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Evaluation of Synergistic Effect of TiO_2 and Al_2O_3 Nanoparticles on Hela Cell Line

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Abstract
Objective: The aim of this study was to analyze and compare the cytotoxic effects aluminium oxide (Al_2O_3) and titanium dioxide (TiO_2) nanoparticles (NPs) and binary mix on them on Human cervical cancer (CC) cell line (HeLa) and healthy mouse fibroblast cell line (L-929) *in vitro*.
Materials&Methods: In the study, TiO_2 nanoparticle was synthesized by sol-gel method. Al_2O_3 nanoparticle is commercially purchased. TiO_2+Al_2O_3, Al_2O_3 and TiO_2 nanoparticles were dissolved in water under certain conditions by probe and characterization analyzes were performed.
Cytotoxicity of the TiO_2+Al_2O_3, Al_2O_3 and TiO_2 was evaluated by MTT assay. HeLa and L-929 cell line were treated with different concentrations of this nanoparticles (0,5-100 µg/ml) for 24, 48 and 72 hours.
Results: In our study, TiO_2+Al_2O_3, Al_2O_3 and TiO_2 nanoparticles were prepared in water. UV-vis spectrofometric analysis and zeta potential measurements of this nanomaterials were performed. For this nanoparticles comparable cytotoxic action on HeLa and L-929 cell line were detected. The effects of TiO_2+ Al_2O_3, Al_2O_3 and TiO_2 nanoparticles on the HeLa and L-929 cell line were compared to the control group and IC_{50} values were found for 24, 48 and 72 hours. We also compared the effect of these nanoparticles on the HeLa cell line with the healthy L-929 cell line. The spectrophotometric readings at 570 nm were recorded and analysed with Graphpad Prism7.
Conclusion: There are many drug methods for CC treatment. However, new treatment methods are needed to minimize unwanted side effects and to overcome drug resistance. In this study, we found that the HeLa cell line of TiO_2+Al_2O_3, Al_2O_3 and TiO_2 nanoparticles had anticancer effects and inhibited cell growth. We found that these drugs were more active in the HeLa cell line compared to the L-929 cytotoxic effects on the HeLa cell line.

Key Words: Cervical cancer, HeLa, L-929, TiO_2, Al_2O_3

1. Introduction

Nanotechnology includes the creation and manipulation of materials at 1 to 100 nanometers levels to create products that exhibit novel properties. Studies on nanotechnology have dramatically risen during the past 10 years [1]. Compared to micrometer sized materials, nanomaterials have specific features such as large surface area, small size, shapes and special structure [2]. Because of the unique dimensional and morphological properties, nanomaterials can be physically and chemically managed and widely used in treatment, diagnosis, monitoring, and controlling of biological systems [3]. Thus, nanotechnology holds promise for a broad variety of new biological, biomedical and biochemical applications. The small size of NPs allows them to participate in cells through endocytosis and therefore may affect cellular function [4]. Among the produced NPs, TiO_2 and Al_2O_3 NPs are the earliest industrially produced nanomaterials and they are commonly used in various applications. TiO_2 is a natural, very insoluble and thermally

stable. This oxide is used as a white pigment in ceramics, cosmetics, food colorants, medicines and a variety of personal care products such as sunscreens and cosmetic creams [5]. Likewise, aluminum oxide or alumina (Al_2O_3) is used in drug delivery systems and many branches of industry especially in the use of wear-resistant coatings [6]. As nanoparticles continue to be widely integrated in the industry as well as in the pharmaceutical industry, research into the cytotoxicity of these nanoparticles has gained importance. As advances in nanotechnology increase, the multidisciplinary study of nanotube, nanotechnology and biomedical promises new prospects for cancer treatment [7-9].The idea that nanoparticles can trigger autophagy in cancer cells is of great interest as it may be a new approach useful for treating cancer [10,11].

Cervical cancer has the highest incidence in gynecological malignancies and is one of the main causes of cancer-related deaths in women, especially in underdeveloped and developing countries [12-15]. CC was associated with persistent Human Papillomavirus (HPV) infection [16]. The development of this cancer is thought to be associated with the integration of the viral genome and expression of two major viral oncogene E6 and E7. The E6 protein binds to the p53 tumor suppressor protein and induces ubiquitin-mediated destruction[17]. CC treatment is specific to the stage of the disease. The early stage of the disease can be treated with radiotherapy or surgery, but the most effective treatment for local and advanced patients is concurrent chemotherapy and pelvic irradiation[18,19]. Although the incidence and mortality rates of neoadjuvant radiotherapy and chemotherapy are reduced due to the use in a group of CC patients, intrinsic and acquired drug resistance to treatment of neoplastic cells still remains a problem in patients [20]. Despite the intensive work of scientists and pharmaceutical industries, efforts to combat CC remain insufficient. In addition to the synthesis of new drugs against CC, it is of great importance to treat existing treatments using new or unusual drug delivery systems [21].

2. Materials and methods

2.1. Chemicals

This study investigated two different NP types including TiO_2 and Al_2O_3 (Table 1) and mixture of them. TiO_2 nanoparticles were synthesized by sol gel, according to the described method [22]. Al_2O_3 was purchased commercially.

Table 1. Commercial source and description of manufactured nanoparticles.

Particles type	Supplier	Description and characterisation
21 nm TiO_2	synthesized by sol gel method	Titanium (IV) oxide nanopowder, 21 nm average particle size, anatase
80 nm Al_2O_3	US Research Nanomaterials, Inc	aluminium oxide nanopowder, 100% alpha-Hydrophilic, Purity: 99+%,15 m^2/g SSA.

2.2. Characterization of TiO_2 and Al_2O_3 NPs

TiO_2 and Al_2O_3 nanoparticles were used as dry powder. The samples were suspended in deionized water at a concentration of 5 mg mL^{-1} (50 µg mL^{-1}) to form a stock solution. The binary mixture was then prepared in equal volume. All samples were ultrasonicated 60 minutes to break any possible aggregation of nanoparticles in a Probe Sonicator (Sonics & materials INC, USA). In an experimental setting this is usually carried out using an ultrasonic bath or ultrasonic probe generally referred to as sonication. However, the probe sonicator is preferred to prevent agglomeration of the nanoparticle. Stability of the samples was determined by measuring their zeta potantial values (Malvern Zetasizer Nano Z) and UV-vis analysis (UV-1280, Shimadzu, Japan). After the probe treatment, the images of the solutions are shown in Figure 1 and no agglomeration is observed at the bottom of the tubes.

Figure 1. Photographic image of TiO_2, Al_2O_3 and TiO_2-Al_2O_3 nanoparticles in water, from left to right respectively.

2.3. Cell culture

In this study, HeLa and L-929 cell lines were cultured in fluids in DMEM medium containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (10 mg/L). This cells were grown at 37 ° C in a 5% CO_2 incubator. For each cell line, 70-80% cell growth was then typsinized in culture fluxes and the cells were plated on 96-well plates.

2.4. Cytotoxic effect of TiO_2, Al_2O_3, TiO_2+Al_2O_3 binary mix nanoparticles and HeLa cell lines

Cytotoxicity measurement of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles on HeLa and L-929 cell lines was performed by Skehan's MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay method [23]. The trypsinized cells were plated in 0.1 mL 96 well plates (Corning, USA) at a density of 1 x 10^5 cells per well and allowed to incubate for 24 hours. Which is in the range of 0,5-100µg/ml, 1µl of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles was added to the cells in each well. Plates were incubated at 37 °C in a 5% CO_2 incubator. After 24, 48 and 72 hours incubation with different concentrations of compound, MTT (5 mg/ml dissolved in PBS) was added per 10µl wells and incubated for 2 hours at 37 °C. The supernatant in each well was then carefully aspirated. 100µl of DMSO was added to each well to dissolve Formazan crystals. The absorbance of plates were recorded at 570 nm on a microplate reader (Bio-Tek, USA). All drug doses were tested in parallel in three replicates.

3. Results and Discussion

3.1. Stability of TiO_2, Al_2O_3, TiO_2+Al_2O_3 binary mix, nanoparticles suspensions

The major disadvantage of working with nanoparticles is their loss of stability in the water and other fluids and their tendency to settle down to form larger particles. Sonication is the best way to prevent particles from becoming agglomerated in suspension. Some hard aggregates formed within the suspensions were broken by ultrasonication [24,25]. Nanoparticle agglomerate size increased with increasing nanoparticle concentration in all media. Therefore, low nanoparticle concentration was selected in the stability tests in this study. In the cell culture study, nanomaterials were first vortexed and then dosed. Zeta potential is a key indicator of the stability of colloidal dispersions. According to Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, the stability of a colloidal suspension is based on the net balance of two forces: the electrostatic repulsion which prevents aggregation and a universal attractive van der Waals force which acts to bind particles together [26,27]. According to the ASTM, colloid solutions with zeta potentials higher than 40 mV are considered to have good stability [28]. In this study, all samples suspended in deionized water at a 50 µg mL^{-1} concentration formed a relatively stable dispersion with a zeta potential of 30 mV. By comparing the value of zeta potential at the pH of 7.0, as shown in Fig. 2, it generally indicates that the stability of solutions is approximately the same but the zeta potential of TiO_2 nanoparticles is slightly higher than the others. Because the diameter of the TiO_2 nanoparticles is smaller than Al_2O_3 its stability is better. As a result, all samples were moderately stable.

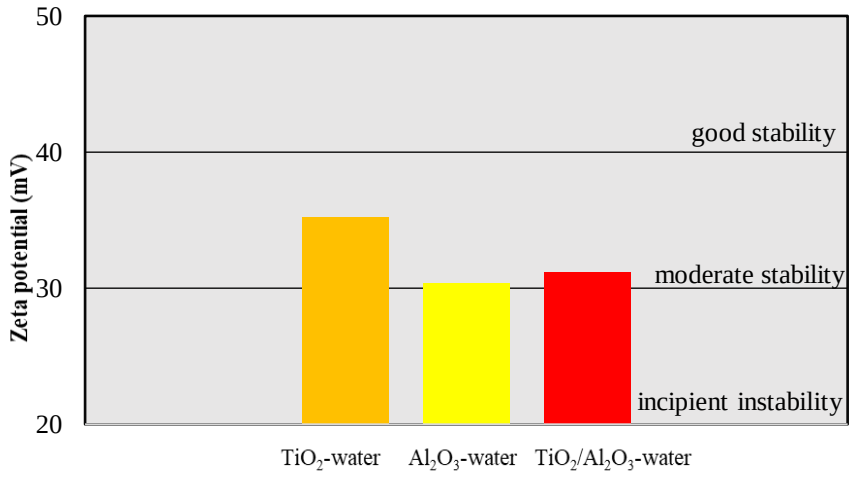

Figure 2. Zeta potential of TiO_2, Al_2O_3 and TiO_2-Al_2O_3 nanoparticles in water

Figure 3 show the UV–Vis spectra of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles in water. From the spectrum, it could be observed that there were obvious absorbance differences in the UV light region and visible region between the samples. In the UV region, sample TiO_2 also exhibited the highest absorbance, followed by TiO_2+Al_2O_3 binary mix and Al_2O_3. From the UV/Vis spectra, the TiO_2 nanoparticle can absorb most light with wavelengths less than 400 nm, this result is consistent with the literature [29,30].

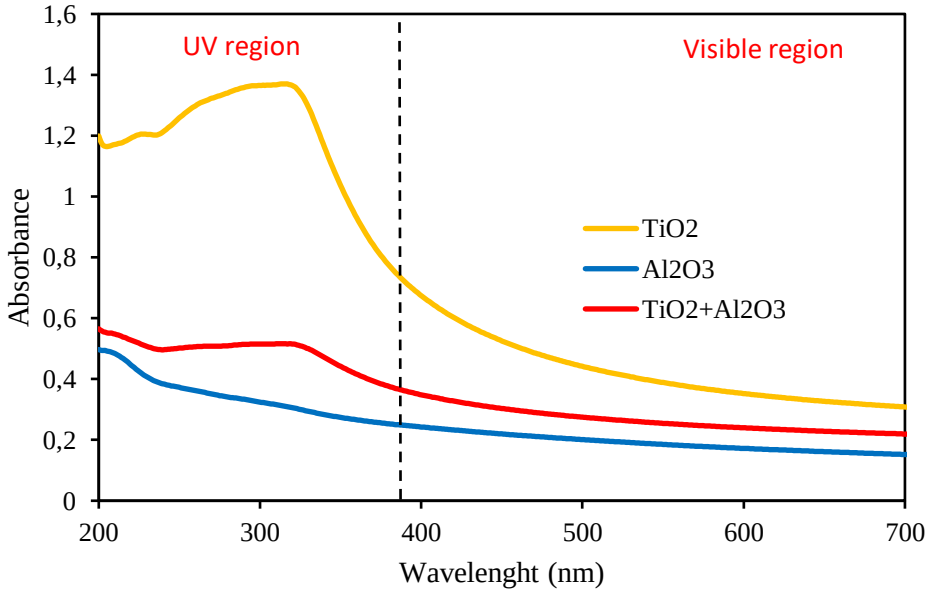

Figure 3. UV-VIS spectrum of TiO_2, Al_2O_3 and TiO_2+Al_2O_3 nanoparticles in water

The peak of Al_2O_3 is seen at approximately 210 nm [31]. Despite the prob sonication, due to the high nanoparticle size of Al_2O_3, the particles may have been agglomerated and a high peak Al_2O_3 was not observed. The peak of TiO_2+Al_2O_3 binary mix contains the characteristic peaks of both particles. Al_2O_3 showed a relatively low absorbance rate due to the increase in particles size. A lower absorbance rate at the UV range led to low responses to the UV light. It can be concluded that as the particles are low in crystallinity and large in particles size, the tendency to absorb UV light is limited, which is due to the smaller surface in the limit areas [32].

3.2. Cytotoxic activity of TiO_2, Al_2O_3, TiO_2+Al_2O_3 binary mix, nanoparticles on HeLa and L-929 cell lines

Nanotechnologically produced particles provide an effective way of developing drugs against cancer [33]. Various cell line models are an important tool in cancer research to investigate the antiproliferative or cytotoxic properties of nanotechnologically synthesized nanoparticles [34]. Prior and final toxicity studies on human cell lines have found a number of nanostructures that can be selectively toxic to certain cell lines, including cancer cells [35,36]. This selective toxicity against specific types of cancer is a promising area of research with potential effects on diagnosis and therapeutics [37,38]. In our study, we determined whether there were cytotoxic effects associated with the presence of intracellular TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles on HeLa and L-929 cell lines were exposed to a range of concentrations of this nanoparticles rate was examined by MTT (Fig.4 and Fig.5). Fig.4 and Fig.5 show changes in cell inhibition for 24, 48 and 72 hours versus increasing concentrations of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles on HeLa and L-929 cell lines. Compared to the control group, TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles treated HeLa cells showed significantly decreased cancer cell survival rate after 24h, 48h and 72h of incubation. TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles on HeLa cells were the most active for 72 h of incubation. In addition, IC_{50} values of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 for 72 hour were 2,81±0,11µg/ml, 83,51±1,71µg/ml and 8,06±0,38µg/ml respectively (Table 2). Similarly, we applied these nanoparticles to the L-929 cell line and determined the cytotoxic dose. These nanoparticles were found to be active in the L-929 cell line compared to the control group. IC_{50} values of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 72 hours were 18,65±0,48µg/ml, 86,32±0,52µg/ml and 16,51±1,31µg/ml respectively on L-929 cells (Table 2). The cytotoxic effect of these nanoparticles was determined to be more active in all three time periods of 24, 48 and 72 hours in HeLa cells compared to HeLa and L-929 cells.

Table 2. Comparison of IC_{50} values between TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles on HeLa and L-929 cells after 24 h, 48 h and 72 h of incubation.

	HeLa			**L-929**		
	IC_{50} (µg/ml±SD*)					
Nanoparticles	24h	48h	72h	24h	48h	72h
TiO_2+Al_2O_3	4,07±0,81	3,02±0,64	2,81±0,11	29,51±1,70	23,05±0,31	18,65±0,48
Al_2O_3	90,42±1,14	86,31±3,42	83,51±1,71	≥100	89,71±6,16	86,32±0,52
TiO_2	16,63±1,41	15,09±0,27	8,06±0,38	20,83±0,55	17,88±0,66	16,51±1,31

*The mean standard deviation values of IC_{50} obtained from three independent experimental repetitions after 24 h, 48 h and 72 h incubation for HeLa and L-929 cell lines.

In our study, we treated HeLa and L-929 cells with TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticle for 48 hour with 50µg/ml. Then we obtained images with a 20x magnification on the microscope (Figure 6). As shown in Fig 6. it was noted that the TiO_2+Al_2O_3 compared to the control and other nanoparticle (Al_2O_3 and TiO_2) was more active on HeLa cells in 48h. There was a similar situation in L-929 cells. However, when we compared HeLa and L-929 cells, we observed that the TiO_2+Al_2O_3 nanoparticle further disrupted the morphology of the HeLa cell. In addition, we found that the TiO_2 nanoparticle acts more than Al_2O_3 on both cell lines.

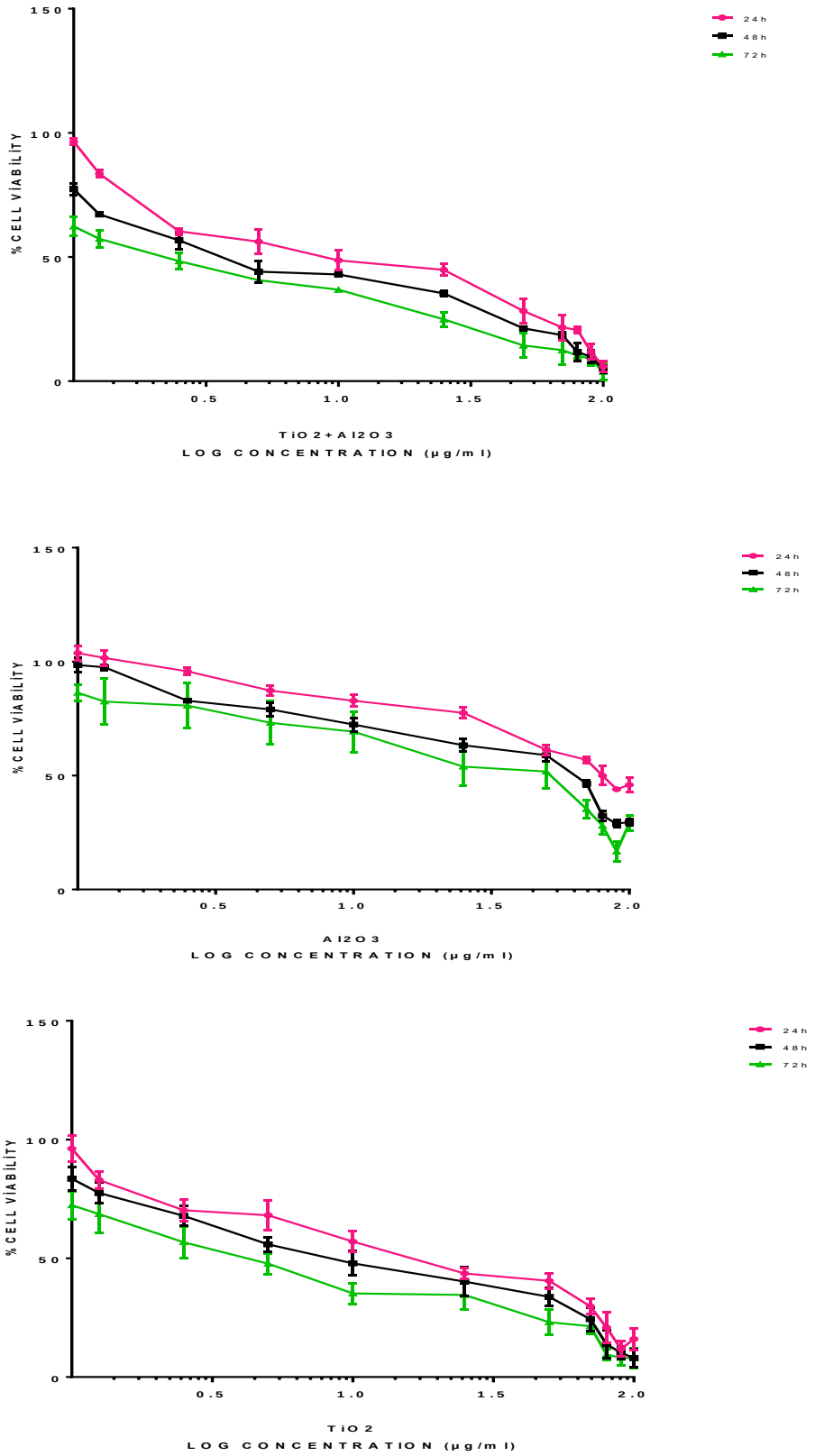

Figure 4. Anti-cancer activity of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles on HeLa cell lines

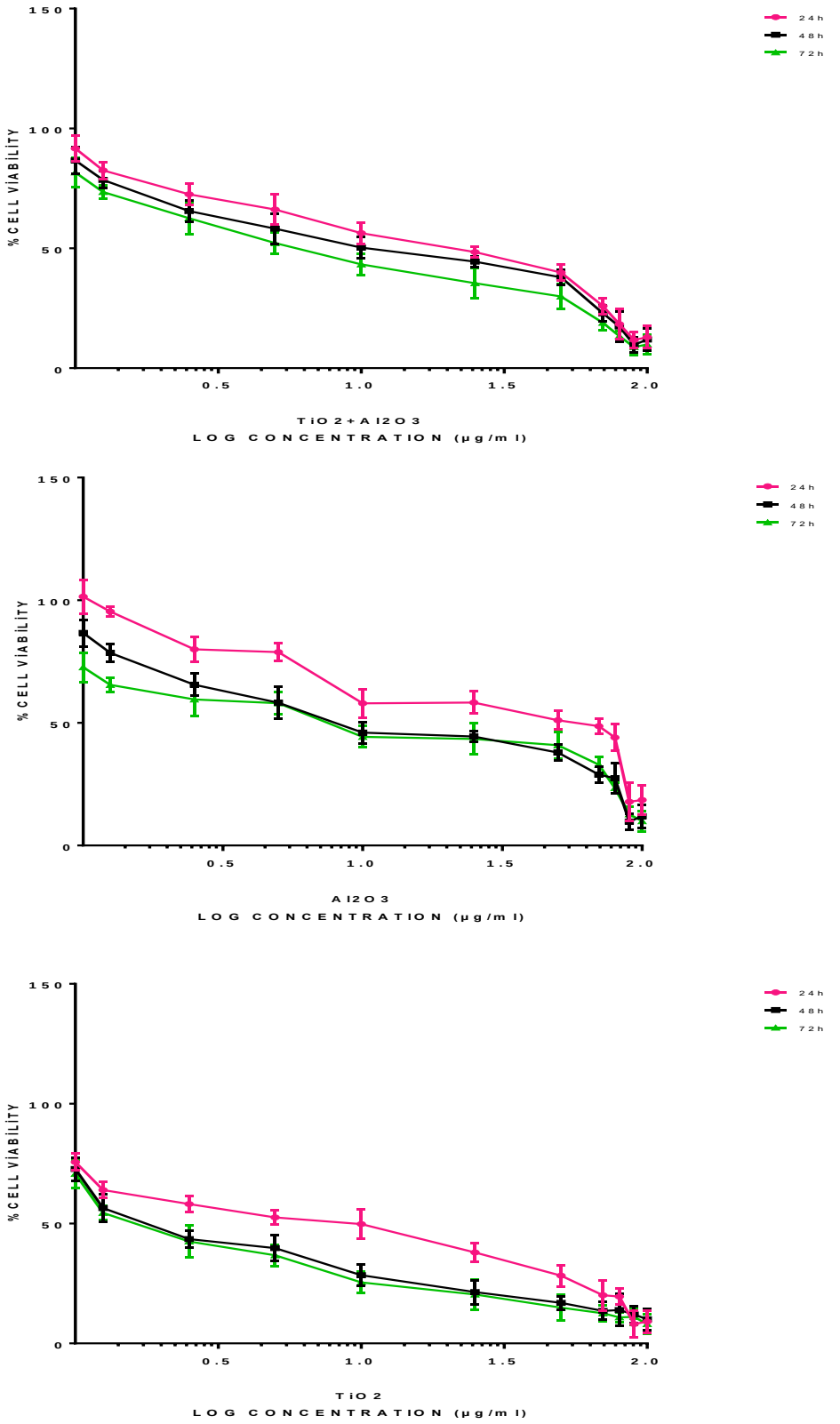

Figure 5. Anti-cancer activity of $TiO_{2+}Al_2O_3$ binary mix, Al_2O_3 and TiO_2 nanoparticles on L-929 cell lines

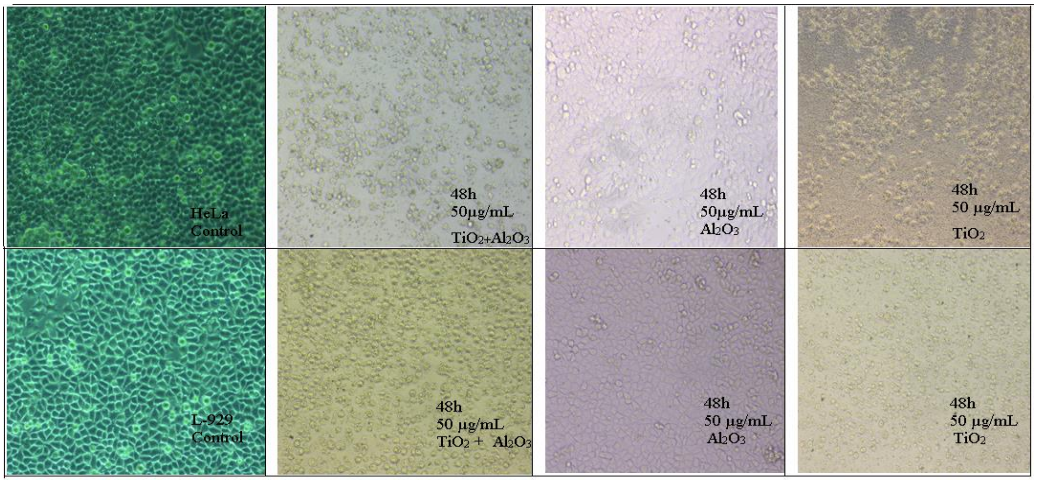

Figure 6. Morphological changes of HeLa and L-929 cells after 48 hour of incubation with concentrations (50 µg/ml) of TiO_2+Al_2O_3 binary mix, Al_2O_3 and TiO_2 nanoparticles. the results presented are from that were carried out and photographed microscopically

4. Conclusions

TiO_2 nanoparticles have been successfully synthesized via a simple sol-gel method at room temperature using acid hydrolysis of titanium tetra-isopropoxide as the precursor material. Al_2O_3 is purchased commercially. TiO_2+Al_2O_3 binary mixture was prepared successfully in equal volume. Zeta potential analysis showed that the stability of all samples was higher than 30 mV. All nanomaterials prepared can be called stable. The biggest disadvantage of the solutions prepared with nanoparticles is their stability. Nanoparticles tend to aggregate and collapse by losing their stability as the time passes in the liquid. In this study, the result of zeta potential analysis is the same value (~30 mV) after 7 days but it started to decrease after 7 days. Cell line studies were performed within the first 7 days after the preparation of the nanoparticles and samples were vortexed at each stage. In summary, at the end of our study, when we compared TiO_2+Al_2O_3 nanoparticle with L-929 we found more active in HeLa cell line. However, when we looked at the toxic dose ratios, we also recorded high activity in L-929 cells.
In future studies, nanoparticles can be chemically modified with a polymer and applied to the cell line. Thus, both toxic effects will be eliminated and at the same time their stability will be higher and they will be able to maintain their stability for longer.

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