Enhancing Antioxidant Cellular Defense by Using Quercetin Loaded TiO$_2$ Nanoparticles in Swiss 3T3 Albino Mouse Fibroblast Cells

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**Abstract**

Living organisms are continuously exposed to a wide variety of oxidative radicals. Therefore, there is a constant demand for exogenous antioxidant supply. In this regard, one of these exogenous antioxidants with well-established potency, quercetin has been utilized in various formulations. Yet due to its poor water solubility, its extensive applications are limited so far. In this study, TiO$_2$ nanoparticles (TiO$_2$-NPs) were employed to improve the cellular penetration of quercetin and to maximize its antioxidant effects on Swiss 3T3 albinio mouse fibroblast cells. Surfaces of TiO$_2$-NPs were modified with Polyethylene glycol (PEG) that enabled better dispersion and enhanced biocompatibility. Toxicity of quercetin and quercetin loaded TiO$_2$-NPs (QL-TiO$_2$-NPs) were evaluated in terms of cell morphology and measured with cell viability assay. For an in-depth cell viability analysis, key markers of apoptosis were investigated by immunoblotting analysis. As an indicator of apoptotic cell death, cleavage of caspase 3 (Cas 3) and poly (ADP-ribose) polymerase proteins (Parp) were detected in quercetin treated cells. Antioxidant capacity of quercetin in the form of QL-TiO$_2$-NPs was measured in the cells in which generation of reactive oxygen species (ROS) and superoxide was induced by pyocyanin. Quantitative ROS measurements were confirmed with confocal microscopy. Further mechanistic insight on upregulation of NF-E2 related factor 2 (NRF2) pathway via QL-TiO$_2$-NPs was also provided in an effort to validate its antioxidant defense. Target enzymes of NRF2, heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase1 (NQO1) and superoxide dismutase1 (SOD1) expressions were increased in the model proposed. QL-TiO$_2$-NPs allowed high bioavailability of quercetin concentration and stability in the cell with maximum antioxidant capacity against formation of ROS without any cytotoxicity. Overall, this paper sheds light on how the efficiency of quercetin in a nanosystem can serve as a safe therapeutic candidate for breaking the vicious ROS cycle in the cell.

**Keywords:** Quercetin; TiO$_2$ nanoparticles; ROS; Superoxide; NRF2; Quercetin topical application

**Introduction**

Oxidative stress is related to many diseases and pathological conditions such as cancer, neurodegenerative diseases, cardiovascular diseases and aging [1]. It also plays an important role in cell signalling, either as an initiator or as a suppressor of the progression of those pathological conditions. Despite these clinical outcomes, living organisms are also exposed to oxidative stress continuously as a result of environmental effects and pollution. Therefore, a spontaneous struggle takes place within the cells to balance the antioxidant defense mechanism against oxidative stress for survival. Many studies have focused on exogeneous antioxidant supplementation from natural resources that are necessary for the restoration of antioxidant defense of cells. One of the readily accesible antioxidant family are flavonoids that are phenolic phytochemical compounds and exist in rich amounts in natural resources. A number of investigations have shown their antioxidant effect on protecting cells against damage by reactive oxygen species (ROS) [2]. Quercetin is the strongest antioxidant among flavonoids owing to three active functional groups in its structure [3]. Despite its highest antioxidant capacity, quercetin applications are considerably inefficient by virtue of its hydrophobic nature (Water solubility less than 0.01g/l) [4]. Recently, quercetin has been formulated in a various nano-sized formulations in order to address its conventional passive delivery and to enhance its cellular penetration into skin cells [5]. Nanoparticles are particularly useful in drug delivery for water-insoluble molecules such as chemotherapeutic drugs [6]. They have many advantages such as small size (≤100 nm), thereby, increasing absorption, ensuring bioavailability and controlled drug release making them most desirable compared to conventional drug delivery.

TiO$_2$ has been commonly used in paints, paper, plastics, textiles, food and pharmaceutical industries for many years. Its applications in medicine and life sciences increased after it was accepted as a biologically inert material in 1990s [7-9]. It has been widely added in sunscreen products as a major component against high ultraviolet rays in cosmetics due to its high-efficiency photoprotective properties [10]. Moreover, Titanium dioxide comprises one of the most preferred nanoparticles (TiO$_2$-NPs) and has extensive application in orthopedic and dental implants by well characterized high biosafety, antibacterial and desired mechanical properties [11].

In the present study, a novel nanosystem was synthesized that utilizes quercetin loaded TiO$_2$-NPs (QL-TiO$_2$-NPs) for its application in enhancing skin defense against oxidative stress. Here, TiO$_2$-NPs were encapsulated with Polyethylene glycol (PEG) that enabled better dispersion and enhanced biocompatibility making the PEGylated surfaces more adhesive to the living cells. The nanosystem was designed for high loading capacity of quercetin molecules provided by nano-sized TiO$_2$-NPs for their effective quercetin delivery into the cell. This nanosystem was subjected to different treatment regimes against swiss...
3T3 albinio mouse fibroblast cells. Total cellular ROS and superoxide levels in treated cells were determined and compared with those of control cells. However, quercetin in a nanosystem shows maximum antioxidant activity by triggering NRF2 pathway, upregulating phase II antioxidant enzymes, heme oxygenase-1 (HO-1), NAD(P)H: quinone oxoreductase1 (NQO1) and superoxide dismutase1 (SOD1). In summary, our study demonstrated that quercetin loaded TiO2-NPs served as an effective nanosystem for delivering quercetin to swiss 3T3 albinio mouse fibroblast cells *in vitro* and provided protection against oxidative stress and cellular toxicity.

**Methods**

**PEGylation of TiO2-NPs**

TiO2-NPs were purchased from Sigma (Sigma, 718467) as a powder with particle size of 21 nm. Surface modification of TiO2-NPs with PEG 6000 (Sigma, 81255) was done according to the protocol described by Ramasamy et al. [12]. The TiO2-NPs were suspended in water and ultrasonicated at 500 watts and 34% amplitude for 30 s pulse with an interval of 30 s off on ice for total 20 min. The solution was mixed with 1% PEG 6000 solution in the mass ratio 1:1 for TiO2-NPs surface modification. The mixture was stirred at 750 rpm overnight at room temperature. The PEG coated nanoparticles were centrifuged at 18000 rpm for 1 h at 10°C. The supernatant was removed and PEG functionalised NPs were washed with distilled water. Thus, obtained PEGylated NPs were frozen by liquid nitrogen and freeze dried for two days. Finally, the PEGylated TiO2-NPs powder was collected and henceforth referred to as TiO2-NPs in this paper.

**Scanning electron microscopy (SEM) analysis**

The size and shape of TiO2-NPs powder before and after PEGylation were analysed by SEM. All TiO2-NPs suspension before PEGylation (control) and after PEGylation was dispersed by ultrasonication for 10 min in water to obtain a homogeneous suspension. This suspension was then mounted on silicon wafer chips and sputter coated with a thin layer of Pd–Au before taking SEM images. The SEM images were acquired using a scanning electron microscope (Carl Zeiss, LEO Supra 35VP, Germany) operated at 3 kV.

**Loading quercetin in PEGylated TiO2-NPs**

Quercetin (AppliChem, A3415) concentration was determined using UV visible spectrophotometer at 255 nm wavelength. Quercetin loading was performed by mixing quercetin solution with PEGylated TiO2-NPs suspension in a mass ratio of 1:1 and stirred at room temperature for overnight. This suspension was centrifuged at 2000 rpm for 5 min at room temperature and the supernatant was removed. Thus obtained pellet was washed and resuspended with the same volume of sterile PBS 1X solution.

Percentage of quercetin loading was calculated according to the relative difference between the concentration in the solution before and after loading using the following equation 1:

\[
\text{Drug loading } \% = \frac{(A-B)}{A} \times 100
\]

Where A and B represent the initial and final quercetin concentration of the solution.

**Cell culture and treatments**

Swiss 3T3 albinio mouse fibroblast cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in Dulbecco’s modified Eagle’s medium (DMEM). The cells were sub-cultured when they reached a 70-80% confluence ratio. Cells were divided into several groups based on the treatment regimes as follows; (i) quercetin loaded TiO2-NPs treated (QL TiO2-NPs) cells, (ii) only quercetin treated (Q-Cntrl) cells and (iii) only PEGylated nanoparticles treated (TiO2-NPs) cells. Untreated cells were used as control groups. Treatment of cells was performed for 6 and 12 h for immunoblotting assays and 24 and 48 h for cell viability assays. The test cell culture medium was amended with TiO2-NPs with a final concentration of 100 µg ml-1 in all regimes.

**Cell viability assay**

Cell viability assay was performed with Cell Proliferation Reagent WST-1 Kit following the manufacturer's instructions (Roche, Indianapolis, IN, USA). Cells were cultured at a seeding density of 50 × 10^3 cells/well in a final volume of 100 µl across 96-well microplates. Cells in 96-well microplates were treated as indicated above and 10 µl of WST-1 reagent was added to each well and the plates were incubated for 4 h at 37°C. Absorbance was measured against the background control as blank at wavelength of 450 nm and a reference wavelength of 655 nm by microplate reader (Bio-rad iMark Microplate Reader, USA). Results were expressed as percentage of cell viability versus controls, taken as 100%.

**Total cellular ROS and superoxide measurement**

ROS measurements were done by using Cellular ROS/Superoxide Detection Assay Kit (Abcam, ab139476). Cells were grown in a 96-well black microplate at a seeding density of 50 × 10^3 cells per well in a final volume of 100 µl/well. Pyocyanin was used as a ROS and/or superoxide inducer at a final concentration of 50 µM and ROS and/or superoxide induction was performed after 45 min of reaction. Negative control cells were incubated in the presence of ROS inhibitor (NAC, N-acetyl-l-cysteine) for 30 min prior to ROS induction. For the ROS/superoxide measurement, ROS and/or superoxide detection reagent was added and the plate was read by microplate reader (Molecular Devices, SPECTRA max GEMINI XPS, California, USA) with a filter set compatible to fluorescein and rhodamine (Ex/Em: 488/520 nm to Ex/Em: 550/610 nm).

**Protein extraction and immunoblotting**

Cells were harvested by centrifugation at 1214 rpm for 5 min for total protein extraction. Cells were then resuspended with ice-cold PBS and spun at 13200 rpm for 30 s. Pellet was lysed by incubation in total cell lysis buffer containing protease and phosphatase inhibitors for 30 min, followed by centrifugation at 13200 rpm for 10 min. Supernatant was collected as total protein extract and stored at -80°C. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Munich, Germany) based on Bradford method. Proteins were mixed with loading buffer and separated on 12-15% SDS-PAGE and blotted onto PVDF membranes. Membranes were then blocked with 5% non-fat dry milk (AppliChem GmbH, Darmstadt, Germany) in PBS-Tween 20, incubated with primary antibody overnight, followed by washing in PBS-Tween 20 and incubation with HRP-conjugated secondary antibody. After final wash with PBS-Tween 20, proteins were analyzed with ECL Advance (GE Healthcare Bio-Sciences, USA) and exposed to Hyperfilm-ECL (GE Healthcare Bio-Sciences, USA), Primary Caspase 3 (#9662, rabbit), cleaved caspase 3 (#9664, rabbit), PARP (#9542, rabbit), NFR2 (D129C) (#12721, rabbit), SOD1 (#2770), NQO1(#42672), HO-1 (P249) (#5061, rabbit), B-actin (#4967, rabbit) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA) and used with 1:1000 dilution in immunoblotting. Secondary HRP-
conjugated anti-rabbit (#7074) and anti-mouse (#7076) antibodies were also purchased from Cell Signaling Technology Inc. (Beverly, MA, USA) and used with 1:5000 dilutions in immunoblotting. To ensure equal protein loading, each membrane was stripped and reprobed with β-actin antibody.

Confocal laser scanning microscopy

Cells were seeded directly onto glass slides inserted bottom of the 12-well plate and examined under the confocal laser scanning microscope (Carl Zeiss, LSM 710, Jena, Germany) as per the manufacturer’s instructions of Cellular ROS/Superoxide Detection Assay Kit (Abcam, ab139476). Pyocyanin was used as a ROS inducer and NAC was added as a ROS inhibitor. Cells were incubated with ROS detection reagent for 1 h at 37°C, washed with wash buffer and fixed onto slide by incubation with a 4% PFA solution for 10 min in the dark condition. The fixed Cells were immediately overlaid with a cover slip in the presence of mounting solution (Life Technologies, Molecular Probes, SlowFade Diamond Antifade Mountant, USA) with DAPI dye (4′,6-diamidino-2-phenylindole). Finally, the Slides were observed under the confocal laser scanning microscope using appropriate filter set for fluorescein (Ex/Em: 488/520 nm).

Confocal images were acquired by ImageJ software after utilizing Corrected Total Cell Fluorescence (CTCF) calculation to measure fluorescence intensity. CTCF values were obtained according to formula: CTCF=integrated density – (area of selected cell X mean fluorescence of background readings). CTCF value was divided by their cell numbers from each image. Cells were counted by the analyze particles option of ImageJ software and each value was rounded off for 100 cells.

Statistical analysis

All in vitro results were representative of at least three independent experiments, while immunoblotting results were representative of minumum two independent trials. All numerical data were shown as mean ± SEM (Standard error of the mean). Statistical significance was analyzed with Student’s t-test. P values less than 0.05 were considered to be statistically significant.

Results

Characterization of TiO₂-NPs and drug loading

Morphological characterization of TiO₂-NPs was carried out by SEM analysis. SEM examination showed that pure TiO₂-NPs without PEGylation tend to agglomerate despite extensive homogenization through ultrasonication over time due to their inherent physico-chemical properties (Figure 1A and 1B). PEGylated TiO₂-NPs appeared moderately dispersed compared to those of non-PEGylated NPs with average size of ~25-50 nm that provided relatively large surface area (Figure 1C and 1D). PEG-functionalized TiO₂-NPs enabled >95% drug loading (quercetin) capacity in QL-TiO₂-NPs calculated according to Eq. 1 as described in experimental section.

Morphological analysis of cell treatments

After treatments, experimental groups were observed using fluorescence microscope (Zeiss, Axio Vert Inverted microscope, Gottingen, Germany) in terms of morphologic changes related to cell viability. All experimental groups were viable, except for the Q-Cntrl groups at both 6 and 12 h of incubation (Figure 2). The effects of quercetin toxicity on these cells at early stage of treatment were clearly observed (Figure 2A). Cell density tend to decline after 6 h of quercetin treatment compared to other groups (Cntrl, QL-TiO₂-NPs and TiO₂-NPs). TiO₂-NPs groups however showed constant cell density probably due to aggregation of cells with TiO₂-NPs. After 12 h, the experimental groups failed to show any significant differences except in Q-Cntrl group where cells indicated dramatic changes as they diassociated by losing their contact with the surface and initiated clumping (Figure 2B). These morphological changes seen in Q-Cntrl group indicated that the cell death was highly present in this group compared with the other groups. This increase in cell death was also preserved to be increased by at 24th and 48th h of treatment (data not shown).
Cell viability and cell death analysis

Viability assays were performed at two different concentrations, 100 and 250 µg ml$^{-1}$ of QL-TiO$_2$-NPs nanosystem and its components. Viability of cells treated for 24 and 48 h against to untreated cells (control groups) are shown in Figure 3A. Cells exposed to 100 µg ml$^{-1}$ of QL-TiO$_2$-NPs for 24 h showed a little increasing change in viability compared to control cells. However, the viability of cells in Q-Cntrl declined by half of control level at the same concentration. Cells exposed to higher 250 µg ml$^{-1}$ of QL-TiO$_2$-NPs doses exhibited 14% reduction in the viability of QL-TiO$_2$-NPs treated cells and pure quercetin treated cells lost viability by 92% compared to control cells. Prolonged exposure for 24 h influenced a sharp decline in cell viability among the groups with both concentrations (100 and 250 µg ml$^{-1}$) except with QL-TiO$_2$-NPs groups. The QL-TiO$_2$-NPs groups however showed 71% of cell viability against 250 µg ml$^{-1}$ of control cells. According to results, 100
µg ml⁻¹ of QL-TiO₂-NPs dose was applied for all remaining experiments with an interval of 24 h exposure.

In order to reveal mechanistic details of induced cell death, levels of some key proteins pertinent to the signalling pathways of programmed cell death were analysed. First, key proteins of apoptosis, caspase 3 (Cas 3) and poly (ADP-ribose) polymerase (Parp) were determined in the experimental groups treated for 24 and 48 h periods with QL-TiO₂-NPs, Q-Cntrl, TiO₂-NPs and untreated control groups. The results showed no significant difference among the groups QL-TiO₂-NPs, TiO₂-NPs and Cntrl with respect to apoptotic protein levels (Figure 3B). However, it was not possible to obtain sufficient amount of protein from the Q-Cntrl groups exposed to quercetin for 24 and 48 h, merely due to cell death, and this result is consistent with the viability assays shown in Figure 3A. To identify cell death, apoptotic markers in the early stage of treatment were examined that showed cleavage of cas 3 and parp proteins in Q-Cntrl groups at 6 and 12 h of treatment times (Figure 3C and 3D). As also shown in Figure 2, these result confirmed more dramatic morphological resembling cell death that were seen in Q-cntrl group at 6 and 12 h of treatments.

Antioxidative defence and NRF2

The NF-E2 related factor 2 (NRF2), a key gene that is expressed under oxidative stress for cell survival, was further analyzed in the experimental model. NRF2 was observed by immunoblotting analysis after 24 and 48 h of treatment among cell groups except for Q-Cntrl group since an extracted insufficient amount of protein from Q-Cntrl cells. The results showed that there was no significant change in NRF2 expression (Figure 4A) for 24 and 48 h of treatment. To detailed investigation of molecular mechanism about the relation between survival mechanism and antioxidant defense, NRF2 expression was checked in the early stage of treatment with QL-TiO₂-NPs nanosystem and quercetin. It was clear that NRF2 gene expression was found to be stimulated by QL-TiO₂-NPs in the 6 and 12 h of treatment (Figure 4B), which tended to increase with time as determined by densitometric analysis (Figure 4B). The TiO₂-NPs group however showed moderate NRF2 protein synthesis compared to control at the initial exposure stage. Following this, we surveyed most common genes that are downstream to NRF2, cytoprotective antioxidative enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxydoreductase1 (NQO1) and superoxide dismutase1 (SOD1). However, it was hard to show expressions of the enzymes with immunoblotting analysis since it required a higher amount of protein for the analyses. For this reason, the differences in the expression levels of the enzymes were detected by densitometric analysis. As shown in Figure 4B, HO-1 enzyme levels

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**Figure 4:** Immunoblotting analysis of NRF2 and its target genes. (A) It was not detected any changing in expressions of NRF2 protein in the treatment of 24 and 48 h. (B) NRF2 protein levels increased significantly in cells treated with QL-TiO₂-NPs by time. (C) HO-1 enzyme expression showed considerable increase in QL-TiO₂-NPs group according to densitometric analysis. (D) NQO1 expressed significantly in cells treated with QL TiO₂-NPs. (E) SOD1 enzyme level increased slightly in QL TiO₂-NPs group. Histograms shown mean values ± SEM of minimum two independent experiments; densitometric measurements were normalized against corresponding B-actin levels. Asterisks denote significant differences (*P<0.05 and **P<0.005) versus control.
increased in QL-TiO$_2$-NPs group after 12 h of treatment. A significant increase in HO-1 expression was seen in densitometric analysis (Figure 4C). However, Q-Cntrl group showed a slight rise in at the expression of HO-1 after 6 h of treatment but declined after 12 h. Expression of NQO1 enzyme increased in the cell groups treated with QL-TiO$_2$-NPs at both 6 to 12 h of treatment compared to Q-Cntrl groups in which minimum levels of NQO1 observed (Figure 4D). SOD1 enzyme levels remained constant or moderately increased in all cell groups except Q-Cntrl during the 6-12 h treatments in which least enzyme levels were found (Figure 4E). This analysis demonstrated that expression of NRF2 and its target enzymes were increased only in the cells treated with QL-TiO$_2$-NPs.

**Total cellular ROS and superoxide measurement**

The highest amount of ROS was measured in pyocyanin treated positive control group (P-Cntrl) compared to control group (Figure 5A). ROS formation was completely inhibited in cells in the presence of QL-TiO$_2$-NPs (> 100%), similar to cells in which ROS formation was inhibited by standard NAC. The lowest level of ROS was detected in Q-Cntrl group. Increased ROS generation was measured in TiO$_2$-NPs group and P-Cntrl group with a moderate difference in their levels. This trend was same with superoxide levels measured earlier as shown in Figure 5B. We observed consistent levels of superoxide in both P-Cntrl and TiO$_2$-NPs groups. The cells in QL-TiO$_2$-NPs group showed prevention of superoxide formation, a phenomenon usually observed with internal control (NAC). The cells in Q-Cntrl group however showed the lowest level of superoxide production. As it was expected, the QL-TiO$_2$-NPs nanosystem was shown high protection in the cells which ROS and superoxide formation were induced.

**Monitoring of total cellular ROS generation**

ROS production in cells were also monitored by confocal microscopy to validate the quantitative ROS measurements. Here, Cells nuclei were stained with DAPI (Figure 6B) and the green fluorescence in the image represented for ROS (Figure 6A). Maximum fluorescence intensity was evident in P-Cntrl group followed by TiO$_2$-NPs group. The fluorescence intensity with Cntrl group, QL TiO$_2$-NPs, Q-Cntrl and NAC appeared similar to those of the control or untreated cells. In the end of 24 h treatment, there was a dramatic decline in the viability of QL-TiO$_2$-NPs treated cell group restored 86% survival rate. However, at higher concentration of quercetin treatment shown to be lethal to Q-Cntrl group cells, while the QL-TiO$_2$-NPs treated group remained 86% survival rate. However, at the end of 24 h treatment, there was a dramatic decline in the viability of cells in all cell groups, except with QL-TiO$_2$-NPs (Figure 3A). This result is consistent with literature regarding cell viability under TiO$_2$-NPs exposure [17-19]. Cell morphology after 6 and 12 h treatment times was examined under fluorescence microscope. We observed no significant effects on cell numbers among all groups with healthy cells.
except the Q-Cntrl cell groups (Figure 2). It is apparent that Q-Cntrl groups lost their viability starting from the very early of treatment process and that cell death rate tended to increase with treatment time. Furthermore, these cells evidently suffered from inhibition and therefore lost their contact with the surface. However, cells in TiO₂-NPs group remained healthy unlike the Q-Cntrl group, but these cells tended to accumulate over the TiO₂-NPs.

Previous studies have reported that flavonoids show pro-oxidant and cytotoxic effects at the high concentration [20,21] and these effects may be related to having hydroxyl groups [22]. Moreover, quercetin has also been reported mutagenic thereby cytotoxic in certain cases, [23,24], which is consistent with the cytotoxicity observed with quercetin in this study. Quercetin treated cell groups showed a dramatic fall in viability over time in a dose dependent manner (Figure 3A). However, it is worth noting that the concentration of quercetin here was much higher than the usual concentrations reportedly used in other studies [16,25]. Hence, the cytotoxic effect of quercetin for cells reported in this study can be regarded excess in amount. A previous report showed that breast cancer cells exposed to 300 µM of quercetin was found to be toxic, and this concentration is lower than that used in present study [26]. The cytotoxic effects of quercetin at concentrations lower than 100 µg ml⁻¹ (330 µM) were not tested in this study, since this work required the high amount of quercetin loadings on NPs to effectively diffuse into the cells. It is clearly showed that quercetin cytotoxicity at high concentration can be eliminated by PEG-assisted modification of TiO₂-NPs surface for loading quercetin in this study (Figure 3A).

Another important process for the cell death is apoptosis. Apoptosis takes place following two major signalling pathways, such as mitochondria mediated intrinsic pathway and the death receptor mediated extrinsic pathway [27]. The common process existing in both pathways is the activation of a caspase cascade. This proteolytic cascade causes the activation of initiator caspases and cleavage of effector caspases 3, 6 or 7 [28]. Once a caspase cascade is activated, there occurs cleavage of target proteins of caspases, such as parp [29,30]. The frequent key proteins involved in apoptosis were analysed by immunoblotting to clarify the molecular mechanism underlying quercetin cytotoxicity in this study. Two major phenomena concerning apoptosis occurred that are related to, cleavage of cas 3 and parp proteins, determined in the Q-Cntrl groups at 6 and 12 h (Figure 3C). Cas 3 is the most common executioner caspase [31] and extinguishes cell by cleaving parp [32]. Our results clearly demonstrated that quercetin caused cell death at high concentration through cas 3 dependent apoptotic pathway (Figure 3C). A similar work previously showed that quercetin enhanced apoptosis occurs through the activation of a caspase cascade on MDA-MB-231 human breast cancer cells [26]. In another study, different concentrations of quercetin were applied to hepatic stellate cells, and demonstrated that occurrence of cleavage of Cas 3 and Parp-1, which is consistent with our results (Figure 3C) where these upregulated by
quercetin dose dependently [33]. Interestingly, our study indicated that while high concentration of quercetin leads to apoptosis, the same concentration of quercetin loaded TiO$_2$-NPs failed to induce cell death. Thus, it can be concluded that apoptotic effect of quercetin can be modulated by incorporating with TiO$_2$-NPs.

In some studies, it is suggested that the prooxidant effect was also responsible for cytotoxic and proapoptotic effects of flavonoids [13]. Furthermore, antioxidant behavior of quercetin can shift to pro-oxidant depending its concentration and oxidative sources [34]. For an in-depth analysis of both quercetin and quercetin loaded nanoparticle effects on cells, this study questioned whether quercetin has a pro-oxidant effect by itself at high concentrations. It was established that the NRF2 gene expression of both quercetin and quercetin loaded nanoparticle effects on cells, this study questioned whether quercetin has a pro-oxidant effect by itself at high concentrations. It was established that the NRF2 gene may be the main regulator of defensive response against oxidative stress. Here, we show that QL-TiO$_2$-NPs activated the NRF2 gene expression over time (Figure 4B) in contrast to pure quercetin treatment. Together, expressions of HO-1 and NQO1 enzymes were significantly increased in QL-TiO$_2$-NPs cell groups after 12 h of treatment (Figure 4C and 4D). Moreover, there was a moderate SOD1 increase in QL-TiO$_2$-NPs treated cell groups for both time intervals as shown in densitometric analysis (Figure 4E). The highest levels of all these phase II antioxidant enzymes, which are targets of NRF2 were detected in QL-TiO$_2$-NPs cell groups. In contrast to this, the lowest levels of the enzymes were seen in quercetin treated cells. These data can be supported by the fact that QL-TiO$_2$-NPs activated NRF2 signalling and NRF2 triggered expressions of its target enzymes found in this pathway. Considering these results, it can be suggested that the QL-TiO$_2$-NPs nanosystem prevents cell death by triggering the NRF2 pathway and additionally, NRF2 signalling is conceivably involved in the survival mechanism (Figure 7).

Further, use of TiO$_2$-NPs (100 µg ml$^{-1}$) has shown to cause cell death in various cell types. For example phytohemagglutinin-stimulated human lymphocytes during 24 h showed parp cleavage was activated by nano-TiO$_2$ [35]. On the contrary, we did not observe any apoptotic signature in TiO$_2$-NPs treated cell groups (Figure 3C). These inconsistent results can be interpreted by the fact that surface modified TiO$_2$-NPs by PEGylation in this study seemed to have supressed the cytotoxic effects of pure TiO$_2$-NPs.

It is well-known that the functionality of a molecule depends on its stability in physiological conditions. We here aimed to embed high amount of quercetin facilitated by PEG-layer on NPs that provided more stability in the proximity of the cells. In the literature, quercetin was formulated in various forms for the same purpose. It has been reported the benefits of using deformable liposomes incorporated with quercetin that had many advantages, such as high solubility and better stability in HaCat cells [16]. The same group showed that quercetin deformable liposomes increased the viability accompanied by decreased ROS and MDA in cells stimulated by UVB-irradiation. In another formulation, quercetin was entrapped in glycerosomes and liposomes to strengthen its antioxidant activity on human keratinocytes in presence of H$_2$O$_2$ [36]. Quercetin was incorporated in different proportions into glycerosomes with 88-91% efficiency. Quercetin in both formulations showed strong antioxidant activity with the glycerosome formulation reaching ~95%.

In another study, quercetin was encapsulated with linoleic acid modified chitosan oligosaccharide/β-lactoglobulin nanoparticles for making it a hydrophobic bioactive compound for food industry [37].

The QL-TiO$_2$-NPs nanosystem showed the highest protection against ROS formation on swiss 3T3 albino mouse fibroblast cells (Figure 5A). Existence of the same protective effect for quercetin treated cell groups could not be interpreted because severe cell death occurred in this group. The data strongly supports that the developed nanosystem allowed safe delivery of a high amount of quercetin into cells and hence, quercetin seemed to remain more stable and enhanced the antioxidant capacity if loaded on surface modified TiO$_2$-NPs. This result was also confirmed by quantitative ROS measurements as well as by confocal laser scanning microscopic examinations (Figure 6A) and a similar trend was obtained in the chart by analysing confocal images with CTCF equation (Figure 6C).

Mitochondria harbours the bioenergetic events of the cell and thus

Figure 7: Purposed molecular mechanism for biological effects of QL-TiO$_2$-NPs and quercetin on swiss 3T3 albino mouse fibroblast cells.
it is the main source of ROS. Mitochondrial ROS is majorly composed of superoxide anion [38]. Therefore superoxide amount was determined and the results were consistent with the total cellular ROS measurements (Figure 5B). Several studies related to protective effects of quercetin for mitochondrial dysfunction have been reported. For example, quercetin protects Caco-2 cells against mitochondrial damage [39]. It is also reported that quercetin provided stable mitochondrial function by modulating cellular antioxidant defense and decreasing ROS in mouse zygotes [25]. In this study, quercetin showed strong antioxidant effect against superoxide generation in mitochondria. However, it also caused cell death via mitochondrial damage since the Wst-1 reagent used in viability assays was specific for mitochondrial damage. Overall, it can be commented that quercetin at high concentration causes mitochondrial dysfunction and cell death. However, when the same amount of quercetin was loaded on to PEG-functionalized TiO2-NPs, it could serve as a strong antioxidant without causing any detectable cytotoxicity (Figure 5). Furthermore, TiO2-NPs together in combination with quercetin may not only provided protection against oxidative skin damage, but also can serve as an effective barrier against ultraviolet B (UVB) damage due to the inherent property of embedded nano-sized TiO2-NPs.

Conclusion

A novel nanosystem composed of quercetin encapsulated on PEGylated TiO2-NPs was designed in this study. This nanosystem provided maximum antioxidant activity against formation of ROS and superoxide by maintaining high concentration of quercetin-stable and functional in swiss 3T3 albino mouse fibroblast cells. Our study demonstrated that pure quercetin alone could be detrimental to cells. However, if pure quercetin loaded onto the TiO2-NPs (QL-TiO2-NPs), though at high concentration, could provide effective quercetin to the cells with no detectable cytotoxicity and thus making QL-TiO2-NPs a promising candidate for breaking the vicious ROS cycle in the cell. This nanosystem formulation proposed in this study can potentially be used as a therapeutic agent in a topical cream for enhancing skin defense against oxidative stress, UV and inflammation.

References


