Effects of the interaction of Nanorutile TiO$_2$ with vincristine sulfate on chromosomal abnormalities \textit{in vivo}

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ABSTRACT
Titanium dioxide nanoparticles are massively fabricated and widely used in daily life. However it is known, that Nano TiO$_2$ can induce various toxicities. In this study, we examined the effects of Nano rutile TiO$_2$ on vincristine sulfate (VCR) -induced chromosomal abnormalities in bone marrow of mice by micronucleus assay. Different concentrations of VCR (3, 5, 7 mg/Kg) and Nano rutile TiO$_2$ (0.1, 1, 3 g/Kg) were injected intraperitonealy. After 24 h, the frequency of micronuclei in polychromatic erythrocyte cells (MN PCEs) were investigated and the effective doses were selected. The doses of VCR (5 mg/Kg) and Nano TiO$_2$ (1 g/Kg) were treated, on its own and together simultaneously. Then the frequency of MN PCEs were detected after 24, 48, 72 and 96 h. The results showed, that the percentage of micronuclei in effective doses on its own decreased during the time, but it increased in simultaneous treated groups ($p<0.05$) and in 96-h led to death. Although both VCR and Nano TiO$_2$ induce chromosomal abnormalities, this paper indicated that Nano rutile TiO$_2$ had significantly synergic effects on VCR activity and this could cause acute cell apoptosis and death in the long run.

Keywords: Nanorutile TiO$_2$, Vincristine sulfate, Chromosomal abnormalities, Interaction, \textit{in vivo}

1. INTRODUCTION
Titanium dioxide, a natural non-silicate mineral oxide, can be found in different forms and is widely used in cosmetics, pharmaceutical and paint industries [1], and biomedical applications such as drug-delivery agents and biosensors [2]. Its special characteristics such as small size, large surface per mass and high reactivity make it easy to enter the body [1,3]. Current reports indicate that Nano TiO$_2$ could be absorbed and distributed in key organs. They can also be found to change cell life style or to induce cell into apoptosis [4]. TiO$_2$ NPs can enter not only into cells, but also into mitochondria and nuclei. Therefore, these particles can interact with cytoplasmic proteins such as microtubules (MTs) and affect their crucial functions in different tissues. Studying the effects of TiO$_2$ NPs on the MTs, a significant tubulin conformational change was observed and it was founded that these particles affect tubulin polymerization and decrease it, leading to tubulin protein and microtubule function changes [5]. Failure of the microtubules leads to mitotic block and apoptosis [6]. The mitotic checkpoint guards against chromosome mis-segregation by delaying cell-cycle progression through mitosis until all chromosomes have successfully made spindle-
microtubule attachments [7]. Failure of the spindle checkpoint results in premature separation of sister chromatids even in the presence of misaligned chromosomes, which directly gives rise to chromosomal instability, the perpetual gain or loss of chromosomes or large parts thereof. This is associated with aneuploidy, which is a major hallmark of human cancer. In fact, in many tumor cells the spindle checkpoint function is weakened and the checkpoint signal is not sustained [8]. Thus, defects in the mitotic checkpoint generate aneuploidy and might facilitate tumor genesis, but more severe disabling of checkpoint signaling is a possible anticancer strategy [7]. Most chemotherapeutic anti-cancer drugs used in the clinic today include agents that target the cell cycle in order to inhibit the hyper proliferation state of tumor cells and — subsequently — to induce apoptosis, which is the desired outcome of chemotherapy [9]. The chemotherapeutic drugs used in the treatment of cancer are usually DNA damaging agents and these drugs invariably result in the DNA strand breakage, chromosome breaks and loss or gain of chromosomes [10]. These drugs bind with and inhibit the function of microtubules of the mitotic spindle apparatus, which leads to a stop of the cell cycle in mitosis and subsequently to the induction of tumor cell death. Such as Vincaalkaloids which are widely used clinically in the treatment of leukemia’s, lymphomas and solid tumors [11], wills, tumor, and uterus and lung cancer [12]. These alkaloids interact with tubulin subunits to prevent microtubule assembly, inducing abnormal chromosome segregation in dividing cells and causing aneuploidy [13].

Vinristine sulfate (VCR) is a known drug from this groups which has anti-tumor activity and exhibits cytotoxic effects both in vivo and in vitro [10], including disrupt microtubule functions of the cell, especially in the mitotic spindle apparatus leading to the arrest of cellular mitotic division in metaphase and apoptosis [14, 15]. Numerical aberrations are often a result of interference with the mitotic apparatus preventing normal nuclear division. A micronucleus is a small structure containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis. Based on the mechanism for micronucleus formation, the mammalian bone marrow chromosomal aberration assay can detect lactogenic or an eugenic effects of a test agent. Because of the relative simplicity and sensitivity, the micronucleus assay has now become the most commonly conducted in vivo assay.

Extensive usage of Nano TiO$_2$ increases the risk of combined exposure of Nano TiO$_2$ with other substances and it may react with a wide range of organic and biological molecules and then exhibit various toxic effects. The complexity of mixture toxicity lies in the potential for interaction between the mix constituents. In this study, we evaluated the effects of Nanorutile TiO$_2$ on vinristine (VCR)- induced chromosomal abnormalities in bone marrow of mice by micronucleus assay. The mixing of different compounds may induce unexpected toxic effects, even if the toxicities of the individual compounds are well known.

2. Materials and Methods

1.1. Chemicals and Preparation

Nano rutile TiO$_2$ was prepared via sol-gel process of titanetraisopropylate (TIPP). The details of the synthesis are as follows: The TiO$_2$ nanoparticles were synthesized by sol-gel technique via hydrolysis and condensation of TIPP in absolute ethanol as a starting solution. Also, acetyl acetone was used to modify the reactivity of TIPP. In a typical experiment, 60 mL of Ti(OC$_3$H$_7$)$_4$ was dissolved in 40 mL of ethanol as a solvent and 40 mL of acetyl acetone was added. The starting solution was heated at 50 °C and for 1 h in the liquid paraffin bath. Then, the temperature was raised to 120-130 °C and the sol was homogeneous for 4 h with reflux system to create optimal condition for the formation of proper links and polymer. The solution at 80 °C was heated to
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1.2. Animals and treatment
Balb/c mice of 80 males (22 ± 2 g) were purchased from the Animal center of Razi Vaccine and Serum Research Institute. Animals were housed in plastic cages with stainless steel in a ventilated animal room. Room temperature was maintained at 21 ± 3 °C, with relative humidity at 55 ± 10%, and a 12-h light/dark cycle. The animals received commercial ration and water during the acclimation period and during the experiment. They were acclimated to this environment for 5-7 days and were treated at eight weeks of age. Animals were randomly divided into 18 groups: negative control group (with no treatment), positive control group (treated with sterile water) and eighteen experimental groups. Experimental groups were injected intraperitoneally with Vincristine sulfate (3, 5, 7 mg/Kg BW) and Nano rutile TiO$_2$ (0.1, 1, 3 g/Kg BW). Also the selected doses of drug and Nano TiO$_2$ were treated on it’s own and together in twelve experimental groups and then, were investigated after 24, 48, 72 and 96 h.

1.3. Micronucleus test
1.3.1. Preparation of smear slides from Mouse Bone marrow
The micronucleus test was performed according to the guidelines of OECD assays (2005). The animals were killed by chloroform 24 h (48, 72, 96 h) after treatment, both femurs were removed and cleaned, and the epiphyses were cut to expose the medullary canal. The needle of syringe containing 5 ml RPMI 1640 Glutamax (GIB co) was inserted into the canal, and the bone marrow was aspirated in a falcon tube and it spread through the RPMI as a suspension. The material was centrifuged (Type Chalice, Wagtech International, England) at 1000 rpm for 5 min. The supernatant was removed with a Pastour pipette, then the sediment was carefully aspirated with a pipette, and one drop was spread evenly on a clean slide. After 24 h, the material was fixed in 90% methanol solution for 5 min and then, was stained with Giemsa diluted 1:20 in distilled water.

1.3.2. Analysis of the slides
Polychromatic erythrocyte cells (PCEs) were analyzed under a light microscope with a 100X immersion objective (Type BAZ10, Motic, China). The number of micro nucleated cells (MNs) was counted in 2000 PCEs per animal. All the slides were analyzed in a blind test.

1.4. Statistical analysis
Statistical analysis was done using statistical software SPSS19. Data were expressed as mean ± SE. One-way analysis of variance (ANOVA) was carried out to compare the differences of means among multi-group data. Multi-comparison C test was carried out when each group of experimental data was compared together and with solvent and control data. Significance level for all hypothesis is 0.05.
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2. RESULTS
2.1. Characterization of TiO$_2$ nanoparticles
XRD pattern of Nano TiO$_2$ powder is shown in Fig. 1. This pattern exhibited strong diffraction peaks at 28° indicating TiO$_2$ in the rutile phase. The average grain size calculated from broadening of the (110) X-ray diffraction peak of rutile using Scherrer’s equation was approximately 28.88 nm.

![XRD pattern of nano TiO$_2$ particles](image1)

Fig. 1 XRD pattern of nano TiO$_2$ particles

TEM was used to further examine the particle size, crystallinity and morphology of the samples. TEM bright field images of TiO$_2$ Nano powders in rutile phase are shown in Fig. 2. It is can be clearly seen that the TiO$_2$ powder in rutile phase has spherical shape. Furthermore, it can be estimated that the particle size of the samples is Nano scale with the grain size between 5-45 nm. These are in agreement with XRD results in Fig. 1.

![TEM photographs of Nano-sized TiO$_2$ particles.](image2)

Fig. 2 TEM photographs of Nano-sized TiO$_2$ particles.

The FT-IR spectra of Nano TiO$_2$ are shown in Fig. 3. In the spectra’s of the Nano TiO$_2$ there is a wide bond of 400-800 cm$^{-1}$ which confirms the net of Ti-O-Ti. The signature bond of Ti-O stretching appears at 460 and 516 cm$^{-1}$ which are two standard peaks in rutile phase. In this spectrum, the presence of metal oxide bond was observed at 516 cm$^{-1}$. In the entire spectra the peaks are observed around 1100 cm$^{-1}$ which shows the presence of polymer molecule, but no peak could be seen there. It confirms the purity of Nano rutile TiO$_2$ product. The properties of produced Nano TiO$_2$ are showed in Table 1.

![FT-IR spectra of Nano rutile TiO$_2$](image3)

Fig. 3 FT-IR spectra of Nano rutile TiO$_2$
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Table 1 The properties of Nano TiO$_2$ synthesized by sol-gel method

<table>
<thead>
<tr>
<th>Purity (%)</th>
<th>Morphology</th>
<th>Grain size (nm)</th>
<th>Phase</th>
<th>Temperature of calcination (°C)</th>
<th>Time of calcination (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96.69</td>
<td>Spherical</td>
<td>28.88</td>
<td>Rutile</td>
<td>550</td>
<td>1</td>
</tr>
</tbody>
</table>

2.2. Effects of Nano rutile TiO$_2$ on MN frequency in PCE cells

The micronucleus frequencies were assayed to evaluate the Nano rutile TiO$_2$ induced chromosomal abnormalities in the bone marrow of mice. Table 2 exhibits the change of percentage of MN frequency in PCE cells after three different doses of Nano TiO$_2$ were injected into abdominal cavity for 24 h.

In lower dose (0.1 g/Kg BW), there were no significant changes compared with the control groups ($p > 0.05$). In higher doses of Nano rutile TiO$_2$-treated (1 and 3 g/Kg BW) groups, however, the MN frequency were significantly higher than the control groups ($p < 0.05$). Because of the first micro nucleated PCE cells were observed at 0.1 g/Kg BW dose (Fig. 4), it was selected to treat for 48, 72 and 96 h. The results of Nano rutile TiO$_2$-treated groups at different long times are shown in Table 3. The percentage of MN frequencies in treated groups after 24, 48 and 72 h were higher than the control groups ($p < 0.05$). The changes of MN frequencies after 24 h were significantly higher than the 48 and 72 h-treated groups ($p < 0.05$), however, the micro nucleated PCE cells in 48 and 72 h-treated groups were not significantly different ($p > 0.05$). No the significant difference was observed in the MN frequency of PCE cells in TiO$_2$-treated group after 96 h compared with the control groups ($p > 0.05$) (Fig. 5).

![Fig. 4](image-url) The changes of MN frequencies in PCE cells after exposure to various doses of Nano rutile TiO$_2$ for 24 h. Values represent mean ± SE, $n = 4$. Columns marked with double asterisk means it is significantly different from the control groups and the other treated groups at the 5 % confidence level.

![Fig. 5](image-url) The changes of MN frequencies in PCE cells in 1 g/Kg BW Nano rutile-TiO$_2$ treatments after 24, 48, 72 and 96 h. Values represent mean ± SE, $n = 4$. Columns marked with asterisk or double asterisk means it is significantly different from the control groups or the other treated groups at the 5 % confidence level, respectively.
2.3. Effects of Vincristine sulfate on MN frequency in PCE cells

The percentage of frequencies of micro nucleated PCE cells induced by three different doses of VCR for 24 h are presented in Table 2. All treatments produced significantly more micro nucleated PCEs than the respective controls ($p < 0.05$) (Fig. 6). The significant differences were not observed in the MN frequencies in the 3 mg/Kg BW VCR-treated group compared with the 7 mg/Kg BW VCR-treatments ($p > 0.05$). However, the MN frequency in the 5 mg/Kg BW VCR-treated group was significantly higher than the other VCR-treated groups ($p < 0.05$). Since the most micro nucleated PCEs were produced in 5 mg/Kg BW dose of VCR, it was selected to treat for 48, 72 and 96 h.

![Fig. 6](image1.png)

**Fig. 6** The changes of MN frequencies in PCE cells after exposure to various doses of VCR for 24 h. Values represent mean ± SE, $n = 4$. *Columns* marked with asterisk or double asterisk means it is significantly different from the control groups or the other treated groups at the 5 % confidence level, respectively.

The results showed that the MN frequency decreased as the increasing treatment time increased (Fig. 7). In the VCR-treated group for 24 h, the MN frequencies were significantly higher than the other treated groups, however, the MN frequencies produced by 48 h-treated group had no obvious difference from the 72 h-treated group ($p > 0.05$) (Table 3). All the VCR-treated mice for 96 h, were dead. They had anorexia and lethargy which resulted in body-weight loss and finally death. However, this result could be correlated with intensive effects of VCR during 96 h-treatment.

![Fig. 7](image2.png)

**Fig. 7** The changes of MN frequencies in PCE cells in 5 mg/Kg BW VCR-treatments after 24, 48, 72 and 96 h. Values represent mean ± SE, $n = 4$. *Columns* marked with asterisk or double asterisk means it is significantly different from the control groups or the other treated groups at the 5 % confidence level, respectively.
2.4. Effects of Nano rutile TiO$_2$ and VCR on MN frequency in PCE cells

The frequencies of micro nucleated PCEs produced in groups treated with nano TiO$_2$ and VCR simultaneously are shown in Table 3. The results showed that there were significant changes for all treated groups compared with the control groups ($p<0.05$). The 24 and 48 h-treated groups induced MN changes had no obvious difference from each other. In the 72 h-treated group, there were MN frequencies higher than those of 24 and 48 h-treated groups ($p<0.05$) and simultaneous treatment of VCR and nano TiO$_2$ for 96 h led to death of mice.

Fig. 8 The changes of MN frequencies in PCE cells in Nano TiO$_2$ and VCR-treatments after 24, 48, 72 and 96 h. Values represent mean ± SE, n = 4. Columns marked with asterisk or double asterisk means it is significantly different from the control groups or the other treated groups at the 5 % confidence level, respectively.

A significant increase in MN formation was observed in PCE cells treated with Nano rutile TiO$_2$ and VCR for 48 and 72 h (Fig. 8), which was higher than the groups treated with Nano TiO$_2$ and VCR on it is own at the same treatment time ($p<0.05$). However, the MN PCEs frequencies induced by Nano TiO$_2$ and VCR treated-group for 24 was lower than VCR-treated group and similar to TiO$_2$-treated group at the same treatment time ($p<0.05$).

Table 2 The percentage of micronucleus frequency in polychromatic erythrocytes cells (MNPECs) exposed to different concentrations of Nano rutileTiO$_2$ and VCR for 24 h.

<table>
<thead>
<tr>
<th>VCR (mg/Kg BW)</th>
<th>TiO$_2$ (g/Kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.187 ± 0.0125</td>
</tr>
<tr>
<td>5</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>0.25 ± 0.035</td>
</tr>
</tbody>
</table>

*ap < 0.05 compared with control and sterile water group.
bp < 0.05 compared with correlated Nano rutileTiO$_2$ groups.
cp < 0.05 compared with correlated VCR groups.

Values represent mean ± SE, n = 4

Table 3 The percentage of micronucleus frequency in polychromatic erythrocytes cells (MNPECs) exposed to different treatment times of Nano rutileTiO$_2$ and VCR.

<table>
<thead>
<tr>
<th>Treatment times (h)</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>St. Water</td>
<td>0.05 ± 0</td>
<td>0.05 ± 0</td>
<td>0.05 ± 0</td>
<td>0.05 ± 0</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th></th>
<th>VCR (5 mg/Kg BW)</th>
<th>TiO$_2$ (1 g/Kg BW)</th>
<th>TiO$_2$(1 g/Kg BW)+VCR (5 mg/Kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.65 ± 0.02$^{ac}$</td>
<td>0.15 ± 0$^a$</td>
<td>0.125 ± 0.014$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.175 ± 0.014$^a$</td>
<td>0.137 ± 0.025$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 ± 0$^{ab}$</td>
<td>0.35 ± 0.035$^{ac}$</td>
</tr>
<tr>
<td>Death</td>
<td></td>
<td>0.75 ± 0.054$^{abc}$</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.412 ± 0.023$^{abc}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$p < 0.05 compared with control and sterile water group.  
$^b$p < 0.05 compared with correlated Nano rutile TiO$_2$ groups.  
$^c$p < 0.05 compared with correlated VCR groups.  
Values represent mean ± SE, n = 4

3. DISCUSSION

Micronucleus shown in plasma treated cells is formed by DNA strand breaks generated during the faulty excision repair process. The remaining unsealed DNA leads to the formation of micronuclei in subsequent mitosis, and cells with micronuclei are found to be associated with the loss of reproductive capacity [16]. The appearance of micronuclei may be an important mechanism of cell death in which no apoptosis was induced [17]. Nontoxicity of TiO$_2$ nanoparticles was assessed in mice. DNA fragmentation as a marker for nontoxicity was determined by micronucleus assay. The results of this study indicate that intraperitoneal injection of higher doses of Nano rutile TiO$_2$ can increase micronuclei in PCE cells of bone marrow of mice. It demonstrates that TiO$_2$ nanoparticles were nontoxic to mice. TiO$_2$ NPs in the rutile phase are found as small clusters or as a single particle in the cytoplasm and are not collected in mitochondria. Besides, Nano rutile TiO$_2$ do not exhibit the coordination or surface properties that allow spontaneous ROS generation [18]. The Nano scale TiO$_2$ particles enhanced cell proliferation, as well as multinuclear, micronuclei and polyplody formation. Moreover, cell cycle progression was disturbed at G$_2$ /M phase and abnormal chromosome segregation were significantly enhanced, therefore resulting in chromosomal instability and multipolar spindles. However, a fraction of deregulated cells might continue cell cycle progression and undergo apoptotic cell death. Nano TiO$_2$ can mediate deregulation of centrosome maturation, spindle assembly checkpoint, and cytokinesis. Further more, effect of Nano TiO$_2$ on deregulated mitotic progressing is possibly by interfering with PLK$_1$ function, which controls several processes during mitotic entry and exit [19]. Aneuploidy can be resulted from abnormal centrosome amplification, deregulated spindle assembly check point or the failure of cytokinesis [20]. In agreement with previous studies, we showed that high doses of Nano rutile TiO$_2$ remarkably induce micronuclei in bone marrow mice cells and induce chromosomal abnormalities (Fig.4). The particles effect cell cycle progression through modulating cellular signaling processes and forces cells to proceed into mitosis despite genetic defects. We also speculated that long-term exposure to Nano rutile TiO$_2$ might interfere with cell cycle progression and show chromosomal instability. Despite this theory, the results showed that micronucleus frequency decreased after 24 hours, and there were no significant changes in 96 h-treated group compared with the control group (Fig.5). Agglomeration and surface properties are important characteristics that affect distribution and toxicity of nanoparticles [21]. Keller et al. [22] showed a higher agglomeration rate of Nano TiO$_2$ at higher initial concentrations due to the increased probability of collisions between particles. Higher Nano TiO$_2$ concentration enhances direct particle-to-particle interaction, which favors the formation of agglomerates. Furthermore, the results of a study *in vitro* clearly showed that a 96-h exposure to Nano TiO$_2$ particles leads to the observed high levels of immobility and mortality, and no difference was detected between the TiO$_2$ and control groups [19]. Therefore, agglomeration is increased with the increase of Nano TiO$_2$ concentration and
binding time. Besides, Prasad et al. [23] demonstrated an association between medium composition, particle uptake, and nanoparticle interaction with cells, leading to chromosomal damage as measured by the MN assay. These results showed that TiO$_2$ nanoparticles induced MN only in medium composition, which facilitated the lowest amount of agglomeration, the greatest amount of nanoparticle cellular interaction, and the highest population of cells accumulating in S phase.

The efficacy of any drug is conditioned by many factors related both to the drug and to the various cell populations on which it acts. In general, cell kill is a function which involves two parameters: drug concentration and cell exposure time. As previous results indicate, between sub lethal and super lethal levels, the dose-response curves were transitional, indicating that cell kill depended on drug concentration. Hirshaut et al. [24] obtained dose-response curves in vitro for VCR. These studies showed that vincristine achieved maximum kill at a particular concentration and cytotoxic activity decreased at doses higher than the lethal dose. In agreement with previous studies, our results showed most micro nucleated cells at 5 mg/Kg dose, however, the highest dose of VCR (7 mg/Kg) produced the same amount of MN as the lowest dose (3 mg/Kg) and we have not observed any clear dose-dependent effect (Fig. 6). The decrease in the frequency of MN at the highest dose would suggest mitotic delay and cell death, and the same phenomenon was reported with the aneuploidy-inducing VCR in other cells. Furthermore, cells with a high level of damage may tend to be eliminated, possibly by apoptosis, prior to completing the nuclear division process required for MN expression [25]. The results of studies in vitro showed that VCR dose requires between two and three days to achieve maximum kill [24]. This is consistent with their intervention only at a particular phase in cell growth. VCR is considered to act by producing metaphase arrest, this agent should be “cycle dependent” and act completely only if it is left in contact with target population for a period equivalent to one generation time. In agreement with previous studies in vitro, our results in this study in vivo showed that long-term exposure to vincristine inhibited cell division, and within 96 hours it became apparent that the treated mice were dying (Fig. 7). This response time of VCR suggests that it may require several cell division to act or that it may be independent of the cell cycle altogether but requires along period for interaction with an unknown substrate in order to kill a cell. In any case, prolonged treatments provide sufficient time for cell apoptosis and less micro nucleated cells are seen.

Nano TiO$_2$ may react with a wide range of organic and biological molecules. Interaction of nanoparticles with organic chemicals were reported to affect physicochemical properties and toxicity of nanoparticles. Electrostatic interaction and ligand exchange were proposed for adsorption of natural organic materials onto mineral surface of Nano TiO$_2$ [26]. TiO$_2$ nanoparticles are agglomerated at high concentration, but agglomeration did not affect adsorption capacity and surface area of Nano TiO$_2$ [24]. The aggregated Nano TiO$_2$ can adsorb and enrich VCR effectively. The VCR-bound Nano rutile TiO$_2$ are taken up into the cells, which will facilitate the movement of both VCR and Nano TiO$_2$ into cells, and increase the intracellular exposure level, and it may enhance toxicity of both VCR and Nano TiO$_2$. According to the mode of action of vincristine, there was no significant change in MN frequencies at 24 hours. However, the changes in MN frequency at 48 and 72 hours significantly increased, and ultimately, in the 96 hours led to death (Fig. 8). Thus, an eugenic effects caused by presence of VCR and Nano-anatase TiO$_2$ around the mitotic apparatus were possible reasons for increasing MN formation in bone marrow cells exposed to Nano TiO$_2$ and VCR together. Therefore, the synergistic effects on toxic levels were strong in simultaneous treatment of both Nano TiO$_2$ and VCR.
3. CONCLUSION
The result of this study shed light to our understanding of interaction of Nano rutile TiO$_2$ with VCR-induced toxicity and chromosomal damage in vivo. We suggest that Nano TiO$_2$ enhance the capability of VCR to induce MN formation. Because VCR-bounded Nano TiO$_2$ can enter the nuclei of cells, mitotic dysfunction caused by enriched intracellular Nano TiO$_2$ and VCR may be one reason to enhance chromosomal abnormalities. Although the synergic effects were observed at the concentration of VCR and Nano TiO$_2$ used in this study, other doses of nanoparticle and drug should be studied at different treatment times.

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