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Cytoskeletal Morphological Changes of Mesenchymal Stem Cells after Oxidant Damage and its Prevention by Thymoquinone

(Perubahan Morfologi Sitokerangka Sel Stem Mesenkima selepas Kerosakan Oksidan dan Pencegahannya oleh Timoquinon)

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ABSTRACT

The functional integrity of the cytoskeleton of mesenchymal stem cells (MSCs) is essential for its differentiation into multiple cell lineages including adipocytes, chondrocytes, and osteoblasts. Abnormalities in the cytoskeletal proteins such as actin and microtubule can cause disrupted cell signalling and irregular movements of organelles leading to cell death. This study investigated cytoskeletal and nuclear morphological changes of the MSC due to oxidative damage by hydrogen peroxide (H_2O_2) and the possible prevention of these changes by the antioxidant thymoquinone (TQ). Bone marrow MSCs from Sprague Dawley rats were cultured and treated with different concentrations of H_2O_2 with or without TQ to observe the potential protective activity. Triple-label fluorescence immunocytochemistry was performed post-treatment to observe the nucleus, actin and microtubules using 4',6-diamidino-2-phenylindole (DAPI), Alexa Fluor 488-labelled phalloidin and Cy3-labelled anti-tubulin antibody, respectively. The normal stem cell cytoskeleton demonstrated intact actin and microtubule structures along with normal appearance of the nucleus. However, oxidative damage by H_2O_2 caused a severe disruption of the cytoskeletal morphology of the actin and microtubule along with apoptosis and necrosis of the nucleus. Interestingly, both immunocytochemical and Fluorescence-Activated Cell Sorting (FACS) results showed that these morphological changes were prevented by TQ at low concentrations while higher concentrations of TQ were harmful. This study suggested that TQ could save MSCs from oxidative-induced cell death.

Keywords: Cytoskeleton; oxidative damage; stem cell; thymoquinone

ABSTRAK

Keutuhan fungsi sitokerangka sel induk mesenkima (MSCs) adalah penting untuk pembezaannya kepada pelbagai keturunan sel termasuk adiposit, kondrosit dan osteoblas. Keabnormalan dalam protein sitokerangka seperti aktin dan mikrotubul boleh mengakibatkan isyarat sel terganggu dan pergerakan organel tidak teratur yang membawa kepada kematian sel. Penyelidikan ini mengkaji perubahan morfologi sitokerangka dan nukleus MSC akibat kerosakan oksidatif oleh hidrogen peroksida (H_2O_2) dan kemungkinan pencegahan perubahan ini oleh antioksidan, timoquinon (TQ). MSC sumsum tulang daripada tikus Sprague Dawley telah dikulturkan dan dirawat dengan kepekatan H_2O_2 yang berbeza dengan atau tanpa TQ untuk mencerap potensi aktiviti perlindungan. Imunositokimia *triple-label fluorescence* telah dilakukan selepas rawatan untuk mencerap nukleus, aktin dan mikrotubul sel menggunakan masing-masing 4',6-diamidino-2-fenilindol (DAPI), faloidin terlabel Alexa Fluor 488 dan antibodi anti-tubulin terlabel Cy3. Sitokerangka sel induk normal menunjukkan struktur aktin dan mikrotubul yang utuh berserta dengan penampilan normal nukleus. Walau bagaimanapun, kerosakan oksidatif oleh H_2O_2 mengakibatkan gangguan teruk ke atas morfologi sitokerangka aktin dan mikrotubul berserta dengan apoptosis dan nekrosis nukleus. Menariknya, kedua-dua hasil imunositokimia dan *Fluorescence-activated Cell Sorting* (FACS) menunjukkan bahawa perubahan morfologi ini dihalang oleh TQ pada kepekatan rendah manakala kepekatan TQ yang lebih tinggi adalah berbahaya. Kajian ini menyarankan bahawa TQ boleh menyelamatkan MSC daripada kematian sel yang disebabkan oleh oksidan.

Kata kunci: Kerosakan oksidatif; sel induk; sitokerangka; timoquinon

INTRODUCTION

Stem cell research has been receiving considerable attention over the last decade; growing steadily as it is giving hope for a promising cure for a lot of diseases. Mesenchymal stem cells (MSCs) are multipotent marrow stromal cells that can differentiate into several lineages. Thus, the MSC has the potential to be used for the regeneration or repair of cells. MSCs can be extracted from many sources, although bone marrow has been used more often. Emerging evidence has demonstrated the potential application of MSCs transplantation from bone marrow to injured tissues, such as the infarcted heart (Emerson et al. 2022). Reports are showing that MSC therapies have demonstrated significant improvements; albeit several important challenges needed to be rectified before widespread clinical use (Chen et al. 2021; Li & Chen 2022).

Mesenchymal cytoskeletal is very important in maintaining the normal function of cell. Cytoskeletal abnormalities may be reflected by defects in cell function such as cytoskeletal assembly and proliferation (Sassoli et al. 2018). Depending on the type of cell, the abnormalities involving the cytoskeleton will cause different diseases and pathologies (Dupré, Boztug & Pfajfer 2021). For example, mechanical roles of MSC and cytoskeletal stiffness are the determining factor of innate immune response in Hutchinson–Gilford progeria syndrome (Mu et al. 2020) and dysregulation of the cytoskeleton can be detrimental to the immune system (Ben-Shmuel et al. 2021).

Damaged tissue normally undergoes oxidative stress, which is defined as the disturbance of the balance between reactive oxygen species (ROS) production and antioxidant defences (Sharifi-Rad et al. 2020). Free radicals, which are highly reactive species, are known to damage cells (Liu et al. 2021), proteins (Stadtman & Levine 2000), and DNA (Marnett 2000). In the human body, H_2O_2 is produced or degraded constantly as needed to maintain a stable environment and is mainly involved in redox and oxidative processes. Extra H_2O_2 will usually be broken down into water and oxygen by catalases. H_2O_2 in the environment of the cells can arise from a few sources, namely NAD(P)H oxidases (Maraldi et al. 2021; Szanto, Marc & Maria 2019), mitochondrial respiratory chain (Robinson et al. 2019) and other diverse oxidases (Sies 2019). This H_2O_2 will then enter the cell through Aquaporin 8 channel protein (Bestetti et al. 2020). In a low and tolerable concentration, H_2O_2 becomes a redox signalling for oxidative stress, which is good for the cells. However, in a higher and non-tolerable concentration, it disrupts the normal redox signalling and causes oxidative stress (Kanner 2020). Cells have developed a comprehensive array of antioxidant defences against oxidative damage or limit the damaging effects (Cheeseman & Slater 1993; Kuang et al. 2020). Thymoquinone (TQ) has been vastly reported on its antioxidant capabilities in combating oxidative stress. TQ upregulates and activates antioxidant cytoprotective enzymes including catalase gene expression (Ayuob et al. 2020), glutathione reductase (Ciftci, Turkoglu & Bas 2021), and glutathione peroxidase (Ates & Ortatatli 2021), which scavenge H_2O_2 and superoxide radicals and prevent lipid peroxidation (Isaev et al. 2020). The SURVIVIN gene expression was increased whereas the pro-apoptotic BAX gene was downregulated in mesenchymal stem cell culture (Kalamegam et al. 2020). TQ has also been shown to be pro-apoptotic by increasing the Bax/Bcl-2 ratio, upregulating p53 expression (Samarghandian, Mohsen & Tahereh 2019), decreasing glutathione and increasing reactive oxygen species (ROS) in stem cell culture (Haron et al. 2018).

Oxidative injury such as one caused by hydrogen peroxide (H_2O_2) has been linked to trigger cell death in MSCs (Mohammadi et al. 2021). Cell death is generally accepted as an event of a cell biological process ceasing its normal functions. This may result from old cells dying naturally and being replaced by new ones or maybe due to diseases and injury. The Nomenclature Committee has recommended a standard for the definition and interpretation of cell death over the years. Cell death has been studied from morphological, biochemical, and functional viewpoints (Galluzzi et al. 2014). It is accompanied by noticeable macroscopic changes to the physiology of the cells. Cell death is divided into three categories namely Type 1 cell death or apoptosis, Type II cell death or autophagy, and Type III cell death or necrosis. Prevention of changes to the cytoskeletal structure, an integral part of cellular communication for nearly all cellular processes, may lead to the possible prevention of stem cell death. Microfilaments (actin), intermediate filaments and microtubules are the three major structural constituents of the cytoskeleton. Each has its unique structure, composition and organisation that can be adjusted to a certain cell type or subcellular location. Decreasing or increasing actin dynamics could affect cell death or prolong the life span of the cell involved (Kocsis et al. 2021). Following that, the cytoskeleton could act as a sensor for oxidative damage before nuclear changes. Thus, comprehensive research on cytoskeleton morphology and nuclear changes was the focus of this study.

Materials and Methods

Cell culture

Adult Sprague Dawley rats (200 to 280 g) procurred from the animal house in Universiti Malaya (UM) were euthanised using the carbon dioxide chamber in UM BioHealth Laboratory, Faculty of Science. It was done under the guidelines of UM Institutional Animal Care and Use Committee, which follows international guidelines for animal experimentation (IACUC NO: S/08032018/10112017-02R). Bone marrow stem cells (BMSCs) were extracted from Sprague Dawley rats as described by Smajilagić et al. (2013). Briefly, BMSCs were collected from cleaned femur and tibia, which were disinfected with 70% alcohol (Systerm, Malaysia). Using a syringe with a needle (Terumo, Japan, 23G X 1 1/4") 5 mL of complete culture media ((89% High Glucose Dulbecco's Modified Eagle Medium (DMEM) (Nacalai Tesque, Japan), 10% Foetal Bovine Serum (FBS)(Tico Europe, Netherlands), and 1% penicillin-streptomycin antibiotic (pen-strep)(Nacalai Tesque, Japan)) was drawn; of which 3 mL was transferred into a new T25 flask (Corning, USA), and another 2 mL was used for flushing the marrow from the bones into a petri dish. The mixture was transferred into the T25 flask, making it to be a 5 mL primary culture (P_0). Incubation was, then, done in a carbon dioxide incubator (Esco, Singapore) at 5% and 37 °C for three days before changing to a fresh complete culture media.

Experimental design and treatments

For the first individual experiment, BMSCs were exposed to various concentrations of H_2O_2 (50, 500 and 5000 µM) and TQ (0.5, 5, 50 and 500 µM) for 3 h and subsequently followed by immunocytochemistry or FACS (BD, USA) procedure. For the combination treatment, following the pre-exposure to TQ of different concentrations (24 h), the cells were treated with the combination of TQ and H_2O_2 (500 µM each), 1:1 ratio for another 3 h. These concentrations were chosen according to previous studies and modified accordingly (Gülden et al. 2010; Shanmugam et al. 2018). All experiments were performed using BMSCs from second to fourth passage at 50% to 70% confluency level. The cells were cultured in serum-free media for 24 h before any treatment was conducted in 8-well plates (Sigma, USA).

Immunocytochemistry

The cells were fixed using 10% Neutral Buffered Formalin (NBF) for 30 min and washed 3 times (5 min each) with phosphate-buffered saline (PBS) (Nacalai Tesque, Japan). Then, permeabilisation was done with 1% Triton X-100 (R&M Chemicals, Malaysia) in PBS for 15 min and washed 3 times (5 min each) using 0.1% PBS with Triton X-100 (PBST). Alexa Fluor 488 Phalloidin (Cayman Chemical, USA) was used to stain the actin at 1:100 dilution in PBST for 30 min. Subsequently, a monoclonal anti-β-tubulin−cy3 antibody (Sigma, USA) at 1:100 dilution in PBST was applied for 1 h. Both stains were washed 3 times (5 min each) with PBST. DAPI (Sigma, USA) at 1:10000 dilution was applied for 1 min under dark conditions. Its dilution and washing were done with MilliQ water. Lastly, mounting was done with Mowiol 4-88 (Sigma, USA) media. Images were captured using a DM6000 B microscope (Leica, Germany).

Annexin V FITC, fluorescence-activated cell sorting (FACS)

FACS was performed with Annexin V FITC and propidium iodide (PI) for identifying apoptosis and necrosis after the various treatments described earlier. The cells were seeded into the 6-well plate for 24 h or up to 3 days until the cells adhered to the surface and at about 50% confluence. After washing with PBS solution twice, the cells were treated with different concentrations of TQ (Sigma, USA) for 24 h and then the media were discarded. Next, TQ: H_2O_2 at a 1:1 ratio was applied for 3 h. After media removal, 2 mL of Accutase solution (Nacalai Tesque, Japan) for detachment purposes was added for 5 min at room temperature. The cells were harvested and stained with Annexin/PI (Invitrogen, USA) according to the manufacturer's protocol and incubated at room temperature for 15 min. After the incubation period, 1X annexin-binding buffer was added, mixed gently and put on ice. The stained cells were analysed within 1 h by flow cytometry.

Image acquisition and data analysis

The images were acquired by a Leica DM6000 B microscope, and further processed by the Photoshop software (Adobe, USA) for minor image colour correction. The cell numbers in the images were determined using ImageJ software (NIH, USA) to access the viability of the cells according to the guidelines provided using cell counter plugin with slight modifications (Grishagin 2015; Schneider, Rasband & Eliceiri 2012). The images were converted to greyscale (Image-Type-16 bit) and the images were adjusted through the threshold option to highlight the structures of interest. Two options were used namely the watershed option to separate any merged

cells and the analysed particle option to count the cells. Numbers are mentioned as mean ± SEM and statistical significance was determined using Excel (Microsoft, USA) for data collection and JASP software (JASP, Netherlands) for graph visualisation and a *p*-value of at least 0.05 was considered significant.

Results

The bone marrow mesenchymal stem cells exhibited normal morphology for actin and microtubule cytoskeleton (Figure 1) with normal looking nuclei (blue) (Figure 1(a) long arrow) and normal distribution of actin (green) and microtubule (red) cytoskeleton. In contrast, the oxidant damaged cells treated with 5000 µM hydrogen peroxide showed nuclear changes such as nuclear condensation, where the nuclei sizes became smaller and nuclear fragmentation could also be seen. The cells disintegrated into apoptotic bodies, which contained parts of the cellular materials in Figure 1(b)(i) short arrow. Blebbing of membrane and detachment could be observed in Figure 1(b)(ii) thin arrow.

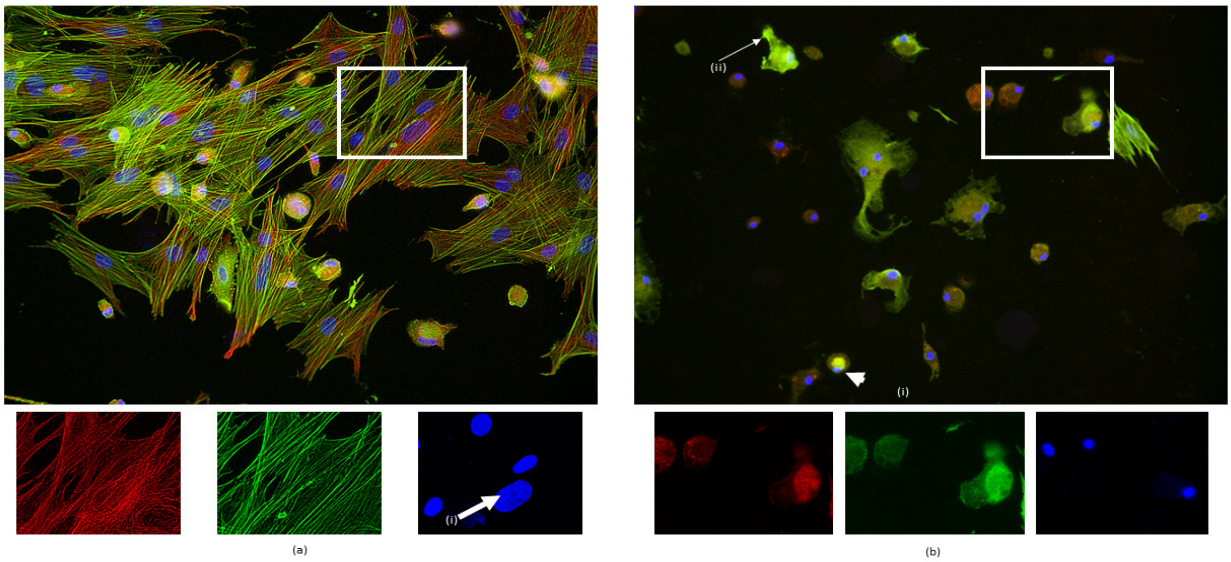


FIGURE 1. Morphology of normal cell on left (a) and damaged on right (treated with 5000 µM hydrogen peroxide) (b), 20×100 magnification

H_2O_2 caused damage to the F-actin and microtubule cytoskeleton along with nuclear changes (Figure 2). Presence of cell death signs started to appear and became more apparent at the highest concentration of H_2O_2 treatment (5000 µM). Nuclear condensation and fragmentation could be seen. Cell membrane also started to bleb and detach while part of the cells had disintegrated into apoptotic bodies after treatment.

TQ caused no damage at lower concentrations (Figure 3). As concentrations increased from 0.5 to 50 µM, the nucleus/cell number increased steadily. However, presence of cell death signs started to appear and became more apparent at the highest concentration of TQ treatment (500 µM). Nuclear condensation and fragmentation could be seen. Cell membrane started to bleb and detach while part of the cells had disintegrated into apoptotic bodies after 500 µM treatment.

TQ prevention of the H_2O_2 damage was shown in Figures 4 and 5. The cytoskeleton and nuclei of the cells were looking normal in the lower concentrations (0.5 and 5 µM) of TQ treatment compared to cells treated with only 500 µM H_2O_2. However, cell death signs started to appear in higher TQ concentrations. While actin (green staining) in H_2O_2 (Figure 2) and TQ (Figure 3) only treatment almost disappeared completely after being treated at the highest concentration, the actin structure could be seen back again after pre-treatment with TQ which suggested the damage destroyed actin first before microtubule and

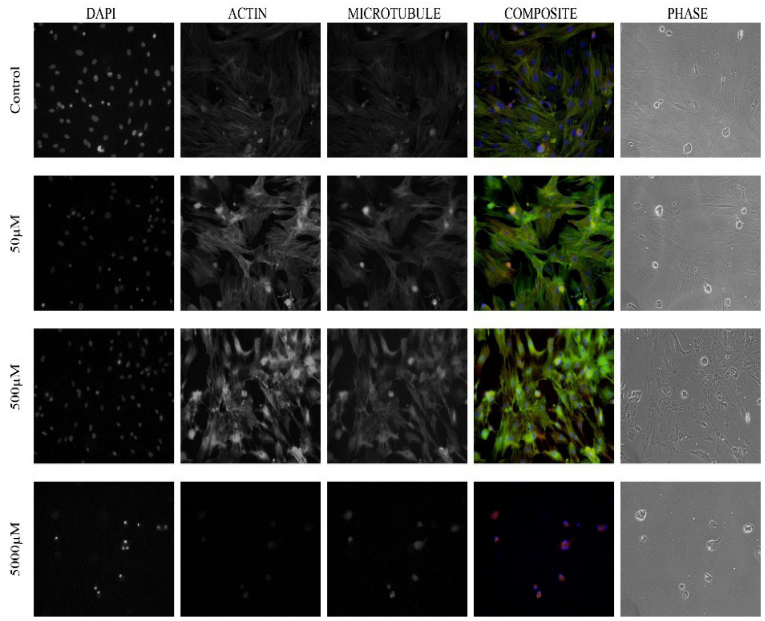


FIGURE 2. Different concentrations of hydrogen peroxide, 20×100 magnification

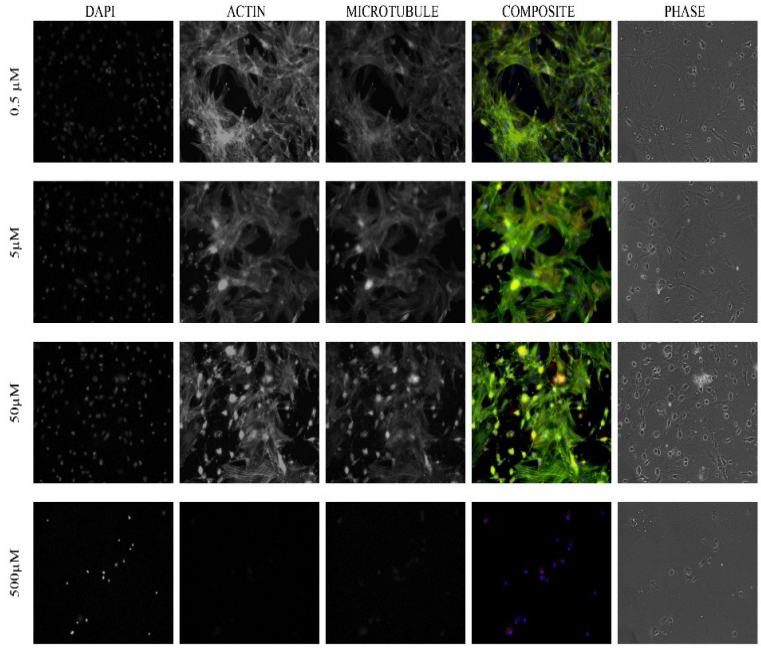


FIGURE 3. Different concentrations of thymoquinone, 20×100 magnification

the interaction of H_2O_2 and TQ might protect the actin structure to some degree. Based on the cell number data analysis using ImageJ, JASP software (Figure 5(a)) and Microsoft Excel (Figure 5(b)), compared to the control, all of the cell counts were significantly different except for treatments for TQ 50 only (102.20 ± 2.0) and TQ 50 combination (96.10 ± 0.89) (p value < 0.05). It was also worth noting that, cell numbers scored were higher than

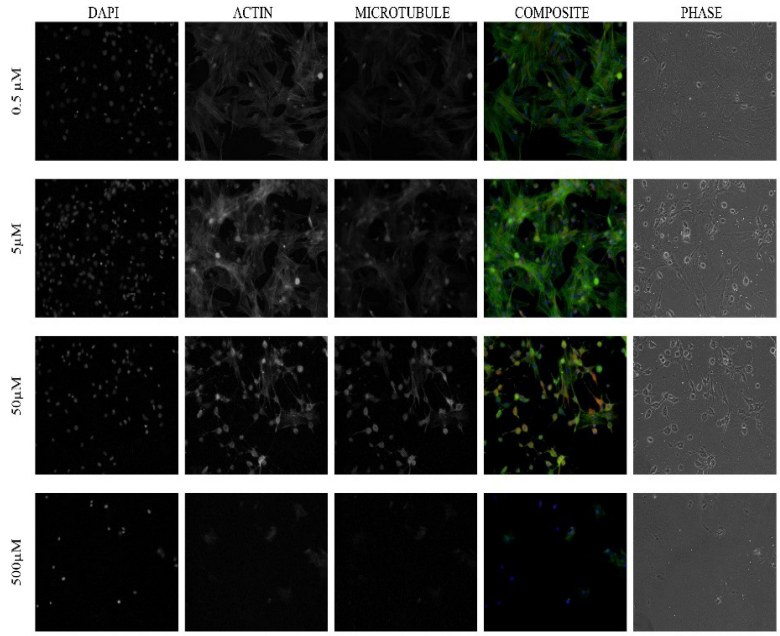
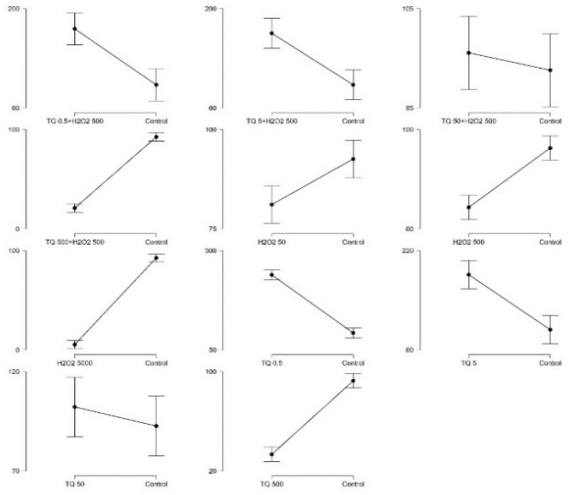


FIGURE 4. Treatment with different concentrations of thymoquinone on 500 µM hydrogen peroxide, 20×100 magnification



(a) Descriptive plots (ImageJ and JASP software)

Paired Samples T-Test

Measure 1		Measure 2	t	df	p
TQ 0.5	-	Control	16.325	29	< .001
TQ 5	-	Control	5.622	29	< .001
TQ 50	-	Control	0.925	29	0.362
TQ 500	-	Control	-14.867	29	< .001
H2O2 50	-	Control	-3.519	29	0.001
H2O2 500	-	Control	-7.020	29	< .001
H2O2 5000	-	Control	-31.849	29	< .001
TQ 0.5+H2O2 500	-	Control	5.052	29	< .001
TQ 5+H2O2 500	-	Control	5.002	29	< .001
TQ 50+H2O2 500	-	Control	0.690	29	0.496
TQ 500+H2O2 500	-	Control	-24.342	29	< .001

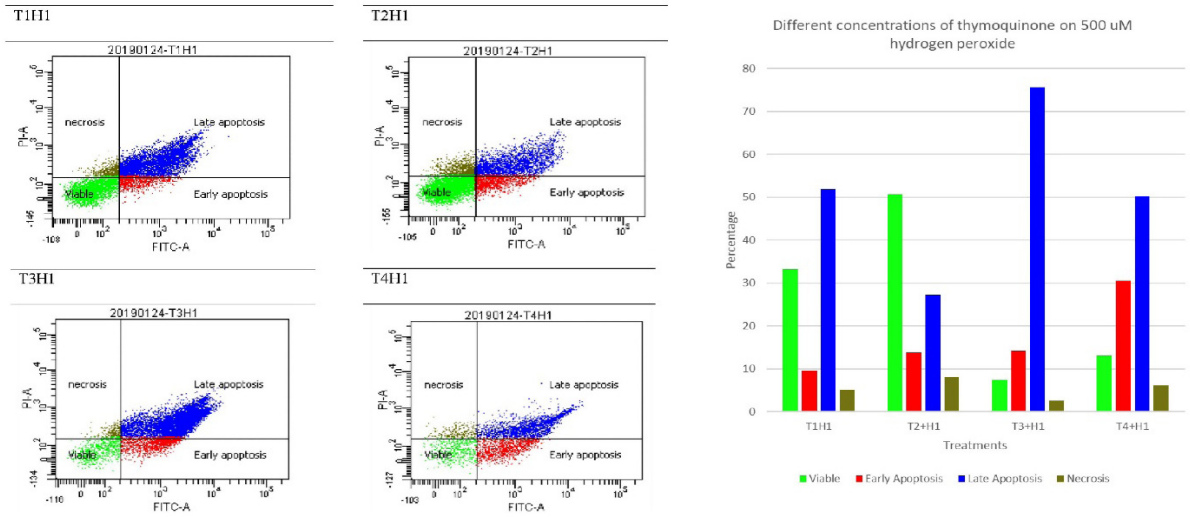
Note. **Student's t-test, *p*-value less than 0.05 is considered significant**

(b) Paired samples t-test (Microsoft Excel)

FIGURE 5. Data analysis on cell numbers of different concentrations of TQ and hydrogen peroxide induced injury

control (92.60 ± 0.47) in TQ 0.5 (238.93 ± 1.62), TQ 5 (181.23 ± 2.77), TQ 50 (102.20 ± 1.99), and combination of TQ 0.5 (171.53 ± 2.70), TQ 5 (165.27 ± 2.63), and TQ 50 (96.10 ± 0.89) with H_2O_2 500 (68.70 ± 0.49).

Oxidative damage was found to cause both apoptosis and necrosis of the BMSCs (Figure 6). The induced apoptotic effect was further confirmed by determination of the percentage of apoptotic cells using



T1 -> T4: Increasing concentration of Thymoquinone (0.5 µM, 5 µM, 50 µM, 500 µM), H1: 500 µM hydrogen peroxide

FIGURE 6. Flow cytometer result of treatments

flow cytometry analysis with Annexin V FITC/PI double staining. T4 combination for H1 showed the highest number of increasing early apoptosis, while for late apoptosis T3 showed the highest score. For the necrosis T2 scored the highest number. All the treatments showed that the cells had undergone late apoptosis (highest percentage) with low necrosis all the time except for the T2 treatment, which resulted in the highest viable cells among the treatments.

DISCUSSION

This study showed that TQ, an active compound derived from *Nigella sativa* (Sahak et al. 2016), is a good candidate for its antioxidant capabilities against oxidative injury specifically from H_2O_2. For centuries, natural products have been used in attempts to treat diseases, ranging from common ailments (Eteraf-Oskouei & Moslem 2013) to deadly cancers (Demain & Vaishnav 2010). These natural products included TQ which is thought to be promising in fighting cancers (Khan et al. 2011; Korak, Emel & Ali 2020; Mondal et al. 2012), and oxidative stress (Ardiana et al. 2020; Fanoudi et al. 2019; Kanter et al. 2004; Leong, Mohd & Kamsiah 2013). Oxidative stress is the cause of numerous diseases, which occur when there is an imbalance between excess ROS or oxidants over the capability of the cells to counteract them with an adequate antioxidant response (Ray, Huang & Tsuji 2012; Uttara et al. 2009). A few models have been used to elucidate the oxidative stress study either *in vitro* (Gille & Joenje 1992a; Hu & Lu 2014) or *in vivo* (Ganie et al. 2011; Kodavanti et al. 2011; Mattia, Ali & Bondy 1993). Since cytoskeleton is an integral part of cellular communication for nearly all cellular processes, it is no surprise that any aberration or changes in the cytoskeleton will lead to diseases. Regulation of microtubule and actin interactions is vital for cellular processes, cell division, cell motility and cell polarity (Kabir et al. 2001; Schaefer, Nurul & Forscher 2002).

TQ is a microtubule-targeting agent (MTA), which binds to the tubulin-microtubule network, preventing microtubule polymerisation and causing mitotic arrest and apoptosis of cancer cells but not of normal cells (Acharya et al. 2014). TQ was shown to decrease F-actin polymerisation and the proliferation of human multiple myeloma cells by suppressing STAT3 phosphorylation and Bcl2/Bcl-XL expression (Badr, Mohany & Abu-Tarboush 2011). Lipid peroxidation causes injury to cells and intracellular membranes, leading to cell destruction (Vijayamalini & Manoharan 2004). There are many antioxidants available to combat oxidative stress, such as Vitamin C (ascorbic acid), glutathione (GSH), and uric acid, but TQ is unique on its own where it is lipid-soluble (Negi et al. 2019; Zakarial et al. 2020).

The current comparative study of three cytoskeletal components of cells (actin, microtubule and nucleus)

was performed to observe the changes due to oxidative stress induced by H_2O_2 in cells *in vitro*. This is similar to other work which have used H_2O_2 to study oxidative stress (Bellion et al. 2009; Gille & Joenje 1992b; Long, Clement & Halliwell 2000). H_2O_2 is produced naturally in the body, and mitochondria are the primary cellular site for its production, a process controlled by the availability of oxygen and nitric oxide to cytochrome oxidase and AD to F1-ATPase (Boveris & Cadenas 2001). In the H_2O_2 toxicity study, mitochondria were shown to be the primary target of damage (Feeney et al. 2008). After cells were introduced to oxidative agents at the higher dosages, the cells started to show morphological changes such as cell shrinkage (Hu & Lu 2014) and degradation of cytoplasm (Kawamori et al. 2003). In the current study, the size of nuclei was also smaller compared to that of the healthy cells, as evidently shown in Figure 1. H_2O_2 permeability of the plasma membrane has been shown through the modulation of the biophysical properties of the plasma membrane (Folmer et al. 2008). According to a study in 2010, H_2O_2 induced apoptosis through two pathways, namely endoplasmic reticulum (ER) and mitochondrial pathways, instead of the extrinsic apoptosis pathways (Wei et al. 2010). The study concluded that H_2O_2 activated p38, which directed the above two pathways in regulating the early apoptosis of MSCs while the c-Jun N-terminal kinase (JNK) pathway was involved in the late apoptosis of MSCs.

TQ and other compounds from *N. sativa* have been shown to exhibit antioxidant capabilities by scavenging anion and ROS in various studies (Abd-Elkareem et al. 2021; Ahlatci et al. 2014; Badary et al. 2003; Sankaranarayanan & Pari 2011). The quinone structure of TQ is partly responsible for having redox properties linked to TQ antioxidant effect. TQ is a pro-oxidant, or it could be an antioxidant at specific concentrations (Mahmoud & Abdelrazek 2019; Zubair et al. 2013). The potential of TQ also depends on the situation where it is present as it can be reduced to semiquinone (pro-oxidant) or thymohydroquinone (antioxidant) (Mansour et al. 2002). In this study, TQ acted differently according to dosage. At low concentrations, no adverse effect could be seen on the morphology of the cells; instead, TQ exerted positive effects by increasing the number of cells and overall healthy-looking cells as shown in Figure 1. However, at a high concentration of TQ, the number of cells decreased significantly, and dead cell signs began to appear (Figure 3). A previous study by Jehan et al. (2020) has shown that TQ could selectively kill abnormal cells while having low toxicity to normal cells. In addition, numerous studies have shown that TQ could produce positive synergy with clinical therapy (Jehan et al. 2020) and might have therapeutical importance in assisting human health and preventing cancer via the modulation of molecular pathways (Rahmani et al. 2014). TQ induces apoptosis through different pathways depending on cell types (El-Mahdy et al. 2005; Hu et al. 2017). For example, in the Siha cervical cancer cell line, apoptosis was regulated through the p53-dependant pathway as shown by an elevated level of p53-mediated apoptosis target genes. In contrast, in the C33A cervical cancer cell line, apoptosis was mainly associated with the activation of caspase 3 (Ichwan 2014; Ng, Latifah & Maznah 2011). In A549 human lung tumour cells, TQ promoted apoptosis by activating both p53 and caspase cascade-dependent pathways (Samarghandian, Mohsen & Tahereh 2019).

After combining both TQ and H_2O_2, the result showed a significantly improved cell number compared to individual treatments (Figures 4 & 5) in the lower concentration. Still, the number of cells was lower in combination compared to higher concentrations of TQ. TQ was able to protect the cells by minimising the damage done by H_2O_2 to the cells. It is worth noting that a higher concentration of TQ proved to be cytotoxic to the normal cells. A higher concentration of TQ was used to find a safe therapeutic dose in the experimental conditions administered to save the cytoskeleton and to see the possible synergistic effect with H_2O_2. The result suggested that there is synergistic effect in possibly damaging the actin cytoskeleton before the microtubules (Figure 4). Based on the data obtained (Figures 1, 2, 3 & 4), cytoskeletal morphology changes could be observed clearly. Cytoplasm shrinkage and retraction, nuclear condensation and disintegration, cell detachment, and blebbing (Häcker 2000) were present in the experiment. Nuclear changes could also be seen in Figure 1 when the morphology of the normal cell was compared to the damaged cells. The size of nuclei was smaller and compacted or known as nuclear condensation. Apoptotic bodies or membrane-bound vesicles were also present as shown in Figure 1b(i). Furthermore, blebbing of the membrane or protrusions from the plasma membrane could be observed in Figure 1b(ii).

The current study showed that oxidative injury was causing apoptosis and necrosis in the cells, which could be prevented or reduced by the TQ compound (Figure 6). The flow cytometry study suggested the cells chose apoptosis as the cell death process compared to necrosis. When cell death occurs, the phosphatidylserine (PS) will

be exposed to the cell outer membrane or external surface. This phenomenon happens during the early apoptosis while the cell membrane itself remains intact. On the other hand, necrosis occurs when the cell membrane integrity is compromised together with the leaking of the cell's constituents (Vermes et al. 1995). Quantitative analysis of the flow cytometer by the Annexin/PI test for TQ indicated that early apoptosis percentage increased as the TQ concentration increased, and the apoptosis rate was higher than necrosis. Quantitative analysis of the flow cytometer for H_2O_2 showed as the concentration used increased, the viable cell number was reduced. In contrast, early apoptosis steadily increased and the same was true for late apoptosis, with necrosis data remained on the lower side. Thus, the data demonstrated H_2O_2 at the used concentrations caused more late apoptosis. Future studies looking at the transcriptional level (qPCR) and transmission electron microscope (TEM) are of interest in elucidating further the biological processes involved.

Conclusions

H_2O_2 and TQ at the higher dosages caused morphological changes such as cytoplasm shrinkage and retraction, nuclear condensation and disintegration, cell detachment and blebbing. TQ pre-treatment successfully mitigated the effect of oxidative damage from $H_2O_{2;}$ thus reducing the number of dying cells. In addition, a lower concentration of TQ (0.5 μM) was also more effective in saving the cells from oxidant injury by acting as an antioxidant in contrast to being pro-oxidant when a higher concentration (500 μM) was used.

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