Endocrine Journal 2020, **67** (4), 439-447

ORIGINAL

Anti-oxidative and anti-adipogenic effects of caffeine in an *in vitro* model of Graves' orbitopathy

JaeSang Ko[1)], Ji-Young Kim[1)], Jae-woo Kim[2),3)] and Jin Sook Yoon[1)]

[1)] *Department of Ophthalmology, Severance Hospital, Institute of Vision Research, Yonsei University College of Medicine, Seoul, Korea*
[2)] *Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul, Korea*
[3)] *Brain Korea 21 PLUS Project for Medical Science, Yonsei University, Seoul, Korea*

Abstract. Oxidative stress and adipogenesis play key roles in the pathogenesis of Graves' orbitopathy (GO). In this study, the therapeutic effects of caffeine on the reduction of oxidative stress and adipogenesis were evaluated in primary cultured GO orbital fibroblasts *in vitro*. Orbital fibroblasts were cultured from orbital connective tissues obtained from individuals with GO. Intracellular reactive oxygen species (ROS) levels induced by hydrogen peroxide or cigarette smoke extract and the expression of anti-oxidative enzymes were measured after caffeine treatment. After adipogenic differentiation and caffeine treatment, cells were stained with Oil Red O and the levels of peroxisome proliferator activator γ (PPARγ), C/EBPα, and C/EBPβ were determined by western blot analysis. Hydrogen peroxide and cigarette smoke extract increased the levels of intracellular ROS and anti-oxidative enzymes, which decreased in a dose-dependent manner upon pretreatment with caffeine in GO orbital fibroblasts. Oil Red-O staining results revealed a decrease in lipid droplets; furthermore, PPARγ, C/EBPα, and C/EBPβ protein expression levels were inhibited upon treatment with caffeine during adipocyte differentiation. In conclusion, caffeine decreased oxidative stress and adipogenesis in GO orbital fibroblasts *in vitro*. These findings may contribute to the development of new types of caffeine-containing pharmacological agents for use in the management of GO.

Key words: Graves' orbitopathy, Thyroid eye disease, Oxidative stress, Adipogenesis, Caffeine

GRAVES' ORBITOPATHY (GO) is an autoimmune disorder of the orbit involving the infiltration of T cells, B cells, plasma cells, and macrophages. The pathogenesis of GO involves inflammation of orbital connective tissue that leads to the enlargement of extraocular muscles and orbital adipose tissue. Such tissue expansion within the bony orbits is thought to be responsible for the progression and complications of GO.

At the center of this pathogenesis lies inflammation and oxidative stress. Infiltration of inflammatory cells to the orbit triggers and propagates tissue expansion. Oxygen free radicals trigger fibroblast proliferation [1] and the abundance of thyroid hormones in Graves' disease renders orbital fibroblasts more vulnerable to oxidative damage [2]. Clinically, cigarette smoking is the most important environmental risk factor for the development and progression of GO [3, 4], suggesting that oxidative stress is one of the key effectors in its pathogenesis.

Caffeine (1,3,7-trimethylxanthine) is a plant alkaloid found in coffee, tea, chocolate, cola, and other soft drinks commonly consumed around the world [5]. It is absorbed rapidly and completely from the gastrointestinal tract and is detectable in all body fluids including in umbilical cord blood [6, 7]. Numerous studies have determined that it acts as a free radical scavenger, taking part in antioxidant activities *in vitro* and *in vivo* in both animals and humans [8-10]. Furthermore, its anti-adipogenic properties in various cell types *in vitro* have shown that caffeine inhibited the expression of adipogenesis-related cytokines and transcription factors, resulting in a decrease in adipocyte differentiation and intracellular lipid accumulation [5, 11]. Given the fundamental role of oxidative stress and adipogenesis in the pathogenesis of GO, we investigated the therapeutic effect of caffeine in an *in vitro* model of GO.

Submitted Nov. 7, 2019; Accepted Dec. 12, 2019 as EJ19-0521
Released online in J-STAGE as advance publication Jan. 16, 2020
Correspondence to: Jin Sook Yoon, MD, PhD, Department of Ophthalmology, Severance Hospital, Institute of Vision Research, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
E-mail: yoonjs@yuhs.ac

Materials and Methods

Reagents and chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamicin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Caffeine was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay solution was purchased from Promega Corporation (Madison, WI, USA). Cigarette smoke extract (CSE) was freshly prepared within an hour of each experiment from commercially available filtered cigarettes [Marlboro 20 class A cigarettes (8.0 mg tar; 0.7 mg nicotine); Philip Morris Korea, Inc., Seoul, Korea] as described in our previous study [12]. Recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN, USA) and Oil Red O was purchased from Sigma-Aldrich Corp.

Cell culture and differentiation protocols

Orbital adipose/connective tissue explants were obtained as surgical waste during decompression surgery in five patients with GO (three females, two males; 29–51 years of age). Normal control tissues were harvested during upper-lid blepharoplasty from the post-septal area of five individuals with no history or clinical evidence of thyroid disease or GO (three females, two males; 31–58 years of age). All five patients with GO achieved stable euthyroidism at the time of surgery, at which point their clinical activity scores were less than three. Furthermore, none of the patients with GO received steroid treatment or radiotherapy for at least three months before surgery. The Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) approved the study and written informed consent was obtained from all participants after explanation of the nature and possible consequences of the study. This study followed the tenets of the Declaration of Helsinki.

Primary cultures of orbital fibroblasts were established as described in our previous study [13]. Briefly, minced tissue was placed directly in 1:1 DMEM:F12 medium with 20% FBS and antibiotics. When the growth of fibroblasts was observed, monolayers were passaged serially with trypsin/ethylenediaminetetraacetic acid solution and cultures were maintained in DMEM with 10% FBS and antibiotics. The strains were stored in liquid nitrogen until further analysis; cells between the second and fifth passages were used.

The anti-adipogenic effect of caffeine was evaluated using our previously reported adipocyte differentiation protocol for GO orbital fibroblasts involving dexamethasone, rosiglitazone (10 μM; Cayman Chemical, Ann Arbor, MI, USA), and IL-1β (10 ng/mL) treatment [13, 14]. Briefly, after 10 days of adipogenic differentiation co-treated with serial concentrations of caffeine (0.1–5 mM), differentiated adipocytes were visualized with Oil Red O staining and expression of adipogenic transcription factors were evaluated with western blotting.

Cell viability assay

To evaluate the effect of caffeine on cell viability of orbital fibroblasts, primary cultured orbital fibroblasts obtained from patients with GO were seeded on 24-well culture plates (1×10^4 cells/well) and treated with various concentrations of caffeine (0.01 mM, 0.1 mM, 0.5 mM, 1 μM, 2 mM, and 5 mM) for 48 h and 72 h. Thereafter, MTS solution was added and the plate was incubated again for 4 h under the same conditions. The absorbance of the dye was measured at 490 nm using an ELISA plate reader. Cell viability is expressed as a percentage relative to untreated control cells.

Intracellular ROS measurement

ROS release was determined with 5-(and 6)-carboxy-20,70-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA), an oxidant-sensitive fluorescent probe, as we described previously [12]. Briefly, primary cultured orbital fibroblasts were pretreated with various concentrations of caffeine for 24 h. The culture medium was then removed, and the cells were washed with PBS, incubated with 10 mM H2DCFDA at 37°C for 30 min, then stimulated with CSE (2%) or H_2O_2 (10 μM) for 30 min [12, 15, 16]. The fluorescently stained cells were examined microscopically at 40× and were quantified *via* flow-cytometric analysis.

Western blotting

Western blot analysis was performed as described previously [17]. To assess the induction of oxidative stress by CSE and H_2O_2 and its inhibition by caffeine, confluent orbital fibroblasts were pre-exposed to serial concentrations of caffeine for 24 h, followed by stimulation with CSE or H_2O_2 for 24 h. Expression levels of manganese superoxide dismutase (Mn-SOD), copper-zinc superoxide dismutase (Cu/Zn-SOD), thioredoxin (Trx), and heme oxygenase-1 (HO-1) were assessed to evaluate oxidative stress levels. To evaluate the expression of adipogenic transcription factors after adipogenic differentiation, the expression levels of peroxisome proliferator activator gamma (PPARγ), C/EBPα, and C/EBPβ were evaluated in GO cells after ten days of adipocyte differentiation. Activation of intracellular signalling proteins was assayed *via* western blot analysis of phosphorylated and total AKT, extracellular signal-regulated kinase

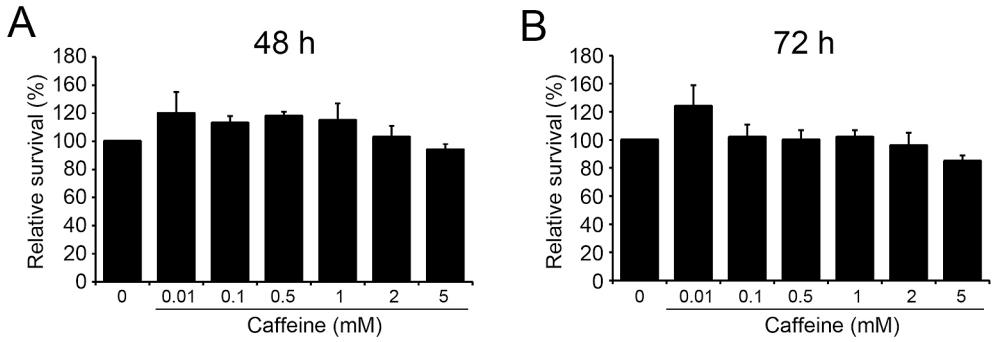

Fig. 1 Effect of caffeine on the viability of orbital fibroblasts

Orbital fibroblasts from individuals with GO were seeded in 24-well culture plates and treated with various concentrations (0–5 mM) of caffeine for 48 h (A) and 72 h (B). After treatment, MTS assays were used to evaluate viability. Assays were performed in duplicate with cells from three different donors. Results are expressed as percentages of untreated control values and are presented as means ± standard deviation.

(ERK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), c-Jun NH(2)-terminal kinase (JNK), and p38 protein. The relative amount of protein in each immunoreactive band was quantified by densitometry and normalised to the concentration of β-actin in the same sample.

Anti-Mn-SOD, anti-Zn/Cu-SOD, anti-Trx, anti-HO-1, anti-peroxisome proliferator activator gamma (PPARγ), anti-C/EBPα, anti-C/EBPβ, anti-NF-κB, p38, and anti–β-actin antibodies were all obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and antibodies against AKT, ERK, and JNK were produced by Cell Signaling Technology (Danvers, MA, USA).

Oil Red O staining

After ten days of adipocyte differentiation of orbital fibroblasts, cells were stained with an Oil Red O working solution as described previously [13, 14]. Stained cells were visualised and photographed at 40×, then quantified using a spectrophotometer at 490 nm according to the manufacturer's instructions. Experiments for the quantitative assessment of adipogenic differentiation were performed in duplicate using cells from different donors; the results were normalised to the absorbance of untreated differentiated control cells.

Statistical analysis

All experiments were performed using cells from at least three different samples and the samples were assayed in duplicate each time. Differences in parameter estimates between the experimental and control groups were assessed by the Student's *t*-test or Wilcoxon rank-sum test using R version 3.1.2 (R Foundation, Vienna, Austria). Values of $p < 0.05$ were considered significant.

Results

Cell viability using MTS analysis

To determine non-toxic concentrations of caffeine in orbital fibroblasts, an MTS assay was performed. Orbital fibroblasts from patients with and without GO were treated with caffeine at concentrations ≤5 mM for 48 h and 72 h. The 0.01 to 5 mM range of caffeine did not decrease cell viability below 95% in both normal and GO orbital fibroblasts in the 48 h treatment (Fig. 1, data for non-GO orbital fibroblasts are not shown).

Caffeine decreased ROS levels induced by H_2O_2 or CSE

To demonstrate the anti-oxidative effect of caffeine in GO orbital fibroblasts, oxidative stress was induced by 10 μM H_2O_2 or 2% CSE with or without 0.01–5 mM caffeine pretreatment for 24 h. Treatment with 10 μM H_2O_2 or 2% CSE for 30 min significantly increased ROS production relative to that in untreated control cells, which was reduced by caffeine pretreatment in a dose-dependent manner (Fig. 2A). Similarly, upon fluorescent staining of cellular ROS, a significant reduction in fluorescently stained cells was observed after treatment with caffeine (40×; Fig. 2B).

Effect of caffeine on SOD, Trx, and HO-1

The relative expression levels of Mn-SOD, Cu/Zn-SOD, and HO-1 increased upon treatment with 10 μM H_2O_2 or 2% CSE in GO orbital fibroblasts for 24 h, while the expression of Trx increased only upon treatment with CSE, not H_2O_2. Upon pretreatment with a serial concentration of caffeine, we found that caffeine concentrations >1 mM decreased the expression of Mn-SOD, Cu/Zn-SOD, Trx, and HO-1 (Fig. 3).

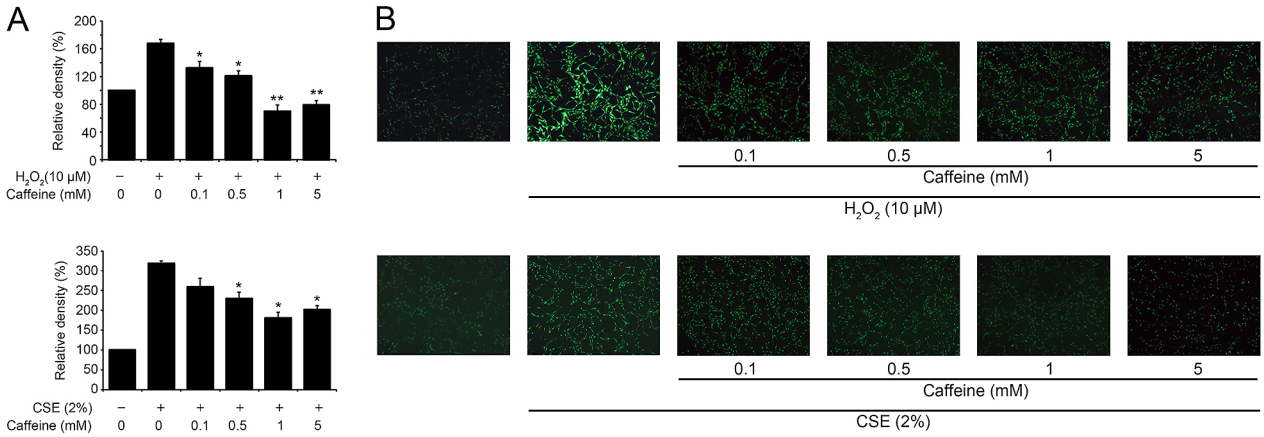

Fig. 2 Effect of caffeine on H_2O_2- and CSE-induced generation of intracellular ROS in orbital fibroblasts from GO patients

Orbital fibroblasts from GO patients (n = 3) were treated with 10 μM H_2O_2 or 2% CSE for 30 min with or without pretreatment with caffeine for 24 h. ROS levels were measured *via* flow cytometry using H_2DCFDA (A) and microscopically visualised at 40× (B). The results are expressed as percentages of the untreated control values and presented as means ± standard deviation. Assays were performed in duplicate with cells from three different GO samples; data from a representative experiment are shown (*p < 0.05 and **p < 0.01 *versus* H_2O_2- or CSE-treated cells without caffeine pretreatment).

Caffeine reduced adipogenesis in GO orbital fibroblasts

To determine the effect of caffeine on adipogenesis in orbital fibroblasts, GO cells were treated with caffeine during adipocyte differentiation. When caffeine (0.5–5 mM) was added to the adipogenic medium, it reduced the number of adipocytes and suppressed the accumulation of lipid droplets. The optical density of Oil Red O-stained cell lysates showed that caffeine-treated cells exhibited decreased absorbance at 490 nm (Fig. 4A). Western blot analysis was then performed to investigate whether caffeine affects the production of the adipogenic transcription factors PPARγ, C/EBPα, and C/EBPβ during adipogenesis. Caffeine had dose-dependent inhibitory effects on the expression of adipogenic transcription factors (Fig. 4B). Each experiment was performed in three GO cells from different patient samples and samples were assayed in duplicate.

Effect of caffeine on intracellular signalling pathways

To investigate the signalling pathways through which caffeine mediates its effects in GO orbital fibroblasts, the expression levels of multiple transcription factors were assessed after caffeine treatment. Both GO and non-GO cells were treated with 1 mM caffeine for various durations. Western blot analysis (Fig. 5) showed that caffeine inhibited the phosphorylation of ERK and p38 protein in a time-dependent manner. The phosphorylation of AKT, JNK, and NF-κB remained unchanged with 1 mM caffeine treatment (data not shown).

Discussion

In this study, we found that caffeine curtailed oxidative stress and adipogenesis, the main pathogenic mechanisms of GO. Based on our results, caffeine appears to have an inhibitory effect on ROS generation in response to oxidative stress, as well as an anti-adipogenic effect.

A state of oxidative stress has been described in Graves' disease and GO [18-20]. In general, an increase in ROS or the reduced elimination of radicals by antioxidative enzymes will result in oxidative damage, inflammation, and loss of function. In the *in vitro* pathogenic mechanism of GO, superoxide radicals stimulate orbital fibroblasts to proliferate and differentiate into mature adipocytes [20]. Based on clinical reports, cigarette smoking—likely the most important environmental factor associated with GO occurrence and progression—may act, among other mechanisms, by stimulating the generation of ROS and reducing antioxidant production [3, 20, 21]. In this regard, several *in vitro* studies demonstrate the therapeutic effectiveness of drugs with antioxidant potential in primary cultured orbital fibroblasts from GO patients [12, 15-17]. Clinical studies on the use of antioxidants for the management of GO have also shown them to be clinically effective in patients experiencing mild severity [22-24].

Although caffeine is known to act as a free radical scavenger—the basis for its antioxidant activities—it should be noted that it exhibits both anti-oxidant and pro-oxidant properties depending on dose: at low concentrations, caffeine shows antioxidant effects, while at high concentrations, it may increase cytotoxicity, leading

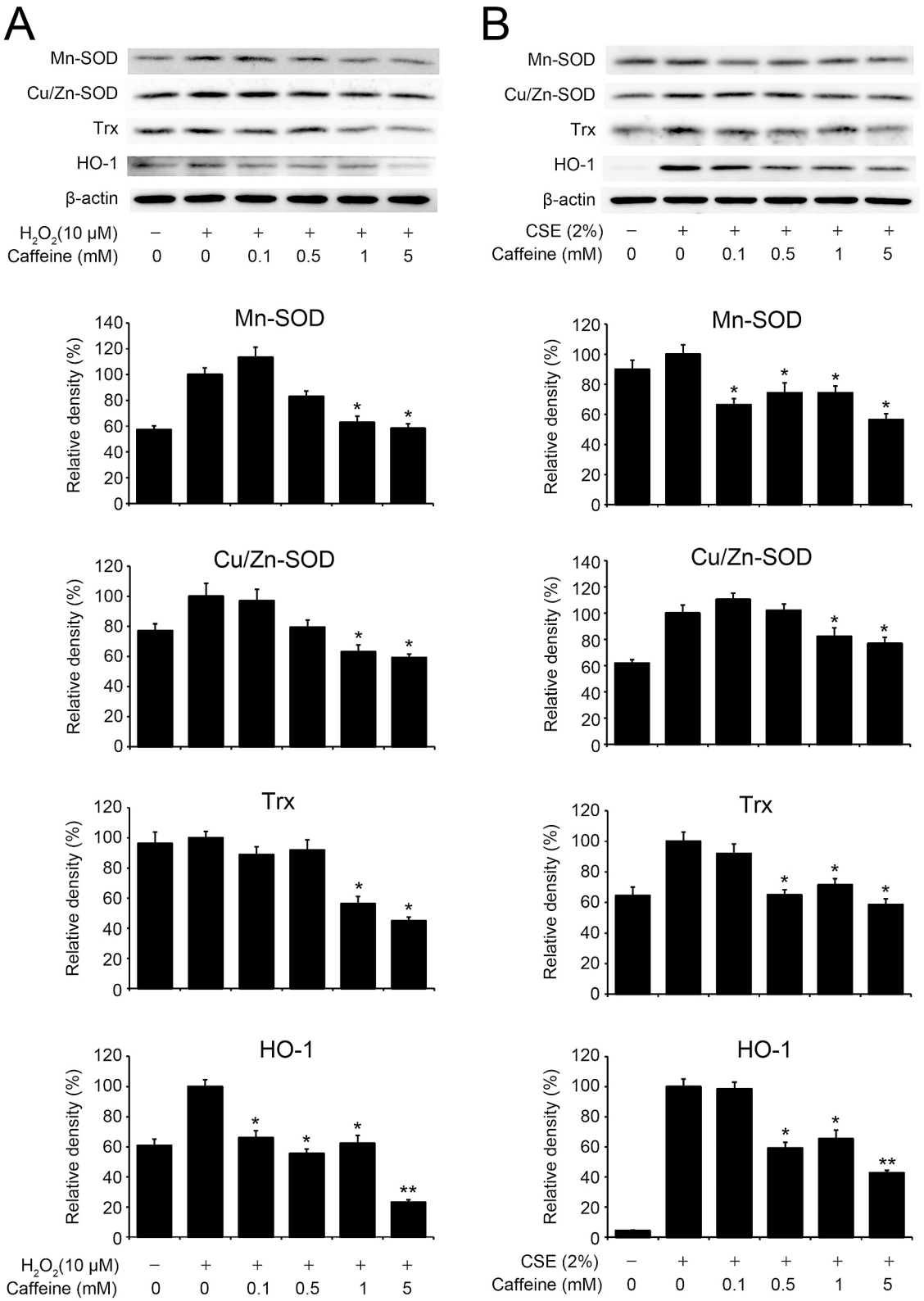

Fig. 3 Effect of caffeine on H_2O_2- and CSE-induced expression of SOD, Trx, and HO-1 in orbital fibroblasts from GO patients

Orbital fibroblasts from GO patients (n = 3) were treated with 10 μM H_2O_2 (A) or 2% CSE (B) for 24 h with or without pretreatment with 0.1–5 mM caffeine for 24 h. The cell lysates were subjected to western blot analysis. Experiments were performed three times using different strains and samples were assayed in duplicate. Results are expressed as the relative density of each protein normalised to the level of β-actin and presented as means ± standard deviation. GO cells treated with neither CSE nor H_2O_2 were used as controls (*$p < 0.05$ and **$p < 0.01$ *versus* H_2O_2- or CSE- treated cells without pretreatment).

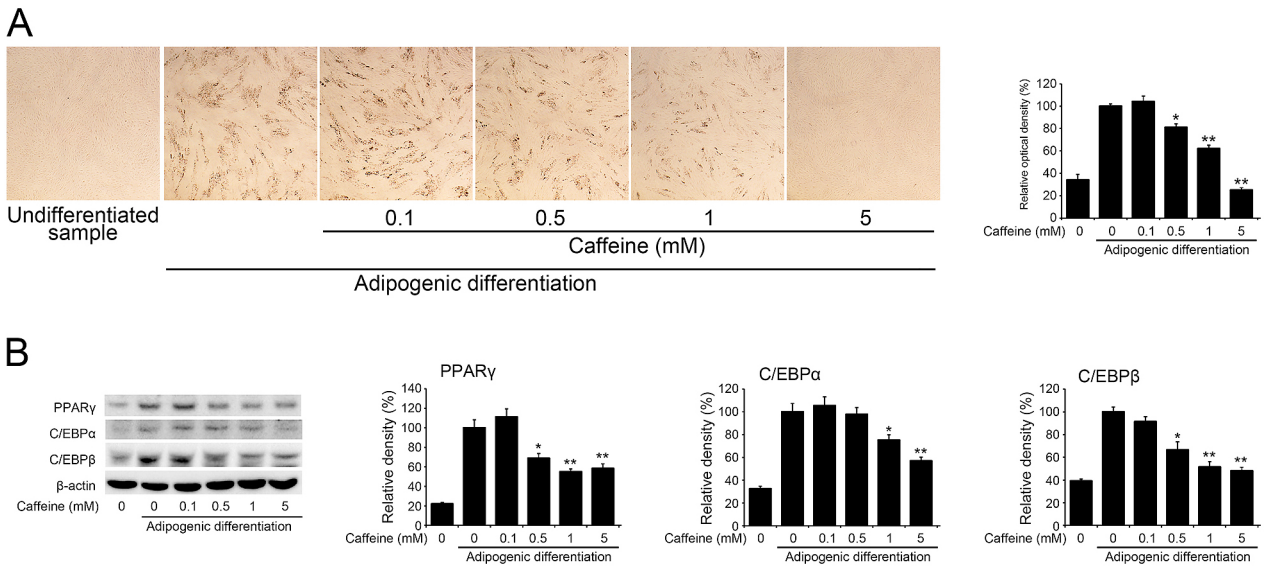

Fig. 4 Effect of caffeine on adipogenesis in GO orbital fibroblasts

(A) Ten days of adipocyte differentiation was induced in primary cultured orbital fibroblasts from GO patients. The addition of caffeine to the adipogenic medium attenuated adipocyte differentiation, as evidenced by microscopic examinations (40×) after Oil Red O staining. The optical density at 490 nm of stained cell lysates also yielded quantitative evidence of the inhibitory effect of caffeine on the accumulation of lipid droplets. (B) Western blot analysis of PPARγ, C/EBPα, and C/EBPβ protein was performed after ten days of adipogenic differentiation. The experiments were performed in duplicate with cells from three different donors. PPARγ, C/EBPα, and C/EBPβ levels determined by densitometry were normalised to the level of β-actin in the same sample. The results are presented as the mean relative density ratios (%) ± standard deviation (*$p < 0.05$ and **$p < 0.01$ *versus* cells without caffeine treatment).

to the subsequent generation of reactive oxygen species (ROS) [5, 25, 26]. In this study, however, orbital fibroblasts were resistant to the generation of ROS at high concentrations of caffeine, which acted as an antioxidant in H_2O_2- or CSE-stimulated orbital fibroblasts even at the highest concentration (5 mM) without causing cell damage.

Adipogenesis, the process by which orbital fibroblasts develop into mature adipocytes, is one of the key pathogenic mechanisms in GO. We found that drugs with antioxidant effects significantly suppressed adipocyte differentiation in primary Graves' orbital fibroblasts [12, 15-17]. Recently, groups studying obesity and metabolism have reported that caffeine inhibits adipogenic differentiation and reduces lipid accumulation [5, 11]. They showed that caffeine may prohibit the activation of C/EBPβ, the first response to adipogenic inducers, blocking the adipogenic cascade involving PPARγ, aP2, or leptin protein. In this study, we showed that ≥0.5 mM caffeine inhibited adipogenic differentiation and lipid accumulation, as well as the expression of adipogenic transcription factors (PPARγ, C/EBPα, and C/EBPβ).

The differentiation of preadipocytes is triggered by hormonal agents and proceeds by the coordination and precise control of transcriptional cascades. The first stage of adipocyte differentiation is growth arrest at a confluent state; proliferating cells will not accumulate lipid droplets in their cytoplasm until they become confluent. Upon induction of differentiation, growth-arrested cells undergo additional rounds of cell division, known as mitotic clonal expansion [27]. A recent study showed that ROS are important in regulating mitotic clonal expansion during adipogenesis and that they facilitate adipocyte differentiation by accelerating mitotic clonal expansion [28]. Moreover, it has been demonstrated that caffeine inhibits adipogenesis through the modulation of mitotic clonal expansion in 3T3-L1 adipocytes [11].

Mitogen-activated protein kinases (MAPKs) constitute a family of protein kinases (ERK, JNK, and the p38-MAPKs in mammals) that play an essential role in relaying extracellular signals from the cell membrane to the nucleus *via* a cascade of phosphorylation events [29]. MAPKs are key participants in signal transduction pathways activated by mitogenic stimuli, environmental stress, and inflammatory agents [30]. Various studies have documented the involvement of the MAPK signalling pathways in redox-stressed cells and tissues [31]. Moreover, it has been shown that the pathogenesis of GO involves activation of the MAPK signalling pathways, especially the p38 and ERK pathways [32, 33]. Our results showed that the levels of phosphorylated p38 and ERK decreased when they were cotreated at increasing

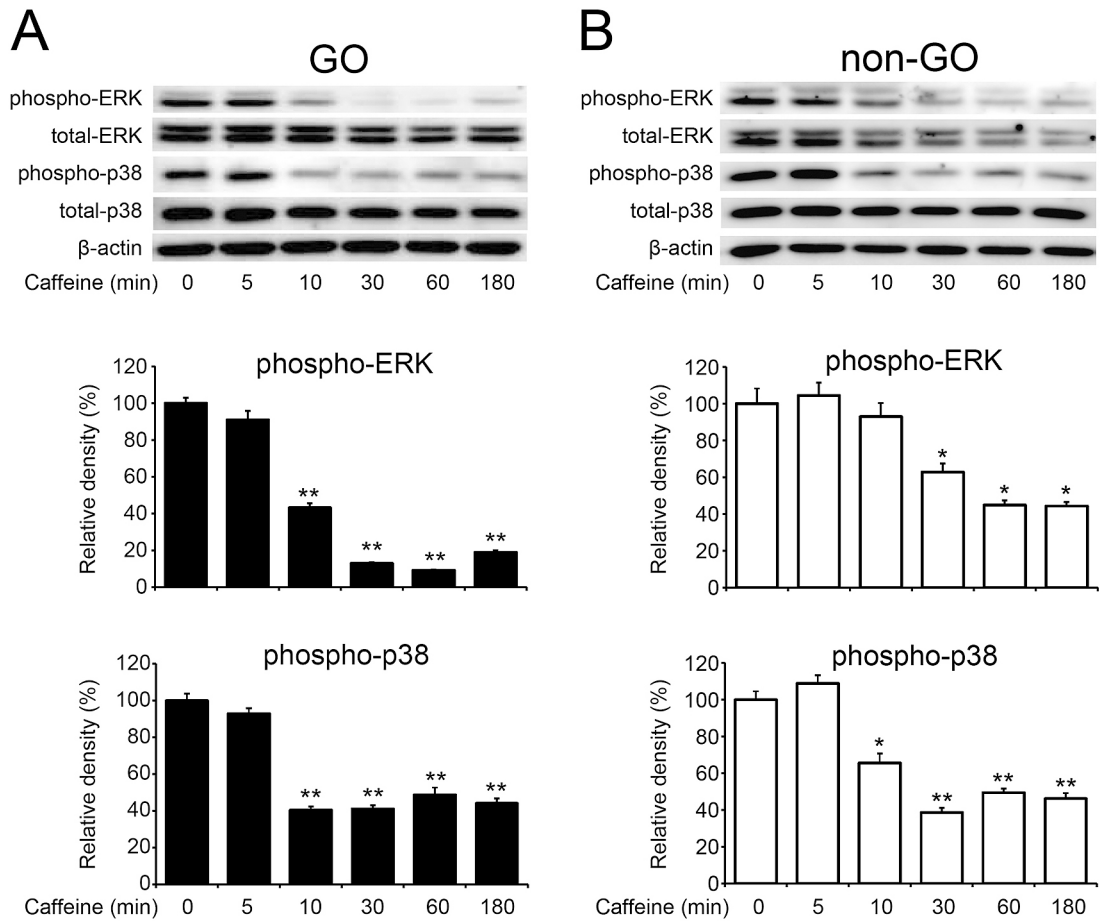

Fig. 5 Effect of caffeine on intracellular signalling pathways

Orbital fibroblasts from GO patients (n = 3) and non-GO individuals (n = 3) were treated with 1 mM caffeine for 0–180 min. The cell lysates were subjected to western blot analysis. Experiments were performed three times using different strains and samples were assayed in duplicate. Results are expressed as the relative density of each protein normalised to the level of β-actin and presented as means ± standard deviation (*p < 0.05 and **p < 0.01 *versus* cells without caffeine treatment).

timeframes of caffeine exposure.

The results of the present study show that caffeine has anti-oxidative and anti-adipogenic properties; however, it should be noted that the concentration of caffeine used in this *in vitro* study is somewhat higher than the normal physiological range. A caffeine concentration of 0.1 mM (19.4 μg/mL) is approximately equivalent to that in a cup of coffee containing 150 mg caffeine (Starbucks© latte) [5]; it has been reported that caffeine causes adverse effects at excessively high blood concentrations (>80 μg/mL) in humans [34]. In this study, caffeine exhibited anti-adipogenic effects beyond concentrations of 0.5 mM and anti-oxidative effects at 0.1 mM. As GO is a heterogeneous autoimmune disease affected by multiple genetic and environmental factors, *in vitro* results may not correlate precisely with *in vivo* conditions. Additionally, we induced pathologic conditions in cells by stimulation using concentrations of chemicals, hormones, and growth factors beyond physiological ranges—for example, our use of oxidants such as H_2O_2 or CSE.

In conclusion, to the best of our knowledge, this is the first study to investigate the effects of caffeine on oxidative stress and adipogenesis in orbital fibroblasts from GO patients. Until now, no clinical or epidemiologic studies have emerged that assess the relationship between caffeine and GD or GO. Our *in vitro* results provide evidence to support further investigation regarding the influence of caffeine on GO progression in clinical settings.

Acknowledgements

Funding: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2017R1A2B4009565).

Disclosure

The authors have no conflicts of interest to declare.

References

1. Lehmann GM, Feldon SE, Smith TJ, Phipps RP (2008) Immune mechanisms in thyroid eye disease. *Thyroid* 18: 959–965.
2. Burch HB, Lahiri S, Bahn RS, Barnes S (1997) Superoxide radical production stimulates retroocular fibroblast proliferation in Graves' ophthalmopathy. *Exp Eye Res* 65: 311–316.
3. Pfeilschifter J, Ziegler R (1996) Smoking and endocrine ophthalmopathy: impact of smoking severity and current *vs.* lifetime cigarette consumption. *Clin Endocrinol (Oxf)* 45: 477–481.
4. Stan MN, Bahn RS (2010) Risk factors for development or deterioration of Graves' ophthalmopathy. *Thyroid* 20: 777–783.
5. Su SH, Shyu HW, Yeh YT, Chen KM, Yeh H, *et al.* (2013) Caffeine inhibits adipogenic differentiation of primary adipose-derived stem cells and bone marrow stromal cells. *Toxicol In Vitro* 27: 1830–1837.
6. Arnaud MJ (1993) Metabolism of caffeine and other components of coffee. In: Garattini S. (ed.), Caffeine, Coffee, and Health. Raven Press, New York, USA: 43–96.
7. Dempsey DA, Partridge JC, Jones RT, Rowbotham MC (1998) Cocaine, nicotine, caffeine, and metabolite plasma concentrations in neonates. *J Anal Toxicol* 22: 220–224.
8. Lv X, Chen Z, Li J, Zhang L, Liu H, *et al.* (2010) Caffeine protects against alcoholic liver injury by attenuating inflammatory response and oxidative stress. *Inflamm Res* 59: 635–645.
9. Jagdeo J, Brody N (2011) Complementary antioxidant function of caffeine and green tea polyphenols in normal human skin fibroblasts. *J Drugs Dermatol* 10: 753–761.
10. Varma SD, Hegde KR, Kovtun S (2010) Oxidative stress in lens *in vivo*: inhibitory effect of caffeine. A preliminary report. *Mol Vis* 16: 501–505.
11. Kim AR, Yoon BK, Park H, Seok JW, Choi H, *et al.* (2016) Caffeine inhibits adipogenesis through modulation of mitotic clonal expansion and the AKT/GSK3 pathway in 3T3-L1 adipocytes. *BMB Rep* 49: 111–115.
12. Yoon JS, Lee HJ, Chae MK, Lee SY, Lee EJ (2013) Cigarette smoke extract-induced adipogenesis in Graves' orbital fibroblasts is inhibited by quercetin *via* reduction in oxidative stress. *J Endocrinol* 216: 145–156.
13. Kim SE, Lee JH, Chae MK, Lee EJ, Yoon JS (2016) The role of sphingosine-1-phosphate in adipogenesis of Graves' orbitopathy. *Invest Ophthalmol Vis Sci* 57: 301–311.
14. Ko J, Kim JY, Lee EJ, Yoon JS (2018) Inhibitory effect of idelalisib, a selective phosphatidylinositol 3-kinase delta inhibitor, on adipogenesis in an *in vitro* model of Graves' orbitopathy. *Invest Ophthalmol Vis Sci* 59: 4477–4485.
15. Kim CY, Lee HJ, Chae MK, Byun JW, Lee EJ, *et al.* (2015) Therapeutic effect of resveratrol on oxidative stress in Graves' orbitopathy orbital fibroblasts. *Invest Ophthalmol Vis Sci* 56: 6352–6361.
16. Rhiu S, Chae MK, Lee EJ, Lee JB, Yoon JS (2014) Effect of tanshinone IIA in an *in vitro* model of Graves' orbitopathy. *Invest Ophthalmol Vis Sci* 55: 5900–5910.
17. Byun JW, Hwang S, Kang CW, Kim JH, Chae MK, *et al.* (2016) Therapeutic effect of protocatechuic aldehyde in an *in vitro* model of Graves' orbitopathy. *Invest Ophthalmol Vis Sci* 57: 4055–4062.
18. Hondur A, Konuk O, Dincel AS, Bilgihan A, Unal M, *et al.* (2008) Oxidative stress and antioxidant activity in orbital fibroadipose tissue in Graves' ophthalmopathy. *Curr Eye Res* 33: 421–427.
19. Komosinska-Vassev K, Olczyk K, Kucharz EJ, Marcisz C, Winsz-Szczotka K, *et al.* (2000) Free radical activity and antioxidant defense mechanisms in patients with hyperthyroidism due to Graves' disease during therapy. *Clinica Chimica Acta* 300: 107–117.
20. Bartalena L, Tanda ML, Piantanida E, Lai A (2003) Oxidative stress and Graves' ophthalmopathy: *in vitro* studies and therapeutic implications. *Biofactors* 19: 155–163.
21. Shine B, Fells P, Edwards OM, Weetman AP (1990) Association between Graves' ophthalmopathy and smoking. *Lancet* 335: 1261–1263.
22. Marcocci C, Kahaly GJ, Krassas GE, Bartalena L, Prummel M, *et al.* (2011) Selenium and the course of mild Graves' orbitopathy. *N Engl J Med* 364: 1920–1931.
23. Bednarek J, Wysocki H, Sowinski J (2005) Oxidative stress peripheral parameters in Graves' disease: the effect of methimazole treatment in patients with and without infiltrative ophthalmopathy. *Clin Biochem* 38: 13–18.
24. Bouzas EA, Karadimas P, Mastorakos G, Koutras DA (2000) Antioxidant agents in the treatment of Graves' ophthalmopathy. *Am J Ophthalmol* 129: 618–622.
25. Azam S, Hadi N, Khan NU, Hadi SM (2003) Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. *Med Sci Monit* 9: BR325–BR330.
26. Tiwari KK, Chu C, Couroucli X, Moorthy B, Lingappan K (2014) Differential concentration-specific effects of caffeine on cell viability, oxidative stress, and cell cycle in pulmonary oxygen toxicity *in vitro*. *Biochem Biophys Res Commun* 450: 1345–1350.
27. MacDougald OA, Lane MD (1995) Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem* 64: 345–373.
28. Lee H, Lee YJ, Choi H, Ko EH, Kim JW (2009) Reactive

oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. *J Biol Chem* 284: 10601–10609.
29. Son Y, Cheong YK, Kim NH, Chung HT, Kang DG, *et al.* (2011) Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? *J Signal Transduct* 2011: 792639.
30. Kyriakis JM, Avruch J (2012) Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol Rev* 92: 689–737.
31. Gaitanaki C, Konstantina S, Chrysa S, Beis I (2003) Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart. *J Exp Biol* 206: 2759–2769.
32. Chen B, Tsui S, Smith TJ (2005) IL-1 beta induces IL-6 expression in human orbital fibroblasts: identification of an anatomic-site specific phenotypic attribute relevant to thyroid-associated ophthalmopathy. *J Immunol* 175: 1310–1319.
33. Kazim M, Goldberg RA, Smith TJ (2002) Insights into the pathogenesis of thyroid-associated orbitopathy: evolving rationale for therapy. *Arch Ophthalmol* 120: 380–386.
34. Nomura M, Ichimatsu D, Moritani S, Koyama I, Dong Z, *et al.* (2005) Inhibition of epidermal growth factor-induced cell transformation and Akt activation by caffeine. *Mol Carcinog* 44: 67–76.