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Zinc Oxide Nanomaterials: Toxicity Mechanism and Biomedical Applications

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المواد النانوية لأكسيد الزنك: ألية السمية لهذه المواد وتطبيقاتها الطبية الحيوية

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Abstract:
Over the last several decades, nanostructured materials have been the focus of intense research, due to their remarkable characteristics suitable for exploitation in electronics, optical, electrochemical, electromechanical and photonics applications. In particular, zinc oxide nanostructures (ZnO-NSs) have been extensively utilized as an efficient intracellular platform to introduce different biomolecules into various types of cells due to their minimal diameter size with a high aspect ratio. Importantly, comprehension of the extraordinary functional properties of ZnO nanomaterials and their promising applications as an elementary unit of future nanodevices is the focus of the current research activities. This work is dedicated to reviewing the outstanding properties of several nanostructured ZnO materials and their functionality in different biomedical and healthcare applications. The potential feasibility of ZnO nanomaterial is further highlighted through its integration with different antibacterial, anticancer, tissue engineering, wound healing, and bioimaging applications as well as potential biomolecule and drug delivery platforms.

Keywords: ZnO nanomaterials, Biomedical applications, Toxicity, Tissue engineering, Healthcare, anticancer.

الملخص
على مدى العقود العديدة الماضية، كانت المواد ذات البنية النانوية محور البحث العلمي المكثف، نظرًا لخصائصها الرائعة المناسبة للاستعمال في التطبيقات الإلكترونية و البصرية و الكهروكيميائية والكهروميكانيكية والضوئية. على وجه الخصوص، تم استخدام الهياكل النانوية لأكسيد الزنك (ZnO-NSs)على نطاق واسع كمادة فعالة داخل الخلايا لإدخال جزيئات حيوية مختلفة في أنواع متنوعة من الخلايا نظرًا لصغر قطرها مع نسبة عرض عالية. الأهم من ذلك، أن فهم الخصائص الوظيفية الاستثنائية للمواد النانوية لأكسيد الزنك وتطبيقاتها الواعدة كوحدة أولية للأجهزة النانوية المستقبلية هو محور الأنشطة البحثية الحالية. هذا العمل مخصص لمراجعة الخصائص البارزة للعديد من مواد أكسيد الزنك ذات البنية النانوية ووظائفها في مختلف تطبيقات الطب الحيوي والرعاية الصحية. الدراسة الحالية تسليط الضوء بشكل أكبر على الجدوى المحتملة لمادة أكسيد الزنك النانوية من خلال تكاملها مع مختلف تطبيقات مضادات الجراثيم والسرطان وهندسة الأنسجة والتئام الجروح والتصوير الحيوي بالإضافة إلى دورها المحتمل في توصيل الجزيئات الحيوية والأدوية داخل الجسم البشري.

الكلمات المفتاحية: المواد النانوية لأكسيد الزنك ، التطبيقات الطبية الحيوية، السمية، هندسة الأنسجة، الرعاية الصحية، مكافحة السرطان.

Introduction

ZnO is an n-type multifunctional material which has various applications in surface acoustic wave filters [1], photonic crystals [2] photodetectors [3], light emitting diodes (LED) [4], gas sensors [5], optical modulator waveguides [6] and solar cells [7]. Furthermore, because of its biocompatible, antibacterial properties, chemical stability, electrochemical activity, high electron mobility, and large surface-to-volume ratio, ZnO-NSs have been used in a wide range of healthcare applications, including medicine delivery platforms, cancer therapy, biological imaging techniques, medical devices, sunblock, skin moisturizers [8-12]. Since the nanomaterial industry has become an essential unit for future nanotechnology applications, therefore the entire promising outcome of potential applications can be achieved only when the fabrication process of nanostructures is

integrated with consistent control of their position by organizing them in the form of controlled patterns of well-ordered nanoarrays on different substrates. For example, in biology and life-sciences applications, the pitch between the nanowires may affect cell adhesion [13], therefore it has been a high demand for accomplishing well-ordered nanopattern structures so that their functional properties could be improved. In general, ZnO has been produced in a wide variety of nanoscale structures. Zero dimensional (0D) nanostructures such as quantum dots or nanoparticles that have near-unity aspect ratio [14]. One-dimensional (1D) nanostructure is the biggest group, including rods, needles, helixes, belts, springs, rings, ribbons, tubes, wires, and combs [15-22]. ZnO can crystallize in (2D) geometries, such as nanoplates, nanosheets, and nanopellets [23, 24, 25]. The last group is (3D) structures such as flowers, dandelions, snowflakes, etc [26, 27, 28]. Acknowledging the biocompatible, antibacterial, and anticancer properties of ZnO nanomaterials allows them to be an intensely bright research area and highly used as environmental monitoring tools, food processing, biomolecule and medicine delivery platforms, tissue generating and wound healing promoters, and medical diagnostic tools. In addition, ZnO nanomaterial has been widely employed for its anti-cancerous properties, and its selective cytotoxicity to cancerous cells in an in vitro environment has been proved by Hanley et al. [29]. They found that ZnO nanoparticles display an intense propitious ability to distinguish and kill cancerous cells compared to normal ones, which could diminish the side effects on normal cells. To be safely used in biomedicine platforms, the biocompatibility and biodegradability of ZnO nanomaterials have always been a concern issue. Luckily, ZnO has been licensed by the Food and Drug Administration (FDA) and recognized as safe for cosmetic uses due to its stability and unique ability to absorb UV radiation [30]. One of the mechanisms of ZnO cytotoxicity against cancer cells is its ability to induce reactive oxygen species (ROS) generation, leading to oxidative stress and ultimately cell death when the anti-oxidative capacity of the cell is exceeded [31].

In the current review, the focus is on the different functional properties of nanostructured ZnO materials and their use for novel biomedical and life science applications. Initially, the findings of recent research on the biocompatibility of ZnO-based nanostructures have highlighted, in particular, the controversial reports related to the concealed toxicological effects of this material and their interaction with biological systems, which are crucial keys for healthy and reliable biomedical devices. Moreover, other properties of ZnO nanomaterials have been pointed out, including antimicrobial, antibacterial, anti-inflammatory and antidiabetic properties. Finally, the scope was to review the recent progress in healthcare and life science applications.

Nanotoxicity mechanism of ZnO-based nanostructures

Due to the high demand for ZnO-based nanostructures in broad potential applications, investigating their biocompatibility and comprehending the nanotoxicity and their interaction with biological environments is a highly bottom-line concern for developing eco-friendly and safe biomedical devices. The toxicological effect of ZnO has been addressed in many kinds of literature. There are some potential causes behind the cytotoxicity and genotoxicity of ZnO-NSs, one of the most reported reasons is the generation of ROS such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and superoxide ions (O^{-2}) [32]. The ROS can pass through the cell membrane, affect the mitochondrial function, and damage DNA [33, 29, 34]. The generation of ROS by a photo-induced oxidation process leads to the high photocatalytic activity of ZnO nanoparticles (ZnO-NPs), which is exploited in diverse biomedical and life science applications [35, 36]. The scientists demonstrated the ZnO bio-efficiency through the development of ROS under absorption of UV radiation and associated a such phenomenon with the antibacterial activity of ZnO-NSs. The chemical mechanism of ROS production has been described in detail in the literature [37, 38]. Briefly, once the energy of incident photons is higher than 3.37 eV (band gap energy of ZnO), it would be absorbed by electrons, then those electrons with sufficient energy transfer to the conduction band, leaving behind positively charged holes in the valence band [37, 39]. Besides, those positive holes act as oxidizing agents or electron acceptors to produce highly reactive and short-lived hydroxyl radicals (OH) in the photocatalytic interaction [40, 41]. It is worth mentioning that under UV light, negatively charged electrons and positively charged holes in ZnO combine, and there is an inverse proportionality between how fast this interaction takes place and the amount of absorbed visible light [42], which is a critical aspect for using ZnO as photocatalyst. The electron-hole couple migrates to the ZnO surface and interacts with adsorbed water and oxygen molecules to generate OH and H^+, while O_2 molecules provide superoxide anion (O^{-2}), which reacts with H^+ to yield hydrogen peroxyl HO_2. Then HO_2 integrates with H^+ to produce H_2O_2 molecules. It has been shown that H_2O_2 produced by ZnO enters the E. coli membrane, resulting in damaging and hindering cell growth or even killing them [43].

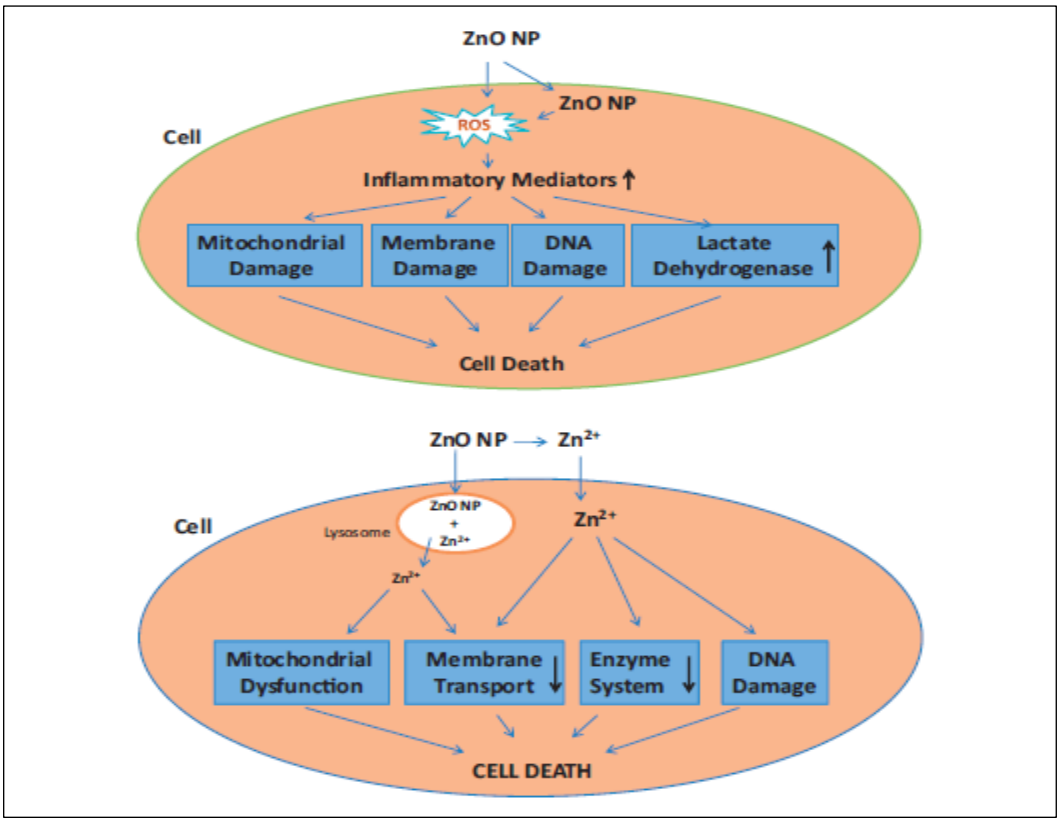

Figure 1: An illustration of ZnO-NPs toxicity induced by ROS and zinc ions (Zn^{2+}). Reproduction from [44].

Another noteworthy mechanism related to ZnO nanotoxicity is the release of zinc ions (Zn^{2+}) from ZnO-NPs, as summarized in Fig. 1. It found that Zn^{2+} can significantly affect the active transport inhibition process, the metabolism of amino acids, and the disruption of enzyme activity. It reported that the incorporation of Zn^{2+} into the cell growth culture obstructs the dynamic transport of membrane, damaging DNA and interrupting catalyst function, which was associated with the toxic effect of ZnO-NPs [45, 46, 47]. The culture medium constituents are highly related to the physical and chemical characteristics of ZnO-NPs and the dissolution of zinc ions. Reported research demonstrated that the Zn^{2+} release procedure is affected by several physical and chemical properties of the nanoparticles, such as shape, size, precursor concentration, and porousness. In addition, other factors related to chemical conditions of the interaction environment, including pH scale, UV irradiation, reaction time, etc, can also contribute to releasing zinc ions [48].

In-vitro assessments of ZnO nanotoxicity

The nanotoxicity of ZnO-NSs has been under debate since those materials are hugely involved in diverse products related to humans, animals, and the environment, such as food additives, cosmetics, sunscreen, medical textiles, pesticides, etc [49- 54]. ZnO-NPs can get through the human body in many ways such as inspiration, chemical absorption via skin, digestion process and parenteral medication. Hence, airborne ZnO-NPs released by industrial products can respire and cause toxicity to human lungs [42]. Furthermore, ZnO NSs can induce cytotoxicity and genotoxicity in different mammalian cells. Hanley and his co-workers [33] manufactured size-controlled ZnO-NPs and determined their cytotoxic effect on various human immune cell subsets. It reported that ZnO-NPs toxicity stimulates cells depending on the intensity of the electrostatic interaction among ZnO-NPs and cell membrane, phagocytic capacity, and intrinsic cellular ability to generate ROS. Based on their study, they found that lymphocytes are the ultimate resistant cells, while monocyte cells show high susceptibility to ZnO-NPs. In addition, it observed that memory and naive lymphocyte cells vary in cytotoxic response to ZnO NP, which could be attributed to the fewer activation signals required to proliferate memory lymphocyte cells. Further, it showed that ROS is a critical cause of ZnO-NPs cytotoxicity and highly depends on the particle size, as nanoparticles of 4 nm display a high concentration of ROS. Lastly, their finding showed that ZnO-NPs can boost the expression of interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukin 12 (IL-12) in primary immune human cells at specific concentrations of nanoparticles. Therefore, it suggested that applying suitable concentrations of ZnO-NPs may improve tumour cell killing by producing cytokines and inflammatory mediators, which increase anti-tumour response. However, extended exposure to cytokines and inflammatory mediators could be harmful, so it is essential to control parameters related to ZnO-

NPs toxicity, including size, concentration, and biodistribution of nanoparticles. Another in-vitro study was carried out to examine the cytotoxicity and genotoxicity of ZnO-NPs at various concentrations (50, 100, 250 and 500 ppm). Human erythrocytes (red blood cells; RBCs) and human lymphocytes have been utilized to investigate the cytotoxic and genotoxic effects respectively [55]. According to their results, the hemolytic activity of RBCs is concentration dependent, as ZnO-NPs at a concentration of 250 ppm cause 65.2% hemolysis, which is cytotoxic to human RBCs. The authors demonstrated that ZnO-NPs disturbed the integrity of RBC membranes and affected cellular hemolysis. In addition, the genotoxic effect of ZnO-NPs has been confirmed through significant DNA damage in human lymphocytes. Finally, it reported that the formation of ROS is a critical factor for inducing cytotoxic and genotoxic effects by ZnO-NPs.

Hence, the oxidative stress process is critically correlated with some physiological conditions like inflammatory disorder, which is related to prominent levels of oxidative stress, so its contribution to cell sensitivity under exposure to ZnO-NPs needs to be investigated. One study introduced by Heng et al. [34] demonstrated that exposure of human bronchial epithelial cells (BEAS-2B) to sub-lethal dosages (5 and 10 µM) of H_2O_2 for 45 min and then exposure to various concentrations of ZnO-NPs size of 10 nm. The results showed that primary exposure of these cells to oxidative stress simulated their following responses to the cytotoxic effect of ZnO-NPs. Therefore, the authors suggested that pre-exposure to oxidative stress could be determined by the defined threshold degree of the nanoparticle's concentration. Thus, at lower concentrations, cells may have high resistance, while cells may show significant susceptibility to the nanoparticle's cytotoxicity at higher concentrations. It suggested that ZnO-NPs could be responsible for activating Endoplasmic reticulum (ER) stress in human umbilical vein endothelial cells (HUVECs), as ER stress might act as an initial index for the cytotoxic effect of ZnO-NPs [56]. Thus, ER is well known for its crucial role in cell homeostasis and viability, so interrupting its function results in developing ER stress state, which is involved in heart and blood vessel diseases such as atherosclerosis. However, the relationship between ER stress and the toxicity of ZnO-NPs, assuming that the existence of ER stress inducer thapsigargin (TG) should simulate the interaction of HUVECs to ZnO-NPs, which means ER stress is related to the nanoparticle's cytotoxicity. According to their finding, they found that ER stress was not correlated to ZnO-NP exposure-induced oxidative stress and inflammatory responses in HUVECs. The mechanism of ZnO-NPs toxicity to Raw 246.7 macrophage cells has been demonstrated by Wang et al. [57]. The impact of these nanoparticles on cellular toxicity has been highlighted in the context of mitochondrial membrane potential (MMP), intracellular level of ROS, total and released lactate dehydrogenase (LDH), cell viability, and Zn^{2+} concentration. Based on their results, ZnO-NPs induced an increase in intracellular concentration of Zn^{2+}, resulting in multiproduction of intracellular ROS, plasma membrane damage, mitochondria defect, and cell necrosis. To examine ZnO-NPs solubility and its release of Zn^{2+} ions, diverse pH values were employed. It observed that an acidic medium (pH 5.5) was more soluble than a physiological condition (pH 7.2). Therefore, the cytotoxicity is significantly interrelated to cellular uptake, intracellular dissolution, and the release of Zn^{2+} from the nanoparticles, which may occur in the cell culture and inside cells. In general, it reported that the higher toxicity of nanoparticles to Raw 246.7 macrophages is a concentration and time-dependent process. According to the author's viewpoint, the toxicity of ZnO-NPs could be associated with outstanding catalysis features of nanoscale architecture, which intervene within diverse physiological intracellular interactions. Besides, the disintegration of nanoparticles and subsequently released ions that are involved with intracellular homeostasis.

Since there has been a lack of literature investigating the cytotoxic activity of ZnO-NPs on the immune system, one study investigated the cytotoxicity of ZnO-NPs on mouse primary peritoneal macrophages. It reported that ZnO-NPs treatment induces inflammation in macrophages, and these nanoparticles could internalize in macrophages through a caveolae mediated path [58]. It found that ZnO-NPs induce ROS formation via diminishing antioxidative enzymes, boosting lipid peroxidation (LPO) and carbonyl contents of protein in macrophages. Additionally, afterwards, the exposure to ZnO-NPs for a period time of 0.5–24 h, the number of autophagosomes and autophagy marker proteins, including microtubule-associated protein 1 light chain 3-isoform II (MAP-LC3-II) and Beclin 1 was raised. Some molecular markers of DNA damage such as γH2Ax and poly (ADP-ribose) polymerase (PARP) proteins, which are well-known as genotoxic indicators, were observed to be stimulated by the expression of p53 and p21/waf1 proteins in macrophages [58]. Furthermore, the authors demonstrated that ZnO-NPs treatment induced oxidative stress in macrophages leads to autophagy and apoptosis concurrently, and also interruption in the cell death mechanism could impede the functions of the immune system and might cause chronic disorders. Lee et al. [59] investigated the toxicity of ZnO-NPs on human epidermal keratinocytes (HaCaT) by employing various concentrations of 0, 10, 20, 40 and 80 µg/mL for 24 h. According to findings, interruption of mitochondria function, and boost membrane leakage of LDH have been reported, as well as induction of oxidative stress and ROS formation were observed. Another in vitro study for short and long-term exposure time of ZnO nanorods to human keratinocytes was demonstrated by Kocbek et al. [60]. Results showed that the morphology of nanoparticles, concentration, and exposure period affected

intracellular response. In addition, short-term exposures with concentrations of 15 µg mL^{-1} resulted in decreasing cell viability, whilst extended time to nanoparticles at a dose of 10 µg mL^{-1} caused a reduction in mitochondrial function, change in usual cell shape, and disruption in the cell cycle. The cytotoxicity of ZnO-NPs is caused by the dissolution of ZnO in the culture medium and the release of Zn^{2+} ions. An investigated study by Guo et al. [61] reported the ZnO-NPs cytotoxicity and the potential molecular mechanisms associated with calcium homeostasis mediated by plasma membrane calcium ATPase (PMCA) in rat retinal ganglion cells (RGC). Based on a Real-time cell electronic sensing system for evaluation of the influence of the nanoparticle concentrations on RGC cell proliferation, it showed that ZnO-NPs of 2.5 µg/mL concentration might exert cytotoxic activity on RGC cells, which hinder the cell growth and division. In addition, the formation of ROS in RGC cells was a concentration-dependent process. Besides, the cytotoxic impact of nanoparticles on the expression level of plasma membrane $Ca2^{+}$ protein (PMCA2) and gene as well as calcium homeostasis has been highlighted through the quantitative polymerase chain reaction Q-PCR and enzyme-linked immunosorbent assay (ELISA) techniques. According to the authors' opinion, ZnO-NPs obstruct the proliferation of RGC cells and increase the production of ROS, which leads to cell injury. Additionally, the multiproduction of ROS could reduce the PMCA2 expression, hinder Ca^{2+}-ATPase function, raise intracellular levels of calcium ions, disturb intracellular calcium homeostasis, and eventually cause RGC cell necrosis.

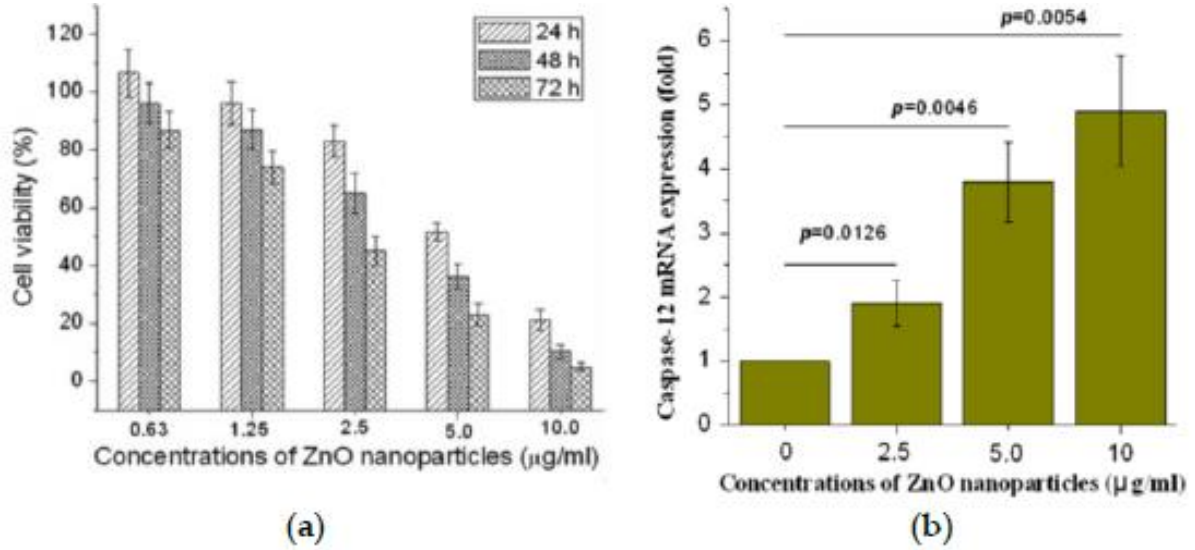

Figure 2: (**a**) The viability of RGC cells versus ZnO-NPs concentrations. (**b**) Real-time Q-PCR measurements of caspase-12 gene expression in RGC cells mediated with concentrations of 2.5, 5 and 10 µg/mL of ZnO-NPs. Reproduced from [62].

Another similar study attempted to understand the mechanisms of the ZnO-NPs cytotoxicity by investigating the contribution of oxidative stress in the formation of ROS after treatment of RGC with ZnO-NPs (60 nm) [62]. MTT assay was applied to test cell viability after ZnO-NPs exposure at various doses for 24, 48 and 72 h (Fig. 2a). It found that viable cells declined by increasing time and concentration of nanoparticles. Moreover, it demonstrated that the levels of nanoparticle-induced H_2O_2 and HO_2 radicals were elevated by increasing the nanoparticle concentrations, leading to shrinkage of MMP and overproduction of ROS. In addition, the caspase 12 gene was also tested by real-time Q-PCR method upon exposure to diverse doses of the nanoparticles, it showed excessive expression of caspase 12 in RGC cells (Fig. 2b). Authors suggested that the mechanism of ZnO-NPs toxicity initially caused by an excessive formation of ROS following by provoking ER stress and subsequently causing cell necrosis (Fig. 3). As mentioned earlier, ZnO-NPs are common elements of cosmetics, sunscreen, and skincare products, but their cytotoxic mechanism to normal skin cells is still under debate. In this regard, one study conducted by Yu et al. [63] highlights the mechanism of ZnO-NPs toxicity in normal skin cells. Results showed that the induction of intracellular ROS in normal skin cells evaluated by electron spin resonance was a concentration and time-dependent process. Further, it detected accretion of abnormal autophagic vacuoles and mitochondria damage in ZnO-NPs-treated cells, which subsequently led to cell necrosis. Another in vitro study regarding the biological risk of ZnO-NPs on human dermal fibroblasts has been introduced by Meyer et al. [64]. It examined the programmed cell death (apoptosis) process stimulated by ZnO-NPs via p38 mitogen-activated protein kinase (P38 MAPK) and cell cycle checkpoint protein p53 pathways in human dermal fibroblasts. Cytotoxicity results reported that ZnO-NPs induced apoptosis in human dermal fibroblasts through upregulating p53 and phospho-p38 proteins. In terms of cell viability, it was observed that

ZnO-NPs treated cells were less viable, especially at nanoparticle concentration of 25 µg/mL, as assessed by MTT assay. Finally, the cell's shape was investigated and based on phase contrast images, less density and a circular form were noticed in ZnO-NPs exposed cells at concentrations of 50 and 100 µg/mL.

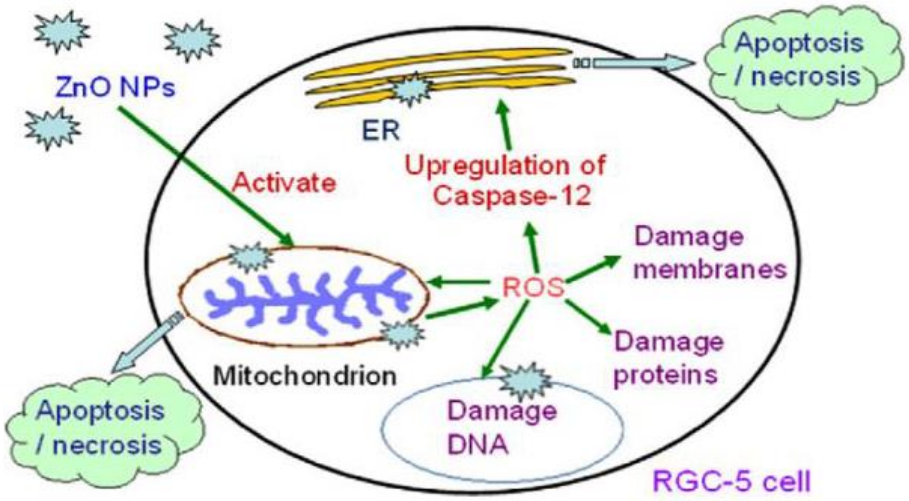

Figure 3: The potential mechanism of ZnO-NPs toxicity in RGC cells. Reproduced from [62].

Moratin et al. [65] investigated cytotoxicity, genotoxicity, programmed cell death process, cell proliferation, and cell cycle changes of malignant and non-malignant cell lines under ZnO-NPs exposure. Hence, as malignant representatives, squamous cell carcinoma of the head and neck (SCCHN) derived from FaDu cells were incubated at ZnO-NPs concentration of 4– 20 µg/ml for 1–48 hr periods. Besides, human mesenchymal bone marrow stem cells (BMSCs) were utilized as a non-malignant representative model to examine oxidative stress-related genotoxicity. For cell viability examination, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was applied to determine the extent of cytotoxic concentration of the nanoparticles. It found that considerable cytotoxicity relating to a decrease in cell viability was observed at all proposed nanoparticle concentrations. Besides, for apoptosis and necrosis measurements, a real-time PCR was applied to evaluate the mRNA levels of caspase-3, which is a crucial enzyme of apoptosis. According to findings, vital alterations of caspase-3 gene expression levels in FaDu cells, besides mRNA levels elevated in a time-dependent effect. In addition, the Authors applied the flow cytometry analysis to gain more details about apoptosis and cell death. The results were in good match with the prior analysis PCR in terms of a decrease in cell viability following nanoparticle treatment. Based on the comparison between the two cell lines, the nanoparticle concentrations of 5, 10, and 15 µg/mL were non-cytotoxic for the BMSCs and did not decline cell viability, whilst the viability of the FaDu cells decreased at the same tested concentrations. However, vital DNA damage was not avoidable in both malignant and non-malignant cell lines therefore it suggested that more in vivo investigations are critically required to estimate the safety issues of ZnO-NPs as anticancer agents.

It reported that ZnO-NPs could be related to the inflammation and degeneration of the nervous system and raised the possibility of degenerative brain diseases. These nanoparticles are capable of getting through the olfactory bulb brain pathways and cause neurotoxicity by triggering astrocytes and microglia in the brain [66]. One study investigated the probable causes of the apoptosis process induced by ZnO-NPs treatment in mouse primary astrocytes [66]. The data showed that nanoparticles can provoke cell apoptosis by stimulating loop phosphorylation, releasing cytochrome c from the mitochondria, decreasing Bcl-2 protein expression, and activating PARP. A study of the neurotoxicological effect of different-sized ZnO-NPs on neural stem cells (NSCs) was demonstrated by Deng et al. [67]. Based on the cell counting kit-8 (CCK-8) assay, it found that viable NSCs number decreased in a concentration-dependent manner. Transmission electron microscopy (TEM) analysis, confocal microscopy, and flow cytometry techniques were applied to examine the apoptosis and necrosis of NSCs under ZnO-NPs exposure. The data showed that apoptosis occurred after treating NSCs to nanoparticle concentrations of 12 ppm for 24 h, which was attributed to the released Zn^{2+} ions in the growth medium or inside cells. However, NSCs treated below 6 ppm of ZnO-NPs concentration for 24 h had no toxic effects. An in vitro investigation to assess the toxicity of ZnO-NPs on mouse Leydig TM3 cells was reported by Shen et al. [68]. According to their findings, it found that the proliferation of mouse Leydig TM3 cells was hindered, whilst apoptosis and autophagy were induced following ZnO-NPs exposure. Furthermore, oxidative stress is also implicated in ZnO-NPs-induced apoptosis and autophagy. An investigation was performed by Yang et al. [69] to evaluate the cytotoxic activity of ZnO-NPs on mouse-derived spermatogonia cells (GC-1

spg). Following ZnO-NPs treatment for a period of 24 h with and without radical scavenger N-acetyl cysteine (NAC) or autophagy inhibitor 3-methyladenine (3-MA), a western blotting analysis and Annexin V-FITC/PI assay were utilized to test apoptosis, whilst autophagy was defined via TEM technique and western blotting analysis. Finally, the levels of oxidative stress such as malondialdehyde (MDA) and Glutathione (GSH), as well as the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), were examined by oxidation-antioxidation assay kits. The data obtained by western blot analysis revealed that the contents of autophagy proteins, including microtubule-associated protein 1A/1B light chain 3 (MAP 1A/1B LC3), autophagy-related 5 (Atg5) and Beclin1 increased (Fig. 4a, b).

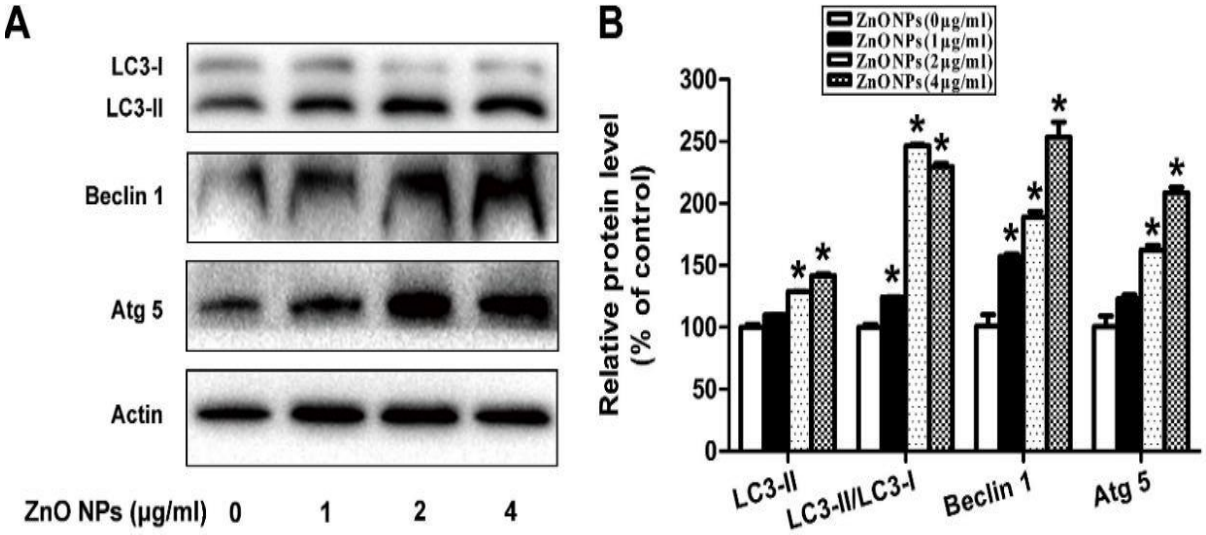

Figure 4: (**a**) Western blot analysis of protein levels for Atg5, Beclin1 and LC3 in the GC-1 spg mediated with 4 μg/mL ZnO NPs for 24 h. (**b**) Quantitation of Atg5, Beclin1 and LC3 levels in Western blotting. Reproduced from [69].

In addition, the MDA level was considerably raised, whilst the levels of GSH and SOD were declined, showing the oxidation impact. These results indicated that ZnO-NPs induced autophagy and apoptosis in GC-1 spg because of ROS formation and induction of oxidative stress. Lastly, the authors pointed out that oxidative stress was implicated in apoptosis and autophagy of GC-1 spg cells treated with ZnO-NPs, and autophagy had a cytotoxic impact in ZnO-NPs-induced cell death.

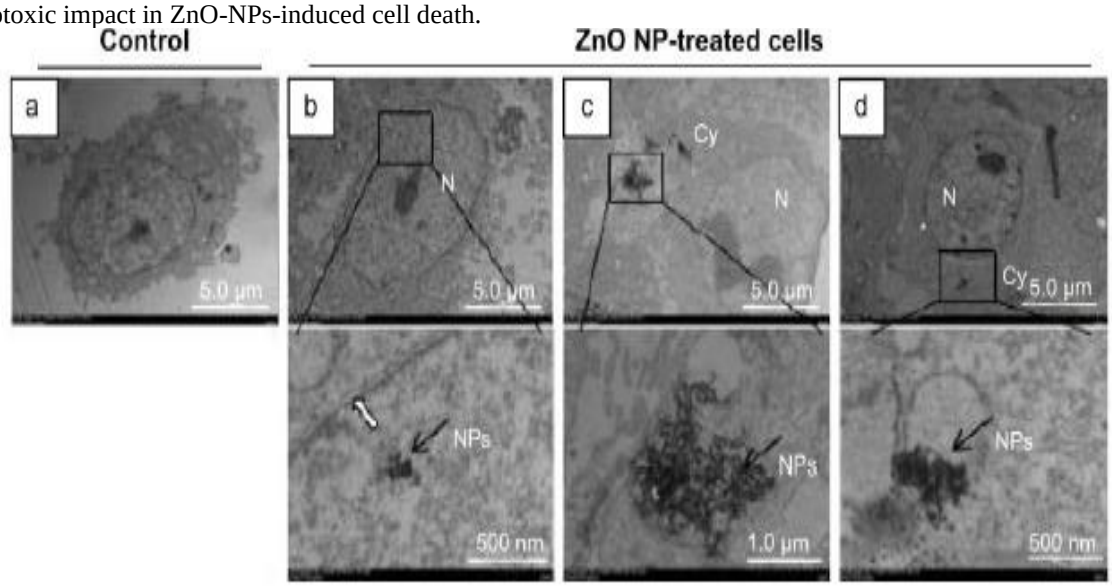

Figure 5: TEM images of (**a**) the control group of Leydic cell and (**b**–**d**) the ZnO-NPs treated group showing nanoparticles were internalized within the nucleus and cytoplasm of the cell. Reproduced from [70].

In vitro measurements of the cytotoxic activity of the ZnO-NPs on Leydig and Sertoli cell lines were carried out by Han et al. [70]. Results demonstrated that nanoparticles were internalized by two kinds of cells, leading to

cell necrosis in a time- and concentration-dependent effect (Fig. 5). According to the MTT assay, the viable cell number for both kinds of cell lines treated with ZnO-NPs of concentration ≥ 15 µg/mL for 12 h and 24 h considerably declined. To evaluate the loss of cell membrane integrity the LDH leakage assay was utilized. It showed ZnO NP cytotoxicity was dependent on the time and concentration of nanoparticles and had a higher impact between (10 - 20 µg/mL) of ZnO-NP doses. All these observations were attributed to excessive production of ROS in two kinds of cells and loss of MMP, which consequently provoked apoptosis through DNA damage (Fig. 6). Lungs, skin, and gastrointestinal tract are organized as three crucial exposure spots that interconnect with the nanoparticle, even in some cases nanoparticles can translocate through blood to other essential organs, such as kidney, lungs, heart, liver, pancreas etc. Despite much literature highlighting the ZnO-NPs toxicity to different biological organs, the cytotoxic impact of these nanoparticles in kidney cells has not been often investigated. One in vitro investigation demonstrated the ZnO-NPs cytotoxicity on human embryonic kidney 293 (HEK 293) cells [71]. To assess the cell viability two characterization methods, including the MTT assay and the neutral red uptake assay (NRU) were applied. Data showed that the cell viability was strongly dependent on the nanoparticle's concentration and time. A high decrease in cell viability was noticed at a nanoparticle dose of 25 µg/ml for all ZnO-NPs exposure time. Authors attributed the toxicity of ZnO-NPs to its high dissolution in the extracellular fluid, leading to boost intracellular Zinc ions levels.

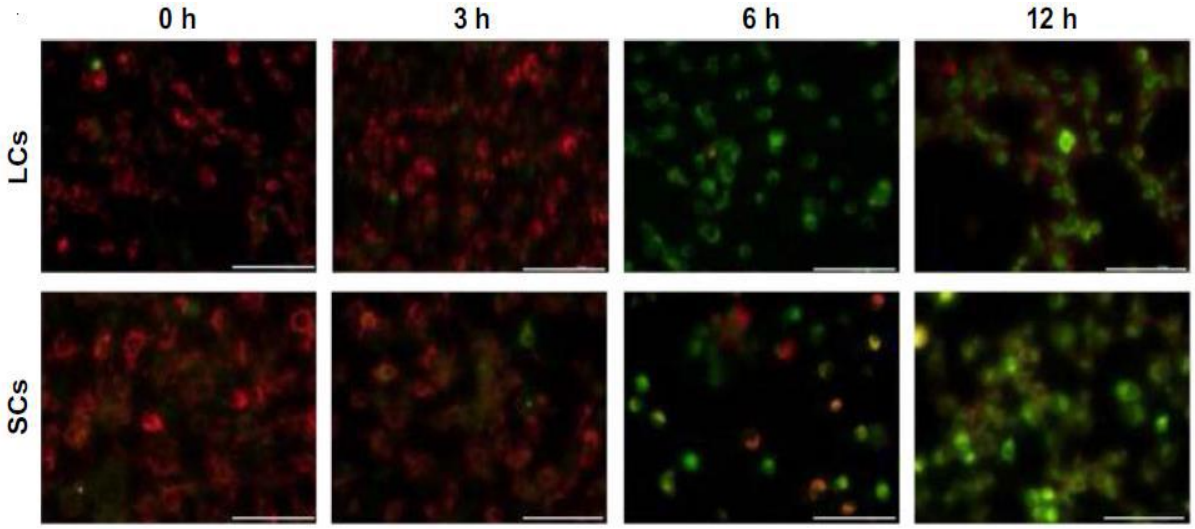

Figure 6: Variations in MMP of Leydic cells (LCs) and Sertoli cells (SCs) mediated with 15 µg/mL ZnO-NPs for 0, 3, 6, and 12 h. Reproduced from [70].

In addition, a considerable decline in mitochondrial function was found in cells treated with a ZnO-NPs dose of 100 µg/ml for 24 h. Moreover, TEM findings illustrated that lower doses of ZnO-NPs had no impact on the shape of HEK 293 cells however, treated cells with ZnO-NPs of 75 µg/ml dose for 24 h resulted in losing their fibroblast morphology. In addition, an excessive increase of intracellular ROS was found upon treating the cells with all ZnO-NPs doses for periods of 3 and 24 h, which was the main cause of ZnO-NPs cytotoxicity. Besides, elevated levels of ROS significantly triggered the loss of MMP and lysosome function, which conclusively caused cell death. In addition, actin as a key cellular cytoskeleton element which is involved in cell reproduction machinery and DNA repair was examined, and changes in its distribution of HEK 293 cells were noticed. It was also noticed a decline in the green fluorescence intensity indicated that the quantity of apoptotic cells essentially raised. Lastly, using acridine orange (AO) staining, autophagy, which is type two of cell necrosis, was measured. Apart from ZnO-NPs, diverse morphological nanostructured materials have attracted enormous attention and have been promising candidates in nanomedicine and diagnosis research areas due to their biocompatible, antibacterial, and anticancer properties. To pave the way for these nanostructures to be safely applied in biomedical and healthcare applications, scientists have extensively investigated their functional physiological properties to evaluate the toxicity impact on different biological systems. One study demonstrated the Nanotoxicology impact of flower-like ZnO NSs fabricated by solution-based growth method in Henrietta Lacks tumour cells (HeLa) and noncancerous human fibroblast L929 cells [72]. The main focus was to highlight the apoptosis and death process, formation of ROS, and cellular uptake. Cells were incubated in various doses of ZnO nanoflowers for 24 h and then cell viability was tested. Data showed a decline in cell viability, which was attributed to the induced oxidative stress with increased intracellular levels of ROS, resulting in cell death. In addition, the cytotoxic responses were measured in both types of cells by determining apoptosis and necrosis through the flow cytometry technique. The findings showed increased cytotoxic responses for cancerous cells,

whilst lower cytotoxic impact was found in the noncancerous cell line L929. Based on these findings, authors pointed out the selective capability of the ZnO nanoflowers, which enable it to induce higher cytotoxic responses in cancer cells compared to normal cells, therefore it might be utilized in cancer treatments with less damage to noncancerous cells. Another study reported the nanotoxicity of the high aspect ratio of zinc oxide nanowires (ZnO-NWs) to human monocyte macrophages (HMMs) at similar concentrations as $ZnCl_2$ [73].

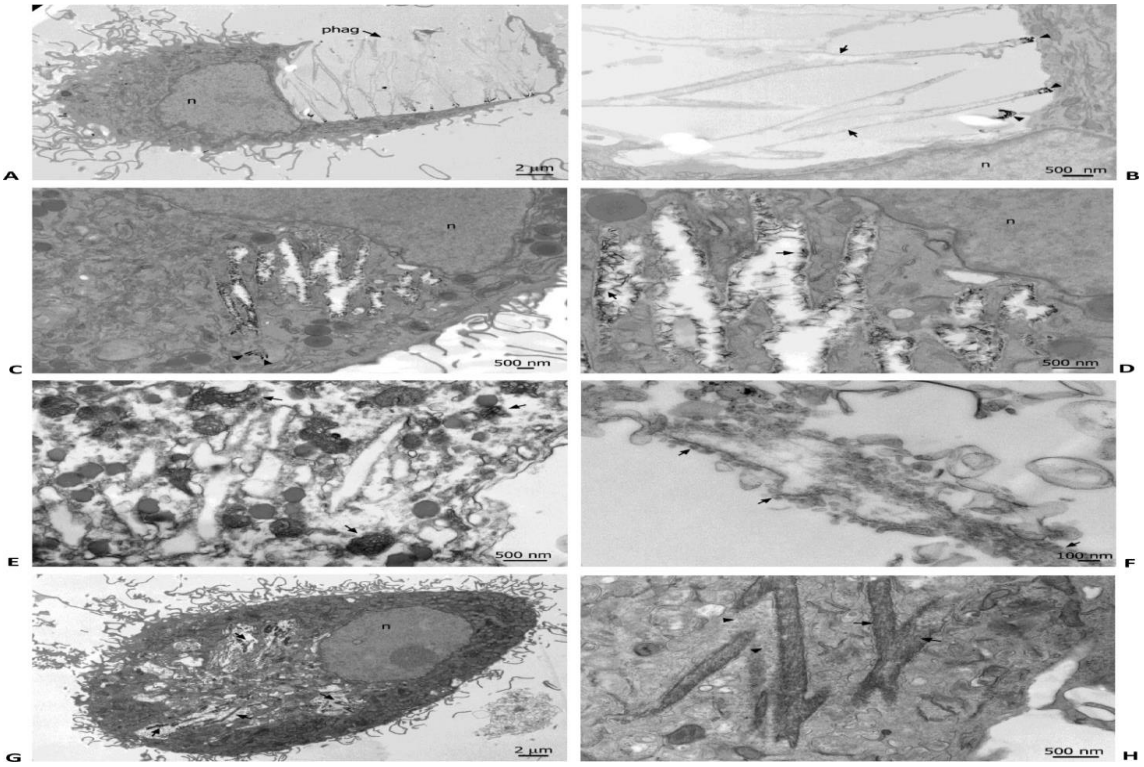

Figure 7: Bright-field TEM of HMMs for treated cells with nanowires for 1 h (A and B) and cultured without nanowires for 1 h (C and D), 4 h (E and F), and 24 h (G and H). Reproduced from [73].

The cytotoxicity of ZnO-NWs and $ZnCl_2$ was examined by the NRU assay, which estimates the accretion of neutral red dye in the lysosomes of viable cells. It was shown that no significant toxic variation between $ZnCl_2$ and ZnO at all tested doses, which demonstrated that the release of zinc ions was the cause of ZnO-NWs toxicity rather than their high aspect ratio. Furthermore, confocal laser fluorescence microscopy and bright-field TEM methods were utilized for further assessment of the cytotoxicity effect (Fig.7).

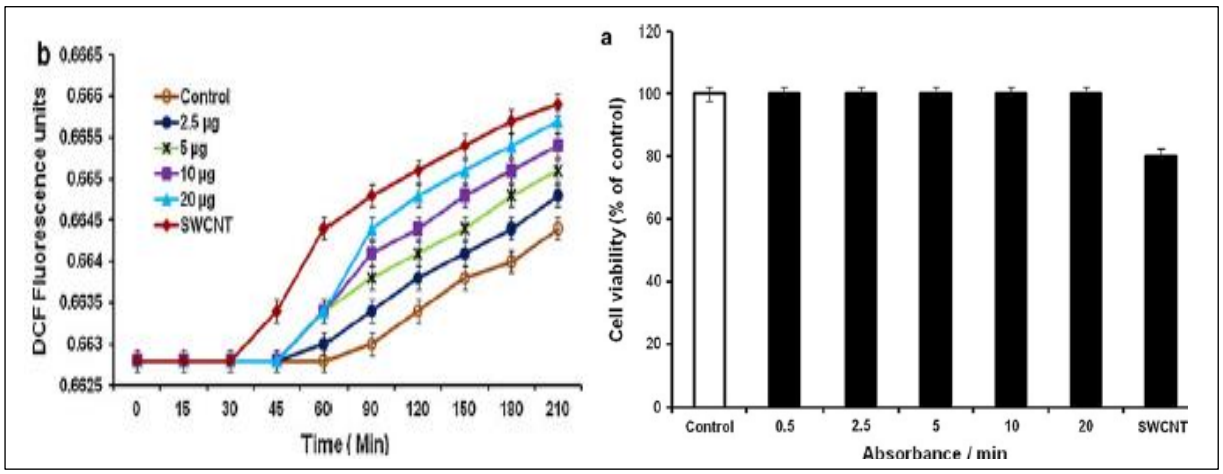

Figure 8: (a) LE cell viability after treatment with different doses of ZnO-NRs for 48 h. **(b)** Time kinetics of ROS production by ZnO-NRs of LE cells mediated with 2.5, 5, 10 and 20 µg/ml of ZnO-NRs. Reproduced from [74].

Confocal microscopy showed a cytotoxic effect through a sharp rise in FluoZin3 fluorescence, which is a zinc ions indicator and displays green fluorescence upon binding with zinc ions inside the cells, the cause attributed to the fast intracellular decomposition of the ZnO, leading to cell necrosis. In addition, to analyse whether decomposition of the ZnO takes place in the extracellular culture before uptake or intracellularly, ZnO-NWs were treated to two various simulated body fluids (SBFs); one SBF has an ionic content of extracellular medium with pH of 7.4 or another SBF which is more similar to lysosomal medium with pH of 5.2, following by performing an inductively coupled plasma mass spectroscopy (ICP-MS). Data showed that ZnO-NWs decomposed quickly in a SBF of lysosomal pH, whilst they were relatively steady at extracellular pH, indicating that decomposition of the ZnO occurred inside the cells provoked by the acidic lysosomal pH. Bright-field TEM exhibited a swift macrophage uptake of the ZnO-NWs aggregates by phagocytosis. Finally, it concluded the possible mechanism of ZnO-NWs cytotoxicity in HMMs was pH-induced, the intracellular release of zinc ions instead of the high-aspect ratio of nanowires. Moreover, zinc oxide nanorods (ZnO-NRs) have been synthesized via a hydrothermal growth process to investigate their biocompatible properties through the interaction with rat lung epithelial cells (LE) [74]. The ROS formation was evaluated using real-time assay upon treating cells with 2.5, 5, 10, and 20 µg/mL of ZnO-NRs, and it found that oxidative stress levels have not increased in a time- and concentration-dependent effect in LE cells (Fig. 8b). Additionally, the ZnO biocompatibility has further proved based on the MTT viability assay, as it showed the cytotoxic impact on ZnO-NRs treated LE cells had no dose and was time-dependent (Fig. 8a). The authors demonstrated that ZnO-NRs do not cause the production of free radicals, the accretion of peroxidative species, antioxidant deficiency, the loss of cell proliferation and DNA damage in LE cells. Therefore, it concluded that ZnO-NRs might be used as a safe candidate material for biomedical applications.

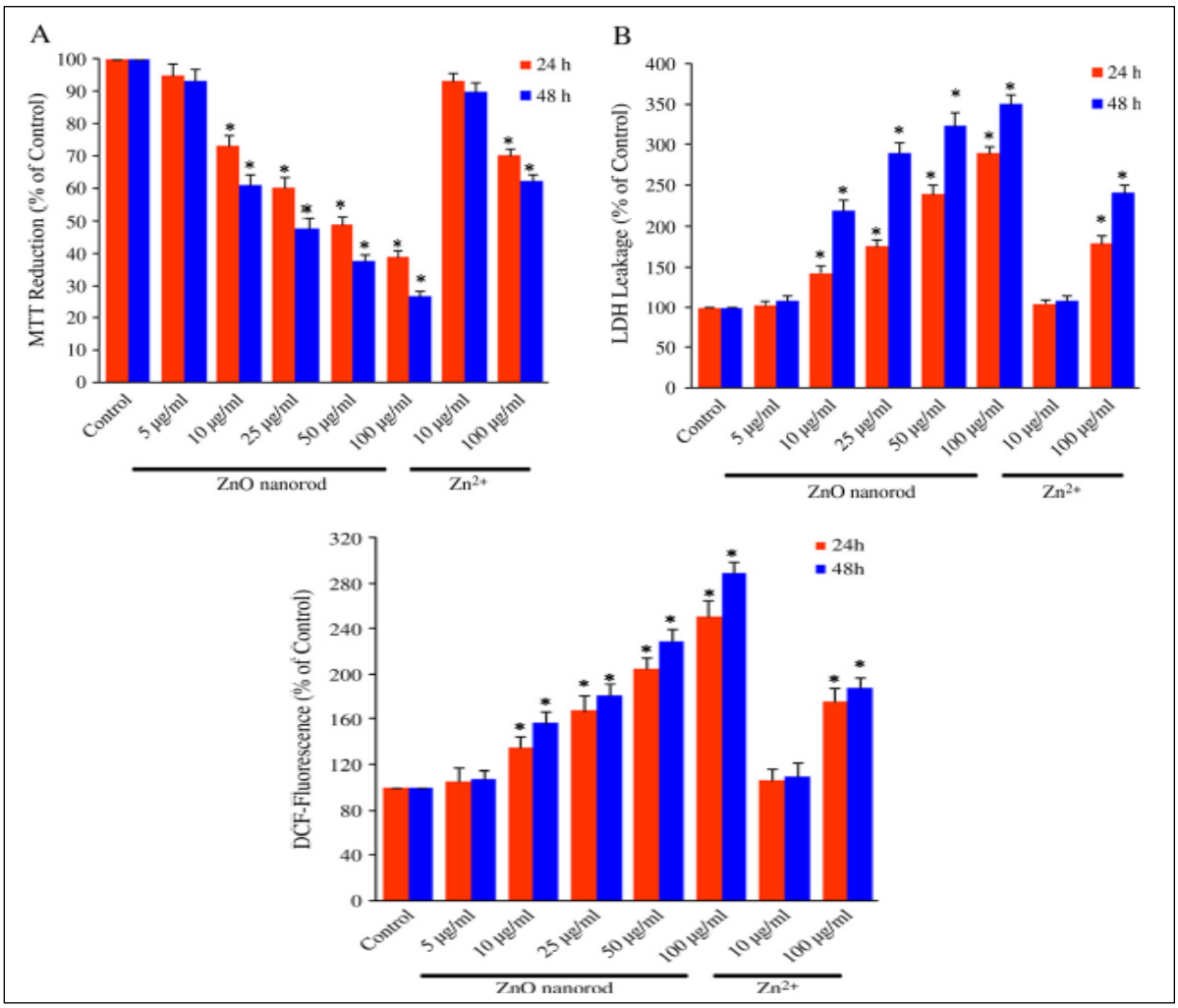

Figure 9: (A) The impacts of ZnO-NRs on mitochondrial efficiency of A549 cells (MTT reduction), cells were exposed to 5, 10, 25, 50 and 100 µg/ml of ZnO-NRs doses. **(B)** The impact of zinc ions on LDH leakage, cells were exposed to 10 and 100 µg/ml of Zn^{2+} for 24 and 48 h. **(C)** The impact of ZnO-NRs and zinc ions on

intracellular ROS formation in A549 cells. Cells were exposed to 5, 10, 25, 50 and 100 µg/ml of ZnO-NRs and 10 and 100 µg/ml of Zn^{2+} for 24 and 48 h. Reproduced from [75].

In addition, ZnO-NRs synthesised by sol-gel growth method have been used to examine their nanotoxicity in human alveolar adenocarcinoma (A549) cells [75]. MTT assay and LDH leakage assays were utilized to analyse the mitochondrial function and membrane damage, respectively. Data illustrated a decrease of cell viability upon treatment of A549 cells with ZnO-NRs doses of 10, 25, 50, and 100 µg/ml for 24 hours (Fig. 9, A), indicating that ZnO-NRs cytotoxicity was highly dependent on time- and concentration and nanorods also induced LDH leakage (Fig. 9, B). Besides, it found that ZnO-NRs induced an excessive formation of intracellular ROS in a time and dose-dependent impact (Fig. 9, C). The ZnO induction of oxidative stress has been confirmed through assessing different oxidative stress biomarkers e.g., MDA level, catalase (CAT), and a marker of LPO were much higher, whilst GSH level was significantly lower in treated A549 cells. Based on these findings authors suggested that oxidative stress could be the main mechanism for ZnO-NRs cytotoxicity in A549 cells. Furthermore, the ZnO-NRs ionization within the cell culture was tested to evaluate their cytotoxic impacts. An atomic absorption spectrometry (AAS) showed a significant decomposition of zinc ions (10 µg/ml) in A549 cells exposure to 100 µg/ml of ZnO-NRs; however, the concentration of these released ions was not enough to provoke cytotoxic impact in human cells excepting in the case of direct contact within the cells. Additionally, caspase-3 and caspase-9, which are critical enzymes in cell apoptosis, were highly activated (Fig. 10, A and B), indicating the role of caspase cascade in -the apoptosis of A549 cells. Besides, the expression of antiapoptotic proteins such as survivin and Bcl-2 were lower, whilst the expression of pro-apoptotic protein Bax was much more significant in ZnO-NRs treated cells. Finally, according to western blot analysis, it found that ZnO-NRs induced the expression of heat shock protein 70 (Hsp70), which is a biomarker of cell damage, in the treated cells. It concluded based on these data, that apoptosis, oxidative stress, and cytotoxicity in A549 cells were induced by ZnO-NRs.

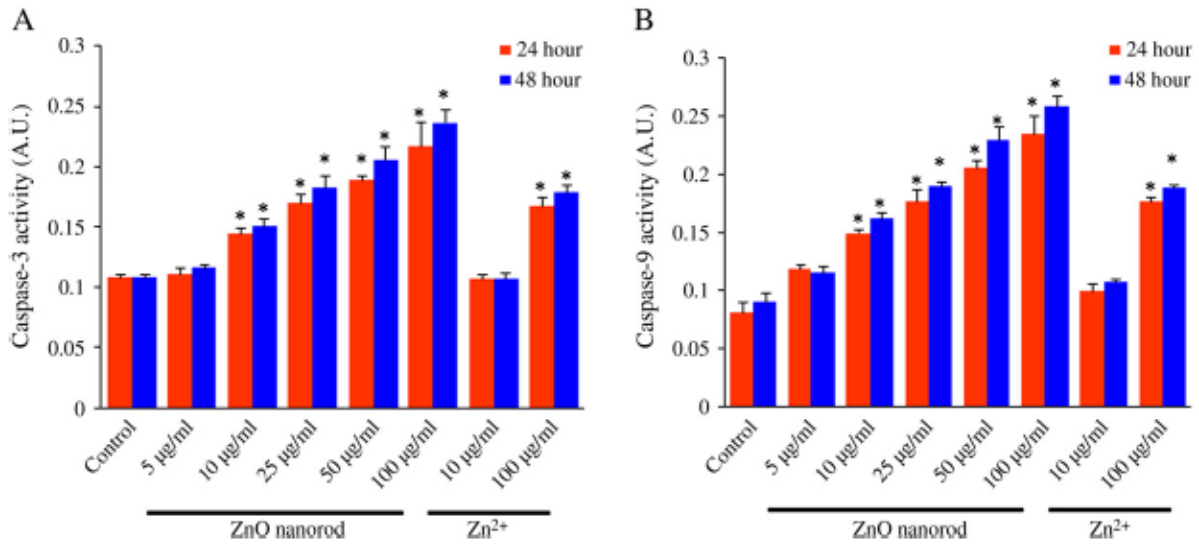

Figure 10: The impact of ZnO-NRs and Zn2+ on the activity of **(A)** caspase-3 and **(B)** caspase-9 enzymes in A549 cells. Cells were exposed to 5, 10, 25, 50 and 100 µg/ml of ZnO-NRs and 10 and 100 µg/ml of zinc ions for 24 and 48 h. Reproduced from [75].

A study was carried out by Wang et al. [76] to evaluate the biocompatibility of ZnO-NWs to three kinds of excitable cells, which are NG108-15 neuronal cell line, HL-1 cardiac muscle cell line, and primary neonatal rat cardiomyocytes. The MTT assays were utilized to investigate the biological impacts of ZnO-NWs and determine the mitochondrial functions of the cells cultured on these nanowires. Data showed that ZnO-NWs had statistically important inhibitory impacts after one day in culture on the metabolism activity of NG108-15 and HL-1 cell lines compared to gold, glass, and polystyrene substrates as well as on the metabolism of neonatal rat cardiomyocytes compared to gold substrate. The diameters of NG108-15 cells, HL-1 cells, and cardiomyocytes were approximately 10–100, 20, and 13 µm, respectively, whilst the nanowires thickness was nearly 300 nm. Since every individual cell was entirely shrouded by the ZnO-NWs, therefore it was demonstrated that the inhibitory impacts might be attributed to the piercing of the cell membrane to the nanowires or the deficient

attachment between the cells and substrate. In addition, the release of intracellular Zinc ions in the lysosomes might be another cause of ZnO-NWs toxicity. Lastly, an interesting finding concluded from this study was the selectivity to cytotoxicity on rapidly dividing NG108-15 and HL-1 cells compared to primary cardiomyocyte cells, as the primary cells were most tolerant to the inhibitory impact of ZnO-NWs. Due to the large-scale industrial manufacture of nano/micro ZnO-tetrapods (ZnO-TPs) and their morphological diversity compared to classical spherical ZnO-NPs, their biosafety on human dermal fibroblasts has been studied by investigating the influence of cell medium parameters and material properties on ZnO-TPs cytotoxicity [77]. According to the results, the cytotoxic efficiency of ZnO- TPs was considerably less compared to that of spherical ZnO-NPs and was also highly influenced by the density of fibroblasts in the cell medium and the number of preceding cell divisions. Moreover, the tetrapod's morphology affected the cellular toxicity in contrast to surface variations functionalized by UV radiation illuminating or O_2 treatment and material age. Besides, the authors noticed that significant toxicity is caused by the direct contact between ZnO-TPs and fibroblast cells compared to transwell culture models, which offer non-direct interaction through the release of zinc ions. In vitro study introduced by Song et al. [78] to assess the cytotoxic impact of commercial ZnO-NPs with diverse sizes and morphologies in mouse macrophages (Ana-1). ZnO-NRs of several sizes (width: 100 nm, length: 107.6 nm; width: 30 nm, length: 70.89 nm), fine ZnO rods (width: 173.48 nm, length: 341.75 nm), and spherical ZnO-NPs (10–30 nm) were utilized to determine the contribution of released zinc ions and cellular ROS in the cytotoxicity of ZnO-NSs. The data showed by the cell viability assay, LDH and ROS levels evaluations revealed that ZnO-NSs provoked concentration-dependent toxicity on Ana-1 cells. In Particular, spherical ZnO-NPs produced more toxicity compared to ZnO-NRs and also induced an excessive ROS formation than fine ZnO rods because of their large surface area and higher surface efficiency. Besides, according to inductively coupled plasma atomic emission spectroscopy (ICP-AES), the toxicity of ZnO-NRs and ZnO-NPs initiated via dissolved zinc ions into the cell culture, and then those ions triggered the ROS production and the LDH leakage from the cell membrane. The concentration of zinc ions was significantly linked with viable cell number and LDH level induced by ZnO NSs hence, the released zinc ions played a critical role in the cytotoxicity of both ZnO-NRs and ZnO-NPs.

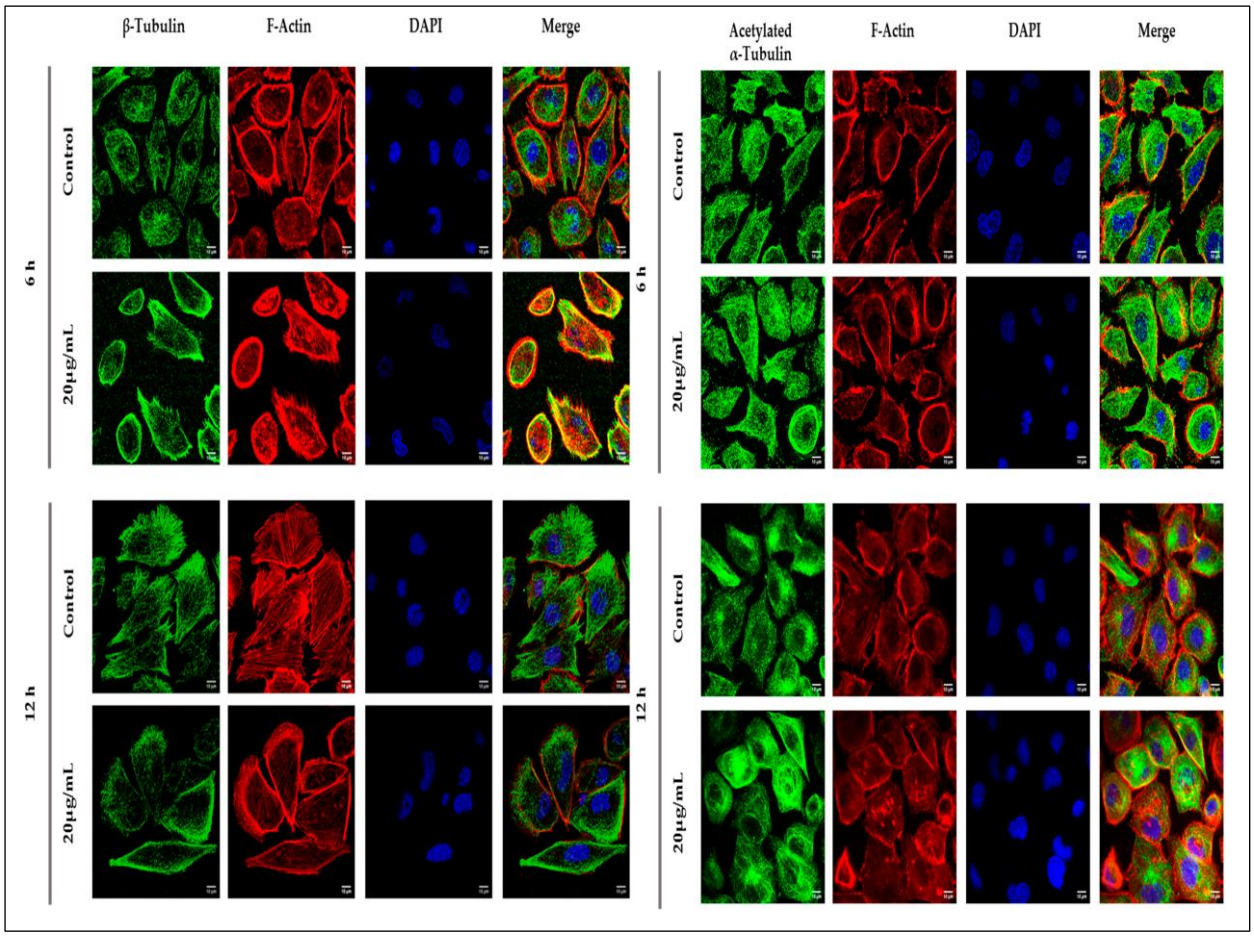

Figure 11: Immunocytochemistry images of β-tubulin, acetylated α-tubulin, and F-actin protein levels. The cells were treated with 0 and 20 µg/mL ZnO-NPs for 6 and 12 h. Reproduced from [79].

An in vitro recent study carried out by Pinho et al. [79] focused on investigating the toxic impacts of ZnO-NPs on mouse-derived spermatogonia cell line (GC-1), especially variations in cytoskeleton and nucleoskeleton. GC-1 cell lines were incubated with various concentrations of nanoparticles for periods of 6 and 12 h, followed by performing cell viability and cell death rate measurements using three different techniques, including the resazurin assay, the trypan blue exclusion approach, and flow cytometry analysis. Besides, evaluating the intracellular ROS levels and DNA damage by the total ROS detection kit. Immunoblotting and immunocytochemistry techniques have been applied to measure potential variations in the cytoskeleton of spermatogonia cells, protein levels of α-tubulin-acetylated and β-tubulin as well as structural proteins of microtubules (Fig. 11). The immunocytochemical findings showed that β-tubulin and F-actin were highly elevated after treatment with 20 µg/mL of ZnO-NPs for 6 h, however, β-tubulin and F-actin levels were dramatically decreased after 12 h at the highest dose of ZnO-NPs. Besides, acetylated α-tubulin levels were increased following 20 µg/mL of ZnO-NPs exposure for 6 and 12 h. In addition, assessments of the complex proteins related to the Nucleoskeleton and Cytoskeleton (LINC), and other intricately connected to nuclear envelope proteins (NE) for spermatogenesis have also been performed by immunocytochemistry. Data clearly showed a significant cytotoxic impact in GC-1 cells upon treatment of higher doses of ZnO-NPs, increasing the intracellular ROS levels, DNA damage, cytoskeleton and nucleoskeleton dynamics changes, and leading accordingly to cell necrosis. The authors concluded that the potential hazard of ZnO-NPs in GC-1 cell lines could not be determined only through studying the dose and time of ZnO treatment in GC-1 cells. Therefore, it would be importantly efficient to evaluate in vivo ZnO-NPs aggregations in the testis at a short exposure time and lower doses.

An in vitro impact of ZnO-NPs toxicity on the sperm motility of rabbits has been investigated via diluting fresh semen extracted from sexually mature rabbits with diverse doses of ZnO-NPs (6–391 mg/mL) [80]. The MTT assay for rabbit spermatozoa cellular metabolic activity was used after three h of incubation time and tested motility parameters including, total motility (MOT), progressive motility (PRO), distance curved line (DCL) and velocity curved line (VCL) examined. According to the obtained findings, motility and progressive motility significantly declined after incubating for three h at a higher ZnO-NPs concentration. Besides, cell viability and spermatozoa membrane integrity were slightly raised at lower doses of ZnO-NPs, indicating that spermatozoa motility and viability parameters were not highly dependent on ZnO-NPs concentrations. Holmes et al. carried out a study to assess the total and labile zinc levels by Dulbecco's Modified Eagle media (DMEM) with various chelators to measure the HaCaT keratinocyte viability and the intracellular labile zinc levels upon adding ZnO-NPs and $ZnSO_4$ [81]. In addition, a comparison between the relative disposition and local cytotoxicity of ZnO-NPs and released zinc ions after treating keratinocyte culture cells and the human epidermis (ex vivo) with ZnO-NPs has been performed. Data demonstrated that ZnO-NPs cytotoxicity to HaCaT cells is significantly dependent on the culture media conditions and the addition of the chelating agents such as bovine serum albumin (BSA) and Ethylenediaminetetraacetic acid (EDTA). Additionally, when HaCaTs were treated with both ZnO-NPs and $ZnSO_4$, it observed ZnO NP induced cytotoxicity effect at lower doses than $ZnSO_4$. For both ZnO-NPs and $ZnSO_4$, cell viability was pertinent to the increased levels of intracellular and extracellular zinc ions, leading to a decline in cell survival. Besides, when zinc species were exposed to viable human epidermis, an increase in labile zinc level was observed, which was attributed to a change in the metabolic condition of the viable human epidermis (Fig. 12).

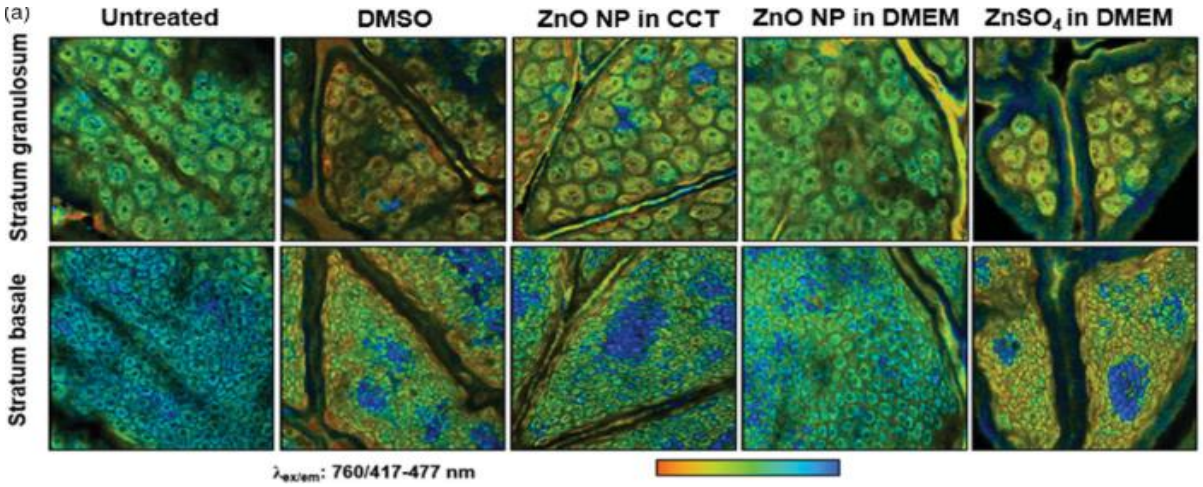

Figure 12: Confocal microscopy of zinc within ex vivo human viable skin tissue after the treatments of ZnO-NPs and $ZnSO_4$. Reproduced from [81].

The authors concluded that zinc ions released from ZnO-NPs could be possibly toxic to the viable epidermis when breaching the outermost stratum corneum, while no toxic effect was found when treating the intact human skin with ZnO-NPs. Recently, Santacruz-Márquez et al. [82] examined the in vitro impact of ZnO-NPs on steroidogenesis and induced oxidative stress in ovarian antral follicles. Antral follicles of an adult CD-1 female mice cultured at ZnO-NPs concentrations of 5, 10, and 15 µg/mL for a period of 96 h. According to the results, all the selected doses of ZnO-NPs did not affect the apoptosis and cell proliferation in antral follicles. Additionally, a low dose of ZnO-NPs (5 µg/mL) was found to elevate the estradiol levels and decline estrogen receptor alpha levels. In addition, ZnO-NPs treatment at 15 µg/mL resulted in an induction of antioxidant responses in the antral follicles as showed via alterations in the levels of antioxidant enzymes, for instance, nuclear factor-erythroid 2-related factor (NRF2), CAT, SOD, Glutathione reductase (GR), GSH-PX and depletion of ROS levels. Moreover, following one hour of treatment, ZnO-NPs decomposed in culture media and integrated into cells, especially at the lower dose, which led to significant levels of internalization to the antral follicles than the higher dose. Another recent study conducted by Li et al. investigated the toxic impact of ZnO-NPs on diffuse large B-cell lymphoma (DLBCL) mitophagy using the DLBCL cell line (U2932) [83]. The human U2932 cells were cultured at various doses of ZnO-NPs (0, 3.0, 6.0, 12.0, 18.0, 24.0, 48.0, and 60.0 µg/mL). Q-PCR assay was performed to explore the role of the PTEN-induced kinase 1 (PINK1) and parkin proteins signalling as a mitochondrial autophagy regulator following ZnO-NPs exposure. Besides, the western blot was applied to evaluate the expression levels of PINK1, Parkin, P62, and LC3. Additionally, monodansylcadaverine (MDC) staining, as a fluorescent probe and a marker of autophagic lysosomes, was utilized to identify the intracellular autophagy in U2932 cells. The oxidative stress in U2932 cells was examined by measuring intracellular ROS concentrations by flow cytometry. The data indicated that the cell viability significantly declined with elevating dose and time of ZnO-NPs.

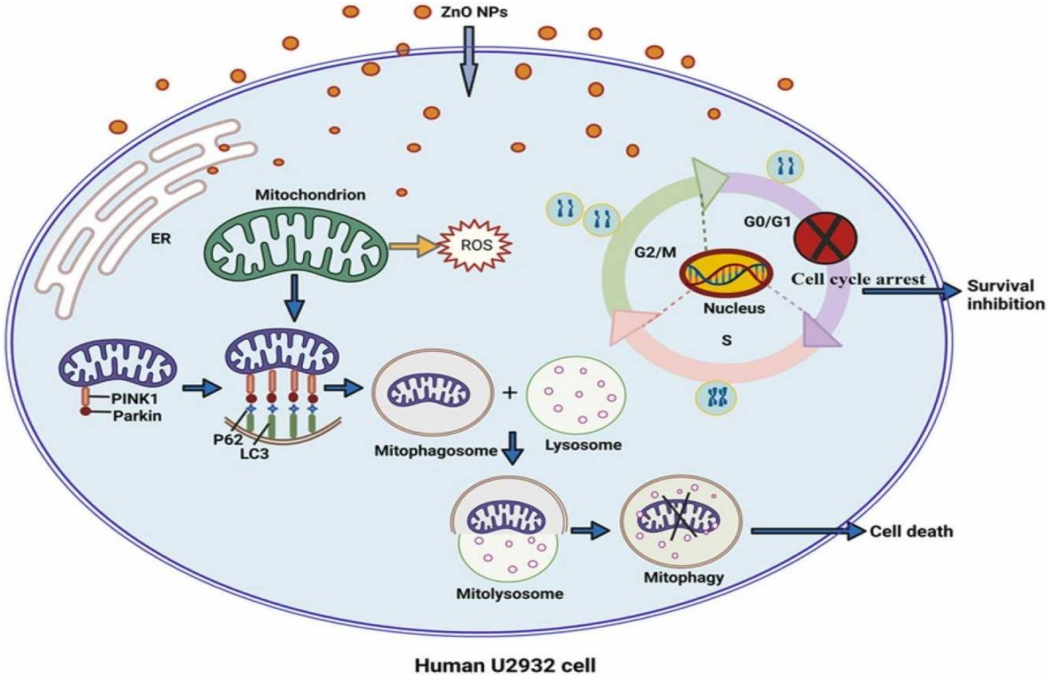

Figure 13: A schematic diagram of ZnO-NPs induce cytotoxicity in human U2932 cells by stimulating PINK1/Parkin-mediated mitophagy. Reproduced from [83].

Furthermore, it found that ZnO-NPs might trigger the PINK1/Parkin-mediated signalling pathway, leading to provoke mitophagy and cell death in ZnO-treated cells, indicated in a significant increase of the PINK1 and Parkin mRNA expression levels in a dose-dependent way. The expression level of P62 declined while it increased for LC3 mRNA, and additionally, higher doses of ZnO-NPs effectively increased ROS formation in U2932 cells. Meanwhile, the MDC fluorescence intensity was increased, indicating the induction of the autophagic pathway by ZnO-NPs. Based on TEM images, higher doses of ZnO-treated cells massively showed many autophagic vesicles and membrane injuries. In general, the toxicity induced by ZnO-NPs in U2932 cells is illustrated in Fig. 13. Farzaneh et al. carried out a short-term in vitro investigation to study the genotoxicity effect of ZnO-NPs exposure at low doses (10 and 20 µg/ml) for a period of 1 and 7 days on a testicular cell culture enriched with Spermatogonial stem cells (SSCs) [84]. Even though the one-day treatment of both ZnO-NPs doses did not considerably affect the cell viability, chromosomal imbalance, and DNA fragmentation of the spermatogonia, induction of ROS production and activation of apoptosis and inflammation-associated proteins i.e., tumour protein (p53), caspase 3 and interleukin-6 (IL-6) were observed. On the other

hand, ZnO-NPs exposure for 7 days at the two doses was found to trigger chromosomal imbalance in the spermatogonia. Liu et al. studied the renal toxicity of ZnO-NPs at varied sizes of 40 and 100 nm by orally administrating a ZnO dose of 34 mg/kg to adult mice for 2 months [85]. The results demonstrated that the cell survival and proliferation of HK-2 cells treated with ZnO (40nm) at 10 µg/mL were effectively declined compared with the control cells, besides the cytotoxic activity of ZnO (40nm) was higher than that of ZnO (100 nm). Additionally, the toxicity of ZnO-NPs was assessed using the LDH leak assay, it found that ZnO-NPs could lead to loss of the membrane integrity, as indicated in a dramatic elevation of the LDH leak. According to flow cytometry examination, ZnO (40 nm) highly increased the cell death of administrated HK-2 cells compared to ZnO (100 nm). Furthermore, dramatic alterations in the expression levels of proteins associated with apoptosis were observed in ZnO (40) (10 and 15 µg/mL) treated cells compared to ZnO (100) group at the same doses (Fig. 14).

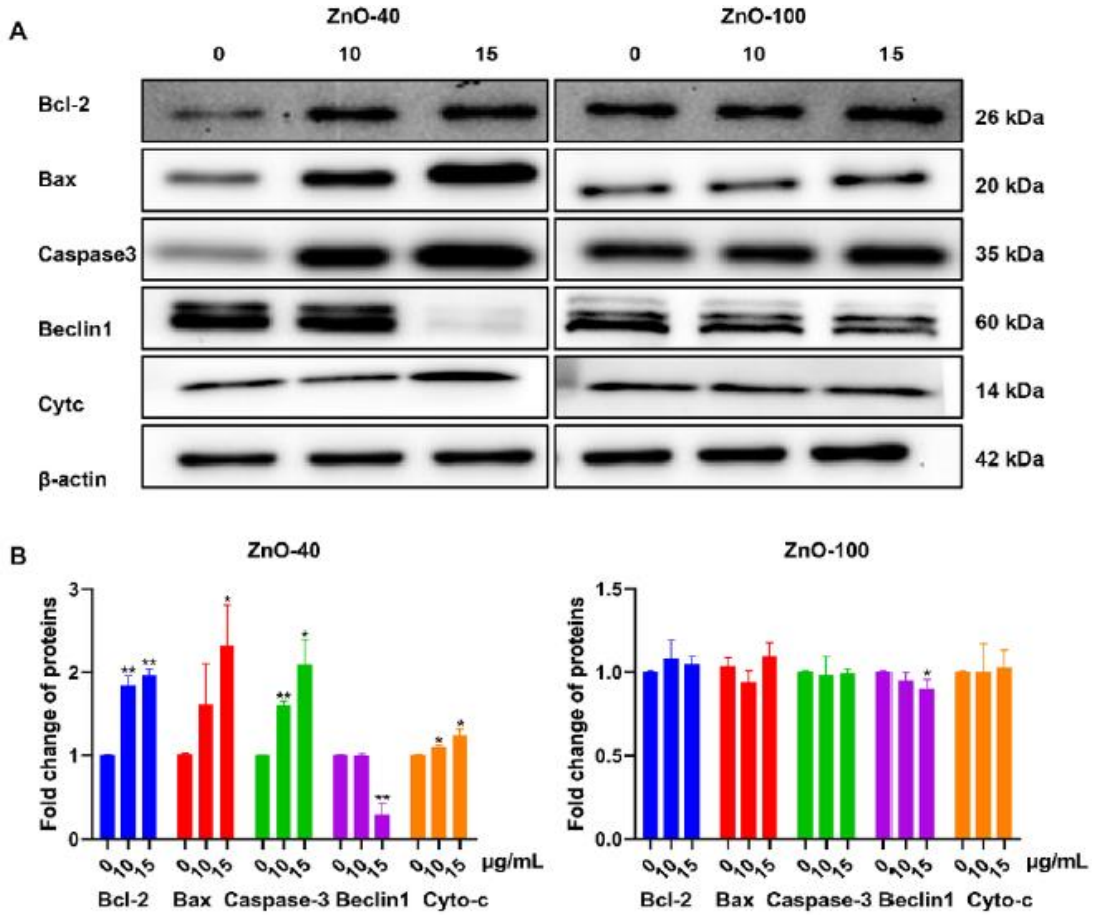

Figure 14: Western blotting of apoptosis-associated proteins (Bax, Bcl-2, Caspase-3, Beclin-1, Cyto-C) in HK-2 Cells exposed to ZnO (40 nm) and ZnO (100 nm). Reproduced from [85]

The intracellular mitochondrial levels of ROS of HK-2 cells exposed to ZnO (40 nm) were remarkably higher than those treated with ZnO (100 nm), besides both selected sizes of ZnO (40, 100 nm) caused mitochondrial damage to HK-2 cells, as showed in the loss of MMP, however ZnO (40 nm) was considerably toxic to the mitochondria compared to ZnO (100 nm). Finally, the authors concluded that ZnO-NPs induced toxicity in human renal tubular epithelial HK-2 cells in a particle size-dependent way, especially the smaller particle size (40 nm). Many researchers have adopted green synthesis, in which no chemical or hazardous elements are involved in the fabrication process, to produce biocompatible and eco-friendly nanostructured materials for medical and diagnostic applications. One study conducted by Mohapatra et al. [86] grown ZnO-NPs using clove and cinnamon extract to address their cytotoxic impact, anti-inflammatory and antioxidant activities applying brine shrimp assay, BSA, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay at the nanoparticle's concentrations of 5 µL, 10µL, 20 µL, 30 µL, 50 µL. The anti-inflammatory activity was assessed through the percentage of the inhibition of protein denaturation, which was significantly higher at ZnO doses of 40 µL (91.1%) and 50 µL (90.5%). The antioxidant activity was presented via the percentage of the inhibition of DPPH free radicals, which was higher (86.2%) at a ZnO dose of 20 µL. Authors concluded that ZnO-NPs synthesized based on clove and cinnamon extract could potentially serve as an anti-cancer, anti-inflammatory and antioxidant agent.

Biomedical Applications of ZnO nanomaterials

Nowadays, nanoscience has been effectively incorporated into a wide range of biomedical applications, providing valuable chances to study the physiological structure of biomolecules and enabling scientists to develop sophisticated and novel devices for diagnostic and therapeutic purposes, contributing to healing different chronic diseases. Recently, the green or biological approach to synthesizing ZnO nanostructures using various plant species has attracted enormous attention as a potential substitute for classical techniques. Most importantly, such a method provides several benefits, e.g., producing safe and eco-friendly materials without involving toxic and hazardous chemicals, which is significantly required for biomedical and clinical applications. Herein, the recent development of ZnO material and their biomedical applications in antimicrobial and antifungal, anticancer, bioimaging, tissue engineering, wound healing, gene, and drug delivery were demonstrated.

Antimicrobial and antifungal applications

The antimicrobial activity of ZnO nanomaterials has been widely investigated to defeat bacterial diseases and develop a new generation for an efficient drug delivery platform. Additionally, various metal nanostructures and compounds such as titanium oxide (TiO_2), copper oxide (CuO) and iron oxide (FeO) have been recognized as anti-biotic agents due to their multifunctional, antibacterial, and antifungal characteristics [87]. Mainly, these materials have been potentially applied to the degradation of water pollution [88], food packaging and preservation, specific self-cleaning textiles [89], super-hydrophobic and antibacterial surfaces [90], etc. Overall, the antimicrobial activities of nano-scaled materials originated from their intrinsic features, i.e., the large surface area to volume ratio, which enables a strong reaction with many bacterial receptors [91]. The effective antimicrobial activity of ZnO-NPs is highly enhanced when these materials are produced by green fabrication methods. It was demonstrated that ZnO-NPs are effective agents for hindering both gram-positive and -negative bacteria. In particular, the concentration of nanoparticles significantly affects their antibacterial effect, whereas morphology and particle size have inconsiderable effects. Such antimicrobial activity against pathogenic microorganisms was related to some proposed mechanisms which attempt to demonstrate the reasons behind those observed behaviours. The formation of ROS and release of zinc ions are potential causes due to the electrostatic interaction of the nanoparticles with the membrane cell wall of the bacteria.

Bacteria and plant-based ZnO-NSs have been extensively reported compared to their conventionally physiochemical synthesized nanomaterials. An impressive synthesis method to produce ZnO-NPs has been adopted by Suresh et al. [92] by applying *Cassia fistula* plant extracts as capping agents to decrease elements, including polyphenols (11%) and flavonoids (12.5%), then efficiently producing a hexagonal wurtzite structure of ZnO-NPs in nanoscale from 5 to 15 nm. Further, significant bactericidal activity in Klebsiella aerogenes, E. coli, Plasmodium desmolyticum and S. aureus has been noticed. Dobrucka et al. [93] evaluated the antimicrobial efficacy of ZnO-NPs (60–70 nm) grown by using Trifolium pratense flower extract against clinical and standard strains of S. aureus and P. aeruginosa and standard strain of E. coli. Overall, the findings confirmed that the prohibitive impact of ZnO has elevated by increasing its concentration. Joseph et al. [94] utilized the extract of Boerhavia diffusa leaves to biosynthesize ZnO-NPs and assess their antimicrobial efficacy against methicillin-resistant Staphylococcus aureus (MRSA) strains. According to the results, ZnO-NPs might hinder the pathogenic effect caused by MRSA strains and hence, act as an effective substitute for traditional cleaning and disinfecting approaches in clinics and medical centers. Presently, Bhatt et al. [95] utilized methanolic seed extract from the eucalyptus grandis plant to synthesise Mg-doped ZnO and ZnO-NPs and test their antibacterial impacts against Escherichia coli. It found that doping Mg with ZnO could improve its characteristics, accordingly, enabling it to be remarkably efficient as an antibacterial agent against gram-negative bacteria. Recently, Krishnamoorthy et al. [96] effectively bio-synthesized Citrus hystrix leaf extract-mediated ZnO-NPs to evaluate their microbial potency. Importantly, the bio-efficient leaf extract provides hydroxyl ions and acts as a capping and stabilizing agent, which efficiently reduces the nanoparticle's dimensions and enhances their physicochemical characteristics. Based on the consequences, it was found that the Citrus hystrix leaf extract mediated ZnO-NPs exhibited great antibacterial efficacies to Escherichia coli and Staphylococcus aureus and antifungal efficiency to Candida albicans and Aspergillus niger. In addition, the significant antioxidant activity of ZnO-NPs has been assessed using the DPPH assay. The authors highly suggested Citrus. hystrix leaves extract mediated ZnO-NPs could serve as a probe in the industry of antifungal and antibacterial drugs.

The biosynthesis of ZnO-NPs has been reported by Rahman et al. [97] using Cocos nucifera leaf extract to examine their antimicrobial and antioxidant activities. The green-prepared ZnO-NPs were found to show highly significant antimicrobial potency against several pathogenic bacteria and fungi, including T. harzianum and S. aureus, within around 14 and 10 mm of an inhibition zone, respectively. Therefore, it is more likely to apply in the progression of antibiotics and pharmaceutic and biomedicine manufacturing. An interesting study of eco-friendly and green synthesis of ZnO-NPs using an Azadirachta indica (Neem) leaf extract has been reported by

Bhuyan et al. [98]. The antibacterial efficacy of the bio-prepared nanoparticles has been evaluated against gram-positive and negative bacteria: Staphylococcus aureus, Streptococcus pyogenes and Escherichia coli showing that the bacterial population effectively declined by increasing the nanoparticles concentration (Fig. 15). However, it was observed that gram-negative bacteria were less sensitive to the nanoparticles compared to gram-positive.

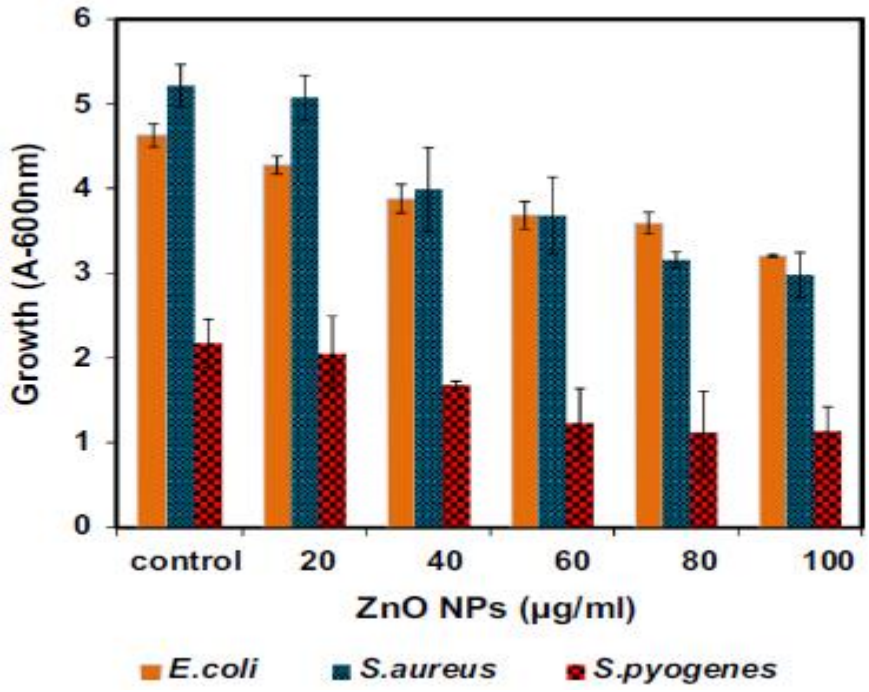

Figure 15: Growth of S. aureus, S. pyogenes and E. coli treated with different doses of ZnO-NPs (20–100 mg/mL). Reproduced from [98].

In addition, among the microorganisms used to produce ZnO-NPs, bacteria were highly considered a great candidate because of their practicability of segregation and the natural detoxification system of metal ions, besides bacteria provide fast and progressive development for mass production of ZnO-NPs [99]. Saravanan et al. [100] bio-synthesized well-shaped ZnO-NPs (45 and 95 nm) using Bacillus megaterium cell-free extracts as a bio-reductant and Zinc nitrate as a precursor. The multidimensional impact of nanoparticles on Helicobacter pylori strains has been investigated, and their biosafety in normal human mesenchymal stem cells (HMSc) was evaluated. The obtained results showed that the growth of bacterial was inhibited at the nanoparticles concentration of 16 and17 μg ml^{-1}, which was possibly attributed to ROS located on the surface of the nanoparticles and electrostatically interacting to the bacterial surface, resulting in internalizing into the bacteria cell membrane and destructing the cellular biomolecules such as lipids, DNA, and proteins. Besides, it was also demonstrated that zinc ions enhanced the antimicrobial activity of the multidrug-resistant Helicobacter pylori strains. In addition, the biosafety of the nanoparticle revealed insignificant toxicity to the mammalian cells at a concentration equal to and less than 12.5 μg ml^{-1}concentration. Recently, Nehru et al. [101] demonstrated an efficient and eco-friendly biogenic fabrication approach to produce ZnO-NPs using an endophytic fungus (*Xylaria arbuscula*) isolated from *Blumea axillaris* Linn. The Biosynthesized nanoparticles have been investigated for their antimicrobial potency against pathogenic gram-positive (Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Enterococcus faecalis) and gram-negative bacteria, and fungi (A. niger, C. albicans). The obtained results showed that ZnO-NPs were an effective inhibitor for bacteria and microbes in a concentration-dependent manner (Fig. 16).

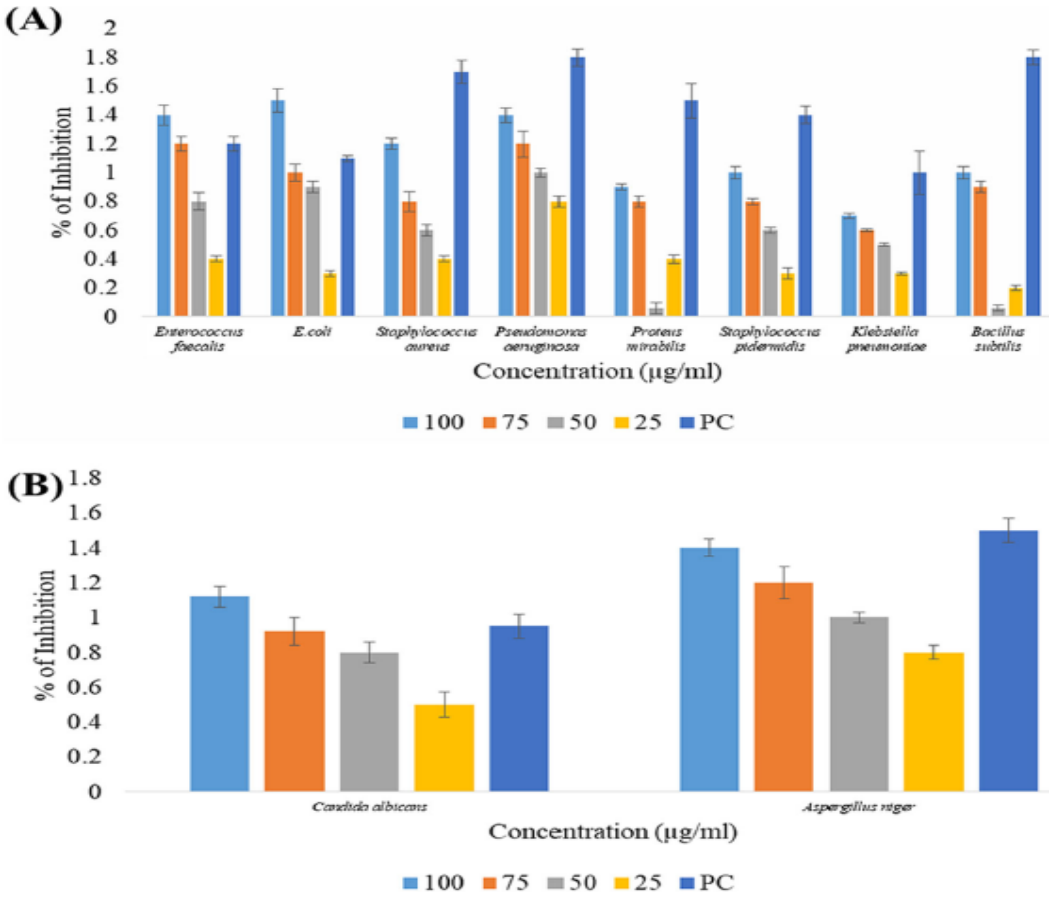

Figure 16: (A) Antibacterial activity of bio-synthesized ZnO-NPs against gram-positive and gram-negative bacteria by disc diffusion method, **(B)** Antifungal activity of ZnO-NPs against Candida albicans and Aspergillus niger. Reproduced from [101].

Anti-cancerous applications

Cancerous diseases have been described by the increasing growth of abnormal cells and tissues, which uncontrollably divide, leading to the development of tumours that can spread out through other organisms, resulting in extreme damage to affected biological parts and even death. It was demonstrated that anti-tumour treatments, including chemotherapy, radiotherapy, photodynamic therapy, and surgery, were significantly unreliable against such a chronic disease and developed critically serious and unintended consequences because of their deselected intractability towards healthy cells [102]. Therefore, biomedical applications for anti-tumour diagnosis and therapy have provided a thriving research area using engineered nanomaterials that have many multifunctional properties, such as efficient and passive targeted delivery, high solubility, biosafety, and biocompatibility [103]. Furthermore, the diameter dimension of the nanowires is smaller than that of cells and biomolecules, such as DNA, RNA, circulating tumour cells (CTCs), and proteins, hence the nanowire tip can penetrate and interrupt the cell membrane function, which leads to potentially extract microorganisms in cells faster than in previous traditional approaches [104]. Additionally, scientists were able to apply nanostructured materials as a probe tip to observe cellular electrical interactions and record changes in living biological systems like cells and tissues [105]. Naturally, zinc is considered one of the most critical trace elements in the human body, playing a crucial role in the immunity system, influencing many features of humoral and cellular immune responses, and preserving essential cellular homoeostasis, whilst a deficiency of it can induce and boost the growth of malignant cells. Moreover, zinc has a significant function in the oxidative stress process, deoxyribonucleic acid (DNA) replication, the damage and repair of DNA, cell cycle progression and apoptosis [30]. Additionally, the gene p53 is well known for its role in suppressing tumours and controlling apoptosis by activating the caspase-6 enzyme, this tumour inhibitor gene is effectively dependent on zinc to be active [106]. Besides, in cancerous cells ZnO-NPs generate an excessive amount of ROS than in normal cells, causing a substantial degree of oxidative stress leading to the cell's death [107]. In addition, when a cell experiences any malignant conditions, a DNA repair mechanism is stimulated to restore the variations in the chemical structure of DNA, otherwise, the cell passes through an apoptosis process to hinder the division of altered cells, which might accordingly develop in the cancerous cells. In whatever way, zinc is involved in all those essential

physical and chemical processes to defend against cancer, and a deficiency of cellular zinc can lead to DNA damage, resulting in destroying the integrity and stability of DNA and increasing the chance of cancerous disease [107]. Ahmed and co-workers [108] showed that ZnO-NPs can efficiently encapsulate chemotherapeutic drugs and carry them straightforwardly to the tumour position, decreasing the probable side effects. A similar study showed that the cytotoxic activity of ZnO-NPs on brain tumour cells was highly selected, without causing any harmful effect on normal human astrocytes [109]. The promising anti-tumour effect of biosynthesized ZnO-NPs against the lung cancer cell line (A549 has been investigated by Murali et al. [110]. The results showed that the nanoparticles showed efficient cytotoxic impacts similar to the effect of a cyclophosphamide drug at smaller doses. Besides, such an anticancer behaviour of ZnO-NPs was concentration-dependent, hence, significant doses of ZnO revealed higher anticancer activity. In addition, the bio-safely produced ZnO-NPs have been investigated for their anticancer properties against breast cancer cells. The results showed strong cytotoxic activity in those cancerous cells at 81.75 μg ml^{-1} of ZnO-NPs concentration [111].

Tissue engineering and wound healing applications

Tissue engineering is a branch of technology that produces structures which are designed from biocomponents and biomaterials that can imitate the natural organ/tissue to develop the functional and physiochemical properties and stimulate the biological interactions needed to assure the compatibility of these materials. Therefore, these structures can be applied for exchanging or repairing injured biological organs, consequently avoiding high-priced, traditional organ/tissue transplant procedures [112]. The ongoing evolution of nanostructure manufacture has developed eco-friendly, biocompatible nanomaterials such as nanoporous scaffolds and nanofiber membranes to be applied in periodontics, neural, bone, and skin tissue engineering [113, 114]. In particular, ZnO nanomaterials provide antineoplastic, wound healing, ultraviolet scattering, osteogenesis, and angiogenic properties, which efficiently were exploited in various tissue engineering applications [115, 116]. However, it has been only a few literature demonstrated the ZnO function in increasing cell growth, and proliferation, promoting the integration of the scaffolds to host tissue/organs, and enhancing the metabolic system of various cell lines for tissue engineering and wound healing applications. In addition, the in vitro and in vivo cytotoxicity of bio-synthesized ZnO-NPs has been comparatively investigated with clinically advocated ZnO-NPs using balb mice 3T3 fibroblasts as essential cell lines of jointed tissue, which help in repair and healing process of damaged tissue cells. The findings indicated that ZnO-NPs have lower toxicity at higher doses compared to commercial nanomaterials [117].

Importantly, the tissue engineering platform requires highly biocompatible nanostructures for cell growth, cell viability, and differentiation processes. These characteristics have been investigated on ZnO nanowires using the PC12 cell line as a typical example for neuronal cells and the H9C2 cell line as a suitable model for muscle cells [118]. In terms of differentiation, PC12 cells revealed a well-progressed neurite network, whilst H9C2 cells displayed less developed regular myotubes and no sequenced dislocations, which ascribed to the various interactions among the cells and the substrate. Khatami et al. [119] demonstrated that the green synthesized ZnO-NPs exhibited significant antibiotic activity when impregnated on cotton wound bandages and conferring patches, therefore they could effectively be utilized for treating and curing infected wounds and injuries, e.g., diabetic or burns wounds. Recently, Nehru et al. [101] investigated in vitro cytotoxicity and wound healing activity of eco-friendly biogenic fabricated ZnO-NPs in L929 cell lines. The results showed that, after 24 h of ZnO-NPs treatment, the wound healing procedure was boosted by approximately $95.37 \pm 1.12\%$ (Fig. 17). Augustine et al. [120] investigated the proangiogenic characteristics of ZnO-doped PCL (polycaprolactone) fibers. To assess the development of new blood vessels, a chicken chorioallantoic membrane (CAM) assay was carried out. It was found that the PCL membranes can effectively boost the development of large, full-grown vessels and highly increase a branched capillary network at a ZnO-NPs concentration of 1 wt %. Besides, it was observed that the angiogenic characteristics of the doped PCL fibers were dependent on the nanoparticle's doses e.g., at ZnO-NPs concentration of 0.5, 2 and 4 wt %, the count of branches in the recently originated blood vessels was considerably less than that measured for PCL fibers doped with 1 wt % ZnO-NPs. Moreover, the biosafety, biocompatibility and angiogenicity characteristics of 3-dimensional nanostructured porous granules of Hydroxyapatite (Hap) and ZnO-NPs have been investigated [121]. An in vivo implantation of the nano-Hap granules into the subcutaneous tissue in rats with and without ZnO-NPs was performed, and then the inflammatory responses were evaluated after 3, 7 and 30 days of the implantation procedure. The results revealed that the structural nanocomposite materials significantly declined bacterial activity at a ZnO concentration of 2 wt %. More impressively, the development of newly formed blood vessels and capillaries around the granule tissues was observed following 3 days of implantation. Besides, after 30 days extremely intense connection between the tissue of fibroblasts and matured blood vessels has been found.

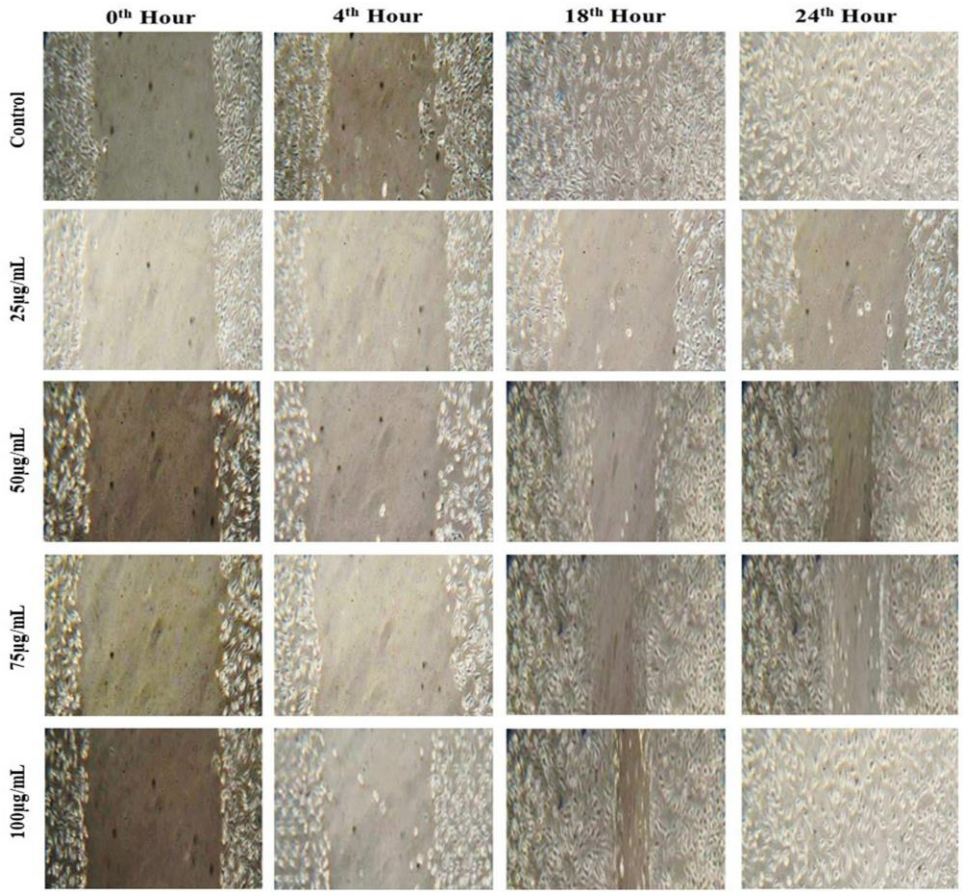

Figure 17: The effect of biologically fabricated ZnO-NPs on wound healing in L929 cells. Reproduced from [101].

Drug and gene delivery applications

It has been an enormous concern to reduce the side effects of chemotherapy through the cancer healing process using specifically located drug delivery. Besides, various techniques have been applied to deliver an active drug or an imaging molecule, including pH level, solubility, temperature modification, and optical and ultrasonic waves for therapeutic applications. Importantly, nanomaterials play a significant role as a bioactive carrier that can adapt medicine via enhancing drug structure and controlling release rate, and consequently, deliver a drug to an intended position at a decided rate and method (Fig. 18) [122, 123]. Consequently, the drug's biophysiological activity would be increased, whilst side effects would be diminished.

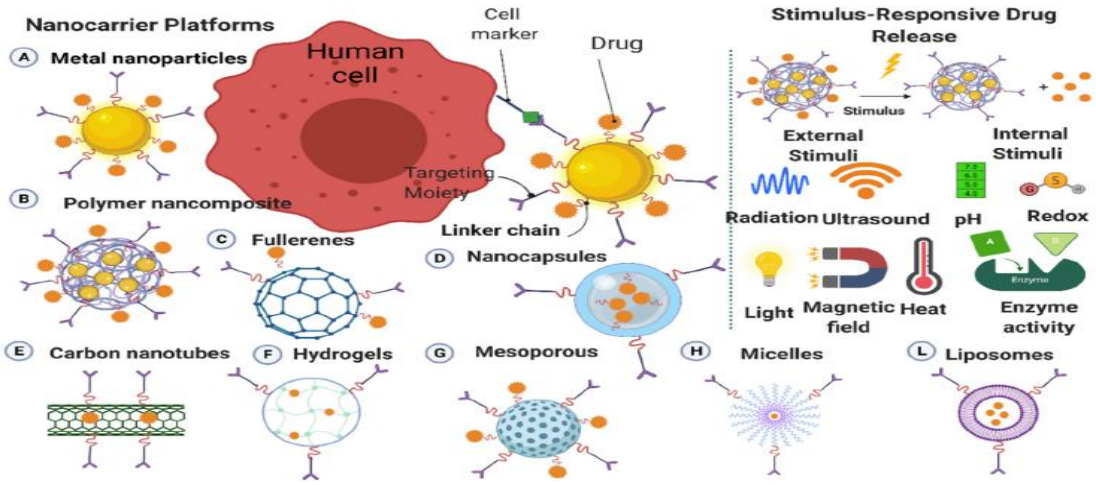

Figure 18: Schematic illustration of the nanomaterial's mechanism as efficient nanocarriers for different medicines and drugs. Reproduced from [124].

There have been many literature reports investigating the performance of nanostructured materials as efficient nanocarriers for various therapeutic medicines and drugs [125, 126]. In particular, nanoparticles as QDs have been applied as an operative vehicle for targeted drug delivery and gene silencing (Fig. 19). In contrast to the traditional drug delivery platforms, more therapeutic efficiency, specificity too high certainty, focusing capacity, and durability at the actual destination can be obtained by drug delivery devices based on nanostructured materials [122, 127]. The key benefits of applying nanoparticles as efficient drug carriers are to minimize the drug side effects and maximize their bioavailability, biocompatibility, in vivo stability and solubility, and target specificity [122, 128].

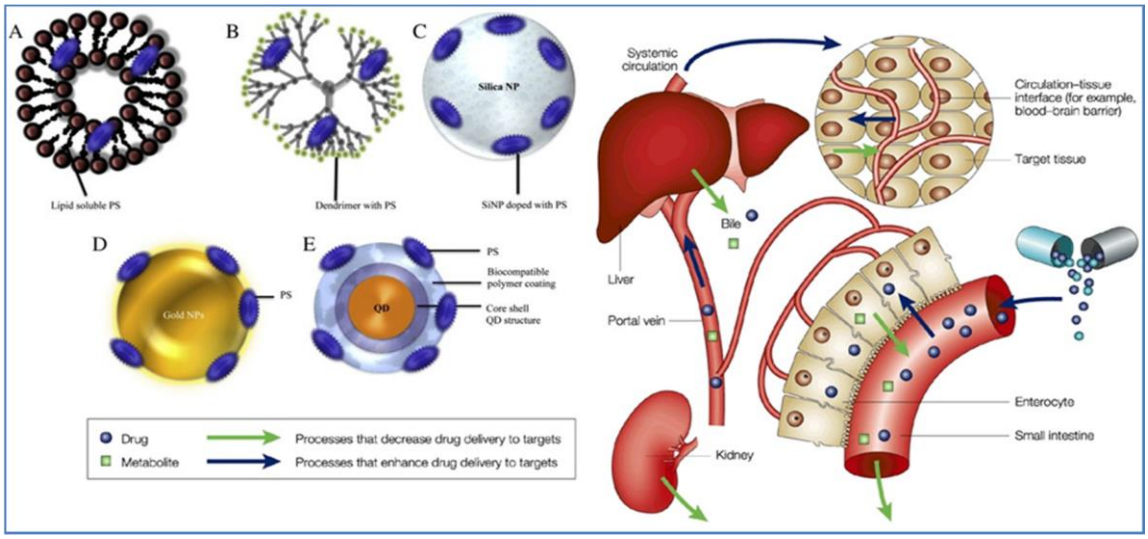

Figure 19: A schematic diagram of various nanoparticles as QDs acting as an efficient vehicle for gene and drug delivery systems. Reproduced from [129].

In addition, nanoparticles as nanocarriers can hinder the drug conversions caused by different enzymes and then expand the drug half-life in blood as well as decrease resistance to treatment by carrying several drugs [123]. The size-dependent adsorption efficiency of ZnO nanoclusters (ZnO-NCs) as potential drug nanocarriers has been investigated through their interactions with the favipiravir drug molecule [130]. The authors showed that the size of ZnO-NCs and the position of the favipiravir on the ZnO-NCs highly affected those interactions, e.g., the adsorption energy between ZnO-NCs and the favipiravir (− 34.27 kcal/mol) was efficiently more promising than the other interactions. Recently, the drug delivery potency of ZnO nanotubes (ZnO-NTs) and their adsorption behaviour as a nanocarrier for hydroxyurea as an anti-cancerous drug has been investigated [131]. Based on the obtained results, it was found that hydroxyurea was highly adsorbed through oxygen atoms onto zinc atoms of ZnO-NTs. The UV/Vis observations showed a significant redshift, indicating that the ZnO-NTs were excellent materials for identifying and monitoring the drug molecule.

Conclusion and future scope

Compared to the bulk structure of ZnO, its material with nanoscale dimensions has been an inspiring and interesting candidate due to its appealing and exceptional physiochemical and biological properties. In general, various morphologies of these materials, including spherical nanoparticles, nanorods, nanowires, nanotubes, nanofibers, nanoflowers, nanobelts etc, have approved their potency in different fields, especially biomedical and healthcare platforms. Researchers have demonstrated the performance of ZnO nanostructured material in boosting the development of various cellular types, accelerating vital biological processes, and stimulating the growth of new cells and tissues, thus enabling these materials to be strongly applied as antibacterial, antibiotic, antitumour, and anti-inflammatory agents. Herin, the first section of the review highlighted the cytotoxicity and genotoxicity of ZnO-NSs and shed light upon the possible mechanisms behind the toxic activity in several mammalian cells and organs. Besides, it is quite critical to evaluate the toxicological effect of ZnO-NSs and investigate their biological compatibility with living organisms to successfully exploit the new generation of sustainable and safe medical devices. Therefore, the second section demonstrated the latest advances in in-vitro studies related to the measurements of ZnO-NSs toxicity upon different biosystems. Finally, the novel applications of ZnO-NSs as an antimicrobial, antifungal, and anti-cancerous promoter, tissue-engineered and wound healing tools, as well as drug and gene delivery systems, are represented in the last section.

Despite the widespread use of ZnO-NPs in pharmaceutical manufacturing, cosmetics and skincare products, textile and food industry etc, there is still a massive debate regarding the potential risky impacts of those materials on human health and the environment. Over the last decade, most of the literature has investigated the underlying mechanisms of Zano-NPs toxicity through in vitro scopes, which evaluate some important mechanistic interactions of nanoparticles with different living cells and biological organisms. However, the in-vitro system has some limitations in terms of addressing complicated biological responses of cell–cell communications, cell and its microenvironment interactions, and hormonal functions. Therefore, there has been a necessity to conduct many more in vivo studies, which are more convoluted and include many biological parameters to produce valuable details regarding the assessments of nanoparticle toxicity in living tissues and organisms. Besides, it is quite practical to initially apply in vitro models for investigating various cell lines and tissues, for example, for monitoring physiological interactions at the cellular level, then followed by suitable in vivo studies to dig deeper into the in vitro consequences, extrapolate the biological signalling through the whole living organisms, and finally concluding good insight on the biocompatibility of ZnO-NSs and their cytotoxic behaviour. Thus, researchers in the biomedical field can determine the most convenient, applicable, and biocompatible dispersion laboratory protocols in both in vitro and in vivo investigations, which help in the reproducibility of findings on specific cell lines under particular experiment conditions.

Moreover, the ongoing issues related to the ZnO-NSs biomedical platforms still require further efforts. One of these issues is the lack of extensive and critical examination for all process parameters of ZnO-NSs synthesis to accurately figure out their potential effects on the structural, physiological, and functional properties, which in turn determine the success and potency of these designed nanomaterials as powerful, eco-friendly, and sustainable biomedicine and healthcare devices. Furthermore, scientists should pay more attention to hidden interactions involved in the nano-bio systems and elicit an underlying and precise clue on how living cells communicate within nano shrouding on intracellular, extracellular, and physio-mechanical points of view. To pave the way in future biomedicine research and pharmacological industries and assess the hazardous impacts of ZnO-NPs extra long-term in vivo toxicological investigations are much required before their mass production and clinical trials.

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