The protective effect of antioxidants on orbital fibroblasts from patients with Graves’ ophthalmopathy in response to oxidative stress

Chieh-Chih Tsai,1 Shi-Bei Wu,2 Shu-Ching Kao,1 Hui-Chuan Kau,1,3 Fenq-Lih Lee,1 Yau-Huei Wei1,4

1Department of Ophthalmology, Taipei Veterans General Hospital and National Yang-Ming University, Taipei, Taiwan; 2Department of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan; 3Department of Ophthalmology, Koo Foundation Sun Yat-Sen Cancer Center, Taipei, Taiwan; 4Department of Medicine, Mackay Medical College, New Taipei City, Taiwan

Purpose: To investigate the biphasic effects of hydrogen peroxide (H2O2) on the orbital fibroblasts of patients with Graves’ ophthalmopathy (GO) and the relation to antioxidants and proinflammatory cytokines.

Methods: Proliferation of cultured orbital fibroblasts from patients with GO and normal controls was evaluated in response to various concentrations of H2O2. The effect of low concentrations of H2O2 (6.25 μM) on the cellular proliferation and induction of intracellular proinflammatory cytokines, and reactive oxygen species of orbital fibroblasts were assessed. Protective effects of N-acetylcysteine and vitamin C on GO fibroblasts in response to 6.25 μM H2O2 stimulation were also investigated.

Results: When the GO fibroblasts were exposed to H2O2 at a concentration of 50 μM or above, significant cytotoxicity was observed. In contrast, lower concentrations of H2O2 (3.125–25 μM) increased the survival of GO fibroblasts with the peak cellular proliferation at 6.25 μM H2O2. However, this biphasic effect of H2O2 on the viability of orbital fibroblasts was not found in normal controls. In addition, 6.25 μM H2O2 led to significant elevation of the levels of transforming growth factor, beta 1, interleukin-1β, and superoxide anion in GO fibroblasts, but no significant change in the normal controls. Pretreatment with N-acetylcysteine or vitamin C reversed the enhanced proliferation capacity and the induction of transforming growth factor, beta 1, interleukin-1β and superoxide anion of GO fibroblasts in response to 6.25 μM H2O2.

Conclusions: These findings revealed the biphasic effect of H2O2 on cellular proliferation of GO orbital fibroblasts. Importantly, a low level of H2O2 can stimulate proliferation of GO orbital fibroblasts and induce the production of proinflammatory cytokines, which can be inhibited by pretreatment with antioxidants. This provides a theoretical basis for the rational use of antioxidant in treating GO at an early stage.

Graves’ ophthalmopathy (GO), the most important and frequent extrathyroidal expression of Graves’ disease, is an inflammatory disorder of autoimmune background [1,2]. The pathogenesis of GO is thought to be a complex interplay between endogenous and environmental factors [3,4]. Recently, increasing evidence has shown that reactive oxygen species (ROS) play an important role in the development of GO [5]. Elevated extracellular levels of ROS have also been noted in the blood [6], urine [7,8], fibroadipose tissues [9], and orbital fibroblasts [10] of patients with GO. However, the contribution of ROS to the pathogenesis of GO has remained elusive. Hydrogen peroxide (H2O2), an ROS naturally produced in human cells during physiologic and pathological processes, has been used as a prooxidant in the study of oxidative stress–related diseases. We recently reported that exposure to a sublethal concentration of hydrogen peroxide (200 μM) resulted in marked cytotoxicity and ROS-elicited oxidative damage in GO fibroblasts [11]. However, superoxide anions, one of the main ROS, have been shown to induce proliferation of orbital fibroblasts obtained from two patients with severe Graves’ ophthalmopathy in a dose–response manner [12]. In the present study, we investigated the possible biphasic effects of ROS on GO orbital fibroblasts, especially the low-dose effect of ROS and its relation to antioxidants and prooxidant cytokines.

METHODS

Culture of orbital fibroblasts: The culture of orbital fibroblasts was established from surgical specimens of seven patients with GO during decompression surgery (two men and five women; mean age: 37.6 years) and from apparently normal orbital tissues in five age-matched patients who received surgery for noninflammatory conditions (one man and four women; mean age: 35.2 years). All patients with GO achieved stable euthyroidism for at least 6 months before...
According to our previous study, the probes from 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) and dihydroethidine (DHE purchased from Molecular Probes) were used to evaluate the intracellular H$_2$O$_2$ and O$_2^-$ content, respectively [11]. After incubation of orbital fibroblasts with H$_2$O$_2$, the cell viability was determined by AlamarBlue assay as described and in our previous study, the probes from 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) and dihydroethidine (DHE purchased from Molecular Probes) were used to evaluate the intracellular H$_2$O$_2$ and O$_2^-$ content, respectively [11]. After incubation of orbital fibroblasts with H$_2$O$_2$, the cell viability was determined by AlamarBlue assay as described and in our previous study, the probes from 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) and dihydroethidine (DHE purchased from Molecular Probes) were used to evaluate the intracellular H$_2$O$_2$ and O$_2^-$ content, respectively [11].
20 μM DCFH-DA or 10 μM DHE at 37 °C for 20 min, cells were trypsinized and then resuspended in 0.5 ml of PBS buffer (pH 7.4) followed by analysis of flow cytometry with a flow cytometer (Model EPICS XL-MCL, Beckman-Coulter, Miami, FL). The excitation wavelength was set at 488 nm, and the intensity of the emitted fluorescence of a total of 10,000 cells at 525 nm was recorded on channel FL1 for the DCFH-DA probe and at 575 nm was recorded on channel FL2 for the DHE probe, respectively. Data were acquired and analyzed using EXPO32 software (Beckman-Coulter, Miami, FL), and the intracellular H₂O₂ or O₂– content in the treated cells is presented as a relative value compared to that of the cells without 6.25 μM H₂O₂ or antioxidant treatment (200 μM NAC or 500 μM vitamin C).

Statistical analysis: Statistical analysis was performed by using the Microsoft Excel 2010 statistical package and SigmaPlot software version 12.3 (Systat Software Inc., San Jose, CA). The data are presented as means ± standard error of the mean (SEM) of the results obtained from three independent experiments. The significance level of the difference between the control and the experimental groups was determined with the Student t test. A difference was considered statistically significant when the p value <0.05 and p value <0.01, respectively.

### Table 1. The expression levels of intracellular cytokines in orbital fibroblasts before and after treatment of the cells with 6.25 mM H₂O₂.

| Cytokine species | Before treatment (mean ± SD) | After treatment (mean ± SD) | Induction ratio (%)* | p-value |
|------------------|-----------------------------|-----------------------------|---------------------|--------|
| **TNF-α (pg per 10⁶ cells)** | | | | |
| Normal | 32.16±5.36 | 35.02±6.17 | 108.71±5.07 | 0.881 |
| GO | 31.60±5.15 | 33.73±9.39 | 106.44±8.13 | 0.603 |
| p=0.755 | | | | |
| **TGF-β1 (pg per 10⁶ cells)** | | | | |
| Normal | 95.73±10.71 | 92.08±12.88 | 96.17±11.73 | 0.631 |
| GO | 126.61±15.04 | 164.82±18.83 | 130.15±18.64 | <0.001 |
| p<0.001 | | | | |
| **IL-1β (pg per 10⁶ cells)** | | | | |
| Normal | 43.32±5.85 | 45.07±3.14 | 104.62±8.84 | 0.537 |
| GO | 52.86±4.13 | 62.59±5.57 | 123.18±12.90 | 0.005 |
| p<0.001 | | | | |

* Induction ratio = the expression of cytokine value after H₂O₂ treatment / baseline value (%)
RESULTS

Effect of various concentrations of hydrogen peroxide on the viability of orbital fibroblasts: The effect of H$_2$O$_2$ on the viability of orbital fibroblasts, as determined with the AlamarBlue cell viability assay, is illustrated in Figure 1. The data show that there was a biphasic effect of H$_2$O$_2$ on the viability of GO orbital fibroblasts. Cytotoxicity was not observed in the concentration range of 3.125–25 μM H$_2$O$_2$ when the GO fibroblasts were incubated with H$_2$O$_2$ for 24 h. In contrast, lower concentrations of H$_2$O$_2$ increased the survival of GO orbital fibroblasts with the peak proliferation (mean increase: 16.4%) at 6.25 μM H$_2$O$_2$ (p=0.0001). When the GO fibroblasts were exposed to H$_2$O$_2$ at a concentration of 50 μM or above, significant cytotoxicity was observed (p=0.0038). Different from GO orbital fibroblasts, control orbital fibroblasts showed no significant proliferation in response to low concentrations of H$_2$O$_2$ (3.125–25 μM). Cell cultures of normal controls exposed to H$_2$O$_2$ at a concentration above 100 μM started to reveal significant cytotoxicity (p=0.0011).

Low concentration of hydrogen peroxide–induced changes of intracellular cytokines in orbital fibroblasts: The changes in the intracellular cytokines upon treatment of orbital fibroblasts with 6.25 μM H$_2$O$_2$ are shown in Table 1. Basal levels peroxide (H$_2$O$_2$) for 24 h, the release of the intracellular levels of interleukin-1β (IL-1β) and transforming growth factor, beta 1 in Graves’ ophthalmopathy orbital fibroblasts. After pretreatment of Graves’ ophthalmopathy (GO) orbital fibroblasts (n=7) with 200 μM N-acetylcysteine (NAC) or 500 μM vitamin C (VitC) for 1 h, followed by the addition of 6.25 μM H$_2$O$_2$ for 24 h, the cell proliferation rate was examined with the AlamarBlue assay. The data were normalized to each control not exposed to H$_2$O$_2$, and the mean values of cell proliferation from GO orbital fibroblasts are shown in the histogram. The pretreatment of VitC at 250 μM and 500 μM in GO orbital fibroblasts significantly inhibited H$_2$O$_2$-induced cell proliferation. The data are presented as mean ± standard deviation of the results from three independent experiments. (p<0.005 and p<0.0001 represented significant decrease.)
of TGF-β1 and IL-1β were significantly higher in the GO orbital fibroblasts compared with those of the control group (p<0.001 and p<0.001, respectively). Low concentrations of H₂O₂ led to significant elevation in TGF-β1 and IL-1β levels in GO orbital fibroblasts compared with the respective controls (p<0.001 and p=0.005, respectively). In addition, the induction ratio of TGF-β1 and IL-1β after treatment with a low dose of H₂O₂ were more pronounced in the GO orbital fibroblasts than those in the normal controls (p<0.001 and p<0.001, respectively). These findings were not observed in TNF-α in the GO orbital fibroblasts. Conversely, there was no significant increase in the intracellular levels of TNF-α, TGF-β1, and IL-1β in the normal controls after treatment with low levels of H₂O₂.

Modulation of low concentration hydrogen peroxide–induced cellular proliferation and changes of intracellular levels of transforming growth factor, beta 1 and interleukin-1β in Graves’ ophthalmopathy orbital fibroblasts with various antioxidants: Figure 2 and Figure 3 show the protective effects of NAC and vitamin C, respectively, in GO orbital fibroblast proliferation in response to 6.25 μM H₂O₂. Preincubation with 100 μM or 200 μM NAC significantly decreased H₂O₂-induced GO orbital fibroblast proliferation (p<0.001 and p<0.0001, respectively). A significant reduction in H₂O₂-induced fibroblast proliferation was also obtained after the cells were preincubated with 250 μM or 500 μM vitamin C (p=0.0048 and p<0.0001, respectively). Figure 4 demonstrates the protective effects of NAC and vitamin C against 6.25 μM H₂O₂-induced expression of intracellular IL-1β and TGF-β1 in GO orbital fibroblasts. Preincubation with 200 μM NAC significantly inhibited 6.25 μM H₂O₂-induced elevations of intracellular IL-1β and TGF-β1 in the GO orbital fibroblasts (p<0.05 and p<0.001, respectively). A significant reduction in H₂O₂-induced elevations of intracellular IL-1β and TGF-β1 was also obtained after the cells were preincubated with 500 μM vitamin C (p<0.005 and p<0.001, respectively).

Low concentration of hydrogen peroxide–induced changes of reactive oxygen species in Graves’ ophthalmopathy orbital fibroblasts: Table 2 shows the 6.25 μM H₂O₂ treatment led to significant elevation in the levels of superoxide anions (mean increase: 14.7%, p=0.00015), but not the intracellular H₂O₂ content in GO orbital fibroblasts (p=0.076). In addition, when we pretreated the GO orbital fibroblasts with 200 μM NAC or 500 μM vitamin C, the low dose of H₂O₂ (6.25 μM)-induced production of the superoxide anions was abolished (Figure 5).

DISCUSSION

Orbital fibroblasts, one of the major cells affected by GO, contribute to many GO-associated pathologic conditions, including cellular proliferation [15]. For the first time, we demonstrated in this study that GO fibroblasts are hypersensitive not only to high concentrations of H₂O₂ but also to low levels of H₂O₂. Interestingly, low concentrations of H₂O₂ stimulated the proliferation of GO orbital fibroblasts but had little effect on the normal controls. The observation in this study of a biphasic effect of ROS on cellular proliferation is consistent with the findings in various cell types [16,17]. Although how human cells respond biochemically to low concentrations of ROS is not well understood, it has been shown that ROS play a role in signal transduction pathways as a second messenger involving cellular growth and protection of cells against apoptosis [18,19]. In addition, recent data also revealed that the H₂O₂ is an important intermediate downstream of adenosine triphosphate receptor pathways leading to enhanced cell proliferation of skeletal myoblasts [20].

Apart from enhanced proliferation of GO orbital fibroblasts, our study also shows that a low level of H₂O₂ induced higher intracellular levels of TGF-β1 and IL-1β than those in the normal controls. We also observed increased production of superoxide anion in GO orbital fibroblasts after the
be important in treating or preventing GO. In a small trial, early blockage of ROS formation in orbital fibroblasts may further exacerbate existing GO [15]. Moreover, accumulating ROS can elicit more oxidative damage and redox imbalance in GO orbital fibroblasts, which further exacerbate existing GO [15]. Therefore, early blockage of ROS formation in orbital fibroblasts may be important in treating or preventing GO. In a small trial, oral antioxidants showed encouraging results in treating mild and moderately severe GO [28]. Recently, selenium (an antioxidant) was successfully applied in patients with mild GO in a large, multicenter, randomized, placebo-controlled trial in Europe [29]. Antioxidants may exert their actions through antioxidative or anti-inflammatory effects. Selenium is an important constituent of the enzyme glutathione peroxidase and thioredoxin reductase, which are responsible for destroying \( \text{H}_2\text{O}_2 \) and lipid-damaging peroxides that are increasingly produced in GO [30]. In addition, selenium could decrease the formation of proinflammatory cytokines, especially the T helper type 1 cytokines, which are predominant early in GO [33-35]. In this study, pretreatment with antioxidants effectively ameliorated the effects of low levels of \( \text{H}_2\text{O}_2 \) on cellular proliferation and induction of proinflammatory cytokines in GO orbital fibroblasts, further suggesting that antioxidants might have a role in treating early GO and preventing the development of GO.

In conclusion, the biphasic effects of \( \text{H}_2\text{O}_2 \) on cellular proliferation of GO orbital fibroblasts may play a role in the pathogenesis of GO. ROS could contribute to the development of GO either by acting directly or inducing the release of proinflammatory cytokines. Moreover, accumulating ROS can elicit more oxidative damage and redox imbalance in GO orbital fibroblasts, which further exacerbate existing GO [15]. Therefore, early blockage of ROS formation in orbital fibroblasts may be important in treating or preventing GO. In a small trial, oral antioxidants showed encouraging results in treating mild and moderately severe GO [28].

### Table 2. Intracellular levels of reactive oxygen species in orbital fibroblasts before and after treatment of the cells with 6.25 mM H\(_2\)O\(_2\)

| Reactive oxygen species | Before treatment (mean ± SD) | After treatment (mean ± SD) | Induction ratio (%)* (mean ± SD) | \( p \)-value |
|-------------------------|------------------------------|------------------------------|----------------------------------|------|
| **H\(_2\)O\(_2\)** (Relative ratio**) | | | | |
| Normal | 101.15±3.46 | 103.64±6.32 | 102.49±2.45 | 0.195 |
| GO | 118.56±4.60 | 117.70±5.52 | 98.80±3.60 | 0.587 |
| | | | | |
| **O\(_2\)** (Relative ratio**) | | | | |
| Normal | 103.26±5.02 | 104.60±5.19 | 101.31±1.24 | 0.477 |
| GO | 112.25±5.49 | 128.80±6.80 | 114.74±3.41 | <0.001 |

* Induction ratio=\( \text{O}_2^- \) or \( \text{H}_2\text{O}_2 \) value after \( \text{H}_2\text{O}_2 \) treatment / baseline value (%) ** Each measurement of cultured orbital fibroblasts was presented as a relative value, which was calculated by taking the intracellular ROS levels of N1 as 100\%.

6.25 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) treatment. Moreover, the low dose of \( \text{H}_2\text{O}_2 \)-induced elevation of the superoxide anions was abolished by the antioxidant treatment. Therefore, we speculate that the increase of TGF-β1 and IL-1β due to the low dose of \( \text{H}_2\text{O}_2 \) is related to the formation of superoxide anions in GO orbital fibroblasts. This result is in line with previous observations in other cell types that had demonstrated that oxidative stress is an important modulator of TGF-β and IL-1β expression [21-23]. TGF-β1, a potent fibrogenic cytokine, has been reported to modulate proliferation of fibroblasts and tissue fibrosis [24,25]. IL-1β is known to stimulate hyaluronan synthesis in orbital fibroblasts [26,27]. Hyaluronan accumulation and fibroblast proliferation are important pathological features in the overt expression of ophthalmopathy in patients with GO. Collectively, these findings suggest that ROS may contribute to the pathogenesis of GO either by acting directly or inducing the release of proinflammatory cytokines.

We previously revealed that GO orbital fibroblasts have accumulated higher basal content of ROS such as superoxide anions and \( \text{H}_2\text{O}_2 \) compared with those of normal controls [10]. Burch et al. also demonstrated that superoxide anions induce the cellular proliferation of cultured GO orbital fibroblasts [12]. In combination with our current observations of biphasic effects of \( \text{H}_2\text{O}_2 \) on the cellular proliferation of GO orbital fibroblasts, we suggest that low levels of ROS may stimulate cellular proliferation and induce more proinflammatory cytokines in GO orbital fibroblasts which promote the development of early GO. Furthermore, accumulating ROS can elicit more oxidative damage and redox imbalance in GO orbital fibroblasts, which further exacerbate existing GO [15]. Therefore, early blockage of ROS formation in orbital fibroblasts may be important in treating or preventing GO. In a small trial,
ACKNOWLEDGMENTS

This study was partially supported by grants (NSC 99–2314-B-075–006-MY3) from the National Science Council of Taiwan, and the grants (V101C-080 and V102C-075) from Taipei Veterans General Hospital, Taipei, Taiwan. We would like to express our appreciation of the technical support and service of the Core Facilities at National Yang-Ming University.

REFERENCES

1. Douglas RS, Gupta S. The pathophysiology of thyroid eye disease: implications for immunotherapy. Curr Opin Ophthalmol 2011; 22:385-90. [PMID: 21730841].

2. Kazim M, Goldberg RA, Smith TJ. Insights into the pathogenesis of thyroid associated orbitopathy: evolving rationale for therapy. Arch Ophthalmol 2002; 120:380-6. [PMID: 11879144].

3. Naik VM, Naik MN, Goldberg RA, Smith TJ, Douglas RS. Immunopathogenesis of thyroid eye disease: emerging paradigms. Surv Ophthalmol 2010; 55:215-26. [PMID: 20385333].

4. Smith TJ, Tsai CC, Shih MJ, Tsai S, Chen B, Han R, Naik V, King CS, Press C, Kamat S, Goldberg RA, Phipps RP, Douglas RS, Gianoukakis AG. Unique attributes of orbital fibroblasts and global alterations in IGF-1 receptor signaling could explain thyroid-associated orbitopathy. Thyroid 2008; 18:983-8. [PMID: 18788919].

5. Bartalena L, Tanda ML, Piantanida E, Lai A. Oxidative stress and Graves’ ophthalmopathy: in vitro studies and therapeutic implications. Biofactors 2003; 19:155-63. [PMID: 14757966].

6. Bednarek J, Wysocki H, Sowinski J. Oxidative stress peripheral parameters in Graves’ disease: the effect of methimazole treatment in patients with and without infiltrative orbitopathy. Clin Biochem 2005; 38:13-8. [PMID: 15607311].

7. Tsai CC, Cheng CY, Liu CY, Kao SC, Kau HC, Hsu WM, Wei YH. Oxidative stress in patients with Graves’ Ophthalmopathy: Relationship between oxidative DNA damage and clinical evolution. Eye (Lond) 2009; 23:1725-30. [PMID: 18849914].

8. Tsai CC, Kao SC, Cheng CY, Kau HC, Hsu WM, Lee CF, Wei YH. Oxidative stress change by systemic corticosteroids treatment of patients with active Graves’ ophthalmopathy. Arch Ophthalmol 2007; 125:1652-6. [PMID: 18071117].

9. Hondur A, Konuk O, Dincel AS, Bilgihan A, Ural M, Hasanreisoglu B. Oxidative stress and antioxidant activity in orbital fibroadipose tissue in Graves’ ophthalmopathy. Curr Eye Res 2008; 33:421-7. [PMID: 18568878].

10. Tsai CC, Wu SB, Cheng CY, Kao SC, Kau HC, Chiou SH, Hsu WM, Wei YH. Increased oxidative DNA damage, lipid peroxidation, and reactive oxygen species in cultured orbital fibroblasts from patients with Graves’ ophthalmopathy: evidence that oxidative stress has a role in this disorder. Eye (Lond) 2010; 24:1520-5. [PMID: 20300129].

11. Tsai CC, Wu SB, Cheng CY, Kao SC, Kau HC, Lee SM, Wei YH. Increased response to oxidative stress challenge in Graves’ ophthalmopathy orbital fibroblasts. Mol Vis 2011; 17:2782-8. [PMID: 22065933].

12. Burch HB, Lahiri S, Bahn RS, Barnes S. Superoxide radical production stimulates retroocular fibroblast proliferation in Graves’ ophthalmopathy. Exp Eye Res 1997; 65:311-6. [PMID: 9268599].

13. Lu CY, Lee HC, Fahn HJ, Wei YH. Oxidative damage elicited by imbalance of free radical scavenging enzymes is associated with large-scale mtDNA deletions in aging human skin. Mutat Res 1999; 423:11-21. [PMID: 10029667].

14. Chen CT, Shih YR, Kuo TK, Lee OK, Wei YH. Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. Stem Cells 2008; 26:960-8. [PMID: 18218821].

15. Feldon SE, Park DJ, O’Loughlin CW, Nguyen VT, Landskroner-Eiger S, Chang D, Thatcher TH, Phipps RP. Autologous T-lymphocytes stimulate proliferation of orbital fibroblasts derived from patients with Graves’ ophthalmopathy. Invest Ophthalmol Vis Sci 2005; 46:3913-21. [PMID: 16249464].

16. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med 1995; 18:775-94. [PMID: 7750801].

17. Mu P, Liu Q, Zheng R. Biphasic regulation of H2O2 on angiogenesis implicated NADPH oxidase. Cell Biol Int 2010; 34:1013-20. [PMID: 20575760].

18. Stone JR, Collins T. The role of hydrogen peroxide in endothelial proliferative responses Endothelium 2002; 9:231-8. [PMID: 12572854].

19. Day RM, Suzuki YJ. Cell proliferation, reactive oxygen and cellular glutathione. Dose Response 2005; 3:425-42. [PMID: 16484617].

20. Sciancalepore M, Luin E, Parato G, Ren E, Giniatullin R, Gorowiec MR, Borthwick LA, Parker SM, Kirby JA, Saretzki G, Bahn RS, Barnes S. Superoxide radical production stimulates retroocular fibroblast proliferation in Graves’ ophthalmopathy orb. Mol Vis 2011; 17:2782-8. [PMID: 22065933].

21. Iglesias-De La Cruz MC, Ruiz-Torres P, Alcamí J, Diez-Marqués L, Ortega-Velázquez R, Chen S, Rodríguez-Puyol M, Ziyadeh FN, Rodriguez-Puyol D. Hydrogen peroxide increases extracellular matrix mRNA through TGF-beta in human mesangial cells. Kidney Int 2001; 59:87-95. [PMID: 1135061].

22. Gorowiec MR, Borthwick LA, Parker SM, Kirby JA, Saretzki GC, Fisher AJ. Free radical generation induces epithelial-to-mesenchymal transition in lung epithelium via a TGF-β1-dependent mechanism. Free Radic Biol Med 2012; 52:1024-32. [PMID: 22240154].

23. Yang ZG, Chen P, Zhou R, Xiang XD. Hydrogen peroxide upregulates interleukin-1beta-induced cyclooxygenase-2
expression in human pulmonary epithelial cells. Zhonghua Jie He Hu Xi Za Zhi 2004; 27:46-50. [PMID: 14989826].

24. Khalil N, Xu YD, O’Connor R, Duronio V. Proliferation of pulmonary interstitial fibroblasts is mediated by transforming growth factor-beta1-induced release of extracellular fibroblast growth factor-2 and phosphorylation of p38 MAPK and JNK. J Biol Chem 2005; 280:43000-9. [PMID: 16246848].

25. Heufelder AE, Bahn RS. Modulation of Graves’ orbital fibroblast proliferation by cytokines and glucocorticoid receptor agonists. Invest Ophthalmol Vis Sci 1994; 35:120-7. [PMID: 8300338].

26. Kaback LA, Smith TJ. Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy. J Clin Endocrinol Metab 1999; 84:4079-84. [PMID: 10566653].

27. Wong YK, Tang KT, Wu JC, Hwang JJ, Wang HS. Stimulation of hyaluronan synthesis by interleukin-1beta involves activation of protein kinase C betaII in fibroblasts from patients with Graves’ ophthalmopathy. J Cell Biochem 2001; 82:58-67. [PMID: 11400163].

28. Bouzas EA, Karadimas P, Mastorakos G, Koutras DA. Antioxidant agents in the treatment of Graves’ ophthalmopathy. Am J Ophthalmol 2000; 129:618-22. [PMID: 10844053].

29. Marcocci C, Kahaly GJ, Krassas GE, Bartalena L, Prummel M, Stahl M, Altea MA, Nardi M, Pitz S, Boboridis K, Sivelli P, von Arx G, Mourits MP, Baleschi L, Bencivelli W, Wiersinga W. European Group on Graves’ Orbitopathy. Selenium and the course of Graves’ orbitopathy. N Engl J Med 2011; 364:1920-31. [PMID: 21591944].

30. Duntas LH. The evolving role of selenium in the treatment of Graves’ disease and ophthalmopathy. J Thyroid Res 2012; 2012:736161. [PMID: 22315699].

31. Li YB, Han JY, Jiang W, Wang J. Selenium inhibits high glucose-induced cyclooxygenase-2 and P-selectin expression in vascular endothelial cells. Mol Biol Rep 2011; 38:2301-6. [PMID: 21052844].

32. Vondrichova T, de Capretz A, Parikh H, Frenander C, Asman P, Aberg M, Groop L, Hallengren B, Lantz M. COX-2 and SCD, markers of inflammation and adipogenesis, are related to disease activity in Graves’ ophthalmopathy. Thyroid 2007; 17:511-7. [PMID: 17614770].

33. Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003; 8:223-46. [PMID: 12946237].

34. Chang Y, Zhang GZ, Piao SL, Gao S, Zheng DM, Song Y, Tsicopoulos A, Ying S. Protective effects of combined micronutrients on islet beta-cells of streptozotocin-induced diabetic mice. Int J Vitam Nutr Res 2009; 79:104-16. [PMID: 20108212].

35. Han R, Smith TJ. T helper type 1 and type 2 cytokines exert divergent influence on the induction of prostaglandin E2 and hyaluronan synthesis by interleukin-1beta in orbital fibroblasts: implications for the pathogenesis of thyroid-associated ophthalmopathy. Endocrinology 2006; 147:13-9. [PMID: 16210363].