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Protective Effects of Annatto Tocotrienol and Palm Tocotrienol-Rich Fraction on Chondrocytes Exposed to Monosodium Iodoacetate

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Featured Application: The study provides important information for developing annatto tocotrienol and palm tocotrienol-rich fraction as potential therapeutic agents against osteoarthritis.

Abstract: Background: This study aimed to compare the chondroprotective efficacy and mechanism of annatto tocotrienol (AnTT) and palm tocotrienol-rich fraction (PT3) using SW1353 chondrocytes treated with monosodium iodoacetate (MIA). Methods: The chondrocytes were incubated with AnTT or PT3 in advance or concurrently with MIA for 24 h. The viability of the cells was tested with an MTT assay. The 8-isoprostane F2-α, extracellular matrix proteins, metalloproteinase and sex-determining region Y box protein 9 (SOX9) levels were determined using immunoassays. Results: AnTT and PT3 reversed an MIA-induced decrease in chondrocyte viability when incubated together with MIA ($p < 0.05$). Prior incubation with both mixtures did not produce the same effects. AnTT and PT3 cotreatment could suppress 8-isoprostane F2-α level in chondrocytes exposed to MIA ($p < 0.01$). Co-exposure to tocotrienols and MIA increased the type II collagen/type I collagen ratio in chondrocytes ($p < 0.01$). In addition, the co-exposure of AnTT and MIA for 24 h significantly upregulated SOX9, type II collagen and aggrecan levels ($p < 0.05$), which was not observed with co-exposure of PT3 and MIA, AnTT or PT3 exposure alone. Conclusion: AnTT and PT3 could prevent a reduction in chondrocyte viability following MIA exposure by reducing oxidative stress. In addition, AnTT might induce self-repair and anabolic activities in chondrocytes challenged with MIA.

Keywords: cartilage; collagen; osteoarthritis; oxidative stress; SOX9; vitamin E

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1. Introduction

Osteoarthritis (OA) is a degenerative disease affecting movable joints of the body. It is characterised by pain, swelling, stiffness, and decreased motion range of the joint, which adversely affect patients' mobility [1]. A recent meta-analysis summarised that the global prevalence of knee OA was 16% in the population aged ≥15 years and 22.9% in the population ≥ 40 years, with a female to male ratio of 1.69 [2]. The Global Burden of Disease Study 2019 reported a 27.5% increase in the prevalence of OA and 27.5% increase in disability adjusted life years due to OA between 2010 and 2019 [3]. OA (except hand OA) is also associated with increased risk of cardiovascular disease and its associated mortality, probably due to shared risk factors and OA-associated physical inactivity [4,5].

Cartilage is one of the joint tissues most affected by OA. Cartilage homeostasis is maintained by chondrocytes, which synthesise and replenish the collagenous extracellular matrix [6]. Accumulated evidence has demonstrated the involvement of the hypocellularity

of cartilage or chondrocyte cell death in OA initiation [6–8]. Chondrocytes with mechanical, oxidative or inflammation-induced damage have poor recovery capacity due to the alymphatic and avascular condition in the cartilage [9,10]. The function and metabolism of damaged chondrocytes will be compromised. Ultimately, the chondrocytes will die off via apoptosis and lead to hypocellularity, matrix depletion and degradation of cartilage [7,11]. Additionally, the leaked or degraded cellular content from dead chondrocytes, as well as the cartilage debris, could activate the inflammatory cascades and the release of pro-inflammatory mediators by joint tissues and immune cells, leading to synovitis [12]. In turn, these mediators induce the release of metalloproteinase by chondrocytes and provoke further cartilage damage [13,14]. Inflammation and oxidative stress are two intricately linked process in OA. Pro-inflammatory cytokines such as interleukin-1 β and tumour necrosis factor-alpha induce nitric oxide production and mitochondrial dysfunction, which contribute to chondrocyte malfunction and apoptosis [15]. A previous study showed that monosodium iodoacetate (MIA), a glycolysis inhibitor, induced proliferation arrest and oxidative stress in chondrocytes in vitro [16]. Therefore, the MIA model may represent a suitable model to study early chondrocyte damage.

The primary pharmacological treatment of OA aims to relieve its symptoms, e.g., non-steroidal anti-inflammatory agents and paracetamol relieve joint pain in OA patients [17]. Intra-articular corticosteroid injection is effective against joint pain, but it may cause local and systemic side effects [18]. Besides, evidence for the efficacy of several common supplements for OA, such as glucosamine, chondroitin and avocado or soybean unsaponifiables for OA management, has not been conclusive according to meta-analysis [19]. The failure of pharmacological interventions allows the condition to progress to end-stage OA, necessitating joint replacement or arthroplasty. Arthroplasty is a costly procedure and introduces the risk of infection, loosening of prosthetics and periprosthetic fractures [20,21]. Therefore, the search for novel agents with better efficacy to treat OA is ongoing.

Tocotrienol, a subfamily of vitamin E found in botanical oil, has been reported to protect chondrocytes and joint health. Palm tocotrienol (30 mg/kg), γ-tocotrienol (5 mg/kg) and δ-tocotrienol (10 mg/kg) isomers have been tested in rat models of rheumatoid arthritis (RA) induced by collagen II [22–24]. They were reported to prevent paw oedema, joint deterioration and oxidative stress in rats with RA [22–24]. In addition, annatto tocotrienol (AnTT; 100 mg/kg/day) has been shown to reduce cartilage damage and its associated cartilage degradation markers in rats administered intraarticularly with MIA [25]. Another study on the effects of palm tocotrienol-rich (PT3, 100 mg/kg) combined with glucosamine sulphate (250 mg/kg) using a similar animal model demonstrated reduced circulating degradation markers and improved grip strength [26]. However, the currently available studies on vitamin E and cartilage damage are predominantly on α-tocopherol, the major isoform of vitamin in the human diet and body [27]. Thus, evidence on the direct action of tocotrienol on chondrocytes is very limited.

This study aimed to determine the effects of annatto and palm-derived vitamin E mixture on chondrocyte damage induced with MIA. Vitamin E derived from annatto is rich in δ-tocotrienol but contains a negligible amount of α-tocopherol [28]. On the other hand, palm vitamin E contains α-tocopherol, along with α-, β-, δ- and γ-tocotrienol [29]. This study assessed whether this compositional difference could give rise to distinct chondroprotective actions in MIA-mediated chondrocyte damage. Both pre-treatment and concurrent treatment models were tested in this study. It was hypothesised that AnTT and PT3 could prevent viability suppression of chondrocytes induced by MIA and preserve their function. The findings of this study could provide further evidence on the use of tocotrienol in preventing chondrocyte damage.

2. Materials and Methods

2.1. Materials

All the chemicals used in this study were purchased from Sigma-Aldrich unless stated otherwise. A 100 mM MIA stock solution was prepared by dissolving it in dimethyl sulfoxide. The stock solution was then aliquoted and kept at −80 °C until use. The AnTT was a gift from American River Nutrition (Hadley, USA) and contained 84% δ-tocotrienol and 16% γ-tocotrienol (Lot Number: #18FA-1270, purity 70%). The PT3 was a gift from Excelvite Sdn. Bhd. (Chemor, Malaysia) and contained 21.9% α-tocopherol, 24.7% α-tocotrienol, 4.5% β-tocotrienol, 36.9% γ-tocotrienol and 12.0% δ-tocotrienol (Batch Number: A1/50/0450_1_220318, purity 50%). Stock solutions of AnTT and PT3 were prepared according to the methodologies used in our previous studies with slight modifications in concentration [30,31]. Briefly, AnTT and PT3 stock solutions were firstly dissolved in absolute ethanol to a concentration of 0.1 and 0.2 g/mL respectively, aliquoted and kept at −80 °C until use. The day before treatment, 45 µL tocotrienol stock solution was mixed with 60 µL sterile foetal bovine serum (FBS) and incubated at 37 °C for 24 h. After that, 105 µL of absolute ethanol was added to the mixture and followed by 90 µL of complete media. The same procedure was employed for the vehicle control (VC) group using 45 µL of absolute ethanol. The entire process of MIA and tocotrienol stock preparation, storage, incubation and treatment were protected from direct light exposure.

2.2. Cell Lines

SW1353 chondrocytes were purchased from American Type Culture Collection (catalogue no. HTB 94) and cultured in a high glucose Dulbecco's modified Eagle's medium (Nacalai Tesque, Japan; catalogue no. 08458-16) that contained 4 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA; catalogue no. 10270-106) and 1% antibiotic-antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA; catalogue no. 15240-062) at 37 °C in an incubator with humidified air and 5% carbon dioxide. These cells were passaged every 2 days to maintain the cells in logarithmic growth. The cells in early passages (3rd–10th passages) were used in the experiment.

2.3. Cytotoxicity Assay

The cytotoxicity of AnTT and PT3 was determined using the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to previous studies with slight modification [16,30,32]. Briefly, 100 µL of SW1353 chondrocytes (5×10^4 cells/mL) were seeded in a 96-well plate with complete media for 24 h. Subsequently, the media were removed and replaced with 100 µL of complete media with a series of concentrations of AnTT or PT3 for another 24 h. At the end of the treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and then further incubated for another 4 h inside a 37 °C incubator. The optical density (OD) of each well was measured at the wavelength of 570 nM using a Multiskan GO microplate reader (Thermo Fisher Scientific, Vantaa, Finland). The viability of the treated cells was calculated by dividing the OD of the treated group with the OD value of the VC and multiplied by 100%.

In certain experiments, SW1353 chondrocytes were challenged with MIA in its half-maximal inhibitory concentration (IC_{50}) value (31.5 µM) determined from our previous study [16], with/without prior or concurrent treatment of tocotrienols. In the preventive model, 100 µL of SW1353 chondrocytes (5×10^4 cells/mL) were seeded in a 96-well plate with complete media for 24 h. Subsequently, the media were removed and the cells were pre-treated with 100 µL of fresh complete media with a series of concentrations of AnTT or PT3 for another 24 h. Lastly, the media were replaced again with 100 µL of fresh complete media with MIA. For the treatment model, 100 µL of SW1353 chondrocytes (5×10^4 cells/mL) were seeded in a similar condition as the preventive model for 24 h. Subsequently, the media were replaced with 100 µL of fresh complete media with a series of concentrations of AnTT or PT3 and MIA (IC_{50}) for another 24 h. An MTT assay was conducted at the end of the treatment. The viability of cells was calculated as a percentage

relative to the VC, as mentioned previously. The effective concentrations of AnTT and PT3 that proved to be protective against MIA-induced reduction in cell viability were used in the subsequent experiments.

2.4. Whole Cell Lysate Preparation

The lysate preparation was conducted according to the methodology used in our previous study [16]. Briefly, SW1353 chondrocytes were seeded in 100 mm culture dishes (5×10^4 cells/mL; 15.5 mL of cells; 3 culture dishes per group) for 24 h. Subsequently, the SW1353 cells were challenged with MIA (IC_{50}) with or without the presence of AnTT (10 and 20 µg/mL) or PT3 (25 and 50 µg/mL) for 24 h. Then, the media with the floated cells were collected and combined with the trypsinised cells. The entire mixture was centrifuged and washed twice with ice-cold phosphate buffer saline at 200× *g* for 5 min. The cell pellets were then lysed with 500 µL of Qproteome mammalian lysis buffer (Qiagen, Hilden, Germany) containing protease inhibitors for 15 min on ice with a gentle intermittent vortex. Subsequently, the contents were centrifuged at 10,000× *g* at 4 °C for 15 min. Finally, the pellets were discarded and only the supernatants were collected as lysates. The lysate preparation was repeated at least three times. The protein concentrations of lysates were determined by using the microtiter format of Bio-Rad Protein Assay concentrated dye reagent (Bio-Rad Laboratories, Hercules, CA, USA). Lastly, the lysates were diluted into 1 mg/mL (final volume 1.2 mL) for the quantitative detection of proteins of interest. All the lysates were kept at −80 °C until further analysis.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The human 8-isoprostane F2-α level (catalogue no. E4805Hu) was determined using sandwiched enzyme-linked immunoassay (ELISA) kit from Bioassay Technology Laboratory (Shanghai, China). Briefly, 40 µL of cell lysates (1 mg/mL; in duplicate) were mixed with 10 µL of free biotinylated human 8-isoprostane F2-α antibody in the 96-well plate pre-coated with human 8-isoprostane F2-α antibody. The standard solutions (50 µL; in serial concentration) were added separately into another 2 rows of wells. Subsequently, all the sample and standard wells (except the blank) were added to with 50 µL of streptavidin-linked horseradish peroxidase (HRP) reagent and the 96-well plate was then incubated at 37 °C for 1 h. Subsequently, the plate was washed 5 times with wash buffer solution and blot-dried with paper towels. Immediately, 100 µL of pre-mixed substrate Solution A and B was added to each well and the plate was further incubated at 37 °C for 10 min. Lastly, 50 µL of stop solution was added to stop the colourimetric reaction and the absorbance was measured using Multiskan GO microplate reader (Thermo Fisher Scientific, Vantaa, Finland) at the wavelength of 450 nm. The absolute levels of 8-isoprostane F2-α were determined based on the standard curve derived from the standard solutions with known concentration.

The human collagen type I α1 (COL1α1; catalogue no. E-EL-H0869), collagen type II α1 (COL2α1; catalogue no. E-EL-H0777), sex-determining region Y box protein 9 (SOX9; catalogue no. E-EL-H1349), a disintegrin and metalloproteinase with thrombospondin type 1 Motif 4 (ADAMTS4; catalogue no. E-EL-H0266) and aggrecan (catalogue no. E-EL-H0294) levels were determined by using the sandwiched ELISA kits from Elabscience (Houston, Texas, USA) according to the manufacturer's protocol. Briefly, 100 uL of standard solution (in serial concentration) and lysates were added (1 mg/mL) in duplicate into the 96-well plates pre-coated with respective specific antibodies. The plates were then sealed and incubated at 37 °C for 90 min. After that, the entire content of the wells was removed and replaced with 100 µL of respective biotinylated detection antibody working solution. Next, the plates were incubated at 37 °C for another 1 h and followed by 3 times of washing with wash buffer solution. Subsequently, 100 µL of HRP conjugate working solution was added, and the plates were incubated again at 37 °C for 30 min. The plates were then washed 5 times with wash buffer solution, followed by the addition of 90 µL of substrate reagent solution and incubated at 37 °C for another 15 min. A 50 µL of stop solution was then

added into the wells, and the absorbance was measured using Multiskan GO microplate reader (Thermo Fisher Scientific, Vantaa, Finland) at 450 nm. The levels of proteins of interest were determined based on the respective standard curves.

2.6. Statistical Analysis

Statistical analysis was conducted by using SPSS software for Windows, version 25. The normality of data was tested and a Box-Cox data transformation was performed on non-normally distributed data before parametric testing [33]. Most of the data (except for COL2α1/COL1α1 ratio, SOX9, aggrecan and ADAMTS4 levels) were not normally distributed, potentially due to the presence of outliers. Mean differences between multiple groups were analysed using a one-way analysis of variance with Tukey or Dunnett's T3 post hoc analysis. A p-value of less than 0.05 was considered statistically significant. At least 3 independent experiments were conducted for all parameters. The data were displayed in mean ± standard error of mean (SEM).

3. Results

3.1. Cytotoxicity of AnTT and PT3 on SW1353 Chondrocytes

The cytotoxicity of AnTT and PT3 on SW1353 chondrocytes over 24 h treatment was determined using an MTT assay (Figure 1). AnTT was cytotoxic to SW1353 chondrocytes at concentrations ≥ 30 µg/mL (all $p > 0.05$), while PT3 was cytotoxic at concentrations ≥ 100 µg/mL (all $p < 0.01$). Therefore, the non-cytotoxic concentrations of AnTT (≤20 µg/mL) and PT3 (≤50 µg/mL) were used in the subsequent experiment.

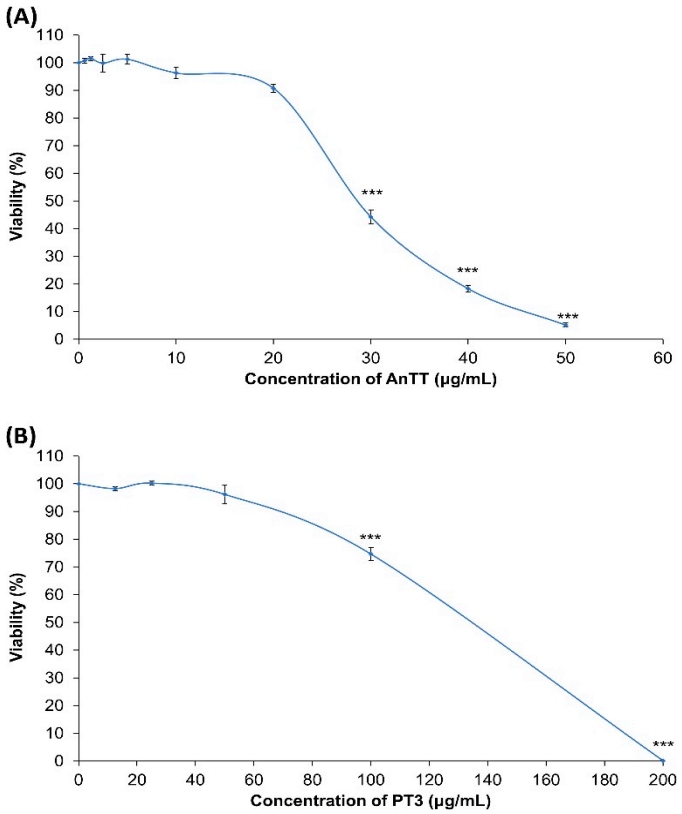

Figure 1. The cytotoxicity of AnTT (**A**) and PT3 (**B**) on SW1353 chondrocytes upon 24 h treatment. The results were obtained from 3 independent experiments with 4 technical replicates during measurement. The data were displayed in mean ± SEM. *** indicates a significant difference ($p < 0.001$) compared to the VC (0 µg/mL).

3.2. The Effects of AnTT or PT3 Pre-Treatment on MIA-Induced SW1353 Cell Death

The viability of SW1353 chondrocytes after an initial 24 h treatment of AnTT or PT3 followed by 24 h treatment of MIA (IC_{50}: 31.5 μM) was tested (Figure 2). Pre-treatment of AnTT and PT3 did not protect the cells from MIA-induced viability suppression. At higher concentrations, both AnTT (≥20 μg/mL) and PT3 (≥12.5 μg/mL) enhanced the toxicity of MIA.

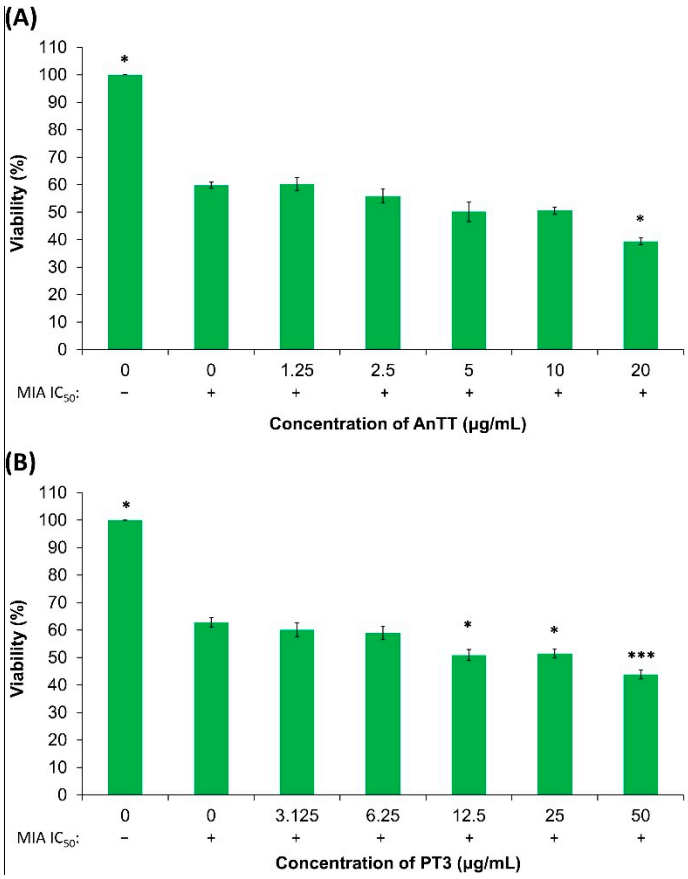

Figure 2. The viability of SW1353 chondrocytes over a 24 h pre-treatment model with AnTT (**A**) or PT3 (**B**), followed by 24 h treatment of MIA at IC_{50} of 31.5 μM. The results were obtained from 3 independent experiments with 4 technical replicates during measurement. The data were displayed in mean ± SEM. * and *** indicate a significant difference of $p < 0.05$ and $p < 0.001$ compared to the MIA control group.

The viability of SW1353 chondrocytes with concurrent treatment of AnTT or PT3 and MIA (IC_{50}: 31.5 μM) for 24 h was also tested (Figure 3). In this model, AnTT (10 and 20 μg/mL) and PT3 (3.125, 25 and 50 μg/mL) protected the cells from MIA-induced viability suppression by significantly improving the viability of SW1353 chondrocytes (all $p < 0.05$ vs. MIA alone). Therefore, these working concentrations of AnTT (10 and 20 μg/mL) or PT3 (25 and 50 μg/mL) were used in the subsequent experiments.

3.3. The Effects of AnTT or PT3 Co-Treatment on 8-Isoprostane F2-α Level

The 8-isoprostane F2-α level of SW1353 chondrocytes treated for 24 h with AnTT or PT3 alone, and in combination with MIA (IC50 31.5 μM) was determined using the ELISA method (Figure 4). The 8-isoprostane F2-α level increased significantly with MIA treatment on its own ($p < 0.05$). Co-treatment with PT3 (50 μg/mL) or AnTT (10 and 20 μg/mL) significantly suppressed the increase in 8-isoprostane F2-α level caused by MIA ($p < 0.01$).

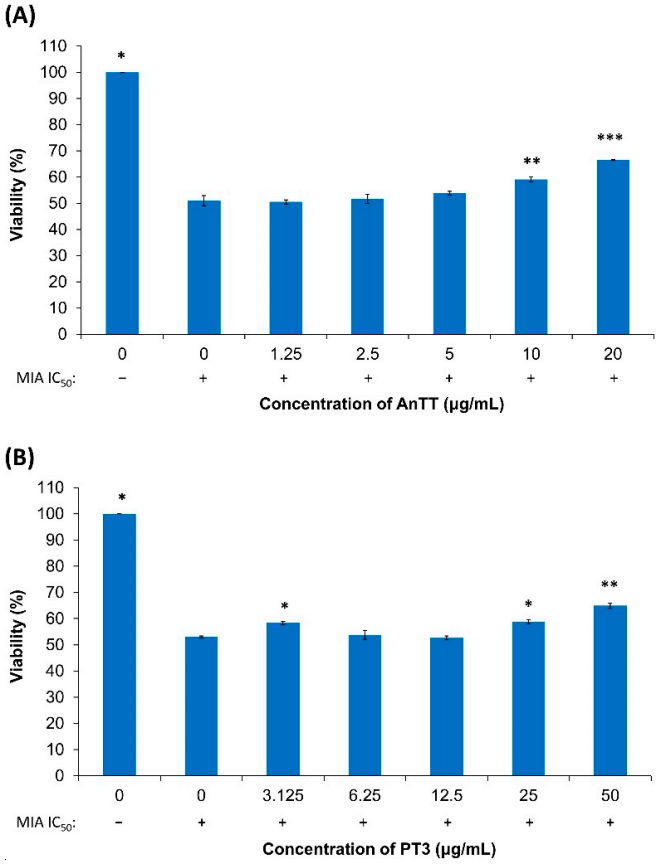

Figure 3. The viability of SW1353 chondrocytes over a 24 h concurrent treatment of AnTT (**A**) or PT3 (**B**) and MIA (IC_{50} 31.5 µM). The results were obtained from 3 independent experiments with 4 technical replicates during measurement. The data were displayed in mean ± SEM. *, ** and *** indicate a significant difference of $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively compared to the MIA control group.

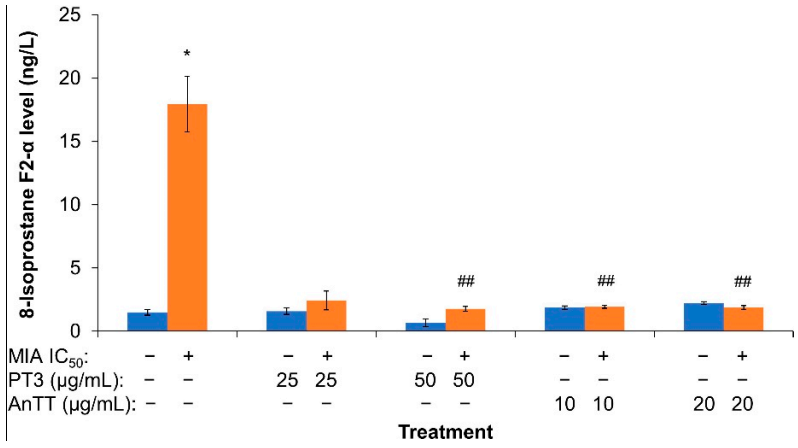

Figure 4. The 8-isoprostane F2-α level of SW1353 chondrocytes treated for 24 h with AnTT or PT3 alone and in combination with MIA (IC_{50} 31.5 µM). All results were from 3 independent experiments. Two technical replicates were used during measurement according to manufacturer's instruction. The data were displayed in mean ± SEM. * indicates a significant difference of $p < 0.05$ compared to VC. ## indicates a significant difference of $p < 0.01$ compared to the group treated with MIA alone.

The protein expression of COL1α1, COL2α1, aggrecan, SOX9 and ADAMTS4 in SW1353 chondrocytes treated for 24 h with AnTT or PT3 alone, and in combination with

MIA (IC_{50} 31.5 μM) were measured using ELISA (Figure 5). MIA (31.5 μM) significantly reduced the COL1α1 and ADAMTS4 levels ($p < 0.05$ vs. VC) without altering the COL2α1, COL2α1/COL1α1 index, aggrecan or SOX9 levels ($p > 0.05$ vs. VC). AnTT and PT3 treatment alone did not alter the baseline levels of COL1α1, COL2α1, aggrecan and SOX9 ($p > 0.05$ vs. VC or MIA alone) but reduced ADAMTS4 ($p < 0.05$ for AnTT vs. VC) and COL2α1/COL1α1 indices at higher concentrations ($p < 0.05$ vs. VC). Neither AnTT nor PT3 restored the MIA-induced reduction in COL1α1 levels ($p > 0.05$ vs. MIA alone) but they did increase the COL2α1/COL1α1 indices for all tested concentrations ($p < 0.05$ vs. MIA alone). Furthermore, PT3 (25 and 50 μg/mL) also restored the MIA-induced reduction in ADAMTS4 levels ($p < 0.05$ vs. MIA alone). Surprisingly, co-treatment with AnTT and MIA significantly increased SOX9, COL2α1 and aggrecan levels ($p < 0.05$ vs. MIA alone).

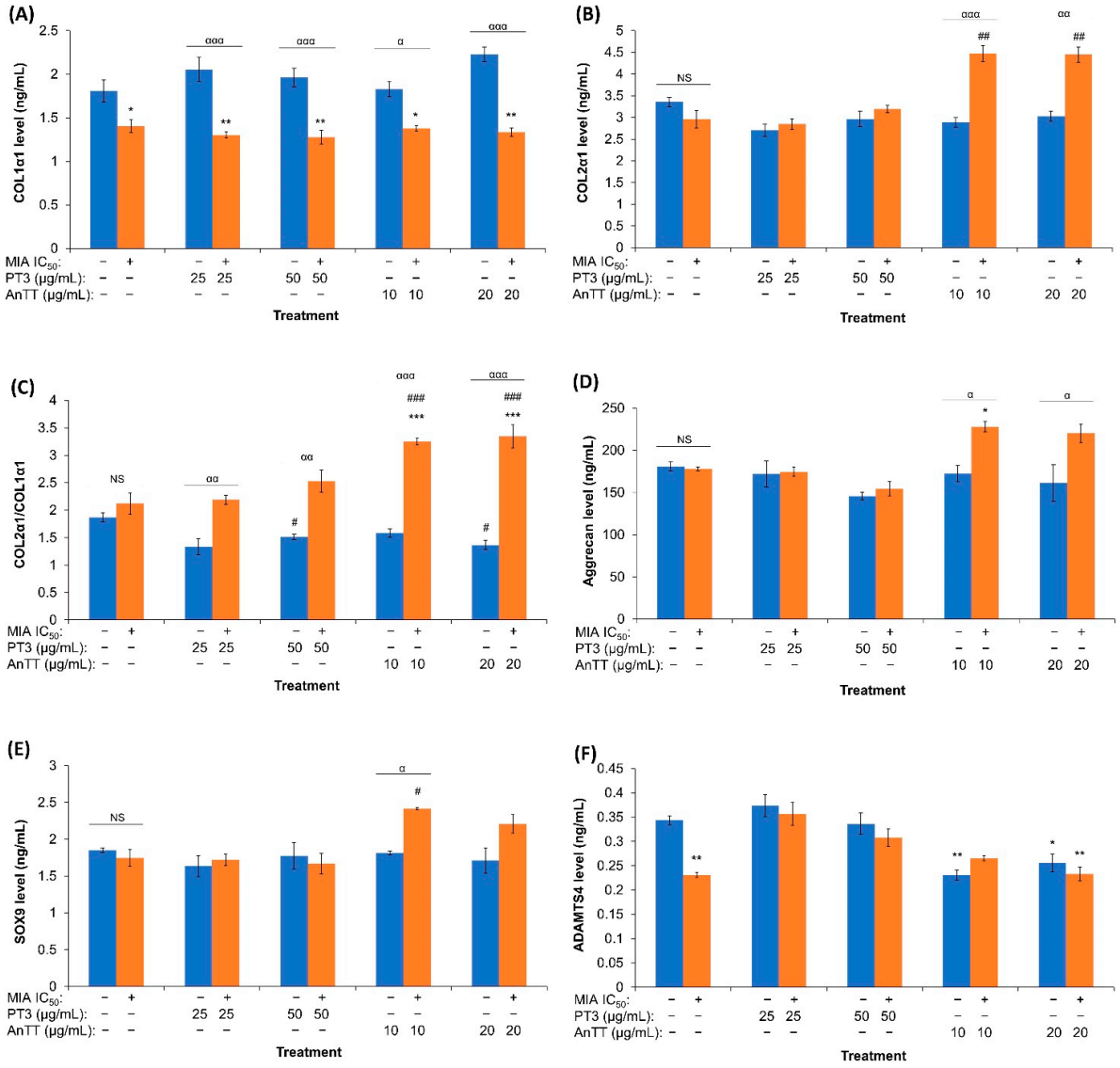

Figure 5. The protein expression of COL1α1 (**A**), COL2α1 (**B**), COL2α1/COL1α1 (**C**), aggrecan (**D**), SOX9 (**E**) and ADAMTS4 (**F**) in SW1353 chondrocytes treated for 24 h with AnTT or PT3 alone, and in combination with MIA (IC_{50} 31.5 μM). All results were from at least 3 independent experiments. Two technical replicates were used during measurement, according to manufacturer's instruction. The data were displayed in mean ± SEM. *, ** and *** indicate a significant difference of $p < 0.05$, $p < 0.01$ and $p < 0.001$ compared to VC; $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ indicate a significant difference of $p < 0.05$, $p < 0.01$ and $p < 0.001$ compared to MIA control; $^{\alpha}$, $^{\alpha\alpha}$ and $^{\alpha\alpha\alpha}$ indicate a significant difference of $p < 0.05$, $p < 0.01$ and $p < 0.001$ between group with and without MIA treatment. NS indicates no significant difference between groups.

4. Discussion

The current study showed that MIA decreased the viability of chondrocytes and suppressed COL1α1 and ADAMST4 levels, but increased the 8-isoprostane F2-α level of chondrocytes. Concurrent incubation with AnTT and PT3 prevented this effect by preventing oxidative damage and improving COL2α1/COL1α1 indices. Prior incubation of chondrocytes with AnTT and PT3 did not achieve the same effects. The concurrent incubation of AnTT and MIA also increased the COL2α1, aggrecan and SOX9 protein levels in chondrocytes.

The inhibitory effects of MIA on chondrocytes' viability found in this study are consistent with findings in previous literature [34,35]. Pang et al. showed that MIA at a similar concentration as the current study (31.5 μM) induced a decrease in the viability of SW1353 chondrocytes by proliferation arrest, not cell death [16]. As a glyceraldehyde-3-phosphate dehydrogenase inhibitor, MIA reduced the cellular energy production and expression of proteins related to glycolysis in SW1353 chondrocytes [36,37]. It has also been suggested that MIA suppresses the mitophagy process, which clears dysfunctional mitochondria [36]. Furthermore, MIA has been shown to disrupt mitochondria membrane potential and trigger autophagy in chondrocytes in primary rat chondrocytes [35]. These findings might explain how MIA causes the number of viable and functioning chondrocytes to decrease.

The chondroprotective effects of AnTT and PT3 were time-sensitive in this study. Concurrent incubation of AnTT or PT3 and MIA reversed the decrease in the viability of chondrocytes. This observation agrees with a previous animal study by Chin et al. (2019), which reported that treating rats with AnTT immediately after MIA induction protected them from cartilage damage [25]. Another study showed that α-tocopherol preserved chondrocytes' morphology in diabetic rats with MIA-induced cartilage damage [38]. It is hypothesised that tocotrienols scavenge free radicals and maintain the intracellular defence against oxidative stress generated by MIA. Co-treatment with zinc, another antioxidant, has also been shown to ameliorate MIA-mediated reduction of chondrocyte viability [28]. However, the present study also showed that pre-treatment of tocotrienol did not offer the same protection. At higher doses, tocotrienol even potentiated the suppressive effects of MIA on cell viability. These findings are in contradiction with previous studies using α-tocopherol. Beecher et al. (2007) reported that pre-incubation with α-tocopherol for 24 h preserved the viability of the chondrocytes in human cartilage explants subjected to mechanical stress [39]. Bhatti et al. (2013) treated rat primary chondrocytes with α-tocopherol for 24 h before exposing them to hydrogen peroxide for 3 h [40]. The intervention preserved viability and the proliferating cell nuclear antigen expression of chondrocytes [40]. It is noteworthy that the MIA-induced chondrocyte damage is mechanistically different from other inducers, such as mechanical stress and hydrogen peroxide, so direct comparison is unattainable. Additionally, since SW1353 is a chondrosarcoma cell line, it is hypothesised that pre-treatment with tocotrienols for 24 h, which are compounds with anticancer properties, could sensitise the cells towards mitochondria dysfunction induced by MIA, independent of their antioxidant properties [41,42]. Thus, the pre-treatment model was not suitable for the objective of this study due to this technical limitation, and the subsequent experiments were conducted using the co-treatment approach.

Oxidative stress contributes to senescence and functional loss of chondrocytes, leading to dysregulation of cartilage haemostasis and subsequent damage [43,44]. In this study, MIA induced significant oxidative stress in chondrocytes, as evidenced by an elevated level of 8-isoprostane F2-α, a stable lipid peroxidation marker [45]. The increased oxidative stress was probably a sequela of mitochondrial dysfunction induced by MIA, consistent with previous reports [34,35]. Additionally, MIA also depleted the intracellular glutathione via non-enzymatic binding, which may explain the increase in the endogenous ROS upon induction [37]. Both AnTT and PT3 were shown to suppress MIA-induced lipid peroxidation completely in this study. The function of tocotrienol as a free radical scavenger has been widely reported [46]. The chromanol ring of tocotrienols readily scavenges ROS by

exchanging electrons with free radicals [47,48], thereby terminating the lipid peroxidation chain reaction. The reduction of oxidative stress by AnTT and PT3 could explain the reversal of MIA-induced viability suppression in chondrocytes.

Collagens and non-collagenous proteins such as aggrecan are the major constituents of the extracellular matrix synthesised by chondrocytes [49]. Type II collagen is a marker of well-differentiated chondrocytes, while type I collagen is a marker of dedifferentiated chondrocytes [50]. Thus, COL2α1/COL1α1 ratio could be used as a marker of chondrocyte differentiation. In this study, MIA caused a significant reduction in COL1α1 levels but did not alter COL2α1 and aggrecan levels. In contrast, previous studies have reported that MIA suppressed type II collagen expression [51,52], while increasing type I collagen expression in articular cartilage samples in rats [53]. Other studies have reported a concurrent reduction of type I and type II collagen and aggrecan protein or mRNA expression in articular cartilage samples of rats induced with MIA [54,55]. It is speculated that MIA at the current dose did not overwhelm the repair capacity of chondrocytes, thus the production of type II collagen and aggrecan was preserved at an extent similar to unstimulated chondrocytes.

In this study, AnTT and PT3 alone or PT3 in the presence of MIA did not significantly alter the COL1α1 and COL2α1 expressions of the chondrocytes. However, concurrent treatment with AnTT or PT3 with MIA increased the COL2α1/COL1α1 indices, and it was found that AnTT treatment was more potent despite being at a lower concentration. Unexpectedly, co-treatment of AnTT and MIA stimulated the COL2α1 and aggrecan expression of the chondrocytes. It is hypothesised that AnTT enhanced the repair response of chondrocytes exposed to MIA. In line with this speculation, it can be suggested that tocotrienols alone did not exert anabolic effects in unstimulated chondrocytes in the current study because the repair response was not activated. By way of comparison, palm vitamin E mixture (Palmvitee) alone was ineffective in preventing the diminished type II collagen expression and the upregulation of type II collagen expression in a monolayer culture of primary chondrocytes from the human nasal septum [56]. However, palm vitamin E mixture promoted type II collagen expression in reconstructed cartilage embedded in vivo [56]. Parallel with our findings, Bhatti et al. demonstrated that pre-treatment of α-tocopherol upregulated the expression of gene coding for COL2α1 and aggrecan, and downregulated gene coding for COL1α1 in primary chondrocytes exposed to hydrogen peroxide for 3 h. This resulted in an increased COL2α1/COL1α1 ratio in the treated cells [40].

The observations above are supported by the results for SOX9 levels in the chondrocytes treated with both AnTT and MIA. SOX9 is a member of the SRY-related high-mobility group (HMG) box (SOX) family of transcription factors. It plays an important role in chondrogenesis, cell survival and activation of transcription for cartilage components, such as type II collagen and aggrecan [57,58]. MIA administration was reported to suppress SOX9, type II collagen and aggrecan expression in the articular cartilage sample of rats [54,59]. In the current study, MIA exposure alone did not lower the SOX9 level in the chondrocytes, supporting the speculation that chondrocytes might be damaged, but their self-repair mechanism was not overwhelmed. Co-treatment of AnTT and MIA stimulated the expression of SOX9, which coincides with the effects of this treatment on COL2α1 and aggrecan level. Taken together, these observations suggest that AnTT enhanced chondrocytes' self-repair mechanism when challenged by MIA.

ADAMTS4 is one of the metalloproteinases responsible for the degradation of type II collagen and aggrecan in the cartilage [60]. ADAMTS4 levels in the serum and synovial fluid increases in patients with early-stage OA compared to intermittent and late-stage OA [61,62]. Intra-articular MIA administration has been shown to increase ADAMTS4 expression in rat cartilage samples [63,64]. In this study, MIA exposure led to a significant decrease in ADAMTS4 levels in chondrocytes, probably due to activation of the self-repair mechanism. PT3 (25 μg/mL) restored ADAMTS4 levels in chondrocytes exposed to MIA. It is postulated that PT3 negated the effects of MIA, and the cellular self-repair mechanism

was not triggered to reduce ADAMTS4 levels. Despite the potential anabolic effects on MIA-induced chondrocytes, as discussed above, AnTT did not normalise ADAMTS4 levels.

Furthermore, we cannot rule out the possibility that the direct chemical interaction between MIA and tocotrienols may partially contribute to the protective effects of tocotrienols during concurrent treatment. Iodoacetate has been demonstrated to bind with nucleophiles such as glutathione or glyceraldehyde-3-phosphate dehydrogenase via electrophilic addition [37]. Furthermore, tocopherol is also reported to bind with mutagenic electrophiles like nitrogen dioxide and peroxynitrite via its electron-rich chromanol ring [65–67]. Tocotrienol, with its similar chromanol ring and a tri-unsaturated aliphatic tail, may serve as a nucleophile in scavenging MIA. Nevertheless, the interaction between tocotrienols and MIA may be minimal, given that concurrent treatment with tocotrienols did not completely abrogate the MIA-mediated cytotoxicity and alteration in metabolism. Therefore, further study is required to confirm the postulated chemical interaction between MIA and tocotrienols.

Comparison between PT3 and AnTT revealed that only AnTT significantly improved the anabolic activities of chondrocytes by increasing the COL2α1/COL1α1 ratio, SOX9, COL2α1 and aggrecan levels. AnTT is postulated to enhance the self-repair mechanism of chondrocytes independently of their antioxidant activities. On the other hand, PT3 lowered the MIA-mediated oxidative stress but did not activate the self-repair mechanism, unlike AnTT. Based on the vitamin E isomer constitution, the AnTT-mediated self-repair mechanism may be contributed to by δ-tocotrienol, given that 20 μg/mL AnTT contains a 2.8-fold higher amount of δ-tocotrienol than PT3 (50 μg/mL). The other isomers in PT3, such as α-tocopherol and α-tocotrienol, may contribute to its antioxidant activities but they did not promote anabolic activities in the chondrocytes. The difference in potency of biological actions for each tocotrienol isomer has been discussed previously [46]. For instance, δ-tocotrienol has been shown to be the most potent isomer in suppressing 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) [68], activating peroxisome proliferating-activated receptor-δ or -γ [69] and improving insulin synthesis [70].

Additionally, the current study demonstrates the absence of anabolic activities for PT3 at 50 μg/mL, despite containing 6 μg/mL δ-tocotrienol, compared to AnTT at 10 μg/mL (8.4 μg/mL δ-tocotrienol). A potential antagonism interaction is postulated between vitamin E isomers. Tocopherol has been shown to antagonise the action of tocotrienols in lowering lipoprotein and inhibiting HMGCR [71,72]. Interestingly, it has been suggested that HMGCR participates in OA pathogenesis, given that its expression is significantly higher in chondrocytes from OA patients [73,74]. Long-term use of statins (HMGCR inhibitors) also significantly reduces the risk of OA among middle-aged adults with cardiovascular diseases [75]. Therefore, the presence of tocopherol in PT3 may hinder the HMGCR-suppressing effects of δ-tocotrienol, which may explain the absence of self-repair and anabolic activities in PT3–MIA co-treated chondrocytes. Further study is required to confirm our speculation and the role of HMGCR in OA initiation, chondrocyte damage and repair mechanisms.

Overall, the current study proposes that AnTT and PT3 prevent the reduction in cell viability of chondrocytes caused by MIA, probably through lowering oxidative stress. AnTT also enhanced the self-repair mechanism of chondrocytes exposed to MIA by activating their anabolic activities, as evidenced by increased COL2α1/COL1α1 ratios, SOX9, COL2α1 and aggrecan levels. PT3 does not share the self-repair activation mechanism possessed by AnTT. A summary of the mechanism of actions of AnTT and PT3 on chondrocytes stimulated with MIA is presented in Figure 6.

Several limitations in this study should be addressed. First, the findings of the current study should be validated in more than one chondrocyte cell line and primary mammalian chondrocytes. Second, cartilage explants should be considered, because chondrocytes might behave differently in monolayer and three-dimensional constructs. Third, the enhanced self-repair mechanism of chondrocytes exposed to AnTT and MIA concurrently should be scrutinised, because the current evidence is preliminary. Some studies have reported an adaptive increase in antioxidant response of chondrocytes exposed

to MIA [34,76]. Forth, silencing of SOX9 could be performed to determine its role in mediating the action of AnTT in promoting chondrocyte repair. Fifth, the ELISA results should be confirmed with a gene expression study or Western blot analysis. Lastly, the chemical interaction between MIA or ROS with AnTT and PT3 should be examined to confirm their upstream protective mechanisms. Nevertheless, the current study suggests the novel action of tocotrienols, especially AnTT, in counteracting chondrocyte damage due to MIA.

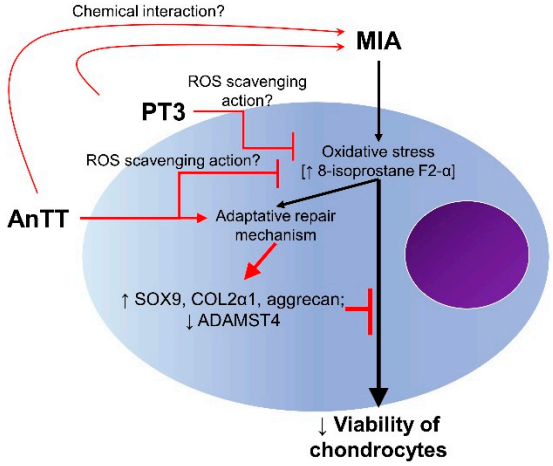

Figure 6. Proposed mechanism of action of tocotrienols on chondrocytes. Both AnTT and PT3 suppress oxidative stress induced by MIA. AnTT also enhances the self-repair mechanism and anabolic activities of chondrocytes exposed to MIA. Abbreviations: ↑, cause or induce; ⊤, inhibit; AnTT, annatto tocotrienols; COL2α1, collagen type II α1; PT3, palm tocotrienol-rich fraction; SOX9, sex-determining region Y box protein 9.

5. Conclusions

Both AnTT and PT3 were found to prevent MIA-induced decreases in the viability of chondrocytes, most likely through their ability to reduce oxidative stress. AnTT possesses additional properties of promoting the self-repair response of chondrocytes exposed to MIA, producing a higher COL2α1/COL1α1 ratio and higher COL2α1, SOX9 and aggrecan levels. AnTT appears to be more effective than PT3 in protecting chondrocytes based on the dose and mechanism of action. Tocotrienol could be developed as a supplement to reduce chondrocyte damage in OA, pending further validation from more comprehensive research.

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