connection with particular inhibitors, kinases, cyclin partners, and phosphatases, CDKs can control their activity (Wohlbold et al., 2006).

The results demonstrate that ZnONPs were responsible for cancer cell death by testing each therapeutic component separately and comparing them to the multi-component platform, which is consistent with other research demonstrating that ZnONPs are highly hazardous to cancer cells. Reactive oxygen species are known to cause oxidative stress, which malignant cells produce at greater inducible levels than normal cells, which is one of the ways hypothesized to mediate toxicity (Valachis et al., 2011, Larner et al., 2015, Hanley et al., 2008).

Furthermore, our study shows that using zinc nanoparticles and laser alone with zinc nanoparticles and laser in combination to reduce the proportion of cell lines in DCFH- compared to DCFH+, highly observed changes and strong significant differences have been recorded. Earlier research has shown that for many different

forms of cancer, a possible therapeutic is photodynamic therapy (PDT). PDT produces ROS by utilizing interactions between a photosensitizer, light, and oxygen (Dolmans et al., 2003a). The amount of ROS would impact the effectiveness of PDT because ROS plays a significant role in tumor cell death by causing vascular shutdown, oxidative stress, and the immunological response (Xue et al., 2015).

The anti-oxidative capacity of cells can be depleted by high and prolonged ROS build-up, which results in cell death. Cancer cells exhibit higher amounts of cellular ROS in comparison to healthy cells due to their increased metabolic requirements and rapid rate of proliferation. Because of their innately high level of oxidative stress, cancer cells are more vulnerable to ZnO-NPs therapy. Proteins may get ubiquitinated as a result of oxidative damage brought on by high ROS buildup. Proteotoxic stress results from an increase in the workload placed on the cell's protein degradation machinery by a buildup of proteins that have been ubiquitinated. In ovarian cancer cells treated with ZnO-NP, we notice a decline in an anti-oxidative capacity as well as an increase in proteotoxic stress. It is known that cancer cells put together specific proteasome isoforms with higher proteolytic capacity as a result of oxidative stress brought on by metals(Padmanabhan et al., 2016).

The mitochondrial electron transport chain is thought to have a role in the creation of intracellular ROS, and it is also thought that cancer-fighting chemicals that enter cancer cells may harm the electron transport chain, which would cause a large-scale intracellular release of ROS (Moghimipour et al., 2018). As a result, increasing ROS levels cause mitochondrial damage, followed by apoptosis caused by an imbalance in protein activity. (Guo et al., 2013). Because of the elevated intracellular amounts of dissolved zinc ions and increased ROS generation caused by ZnO NPs, cancer cells are cytotoxic and die through an apoptotic signaling pathway (Jiang et al., 2018a).

In addition to controlling the p53 tumor suppressor gene's activity, zinc plays a crucial role when the apoptosis-inducing enzyme caspase-8 is stimulated (Ng et al., 2011). Another crucial zinc target is caspase-9 (Velázquez-Delgado and Hardy, 2012), Caspase-3, and other cellular death-causing enzymes that rupture nuclear membranes are powerfully induced by it. Therefore, when trying to treat cancer, Bax, p53, and caspases are regarded as important apoptotic markers. ZnO NPs were introduced to MCF-7 to assess this, and the ZnO NPs demonstrated dose-dependent suppression of cancer cells. Markers like Bax, JNK, p21, and p53 were elevated, which caused MCF-7 to undergo apoptosis (Boroumand Moghaddam et al., 2017).

Caspase-3, a crucial executioner protein in apoptosis, results in the cleavage of PARP and cell death (Sheridan and Martin, 2010). The primary regulators of the apoptotic process are members of the Bcl-2 family proteins, which contain both pro- and anti-apoptotic proteins. Bcl-2 and Bax are pro- and anti-apoptotic proteins, respectively (Gross, 2016). In the current study, PpIX-PDT boosted caspase-3, PARP, and Bax expression while lowering Bcl-2 levels. To inhibit activation of the downstream mitochondrial death cascade, Bcl-2 may bind to and sequester Bax (Figure 8), making the Bcl-2/bax ratio a crucial factor in controlling caspase-dependent apoptosis (Lin et al., 2017). Reduced Bcl-2 expression was observed (Xiong et al., 2017).

This study's findings show a statistically significant difference between adopting zinc nanoparticles and a diode laser to prevent the progression of cancer by promoting the expression of the p53 gene. Additionally, the use of both nanoparticles and lasers affects the expression of the BAX gene, ramps up and increases the rate of the gene, and ultimately results in the death of cancer cells in the treatment of cancer cell lines employing BAX gene expression (the apoptosis regulator BAX).

However, when applying a combination treatment of laser and zinc nanoparticles, there was no visible alteration in the Bcl2 gene.

Figure 8 shows the probable mechanism underlying the anti-cancerous properties of ZnO NPs. Cancer cells can absorb ZnO NPs by an endocytic pathway, and the entrance pathway may change depending on the kind of cell. ZnO NPs are restricted to vesicular structures, endosomes, and finally lysosomes through energy-dependent NP uptake processes. Because of the lysosome's acidic pH, ZnO NPs and Zn2+ ions may be released into the cytosol, preferentially producing toxicity that leads to apoptosis, necrosis, cell cycle arrest, and membrane damage because of excessive ROS production. Zn2+ ions can also enter cells through ion channels that prevent Bcl-2 indicators from functioning. This, in turn, causes the production of Bak/Bax, two pro-apoptotic proteins that enhance cell permeabilization and cytochrome c release. The creation of a complex including cytochrome c, apoptotic protease activating factor (Apaf-1), and pro-caspase 9 activates the apoptosome. Caspase 9 activation results in caspase 3 and caspase 7 gene expression and activity, which eventually induces apoptosis in cancer cells (Anjum et al., 2021).

CHAPTER SIX

Conclusions and Recommendations

6.1. Conclusions

1- The current finding revealed the significant synergistic potency of combination therapy on the number of cells experiencing early apoptosis compared to live, late apoptosis, and necrosis (p-value<0.05). Beside the cell cycle arrest, apoptosis, and raising of ROS levels in the treated cell line.

2- Regarding the cell cycle arrest analysis, malignant Caco2 cells were inhibited by a combination therapy using zinc-oxide nanoparticles and diode laser (IC50 = 20 µg/mL), and there was a significant difference between the percentage of cell lines decreasing in the G2 phase of the cell cycle compared to the S and G1 phases (*p*-value<0.05).

3- Highly noticed modifications and significant differences have been reported when the proportion of cell lines in DCFH- compared to DCFH+ has been reduced using laser and zinc nanoparticles alone or together (*p*-value<0.05).

4-The results suggest that the combination therapy may be a promising treatment approach for colorectal cancer, also up-regulated the pro-apoptotic gene Bax and at the same time resulted in a downregulation of the anti-apoptotic gene Bcl-2.

6.2. Recommendations

Suggestions for further studies

1. It is advised that additional research be done to examine the possibilities of combining zinc oxide nanoparticle therapy with diode laser therapy for the treatment of colorectal cancer in light of the study's findings.
2. In particular, preclinical and clinical studies are needed to evaluate the safety and efficacy of this approach in vivo.
3. More researches are needed to understand the cytotoxic effects of the combination of zinc oxide nanoparticles and diode laser on Colorectal cancer in vivo and in vitro.
4. More advanced techniques are needed to detect the mode of tumor cell death in response to combination therapy.
5. In vivo evaluation of the effect of combination therapy against tumors as therapy or with other chemotherapy.

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خانەی B 2، دەروازەیەکی بنەڕەتییە بۆ تێکچوونی کارکردنی مایتۆکۆندریال و بە شێوەیەکی بەرچاو لە توێژینەوەکەدا دەربڕدرا (بەهای $p<0.05$) دوای چارەسەرکردنی خانەکان.

بەکارهێنانی نانۆگەردیلەکانی لەیزەر و زینک پێکەوە هیچ جیاوازییەکی بەرچاوی لە جینی Bcl2دا دەرنەخست (بەهای $p>0.05$). ئەنجامی تەکنیکە جیاوازەکانی بەکارهێنراو لە توێژینەوەکەدا پشتگیریان لەوە کردووە کە چارەسەری تێکەڵاوی لەیزەری ZnO2 و Diode ژەهراویکردنی خانەیی بۆ شێرپەنجەی کۆڵۆن و ڕیخۆڵە لە ناو ئامێری پشکنیندا و ئەگەری پەرەپێدانی ماددەیەکی چارەسەری کاریگەر هەیە دژی شێرپەنجەی کۆڵۆن و ڕیخۆڵە. دۆزینەوەی ژەهراویبوونی خانەیی جیاوازییەکی بەرچاوە لە چارەسەری تێکەڵاودا لەسەر ژمارەی ئەو خانانەی کە تووشی ئەپۆپتۆزی زوو دەبن بە بەراورد بە ئەپۆپتۆزی زیندوو، ئەپۆپتۆزی درەنگ و نەکرۆزی (بەهای $p<0.05$). گۆڕانکارییە زۆر سەرنجڕاکێشەکان و جیاوازییە بەرچاوەکان ڕاپۆرتکراون کاتێک ڕێژەی هێڵەکانی خانە لە DCFH- بە بەراورد لەگەڵ DCFH+ کەمکراوەتەوە بە بەکارهێنانی نانۆگەردیلەکانی لەیزەر و زینک بە تەنیا یان پێکەوە (بەهای $p<0.05$)، ڕێژەی سەدی خانەکان لە کۆنترۆڵەکەدا لە بەرزیدا دەمێنێتەوە ڕێژەکان و کەمبوونەوەی لە هەموو حاڵەتە چارەسەرکراوەکان بە ڕێژەی جیاواز.

ئەم توێژینەوەیە دەریخست کە تێکەڵکردنی نانۆگەردیلەکانی ئۆکسیدی زینک و چارەسەری لەیزەری دایۆد لەسەر خانەکانی شێرپەنجەی کۆڵۆن و ڕیخۆڵە (Caco-2) لە ناو ئامێری پشکنیندا، کاریگەرییەکی هاوبەشی دژە شێرپەنجەی نیشان دا.

پوختە

شێرپەنجەی کۆڵۆن و ڕیخۆڵە (CRC) سێیەم وەرەمە کە زۆرترین دەستنیشانکراوی هەیە لە سەرانسەری جیهاندا، ڕێژەی مردنی زۆر بەرزە. ستراتیژییەکانی چارەسەرکردن، وەک نەشتەرگەری، چارەسەری کیمیایی، یان چارەسەری تیشکی، بەپێی پێویست کاریگەر نین و چەندین سنووردارکردن نیشان دەدەن. بۆیە ستراتیژییە سەرهەڵداوەکان، وەکو نانۆپزیشکی ئامرازێکی زۆر بەهێز پێشکەش دەکات بۆ چارەسەرکردنی شێرپەنجە. لەم دواییانەدا، تێکەڵکردنی کاریگەری دژە وەرەمی نانۆگەردیلە لەگەڵ هاندانی دەرەکی دەستپێکەر داڕێژرا بۆ بەرزکردنەوەی چالاکیی ژەهراویکردنی خانەیی. لەم لێکۆڵینەوەیەدا، کارکردنی هاوبەشی نانۆگەردیلەکانی ئۆکسیدی زینک و لەیزەری دایۆد لە چارەسەرکردنی هێڵی خانەکانی شێرپەنجەی کۆڵۆن و ڕیخۆڵە (Caco2) لە ناو ئامێری پشکنیندا هەڵسەنگێندرا.

چڕیی جیاوازی ZnO2-NP (0.5، 1، 5، 10، 20، 40، 75، 100، 200، 1000) میکرۆگرام/میلی لیتر بۆ کرداری ژەهراویکردنی خانەیی بەکارهێنرا. سەرەڕای ئەوەش، هێزی لەیزەری ئامانجدار (5 j/cm2). تێکەڵکردنی ZnO2-NP و لەیزەری دایۆد بۆ خانەکە بە شێوەیەکی دروست خۆدزینەوە. IC50 بۆ 20 CaCo2 میکرۆگرام/میلی لیتر بوو. کرداری ژەهراویکردنی خانەیی لە ڕێگەی تاقیکردنەوەی MTT بۆ ماوەی (24،48 و 72 کاتژمێر) پێوانە کرا، لە کاتێکدا فلۆسایتۆمێتەری (Propidium Iodide, Annexin VAssay) بەکارهێنرا بۆ دیاریکردنی ئەپۆپتۆزی، لە کاتێکدا، شیکاری ڕاگرتنی خولی خانەکان بە فلۆسایتۆمێتری پێوانە کرا. سەرەڕای ئەوە، ئاستی دەربڕینی ڕێژەیی (P53، Bax، و Bcl2) بە بەکارهێنانی PCRی کاتی ڕاستەقینەی چەندایەتی (qPCR) پێوانە کرا. ئاستی جۆری ئۆکسجینی کارلێککەر بە شێوازی تاقیکردنەوەی دیکلۆرۆفلۆرۆسێین دیاسیتات (DCFDA) دیاریکرا.

ڕێژەی سەدی ئەو خانانەی کە لە قۆناغی G2ی خولی خانەکان کەمبوونەتەوە بە بەراورد بە قۆناغەکانی S و G1 جیاوازییەکی بەرچاوی هەبووە کاتێک نانۆگەردیلەکانی لەیزەر و زینک تێکەڵکران (بەهای p<0.05). گۆڕانکارییەکی بەرچاو لە بەکارهێنانی لەیزەری دایۆد و نانۆگەردیلەکانی زینک بۆ وەستاندنی بڵاوبوونەوەی شێرپەنجە بە بەرزکردنەوەی دەربڕینی جینی p53 (بەهای p<0000). باکس، پێکهاتەیەکی سەرەکی لایەنگری ئەپۆپتۆزی لیمفۆمای

کارکردنی ژەهراوی و مردنی خانەیی In vitro بە هۆی نانۆگەردیلەی ئۆکسیدی زینک، لەیزەری دایۆد و تێکەڵکردنیان دژی شێرپەنجەی کۆڵۆن و ڕیخۆڵە

نامەیەک

پێشکەش بە ئەنجومەنی کۆلێژی تەکنیکی تەندروستی و پزیشکی - لە زانکۆی پۆلیتەکنیکی هەولێر کراوە وەک بەشێک لە پێداویستیەکانی بەدەستهێنانی بڕوانامەی ماستەر لە شیکاری نەخۆشیەکان

لەلایەن

چراخان صالح رحمان

بەکالۆریۆس لە شیکاری نەخۆشیەکان-کۆلێژی تەکنیکی تەندروستی و پزیشکی هەولێر-٢٠١٨

بە سەرپەرشتی

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هەولێر، کوردستان

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