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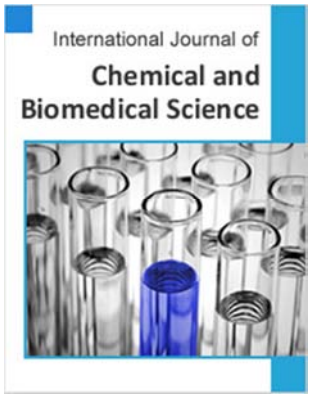

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In vivo Study: Hepatotoxicity, Nephrotoxicity, Antioxidants and Genotoxicity of Quercetinloaded Magnetite Nano Particles

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Abstract
Magnetic Nano-particles (MNPs) is a rapidly growing field having potential applications in many areas. QMNPs have been studied for Hepatotoxicity, Nephrotoxicity, Antioxidants and Genotoxicity. Cell toxicity response induced by QMNPs is checked by measuring complete blood count, liver function, kidney function, oxidative marker, antioxidants enzymes and cell cycle analysis in female albino rats. The biological activity of QMNPs showed significant alteration in white blood cell fractions, significant elevation in ALT and AST was observed, insignificant change in the levels of creatinine and urea. A significant brought down in MAD concomitant with significant elevation of GSH and SOD was recorded. QMNPs enhanced the cell arrest in G1/S phases suggesting slight hepatic toxicity, no toxicity of renal, blood differentiation, DNA analysis and antioxidants system and more investigation is ongoing on the molecular levels.

1. Introduction

Radiotherapy has become an integral part of modern treatment strategies for breast cancer and is used to treat approximately 50% of all cancer patients. However, it is associated with a risk of long-term adverse effects. Strategies to improve radiotherapy in order to increase the effect on the tumor and at the same time, to decrease the effects on normal tissues, which must be achieved without sensitizing the normal tissues in the first approach and without protecting the tumor [1].

A pressing need is to combine conventional radiotherapy with a new strategy to maximize the efficacy of radiotherapy by enhancing drug delivery and increasing the drug concentration reaching the tumor site [2].

Quercetin (3,3',4',5,7-pentahydroxyflavone), a topical antioxidant, is known to have the ability to delay the radiation-mediated oxidant injury and cell death by scavenging oxygen radicals, protecting lipids against peroxidation to terminate the chain-radical reaction, and chelating metal ions to form inert complexes that prevent conversion of

superoxide radicals and hydrogen peroxide into hydroxyl radicals [3]. Quercetin has superior antioxidant potency compared with many other well known antioxidant molecules, owing to the optimized number and distinctive positions of the free hydroxyl groups in this molecule [4]. It is also, known to have antitumor properties. Several studies have shown that its mechanism of action is likely to involve interference with tumorcell signal transduction, inhibition of proliferation and metastasis [5], antiangiogenic effects [6] and reversal of multidrug resistance [7]. However, application of quercetin in pharmaceutics still limited due to its poor bioavailability, hydrophobic nature and low stability [8]. More and above, large quantities of this compound must be administered in order to achieve the desired therapeutic effect which can be lead to acute toxicity or low patients compliance [9].

Nowadays, nanoparticles gaining interest in the field of radiotherapy due to their ability to offer protection against radiation damages [10]. It is worth mentioning that their long blood retention time, biodegradability and low toxicity, magnetic nano particles have emerged as one of the primary nanocarriers for biomedical applications in vitro and in vivo[11]. Furthermore, nanoformulation of quercetin have shown promising results in its uptake by the epithelial system as well as enhanced delivery to the target site [12].

In our previous work [13], we synthesized and characterized quercetin loaded magnetite nanoparticles-Fe_3O_4 (QMNPs-Fe_3O_4) using *Aspergillusoryazae* extract via green method and used experimentally as a targeted drug for breast cancer therapy in animal models. The female rats delivered the formulated nano-complex at a dose of 20 ml/kg each 1ml of the solution contains 11nmol of QMNPsto increase its therapeutic efficacy.

When nanoparticles enter in to the bloodstream, they immediately encounter by the plasma proteins and immune cells. The uptake of nanoparticles may occur through various pathways like hemolysis, thrombogenicity and complement activation. These pathways include numerous activities like reduced number of blood cells, anti-mitotic properties, stimulation of oxidative stress and reduction in cellular antioxidants [14].

Although the nanoscale drug delivery systems are designed to reduce toxicity of the drug and to increase thebiocompatibility, there are some risks owing to their unique characteristics which increased their potential human toxicity. On the other hand the *in vivo* toxicity of IONPs in animal models usually need long-term monitoring investigations for months or even years [15] due to prolonged circulation of the degraded IONPsin the body.

In the view of the aforementioned, and however, toxicity data for magnetite NPs in relation to human health are limited and only a few results from basic studies have been published. It is of importance to examine the possible toxicity of our formulated QMNPs-Fe_3O_4 on the vitality normal tissues and organs (blood, liver, and kidney) as well as endogenous antioxidants and lipid peroxidation. Moreover, cell cycle phases were checked as a biochemical indicator for the possible genotoxicity of the nanocomplex (QMNPs-Fe_3O_4) in treated rats compared to untreated control rats.

2. Materials and Methods

Quercetin (Q4951) was purchased from Sigma Aldrich. All other chemicals of high grade and purity used in this study were purchased from Sigma.

KH2PO4; K2HPO4; MgSO4; $(NH4)_2SO_4$; $K4Fe(CN)_6$ and $FeSO_4$ (Sigma Aldrich, Egyptian International Center for Import Cairo, Egypt).

The formulated Quercetin magnetic nano particles (QMNPs) was prepared and characterized [13].

2.1. Animals and Experimental Design

The present study has been carried out at the Radiation Biology Department and Healthy& Radiation Department, National Center for Radiation Research & Technology (NCRRT), Atomic Energy Authority (AEA), Cairo, Egypt.

The experimental protocol was approved by the animal Ethical committee in accordance with the guide lines for the care and the use of laboratory animals of National Center for Radiation Research & Technology (NCRRT), Atomic Energy Authority (AEA), Cairo, Egypt.

2.1.1. Estimation of the Acute Toxicity and LD50 of QMNPs

In our previous study [13], the collected data revealed that the formulated QMNPs-Fe_3O_4 exhibited a potent cytotoxicity against MCF-7 at IC50 11nmol/ml. In this study, we investigated if the formulated QMNPs-Fe_3O_4 exhibited any hazardous impacts to the normal tissues of the vital organs of female rats.

At first the lethal toxicity of QMNPs was examined, and LD50 which is the dose of our nano formulation that cause 50% mortalities within 72 hrs was estimated. For this purpose, five groups of normal animals (n=10 animals in each) received different concentrations as indicated in the table below to examine the lethal dose (LD50) and incidence of mortalities in each group.

Table 1. *Estimation of acute toxicity and LD50 of QMNPs.*

QMNPs Doses (ml/kg)	Mortalities/group (n=10)
11 nmol/ 1 ml/kg	0/10
110 nmol/ 10 ml/kg	0/10
220 nmol/ 20 ml/kg	0/10
440 nmol/ 40 ml/kg	0/10
880 nmol/ 80 ml/kg	0/10
Lethal dose 50 (LD_{50})	> 880 nmol/80 ml/kg

n =number of animals/ each group

2.1.2. Experimental

Twenty (20) immature female Westar-Albino rats were randomly assigned to tow groups as the following:

Treated Group (n=10): rats injected *ip* with Quercetin magnetic nano-particles at a dose of 20 ml/kg each 1ml of the

solution contains 11nm of QMNPs-Fe_3O_4 twice /week for 8 weeks.

Control Group (n=10): normal rats received only 0.9 % Nacl at a dose equal to that of QMNPs-Fe_3O_4 by *ip* injection.

During the entire experimental time, all animals were observed daily for any toxicity symptoms including the general appearance (fur, skin, eyes) and animal behavior (lethargy, tremors, convulsion, salivation, diarrhea and sleeping) were monitored during the experiment.

The experiment was terminated at the end of the 8thweek. 24 hr after last applied dose, the animals of the two groups were sacrificed by decapitation under light anesthesia using Phenobarbital (30mg/kg, *ip*). The blood was collected from carotid artery into heparinized test tubes and divided into three portions. A portion was snaped-frozen in liquid nitrogen for cell cycle analysis and apoptosis. The 2nd portion was used for complete blood picture. The plasma was separated from the 3rd portion of the blood by centrifugation at 3000 rpm for 15 min. then stored at -20^{o}C until biochemical analysis. The gross examination of the internal organs was taken into consideration.

2.2. Complete Blood Count Assay

Complete blood count was determined by [16]. They were analyzed using the CELL-DYN 1700 (Abbott Diagnostics, Abbott Park, IL, USA) is a multiparameter, automated haematologyanalyser system capable of producing 22 haematological parameters including a screening five-part differential.

2.3. Hepatic Enzymes and Metabolic Activity

The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated in blood plasma of animals colorimetrically using Spectrum Diagnostic kits, supplied by Egyptian Company of Biotechnology (S.A.E) using the method of[17] and according to manufacturer's instructions. The albumin level was evaluated as an indicator for monitoring the hepatic metabolic activity, using a spectrum Diagnostic kit supplied by Egyptian Company of Biotechnology (S.A.E), based on the method of[18] and following the manufacturer's instructions.

2.4. Kidney Function Assay

The kidney function was monitored in blood plasma of rats by evaluation of the kidney metabolites, creatinine and urea using a spectrum Diagnostic kit was supplied by Egyptian Company of Biotechnology (S.A.E), according to the method of [18,19] and following the manufacturer's instructions.

2.5. Assessment of the Oxidative and Antioxidants Markersin Blood Plasma

2.5.1. Assessment of Lipid Peroxidation

Malondialdehyde (MDA) levels were measured (as an important indicator of oxidative stress) according to the previous method [20]. The principle of the method is based on the spectrophotometric measurement of the colour that occurred during the reaction of thiobarbituric acid with MDA.

2.5.2. Superoxide Dismutase (SOD)

The enzyme activitywas estimated according to the method [21] which is based on the inhibition by SOD of the nitro blue tetrazolium (NBT) produced by superoxide radicals. One unit of SOD is defined as the amount of enzyme that causes 50% inhibition of the autoxidation of pyrogallol under assay conditions.

2.5.3. Reduced Glutathione (GSH)

Glutathione (GSH) concentration was also measured with a spectrophotometric method based on the development of a relatively stable yellow color with 5, 5-dithiobis-(2-nitrobenzoic Acid) (DTNB) with GSH [22]. Di-sodium hydrogen phosphate and 5,5-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) solution were added, and the colour formed was read at 412 nm.

2.5.4. Catalase (CAT)

The enzyme activity was also estimated according to the method which is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide [23]. Methanol was used as the hydrogen donor and the formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-trizole (Purpald) as a chromogen.

2.6. Cell cycle Analysis

Blood samples, previously snap-frozen in liquid nitrogen, were thawed in cold PBS/ EDTA [PBS (pH 7.4), containing 0.1% (w/v) EDTA] (approx 1–3 min). The thawed samples were mechanically dispersed, sequentially using 100 µm and 35 µm nylon cell strainers (BD Falcon #352360 and #352235). Peripheral Blood Mononuclear cells (PBMCs) were isolated and washed with PBS followed by propidium Iodide (PI) staining. To stain with propidium Iodide (PI), cells were suspended in 425 µl of PBS and 25 µL of PI (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). The cell pellets were then resuspended in 1mg/ml PI/ Triton X-100 staining solution (0.1% Triton X-100 in PBS, 0.2mg/ml of RNase A (Sigma-Aldrich, St. Louis, MO, USA), and incubated on ice for 20 min. The DNA content of 10000 cells was analyzed by FACS Caliber flow cytometery (Becton Dickinson, CA, USA) to calculate the percentages of cells occupying the different phases of the cell cycle determined by using the Mod Fit LT software [24], samples were run in triplicate and each experiment was repeated three times.

Statistical Analysis

The statistical significance of the data was determined using one-way analysis of variance (ANOVA). The significant difference between the treatments was evaluated by Duncan's multiple range tests. The results were recorded as the mean ± SD and $P < 0.05$ accepted as the minimum level of significance.

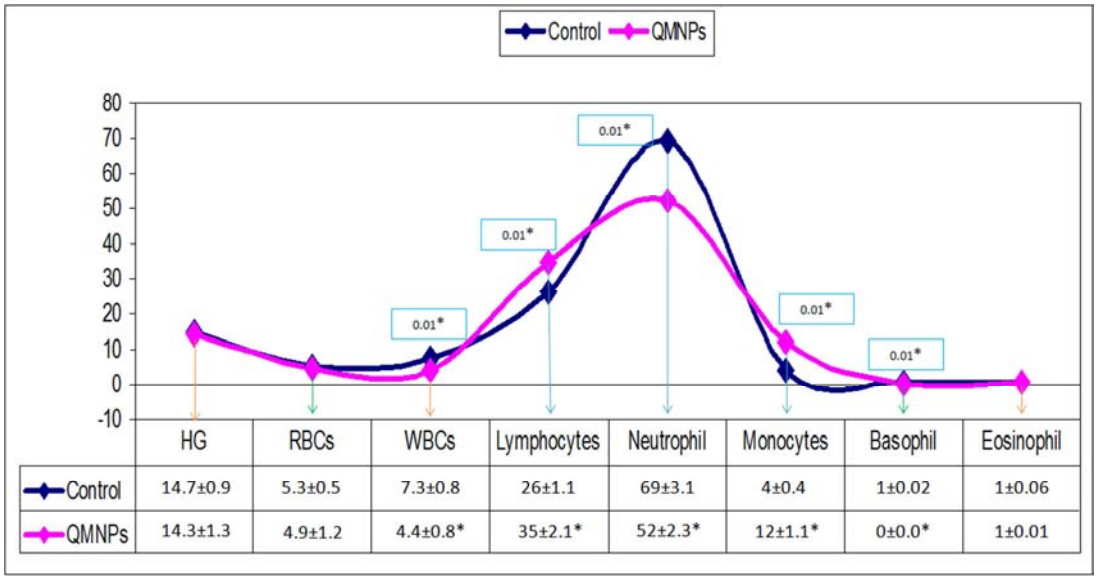
3. Results

All rats survivedalong the entire experimental period and showed no change in their appearance and behavior. The gross examination indicated that the internal organs appeared normal, indicating no significant toxicity.

The preliminary toxicity study revealed that QMNPs didn't exhibit any toxic or pathological symptoms up to the high concentration (880 nmol/ 80 ml/kg of body weight) was administered to the animals, suggesting that the lethal dose is greater than the higher dose applied in the current study. A dose of 20ml/kg of QMNPs-Fe_3O_4 solution was recommended for further in vivo studies each 1ml of the solution contains 11nm of QMNPs-Fe_3O_4.

3.1. Hematological Analysis

Treatment of the female rats with 16 doses QMNPs-Fe_3O_4 (20mh/kg each 1ml contain 11nmol of QMNPs) (twice/ week) showed nonsignificant fluctuation in Hb, RBCs ($P>0.05$). Meanwhile a significant ($P<0.01$) alteration was observed in WBCs, and its fractions Lymphocytes, Neutrophils and Monocytes in treated group compared to the control values (Fig. 1).



	HG	RBCs	WBCs	Lymphocytes	Neutrophil	Monocytes	Basophil	Eosinophil
Control	14.7±0.9	5.3±0.5	7.3±0.8	26±1.1	69±3.1	4±0.4	1±0.02	1±0.06
QMNPs	14.3±1.3	4.9±1.2	4.4±0.8*	35±2.1*	52±2.3*	12±1.1*	0±0.0*	1±0.01

Figure 1. *The effect of QMNPs-Fe_3O_4 administration on hematological indices of treated rats compared to control animals.*

3.2. Biochemical Analysis

Biochemical analysis included liver enzyme markers (ALT, AST)as well as albumin and kidney metabolites (creatinine and urea) were demonstrated in blood plasma of rats.

The results revealed that QMNPs-Fe_3O_4 resulted in significant elevations in AST and ALT activities ($P<0.01$), meanwhile, albumin level was insignificantly ($p>0.05$) changed compared to the control values (Fig. 2). Marginal and statistically insignificant ($P>0.05$) changes were observed in creatinine and urealevels compared with the control values (Fig. 3).

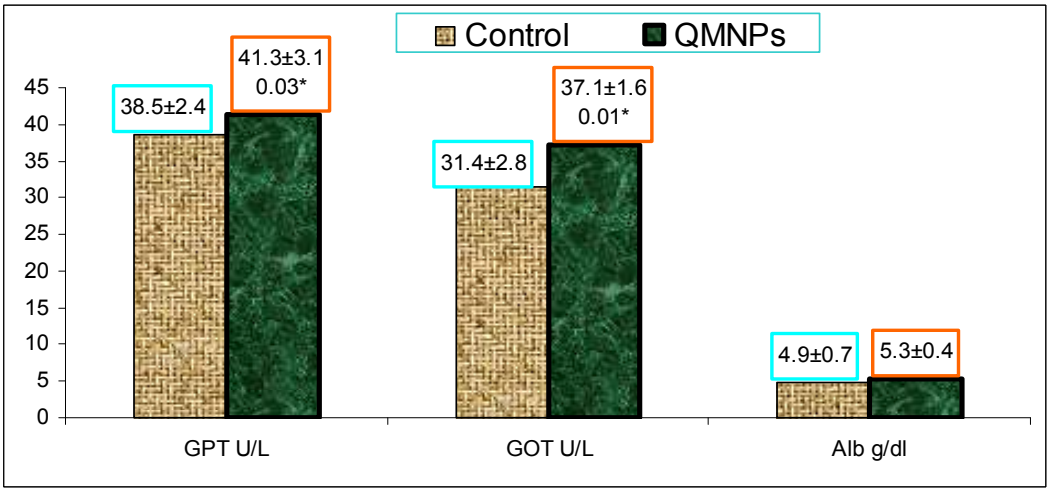


Figure 2. *The changes in the levels of ALT, AST and Albumin in plasma of rats in response to QMNPs-Fe_3O_4 compared to control.*

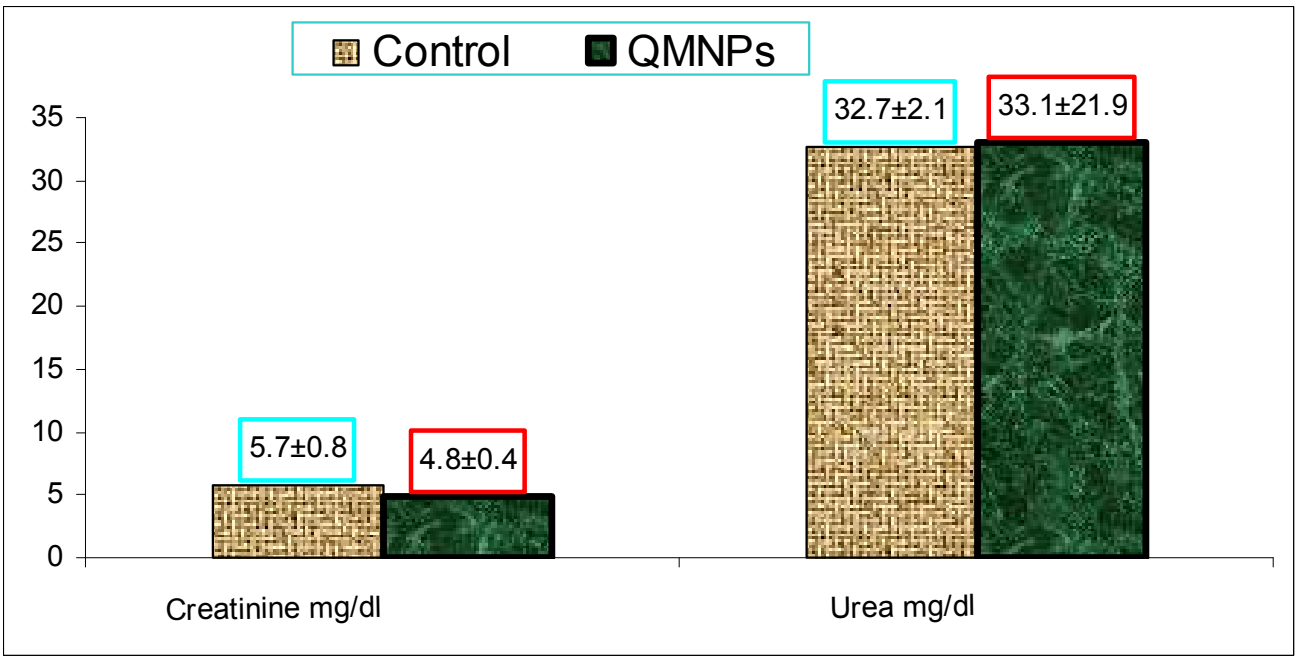


Figure 3. *The changes in the levels of creatinine and urea in plasma of rats in response to QMNPs-Fe_3O_4 compared to control.*

A significant (P<0.001) decrease in LPO (measured as MDA), coupled with significant (P<0.002) decrease in GSH content and significant (P<0.001) in SOD activity inQMNPs-Fe_3O_4 treated rats compared to the control untreated group (Fig. 4).

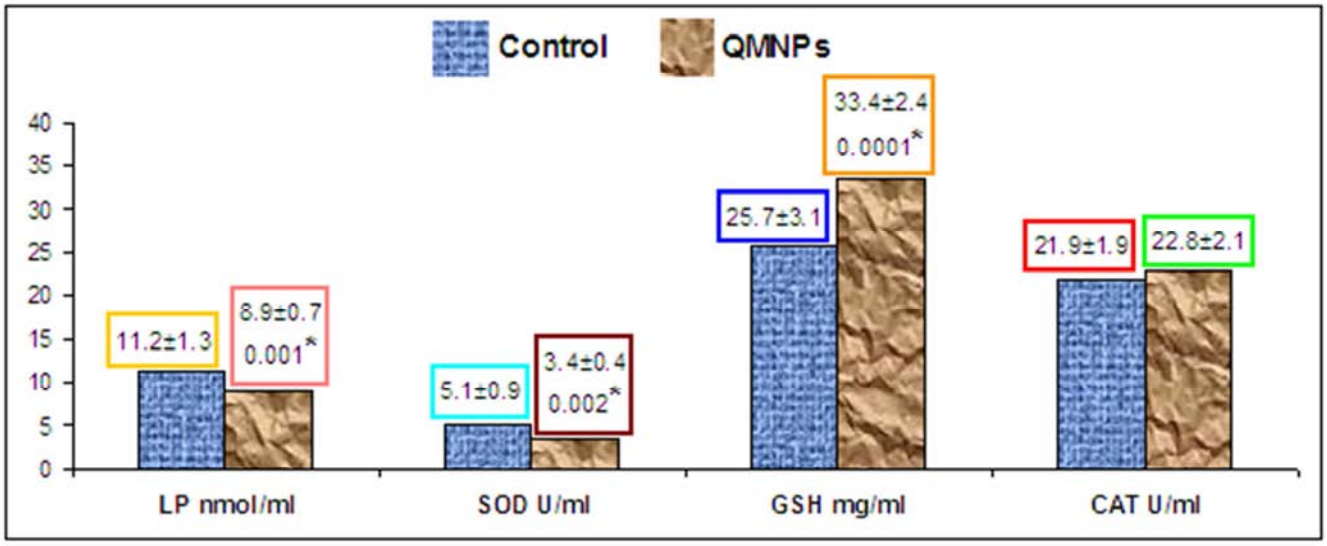


Figure 4. *The alterations in the levels of oxidative parameters in plasma of rats in response to QMNPs-Fe_3O_4 treatment compared to control.*

3.3. Cell Cycle Analysis

The effect of QMNPs-Fe_3O_4 on cell cycle progression and population distribution in mononuclear cells of normal and treated rats by wasdetermined using flow cytometry, by comparing the cell cycle profiles between QMNPs-Fe_3O_4 treated and -untreated rats. The differences were measured in the populations of cells in each phase. The results demonstrated no evidence of apoptotic cell population was observed in sub G1 phase in QMNPs treated rats compared to the control. Treatment of the female rats with QMNPs-Fe_3O_4 resulted in enhancement of cell distribution in the G_1 and S phase compared with the control group; however the number of cells in the was increased significantly (P<0.03) in G1 phase and (P<0.01), but there was no significant difference between QMNPs-Fe_3O_4 and control group in the G2/ M phase (P > 0.05) (Fig. 5).

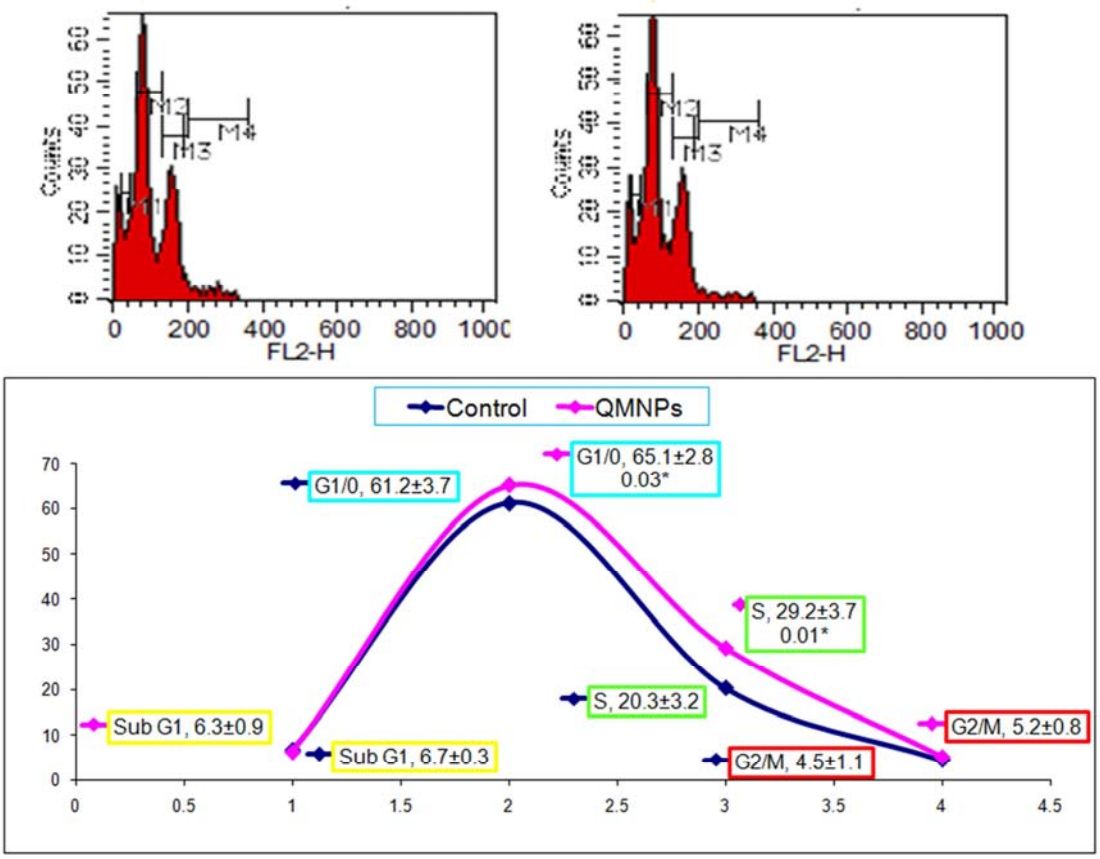


Figure 5. *Cell cycle analysis of the blood mononuclear cells of QMNP-fe$_3$O$_4$ treated rats for 8 weeks. G1/G0, S and G2/M indicate cell cycle phases. Sub-G1 refers to population of apoptotic cells.*

4. Discussion

Nanoparticles have a large surface area to volume ratio which leads to an alteration in biological activity compared to the parent bulk materials [25].

NP toxicity refers to the ability of the particles to adversely affect the normal physiology as well as to directly interrupt the normal structure of organs and tissues of humans and animals. It is widely accepted that toxicity depends on physiochemical parameters such as particle size, shape, surface charge and chemistry, composition, and subsequent NPs stability [26]. Toxic effects caused by Fe_3O_4 (MNPs) as an iron oxide NPs have attracted much attention not only because of their superparamagnetic properties, but also because they have shown to be associated with low toxicity in human body [27].

When nanoparticles enter in to the bloodstream, they immediately encounter by the plasma proteins and immune cells. The uptake of nanoparticles may occur through various pathways like hemolysis, thrombogenicity and complement activation. These pathways include numerous activities like reduced number of blood cells, stimulation of oxidative stress and reduction in cellular antioxidants and increasing the number of cells involved in the immune processes [14].

In the present study Quercetin loaded MNPs-Fe_3O_4 was administered *ip* twice/week at a dose of20ml/kg (each one ml of the solution contain 11nmol). The rats were observed daily for 8 weeks for any toxic symptoms. No any adverse toxic effects and no mortalities were observed during the entire experiment. Moreover, the gross examination indicated that there was no any abnormality detected in the internal organs.

In the present study, the effect of *ip* repeated doses (20ml/kg each one ml contain 11nmol of QMNPs) of QMNPs on the hematological factors such as Hb, RBCs, WBCs (lymphocytes, monocytes, eosinophils and basophils) was assessed. The results showed different changes in blood cells, and the changes were significant for white blood cells (lymphocytes, neuotrophils monocytes, and basophils). Our results are line with other reports [28]. They reported that different concentrations of TiO2 NPs (25nm size) caused significant alterations in white blood cells (lymphocytes, monocytes, eosinophils and basophils) when administered orally for 14 days. Other investigators demonstrated the increase of Neutrophils and Monocytes after administration of different doses of iron oxide nanoparticles (IONPs) [14].

It has been emphasized that magnetic NPs distributes (80-90%) liver, spleen (5-8%) and (1-2%) in bone marrow [29]. Tsuchiya and colleagues elucidated that *ip* administration of MNPs mainly distributed in liver, lungs and lymph nodes [30].

Accordingly theliver is the most important detoxification organ; it is reached by the highest level of NPs concentration over all of the other tissues [31]. Therefore, the liver can be seen as a target organ to identify adverse effects of in vivo

exposure to QMNPs-Fe_3O_4 in our study.

Then the levels of the liver enzymes Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) are two of the most reliable markers of hepatocellular injury [32]. Increased levels of these enzymes in liver tissue as well as serum might be as adaptive mechanism due to the stress of nanoparticles stress [33]. Therefore, the possible hepatictoxicity of QMNPs-Fe_3O_4 was demonstrated by evaluating the change in the levels of ALT and AST and albumin (Alb) as a sensitive biomarker of liver function.

These results indicated that repeated administration of QMNPs-Fe_3O_4 showed significant elevation in ALT and AST, meanwhile Alb level showed insignificant change. Regarding the ALT and AST activity, our observations are consistent with the prior investigators [34]. They stated the increase of hepatic activity of both enzymes 15 days after administration of Fe_3O_4 NPs. The possible underlying toxicity mechanisms of Fe_3O_4 NPs could be lie in accumulation of iron in liver due to the repeated doses delivered in our study and induction of oxidative stress due to the generation of ROS. Regarding the insignificant change of Albumin, this observation agrees with Wang and co-workers. They reported that after administration of APTS-MNPs, total protein (TP) level stay within normal range (Wang, et al 2014). The results of the present study revealed that QMNPs-Fe_3O_4 did not induce Nephrotoxicity as manifested by the insignificant change was detected in creatinine and urea levels. In a study conducted by Aliahmad and colleagues, the stated the elevation of blood nitrogen urea level and serum level of creatinine [35].

These contradicting results could be due to different species of animals (mice/ rats) used in the study, the route of administration (oral or *ip)* and methods for preparation of the nanoformulation in each study. Wang and coworkers reported that the liver and kidney of mice might be acutely influenced after exposure to MNPs but could return to normal in a short time. They also reported that the influence was dose-dependent [36].

Findings from the majority of nano toxicity studies conducted with IONPs have found that overproduction of reactive oxygen species (ROS) could be one of the possible underlying toxicity mechanism. Cells respond to increased levels of ROS by a "detoxification" mechanism, which involves reduced Glutathione and enzymes such as superoxide dismutase (SOD) which considered to play a major role in defense against ROS [37].

Overproduction of ROS has been linked to lipid peroxidation [38]. So oxidative stress is an important mechanism of toxic action of NPs. In a study conducted by Gaharward and Paulrgi, 2015 reported a dose dependent increase in MDA indicating lipid peroxidation coupled with significant brought down in antioxidant enzymes activities (GSH, SOD and CAT) after IONPs treatment. In the current study, there was a significant decrease in lipid peroxidation (as MDA level) coincides with significant elevation of GSH and SOD observed in the QMNPs-Fe_3O_4 treated group meanwhile the activity of CAT did not affect when compared to control. This effect is most likely due to the scavenging and antioxidant properties of quercetin incorporated in the nano-complex. In the contrary with this result, regarding the cell cycle analysis, our results demonstrated that no evidence of apoptosis observed in the sub G1 phase. This result indicated that QMNPs-Fe_3O_4 did not induce endoneocleosome activation and consequently DNA leakage. In a study conducted by Namvar and colleagues, they have reported sub-G1 population of apoptotic cells after MNPs-Fe_3O_4 treatment [39]. Other investigators have elucidated the cell arrest in G1/S and in both G1/S and G2/M phases [40]. These conflicting results could attribute to the fundamental difference between cell types [39].

5. Conclusions

The aforementioned data permit to conclude that the biological activity of QMNP-Fe_3O_4 showed significant alteration in white blood cell fractions. Also, a significant elevation in ALT and AST was observed. Otherwise, there was no renal toxicity as manifested by the insignificant change in the levels of creatinine and urea. A significant brought down in MAD concomitant with significant elevation of GSH and SOD was recorded. QMNPs-Fe_3O_4 enhanced the cell arrest in G1/S phases. All together the results recommended slight hepatic toxicity of the formulated nano complex in the cellular level. More investigation is ongoing on the molecular levels.

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