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Anti-glycation and anti-oxidative effects of a phenolic-enriched maple syrup extract and its protective effects on normal human colon cells

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Abstract

Oxidative stress and free radical generation accelerate the formation of advanced glycation endproducts (AGEs) which are linked to several chronic diseases. Published data suggest that phenolic-rich plant foods, show promise as natural anti-AGEs agents due to their anti-oxidation capacities. A phenolic-enriched maple syrup extract (MSX) has previously been reported to show anti-inflammatory and neuroprotective effects but its anti-AGE effects remain unknown. Therefore, herein, we investigated the anti-glycation and anti-oxidation effects of MSX using biochemical and biophysical methods. MSX (500 μg/mL) reduced the formation of AGEs by 40% in the bovine serum albumin (BSA)-fructose assay and by 30% in the BSA-methylglyoxal (MGO) assay. MSX also inhibited the formation of crosslinks typically seen in the late stage of glycation. Circular dichroism and differential scanning calorimeter analyses demonstrated that MSX maintained the structure of BSA during glycation. In the anti-oxidant assays, MSX (61.7 μg/mL) scavenged 50% of free radicals (DPPH assay) and reduced free radical generation by 20% during the glycation process (electron paramagnetic resonance time scan). In addition, the intracellular levels of hydrogen peroxide induced reactive oxygen species were reduced by 27-58% with MSX (50-200 μg/mL) in normal/non-tumorigenic human colon CCD-18Co cells. Moreover, in AGEs and MGO challenged CCD-18Co cells, higher cellular viabilities and rapid extracellular signal–regulated kinase (ERK) phosphorylation were observed in MSX treated cells, indicating its protective effects against AGEs-induced cytotoxicity. Overall, this study supports the biological effects of MSX, and warrants further investigation of its potential as a dietary agent against diseases mediated by oxidative stress and inflammation.

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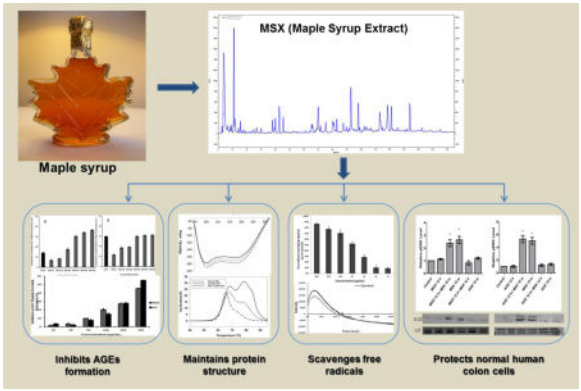
Conflict of interest

The authors have declared no conflict of interest.

Graphical abstract

Phenolic-enriched maple syrup extract (MSX) inhibits the formation of AGEs and protects normal/non-tumorigenic human colon cells from oxidative stress.



Keywords

Maple syrup extract (MSX); phenolic; anti-oxidant; advanced glycation endproducts (AGEs); free radical; extracellular signal; regulated kinase (ERK)

1. Introduction

Advanced glycation endproducts (AGEs) are a heterogeneous group of compounds formed through the non-enzymatic glycation of proteins with reducing sugars and α-carbonyl compounds. The formation of AGEs occurs endogenously and accumulates in many tissues during aging.[1] Recently, it has been shown that AGEs are involved in the pathology of major diabetic complications including neuropathy, nephropathy, and retinopathy[2], as well as in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease.[34]

The initial reaction in the formation of AGEs is the reversible addition of a carbonyl group onto the free amino of a protein, such as the ε-amino group of the lysine residue to form a Schiff base adduct. This Schiff base subsequently undergoes rearrangement to form a more stable ketoamine structure typically referred to as an Amadori product.[1, 5] Following early glycation, there are multiple pathways that can lead to the formation of stable and irreversible AGEs. First, α-dicarbonyl species such as methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG) can arise through the oxidative fragmentation of Schiff bases, the dehydration and rearrangement of Amadori products, and the auto-oxidation of D-glucose. These highly reactive carbonyl compounds, also called reactive carbonyl species (RCS) or AGEs precursors, are key intermediates in advanced glycation and can directly modify intracellular and extracellular proteins to form AGEs.[6] Moreover, certain AGEs species, such as Nε-(carboxymethyl)lysine (CML) and Nε-(l-carboxyethyl)lysine (CEL), are formed directly from the oxidative degradation of Amadori adducts.[5]

Multiple factors contribute to the acceleration of the formation of AGEs. Elevated D-glucose levels under hyperglycemic conditions is indeed one of the essential factors which can lead

to the abnormal accumulation of AGEs in vivo. However, growing evidence suggest that several other factors also play crucial roles in the generation of AGEs, e.g. oxidation, oxidative stress, and elevated levels of reactive oxygen species (ROS).[7–9] For example, free radicals and transition metals can facilitate the formation of α-dicarbonyls including GO and MGO, and these AGEs precursors are much more reactive than D-glucose. Moreover, Schiff bases, Amadori products, and some advanced glycation structures can undergo autoxidation, causing the fragmentation of these intermediates and ultimately, leading to the formation of AGEs.[10] On the other hand, oxidation is believed to be one of the most important biological mechanisms of the harmful effects of AGEs to both tissues and cells. AGEs bind to a multiligand transmembrane receptor known as RAGE (receptor for advanced glycation endproducts), which is widely expressed in tissues including brain, heart, lung, and skeletal muscles. The binding of AGEs to RAGE upregulates inflammatory cytokines, activates NADPH oxidase, and promotes the generation of ROS. The activation of RAGE, therefore, leads to chronic oxidative stress, inflammation, and tissue damage.[11] For example, the participation of AGE-RAGE interaction has been confirmed to play a key role in the pathogenesis of AD.[12]

A growing body of data suggest that several plant foods including phenolic-rich fruit, vegetables, and their derived beverages and extracts, show anti-AGEs effects through their abilities to scavenge free radicals and inhibit oxidation.[13, 14] While the majority of these studies have focused on these aforementioned plant foods, no data is currently available on plant derived natural sweeteners which contain phenolic compounds such as maple syrup.[15]

Maple syrup is a plant derived natural sweetener produced by concentrating the watery sap collected from mainly the sugar maple (*Acer saccharum* Marsh) species. During the evaporation process, natural phenolic compounds originally present in the tree xylem sap, as well as process-derived compounds, are concentrated in maple syrup along with vitamins, amino acids, organic acids, simple sugars (primarily, sucrose,) as well as complex carbohydrates such as inulin.[15, 16] Maple syrup contains a wide variety of phenolic constituents[17–19] and in our group's ongoing research on this natural product,[16, 18–21] we have developed a phenolic-enriched maple syrup extract (MSX) for nutraceutical applications which is well-tolerated and non-toxic to rats (at doses of up to 1000 mg/kg/day).[20] Phenolic-enriched extracts of maple syrup have been reported to show antioxidant[18], anti-diabetic,[20–22] anticancer,[23] anti-inflammatory[24, 25], and neuroprotective[26, 27] properties in vitro. More recently, our group has also reported on the neuroprotective effects of MSX on murine microglia BV-2 cells, human neuronal SH-SY5Y cells, and against neurotoxicity in *Caenorhabditis elegans* in vivo.[28] However, to date, there have been no anti-AGE related studies on this natural product.

In light of the aforementioned factors, herein, we focused our attention on the anti-AGE effects of MSX by targeting its anti-oxidative capacity given that it's a phenolic-enriched extract. The inhibitory effects of MSX on the formation of AGEs was investigated using a series of biochemical and biophysical methods including intrinsic fluorescence AGE assays, G.K. peptide assay, circular dichroism (CD), and differential scanning calorimetry (DSC). Next, using the DPPH free radical scavenging and electron paramagnetic resonance (EPR) assays, we evaluated the ability of MSX to scavenge free radicals. In addition, we sought to

investigate whether MSX could ameliorate normal/non-tumorigenic human colon (CCD-18Co) cells from MGO and MGO-derived AGEs induced cytotoxicity. Lastly, we examined whether the effects of rapid phosphorylation of two mitogen-activated protein kinases (MAPK), namely, extracellular signal–regulated kinase (ERK1/2) and p38, in the CCD-18Co cells, contributed to the proliferative effects of MSX. This is the first report of the protective effects of this natural sweetener extract on AGE-induced cytotoxicity and its scavenging properties against AGEs.

2. Materials and methods

2.1. General materials

Bovine serum albumin (BSA), D-fructose, aminoguanidine hydrochloride (AG), methylglyoxal (MGO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-alanine, resveratrol (RESV), quercetin, and 2′,7′-dichlorofluorescin diacetate (DC-FDA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). G.K. peptide was purchased from Bachem (Torrance, CA, USA). DMEM, phenol red-free DMEM medium and Trypsin-Versene were purchased from Life Technologies (Grand Island, NY, USA). Phosphatase inhibitor cocktail was purchased from Roche (Indianapolis, IN, USA). Laemmli buffer and blotting buffers were obtained from Bio-Rad (Hercules, CA, USA). Primary antibodies for extracellular signal–regulated kinase (ERK1/2), phospho-ERK (Thr 202/Tyr 204), p38 and phosphorylated-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). The cell counting kit (CCK-8) reagent was purchased from Dojindo Molecular Technologies (Rockville, Maryland). The preparation and chemical composition of the food-grade phenolic-enriched maple syrup extract (MSX) has been previously reported by our group (see Fig. S1 and Table S1 in the Supplementary Information).[20, 28]

2.2. BSA-Fructose assay

The BSA-fructose assay was used to evaluate the inhibitory ability of MSX on AGE formation according to the methods described previously.[29] To confirm that MSX alone will not induce BSA glycation, blank solutions containing 10 mg/mL BSA and different concentrations of MSX (50-500 μg/mL) were incubated at 37 °C for 7 days. For the experimental groups, each reaction mixture contained 10 mg/mL BSA and 100 mM D-fructose with or without MSX at varying concentrations (50, 100, 200, 300, 400 and 500 μg/mL). Synthetic and natural antiglycating agents, aminoguanidine (AG) and quercetin, respectively, at concentrations of 50 μg/mL each, served as the positive controls. All reaction mixtures were prepared in triplicate under sterile conditions and incubated at 37 °C for 7 days. After incubation, the intrinsic fluorescence measurement was performed on a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 360 nm and an emission wavelength of 430 nm, which are the characteristic fluorescence wavelengths for AGEs derived from BSA.[30] For all measurements, the background absorbance generated from MSX (with corresponding concentrations) was subtracted. All data were compared to a negative control containing BSA and D-fructose only and the AGE inhibition level was calculated using the following equation: % inhibition = [1-(fluorescence intensity of solution with treatment/fluorescence intensity of negative control solution)] × 100%.

2.3. BSA-MGO assay

In this assay, MGO was used as the glycating agent to induce the formation of BSA AGEs following published methods with minor modifications.[31] Each reaction mixture contained BSA (10 mg/mL) and MGO (5 mM). Different concentrations of MSX ranging from 50 to 500 μg/mL were added to the BSA-MGO solutions and blank solutions included either 10 mg/mL BSA or different concentrations of MSX. AG and quercetin, at concentrations of 50 μg/mL each, served as the positive controls. After incubation at 37 °C for 72 h, samples were analyzed for the formation of MGO induced AGEs using a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 340 nm and 430 nm, respectively. The aforementioned wavelengths are optimal for the detection of MGO derived AGEs and the inhibition level was calculated using the following equation: % inhibition = [1-(fluorescence intensity of solution with treatment/fluorescence intensity of negative control solution)] × 100%.

2.4. G.K. peptide-MGO assay

The G.K. peptide-MGO assay was performed to evaluate the formation of peptide cross-linking structures produced in the late stage of glycation as described previously.[29] Briefly, 40 mg/mL G.K. peptide was incubated with 5 mM MGO in 0.1 M phosphate buffer, pH 7.4 at 37 °C. Different amounts of MSX or the positive control, AG, were added to the G.K. peptide-MGO mixtures with their final concentrations adjusted to 10, 20, 50, 100, 200 and 500 μg/mL. After incubation for 3 h, the fluorescence of each sample was obtained using a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 340 and 420 nm, respectively.

2.5. Circular dichroism (CD) experiments

CD analysis was performed on a Jasco J-720 spectropolarimeter (Tokyo, Japan) based on the previously published method[32] and the interpretation of results was performed by the Spectra Manager software. CD spectra were obtained for the following samples: 1) native BSA, 2) glycated BSA without MSX treatment (negative control), 3) glycated BSA treated with 500 μg/mL MSX, and 4) glycated BSA treated with AG (50 μg/mL, positive control). Prior to the CD measurements, all samples were incubated in the dark at 37 °C for 7 days and then diluted with 0.2 M phosphate buffer to obtain a final BSA concentration of 0.2 mg/mL. Using a quartz cuvette with 1 mm path length, the CD spectra from 190 to 250 nm were obtained by averaging 5 consecutive scans.

2.6. Differential scanning calorimetry (DSC) measurements

Differential scanning calorimetry (DSC) measurements were carried out using a Nano-DSC from TA Instrument (Lindon, UT, USA) on the following samples: 1) native BSA, 2) glycated BSA without MSX treatment (control), and 3) glycated BSA treated with 500 μg/mL MSX. Prior to analyses, BSA concentrations were adjusted to 2 mg/mL. Temperature scans between 25 °C and 95 °C were performed at heating rates of 2 °C/min. All of the samples were degassed for 15 mins before loading on to the cells. Raw data was collected in the form of microwatts vs. temperature. The calorimetric data baselines were corrected by

subtracting PBS buffer (0.1 M, pH 7.4) and DSC curves were analyzed with Nano Analyzed software V3.6.0 by two-state scaled model as previously reported.[33]

2.7. DPPH free radical scavenging assay

The anti-oxidant activity of MSX was evaluated using a free radical scavenging (2,2-diphenyl-1-picrylhydrazyl, DPPH) assay based on our previously reported method with slight modifications.[34] Briefly, DPPH (0.25 mM) was dissolved in 50% aqueous methanol and MSX solutions were prepared in concentrations ranging from 8 to 500 μg/mL in 70% aqueous methanol. Then a reaction mixture consisting of MSX and DPPH solution (100 μL each) was placed at room temperature for 30 min in a 96-well plate. A solution of 70% aqueous methanol served as the control. The absorbance was then read at 520 nm using a micro-plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA). The scavenging capacity of the sample was calculated as follows: [(ΔAcontrol − ΔAsample)/ΔAcontrol] × 100%.

2.8. Electron paramagnetic resonance (EPR) measurements

Electron paramagnetic resonance (EPR) measurements were carried out based on our previously reported method with slight modifications.[34] Prior to analyses, freshly prepared MGO (0.2 M) were mixed with L-alanine (0.2 M) with or without MSX (500 μg/mL). Upon mixing, the reaction solutions were immediately transferred into a bent 0.86 Teflon capillary (Zeus Scientific Inc., Somerville, NJ, USA) and inserted into the EPR cavity. The EPR spectra were obtained on a Bruker EMX spectrometer (Bruker Inc., Billerica, MA, USA) and recorded using the WIN-EPR software. Spectral determinations were obtained at 9.8 Ghz with 100 khz modulation frequency and 20.8 mW microwave power. Two modulation amplitudes, namely, 1 G for radical identification and 10 G for quantification, were used. While some experiments were conducted at a constant time of 81.9 ms and sweep time of 41.9 seconds, other spectra were accumulated at 40 sweeps of 1342 seconds each to minimize noise. Timed comparisons were conducted at 3470 G which is the maximum for the 10 G spectra.

2.9. Cell culture

The normal/non-tumorigenic human colon CCD-18Co cell line was obtained from ATCC (Manassas, VA, USA) and cultured in DMEM medium in the presence of 5% CO_2 at 37°C. The medium was supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and non-essential amino acids.

2.10. Measurement of reactive oxygen species (ROS) levels

The CCD-18Co cells were seeded into 96-well plates at a density of 10^4 cells/well in DMEM with 10% FBS. After 24 h, the cells were pretreated with MSX (50–200 μg/mL) or the natural phenolic compound, resveratrol (RESV; 10 μM), used as positive control, for 24 h. Subsequently, the cells were challenged with H_2O_2 (100 μM) for 1 h and the medium was replaced with DMEM/F12 containing DC-FDA (20 μM) reagent. The formation of ROS was determined at excitation and emission wavelengths of 485 nm and 535 nm, respectively, after subtracting the background.

2.11. Cell proliferation assay

The CCD-18Co cells were seeded into 96-well plates at a density of 8×10^3 cells/well in phenol red-free DMEM with 10% FBS. After 24 h, the cells were treated with 50 μM MGO or 200 μM AGEs for 12 h. The medium was replaced with FBS-supplemented DMEM medium containing MSX (0–500 μg/mL) and incubated for 48 h. At the end of the treatment periods, the cells were incubated with CCK-8 (for the AGE treatment) or 10 μL (5 mg/mL) of the MTT reagent for 3 h (for the MGO treatment). Cell viability was determined by measuring absorbance at 490 nm or 570 nm (background absorbance was subtracted at 690 nm) for the CCK-8 or MTT reagents, respectively.

2.13. Western blotting

The CCD-18Co cells were seeded in 6-well plates at a density of 10^5 cells/well and treated with 50 μM MGO or 200 μM AGEs. To detect ERK1/2 or p38 phosphorylation, the cells were treated with MGO or MSX for 15 min in serum-free DMEM. The cells were washed with PBS before harvesting and lysed in RIPA buffer supplemented with protease inhibitor, 2 mM PMSF, and phosphatase inhibitor. The lysate was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was heated to 95°C with Laemmli buffer containing 5% β-mercaptoethanol. The same amount of protein was applied to each well before electrophoresis. Semi-dry Transfer Cell was used to transfer protein to nitrocellulose membrane at 20 V for 40 min. The membrane was blocked in 5% non-fat milk for 1 h and incubated with primary antibodies for ERK-1/2, phospho-ERK1/2 (Thr 202/Tyr 204), p-38 or phospho-p38 at 4°C overnight. The membrane was washed three times for 10 min with 0.1% PBST (PBS supplemented with 0.1% tween 20) before incubation with goat anti-rabbit secondary antibody (Licor, Lincoln, NE, USA) for 1 h. The membrane was scanned in an Odyssey Infrared Imager after washing with PBST for 3×10 min.

2.14. Data analysis

All experiments were repeated at least three times. Western blot data were analyzed and quantified by Odyssey Infrared Imaging Software. One-way ANOVA was performed by GraphPad Prism 5 with Tukey's post hoc test on all data and $p<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Preparation and standardization of MSX

Our group has previously reported on the development and chemical characterization of a food-grade phenolic-enriched maple syrup derived extract (MSX) which contains over 40 phenolic compounds predominantly belonging to the lignan sub-class (HPLC-DAD chromatogram and identification of the compounds in MSX are provided in the Supplementary Information Fig. S1).[20] We also reported that MSX was well tolerated and non-toxic in rats (at doses of up to 1000 mg/kg/day for 7 days)[20] and that it showed in vitro anti-inflammatory effects superior to any of its individual phenolic constituents alone.[25] Therefore, we have taken a whole-food approach towards studying the biological effects of this botanical natural product as we reported in our recent evaluation of the neuroprotective

effects of MSX.[28] With regards to the phenolic content of the extract, we have previously reported on the development of a maple product specific standard, named MaPLES (maple phenolic lignan-enriched standard), for the quantification of phenolic content of maple-derived food products since MSX contains predominantly lignans which cannot be adequately quantified based on gallic acid equivalents (GAEs).[35] Therefore, MSX contains 92.4% phenolic content based on MaPLES equivalents; see Supplementary Information Table S1). Notably, MSX contains a similar chemical compositional profile of bioactive substances as the whole food natural sweetener, maple syrup, but with reduced sucrose content (<0.1%).

3.2. MSX inhibits fructose and MGO induced AGE formation

In the early and middle stages of glycation, the aldehyde groups of reducing sugars non-enzymatically condense with free amino groups of proteins, forming unstable Schiff bases and Amadori compounds. These glycation intermediates could react with reactive carbonyl species such as MGO, which are generated from either sugar degradation or cellular metabolism. The rapid and irreversible reaction between MGO and protein glycation intermediates consequently leads to the formation of AGEs which exhibit characteristic fluorescence absorbance at certain wavelengths (360/430 nm).[30] To evaluate the effect of MSX (from 50-500 μg/mL) on the formation of AGEs, we used the BSA-fructose and BSA-MGO solutions as model reaction systems with fluorescence measurement carried out at the aforementioned wavelengths. It should be noted that the blank solutions containing BSA and MSX alone did not generate AGE related fluorescence, confirming that MSX alone did not induce BSA glycation (data not shown).

As shown in Fig. 1A, MSX inhibited the formation of AGEs in a concentration dependent manner with the highest inhibition occurring at 500 μg/mL MSX. At this concentration, MSX reduced the production of AGEs by about 40%. As previously noted (see Sec. 3.1), MSX contains 92.4% phenolics, based on MaPLES equivalents (see Supplementary Information, Table S1), given that its major polyphenolic constituents are lignans. However, because flavonoids are also widely regarded as a polyphenol sub-class with well-known antiglycating effects[36], we included the natural flavonoid, quercetin, as a positive control. At equivalent concentrations of 50 μg/mL, quercetin showed better anti-glycative effects compared to MSX (38.5% vs 7.2%, respectively) which was not surprising given that quercetin is a pure compound whereas MSX is an extract containing multiple constituents present in the whole food maple syrup. Notably, the anti-glycation effect of MSX (lowest concentration of 50 μg/mL) was comparable to AG, a synthetic agent designed to control AGE related complications.

Next, the BSA-MGO assay was used to evaluate the inhibitory effects of MSX on MGO induced AGE formation. Since MGO is much more reactive than reducing sugars[6], a lower MGO concentration (5 mM) was used and the samples were incubated for a comparatively shorter period (72 h). As shown in Fig. 1B, the highest inhibition (30%) was observed when the concentration of MSX was 300 μg/mL (equals to 282.6 μg of phenolics based on MaPLES equivalents) or higher. As expected, because AG is a pure synthetic compound with known antiglycating effect, an equivalent level of inhibition was observed but at a

lower concentration of 50 μg/mL. At a lower concentration of 50 μg/mL, MSX also showed an inhibitory effect and reduced AGE formation by more than 10%.

Since reactive carbonyl species, including MGO, could contribute to the cross linking of peptides that containing lysine and arginine residues thereby leading to the generation of late stage AGEs[37], the anti-cross-link effects of MSX was evaluated using the G.K. peptide model in the presence of MGO as the glycator. As shown in Fig. 2, 1.6, 3.3, 10.0, 20.3, 27.3, and 45.4% of cross-linked AGE production was inhibited with the treatment of 10, 20, 50, 100, 200, and 500 μg/mL MSX, respectively. Compared to MSX, the positive control, AG, which is a known peptide cross-linking inhibitor, had equivalent potency in the inhibition of late stage AGEs production. At 10, 20, 50, 100, 200, and 500 μg/mL concentrations, AG exhibited inhibitory effects of 3.4, 2.2, 7.8, 15.1, 28.0, and 55.1%, respectively, against the formation of AGEs. These results demonstrate that MSX can effectively inhibit the formation of MGO-induced late stage AGEs.

3.2. MSX protects the secondary structure of BSA

Advanced glycation can alter protein secondary structure, cause structural aggregation, and ultimately lead to the loss of their function.[38] Thus, in this experiment we evaluated the protective effects of MSX against AGE formation induced structural alteration of model protein BSA using CD and DSC. As shown in Fig. 3A, native BSA yielded a CD spectrum with two prominent far-UV CD signals at 208 nm and 224 nm, which represented secondary constituents of α-helical structures. The CD spectral profile of a mixture containing glycated BSA showed similar CD patterns as that of natural BSA, but with less pronounced signal intensities. Such a change suggested that the α-helical structure of BSA decreased due to non-enzymatic modification by glycation. However, while glycation occurred in the presence of MSX, a much smaller change in the CD spectrum was observed, indicating that the secondary structure of the protein was stabilized and protected by MSX. The positive control, AG, also showed similar protective effects as MSX, yielding a CD spectrum almost superimposed to that of MSX treated BSA (data not shown).

Next, we used DSC to compare thermal denaturation patterns of native BSA, glycated BSA, and MSX treated glycated BSA. The areas under the curve (AUC) represent transition enthalpy values (ΔH). The DSC results are independent of concentration and thus provide reliable thermal and thermodynamic parameters. As shown in Fig. 3B, native BSA is relatively stable toward thermal unfolding and undergoes two transitions showing bimodal DSC curves with two transition midpoint temperatures (Tms) around 65 °C and 74 °C, which is in agreement with published data.[39] However, the glycated BSA, with or without MSX, both showed two distinguishable Tms around 67 °C and 78 °C (shoulder) respectively, indicating that glycation took place in both cases. However, significant differences in enthalpies (ΔH) were observed for the two unfolding transitions. For native BSA the ΔH of Tm_1 and Tm_2 were 150.6 kcal/mol and 56.2 kcal/mol, respectively.[40] For the glycated BSA samples, ΔH showed dramatic increase compared to the Tms values of native BSA (Tm_1 233.7 kcal/mol; Tm_2 488.8 kcal/mol). However, when treated with MSX, glycated BSA showed ΔH values between native BSA and glycated BSA (Tm_1 168.1 kcal/mol; Tm_2 230.9 kcal/mol), indicating a smaller structural change in MSX treated BSA.

Moreover, glycation of BSA caused a large broadening of the second unfolding transition, which indicated that glycated BSA exhibited a noticeable unfolding behavior with reducing folding stability. However, after the treatment with MSX, the folding stability was partially recovered. The thermal parameters for all sample measurements are summarized in the Supplementary information Table S2.

3.3. MSX shows anti-oxidative effects in the DPPH, EPR, and cellular assays

MSX may inhibit the formation of AGEs through multiple pathways. Free radicals generated from early glycation is known to accelerate protein modification and AGE formation[41] and thus, the scavenging activity of MSX against free radicals may contribute to its anti-glycation effect. Therefore, we sought to characterize the anti-oxidative and free radical scavenging capacities of MSX. First, the anti-oxidant effects of MSX were evaluated in the DPPH free radical scavenging assay. As shown in Fig. 4, MSX scavenged free radicals in a concentration-dependent manner. At concentrations ranging from 8 to 500 μg/mL, MSX trapped 9.2 and 86.8% of free radicals, respectively, with an IC_{50} value of 61.7 μg/mL which was in agreement with previously published data.[20]

The free radical trapping capacity of MSX was measured during the glycation process, where free radicals are generated as intermediates and further catalyze the formation of AGEs. In order to rapidly produce verifiable yields of free radicals, MGO was reacted with L-alanine under basic conditions to produce free radical species which act as AGE precursors and facilitate the formation of AGEs.[42] As shown in Fig. 5, the EPR signal continued to increase in both treated and non-treated groups for 3 mins then decreased, a typical pattern observed in the production of carbon centered crosslink structures. However, from comparison of the EPR signal with time at 3470 G, the MSX treated sample showed a reduction of the maximum amplitude of about 20% compared to the non-treated group, indicating a decrease in free radical formation. After about 8 minutes, the two curves became essentially identical, probably because the MSX was depleted. These observations demonstrated that MSX has the ability to scavenge free radicals produced in the course of glycation thus largely contributing towards its anti-AGE capacities.

The results of the DPPH and EPR assays confirmed that MSX exerted anti-oxidation effects in a cell free system. To further evaluate its anti-oxidation effect in cells, we next used the DC-FDA assay to measure the intracellular ROS levels in MSX-free and MSX-treated normal/non-tumorigenic human colon CCD-18Co cells. As shown in Fig. 6, in CCD-18Co cells, 50-200 μg/mL MSX treatment decreased ROS level in a concentration-dependent manner. MSX (at 200 μg/mL) decreased ROS levels by 58%, as compared to the unprotected cells, which was comparable to the positive control, RESV (at 10 μM).[43]

3.4. MSX protects normal/non-tumorigenic human colon CCD18 Co cells against AGE and MGO induced cytotoxicity

MSX has previously been shown to be non-toxic in animal studies at doses of up to 1000 mg/kg/day for 7 days.[20] Moreover, MSX shows neuroprotective effects in *Caennorhabditis elegans* and alleviate lipopolysaccharide-induced inflammation and cell stress in murine microglial and human neuronal cells.[28] As previously discussed, AGEs could trigger cell

stress by inducing oxidative stress and activating inflammatory signaling pathways.[43] Therefore, in the current study, we evaluated the protective effects of MSX against AGE-induced cytotoxicity on normal/non-tumorigenic colon CCD-18Co cells. As we previously reported, the cell proliferation assay was validated by using a known ERK inhibitor, namely, PD 184161.[44] We confirmed the cytotoxicity of MGO and AGE on the CCD-18Co cells by this assay where the untreated cells showed the highest viability (see Supplemental Information, Figure S2). In the pre-treatment groups, the cells were first challenged with 200 μM AGE for 12 h, then incubated with MSX for 48 h. As shown in Fig. 7, MSX (at 10–100 μg/mL) increased cell viability by 21 - 32%. In the co-treatment groups, cells were treated with MSX and AGE together for 60 h and the co-treated cells showed higher viability than the cells treated with AGE alone. In a separate experiment, the cells were challenged with MGO and, similarly, MSX (at 10-100 μg/mL) exhibited protective effects against MGO induced cytotoxicity by increasing cell viability by 12 - 29%, respectively. Therefore, the cell viability data demonstrated that MSX alleviated cytotoxicity induced by AGE and the AGE precursor, MGO, in normal human colon CCD-18Co cells.

3.5. MSX activates ERK 1/2 phosphorylation

ERK1/2 is a member of the MAPK signaling pathway and the ERK1/2 cascade functions in responding to extracellular stimulant induced cell signaling. Furthermore, the rapid activation of ERK1/2 is associated with cell proliferation, cell transformation, and protection against cell apoptosis.[45] It has been reported that reducing oxidative stress markedly increased extracellular signal-regulated kinase-1 and 2 (ERK1/2) activation.[45] Therefore, we next evaluated the effects of MSX on ERK1/2 activation in the normal human colon (CCD-18Co) cells. As shown in Fig. 8, ERK1/2 was not activated upon AGE (200 μM) or MGO (50 μM) treatment in the CCD-18Co cells at 15 mins or 12 h. However, MSX treatment for the equivalent time of 15 mins induced ERK1/2 phosphorylation which was 3–4 fold higher than the non-treated control cells. Given that p38, another member of the MAPK signaling pathway, is also known to play an important role in oxidative stress,[46] we also evaluated the effects of MSX on phosphorylation of p38. However, after treatment with MSX for 15 mins and 12 h, p38 phosphorylation was not observed in the CCD-18Co cells (see Supplementary Information, Figure S3). We next evaluated whether MSX had a similar effect on ERK1/2 activation in the AGE or MGO pretreated cells. Therefore, using the same AGE or MGO treatment conditions (as the cell proliferation assay), CCD-18Co cells were pretreated with MGO or AGE for 12 h and then treated with MSX for 15 mins. Notably, MSX increased ERK1/2 phosphorylation of the AGE or MGO-pretreated cells by 3-5 fold compared to non-treated control cells. Additionally, we did not observe sustained ERK1/2 phosphorylation in the 12 h co-treatment group of AGE/MGO and MSX which was expected because proliferative ERK1/2 phosphorylation will disappear in hours and constant ERK1/2 phosphorylation will result in cell apoptosis.[39] Notably, similar to our current observations with MSX, the activation of ERK1/2 has also been observed with other phenolic-rich natural products.[47, 48]

4. Conclusion

The formation and accumulation of AGEs is associated with several chronic human illnesses including diabetes and neurodegenerative disorders. Natural products and dietary agents, including phenolic-rich foods such as berries, fruit, vegetables, and their derived beverages, have been reported to show anti-AGE effects.[13, 14] However, there is lack of similar data on phenolic-containing natural sweeteners such as the plant-derived natural product, maple syrup. Given the wide popularity and consumption of this sweetener, we initiated this study on MSX, a standardized food-grade phenolic-enriched maple syrup derived extract (previously developed by our group for nutraceutical applications), which contains the natural chemical composition of the whole maple syrup food but with reduced sucrose content.[20, 28]

In summary, we found that MSX inhibited the formation of AGEs, scavenged free radicals, decreased cellular ROS levels, and protected normal human CCD-18Co colon cells from AGE-induced cytotoxicity in vitro through the stimulation of ERK1/2 phosphorylation. The current study has limitations since it does not consider critical physiologically relevant issues such as bioavailability and metabolism that can only be obtained from in vivo studies. Nevertheless, the current data provide valuable insights into the mechanisms underlying the anti-AGEs effects of MSX. Overall, this study supports the anti-AGE effects of phenolic-rich plant foods including the natural sweetener, maple syrup.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MSX	Maple syrup extract
AG	Aminoguanidine
BSA	Bovine serum albumin
AGEs	Advanced glycation endproducts
RCS	Reactive carbonyl species
ROS	Reactive oxygen species
MGO	Methylglyoxal
CD	Circular dichroism

EPR	Electron paramagnetic resonance
MAPK	Mitogen-activated protein kinase
ERK	extracellular signal–regulated kinase
DSC	Differential scanning calorimetry

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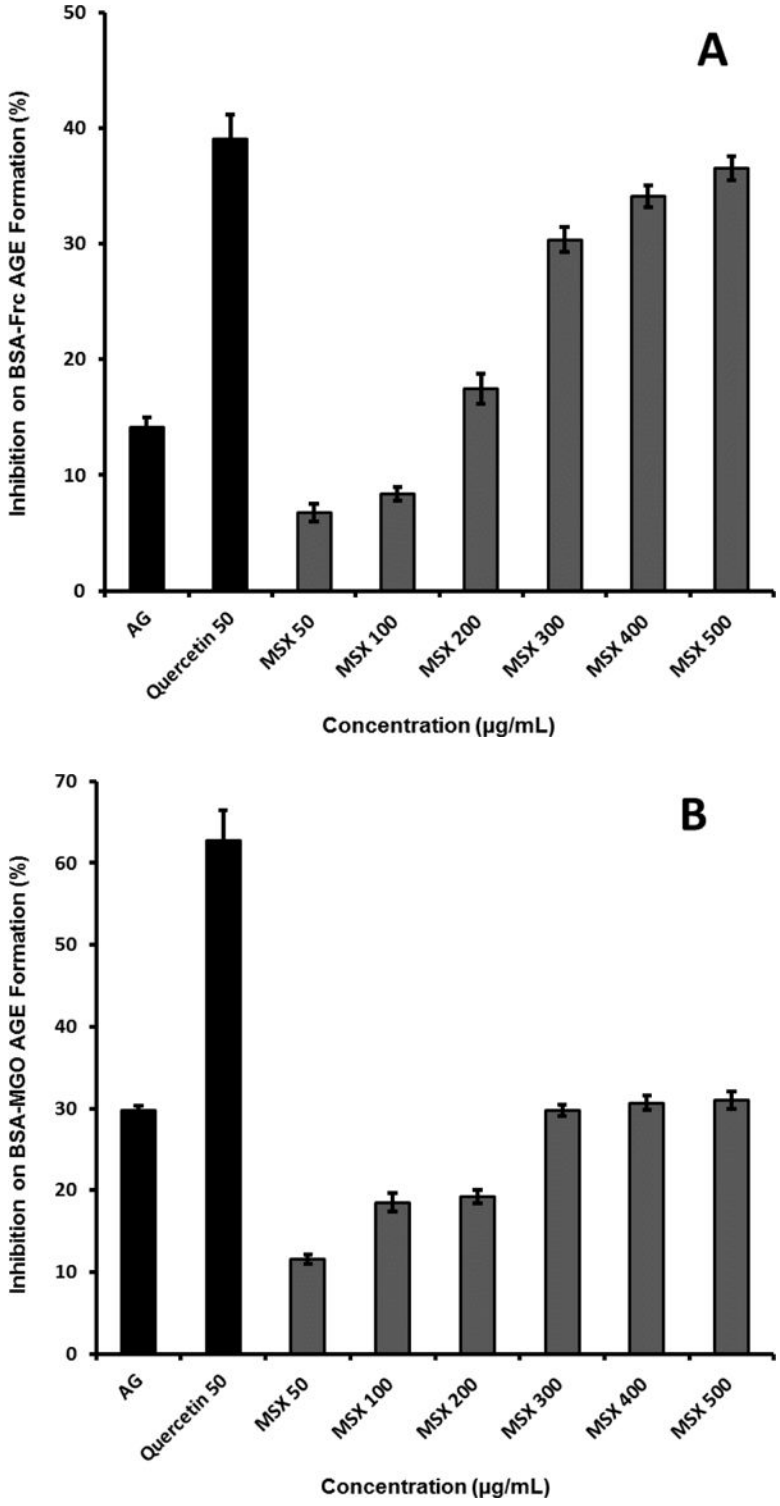

Figure 1.

Inhibition of different concentrations of MSX on the formation of D-fructose (A) and methylglyoxal (B) induced AGEs. Inhibition was determined on the basis of intrinsic fluorescence intensity of each sample relative to that of the negative control solution set at

100%. All data points represent the average of triplicate measurements with the bars at each point representing the respective standard derivation. AG and quercetin (50 μg/mL) served as positive controls.

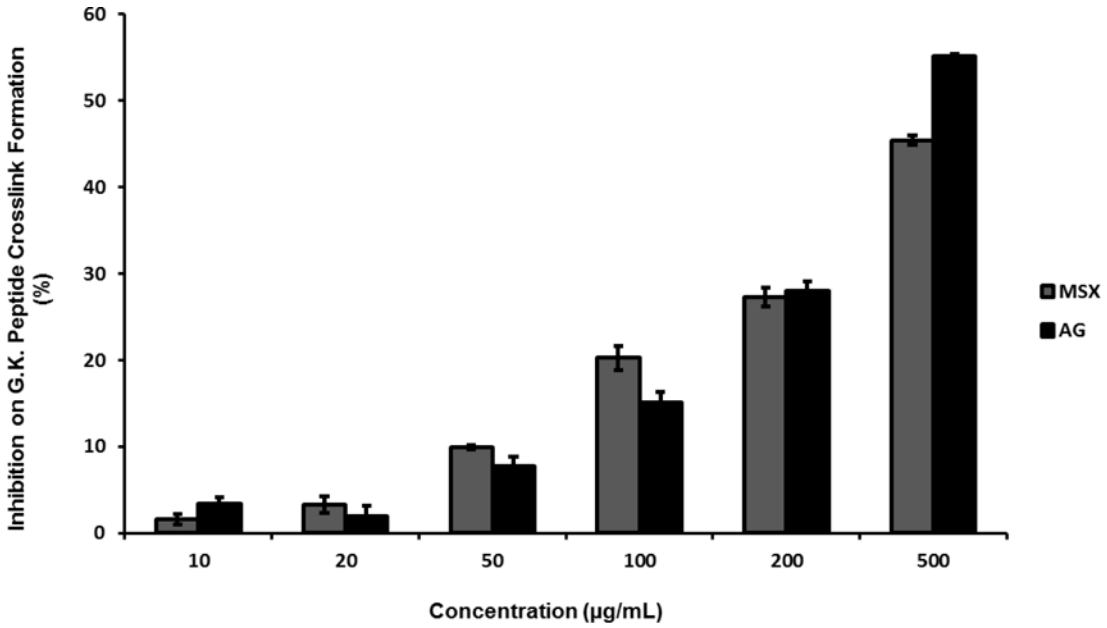

Figure 2.

Inhibitory effects of different concentrations of MSX or AG (positive control) on the formation of AGE crosslinks in the G.K. peptide system. Intrinsic fluorescence of each sample was measured at an excitation and emission wavelengths of 340 and 420 nm, respectively. Results are means ± SD for three independent tests.

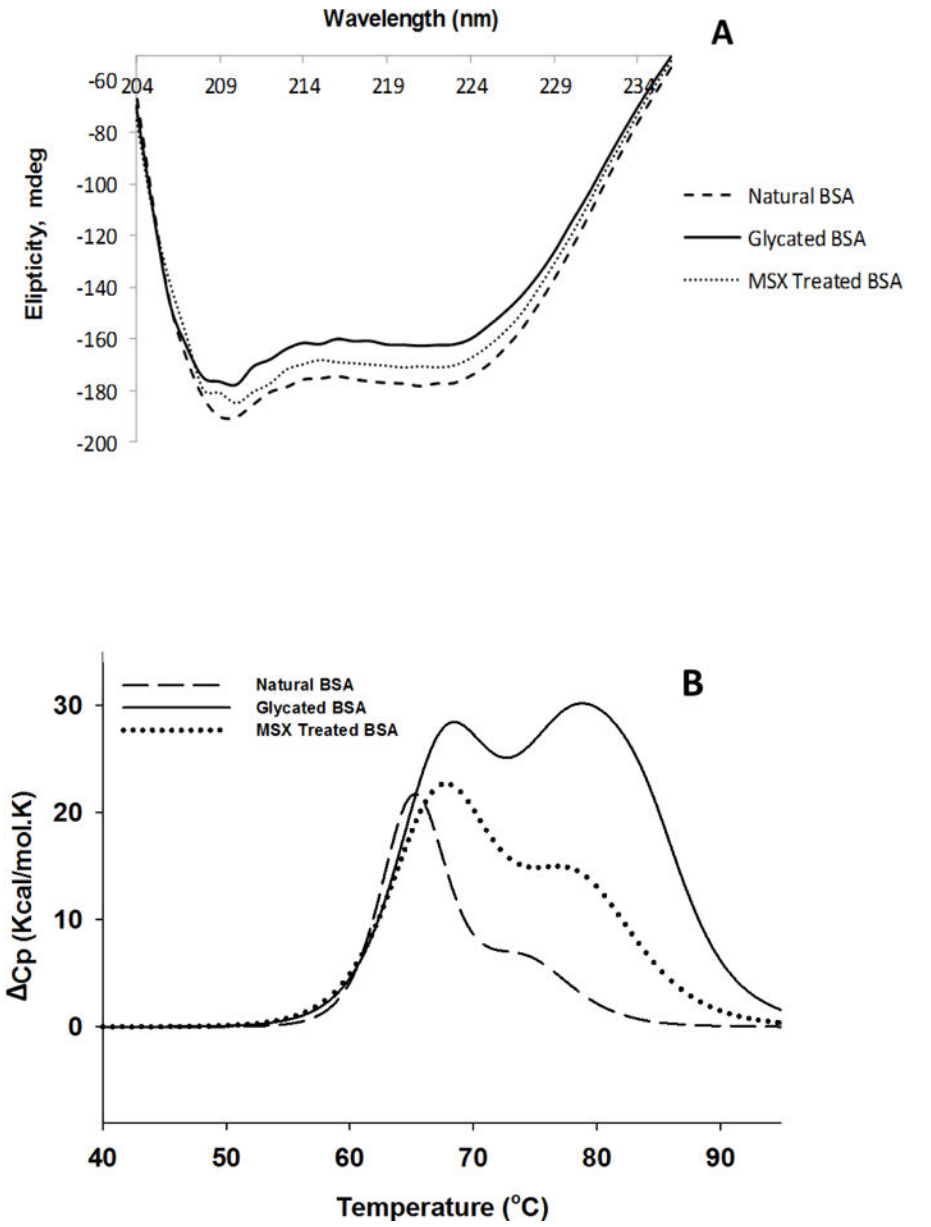

Figure 3.

The inhibitory effects of MSX on glycation induced structural change of BSA characterized by (A) CD spectrum and (B) DSC. Analyses were conducted on natural BSA, glycated BSA, and MSX-treated glycated BSA. Repeat scans revealed no significant difference in the spectra.

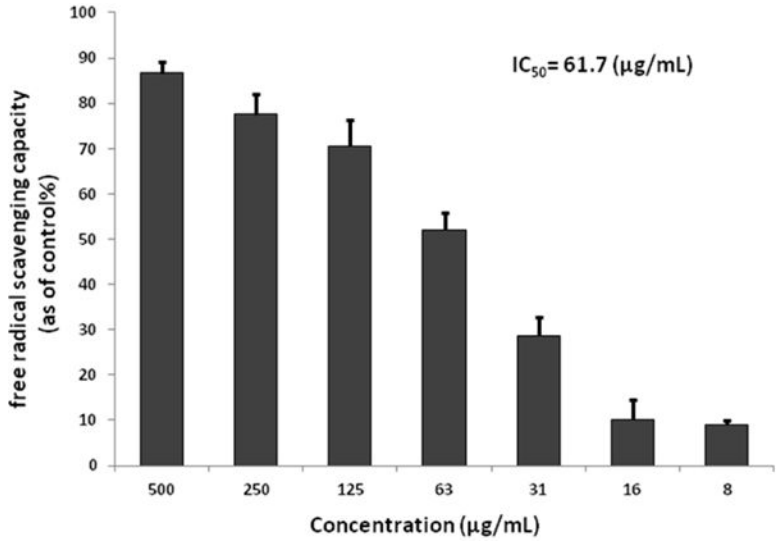

Figure 4.
Free radical scavenging activity of MSX in the DPPH assay.

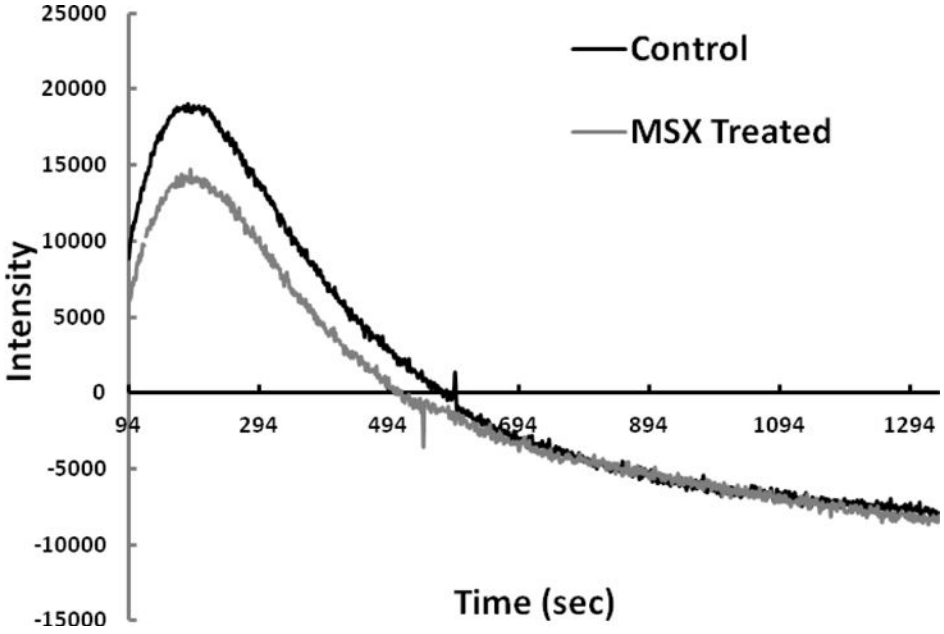

Figure 5.

EPR time scan monitoring the formation of free radicals generated from the glycation reaction of L-alanine and methylglyoxal (MGO). Control solution contained L-alanine and MGO only while treatment included 500 μg/mL MSX.

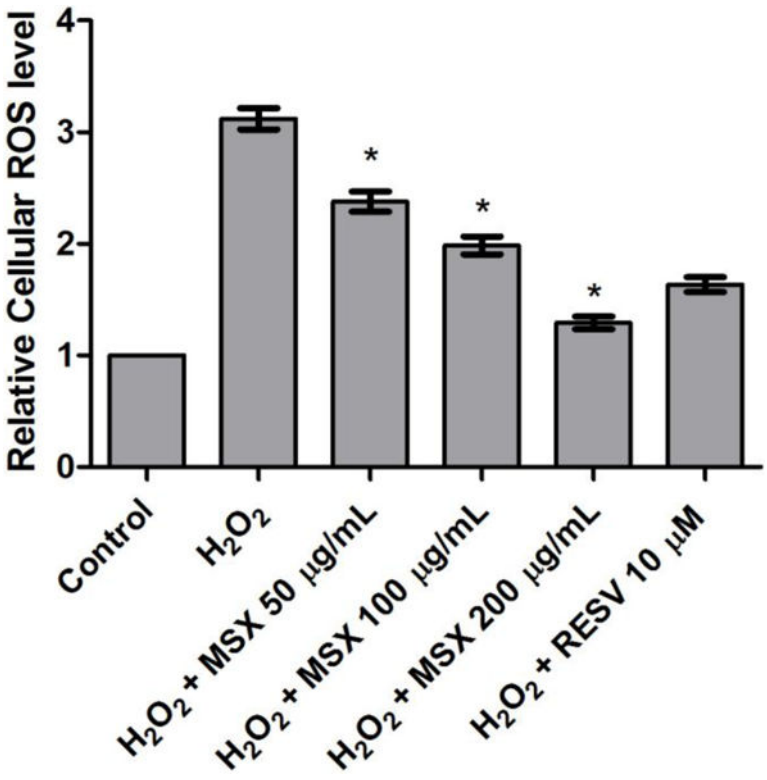

Figure 6.

MSX decreased H_2O_2 -induced ROS levels. CCD-18Co cells were pretreated by 50-200 μg/mL MSX or 10 μM resveratrol (RESV) then were treated with 100 μM H_2O_2 for 1 h. Intracellular ROS levels were detected by the DC-FDA assay. The relative cellular ROS levels of the H_2O_2 and treatment groups were normalized to untreated control and plotted as mean ± SE (n=4).

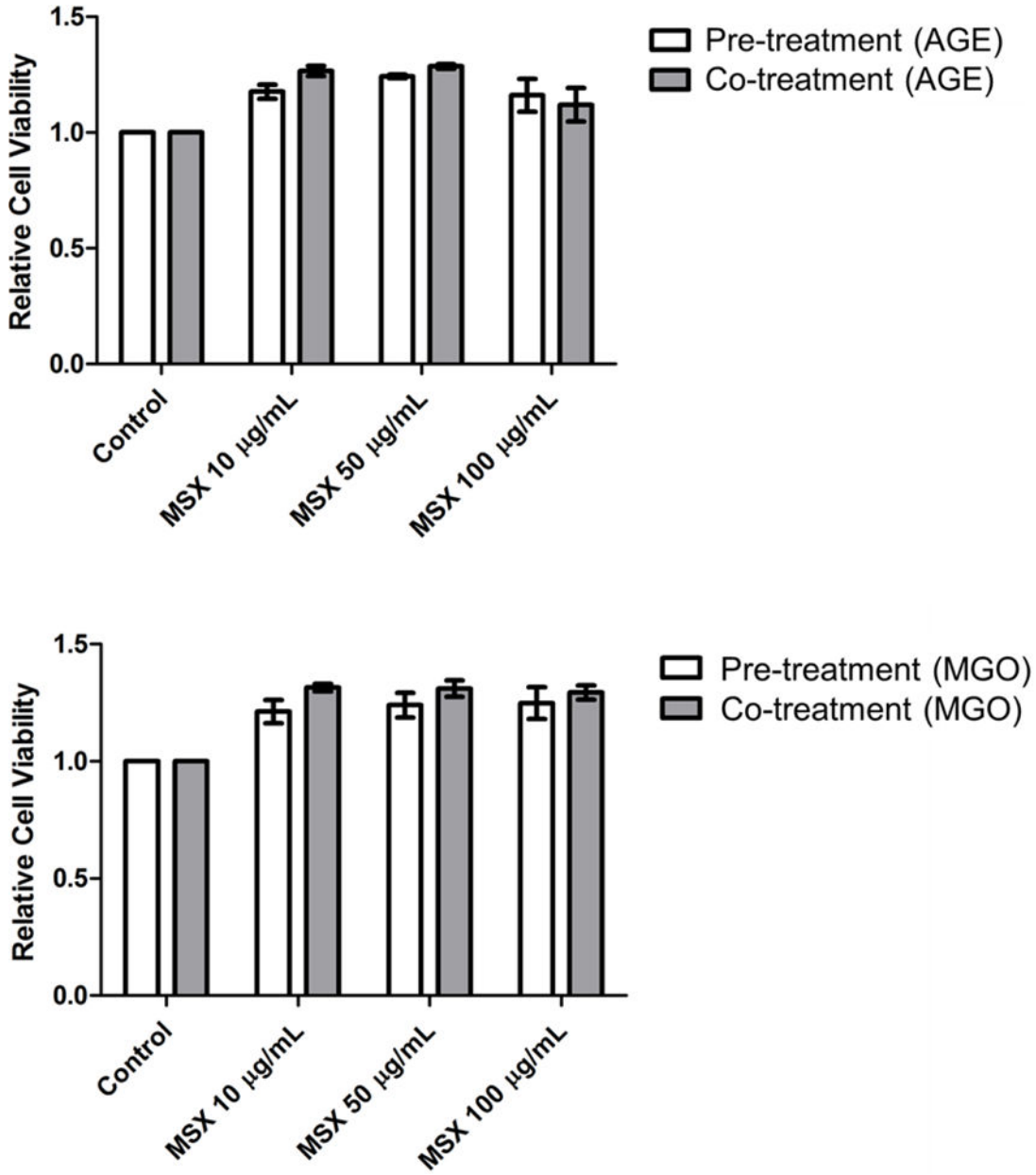

Figure 7.

Protective effects of MSX on AGE or MGO-treated normal human colon (CCD-18Co) cells. Pre-treatment groups: After 12 h treatment with AGE (200 μM) or MGO (50 μM), the medium was replaced with FBS-supplemented DMEM containing 0–100 μg/mL MSX and incubated for 48 h. Co-treatment groups: The cells were incubated with AGE (200 μM) or MGO (50 μM) in FBS-supplemented DMEM medium with 10–100 μg/mL MSX for 60 h. Cell viability was measured by the CCK-8/MTT assay. Relative cell viability of treated groups were normalized to untreated control and plotted as mean ± SE (n=3).

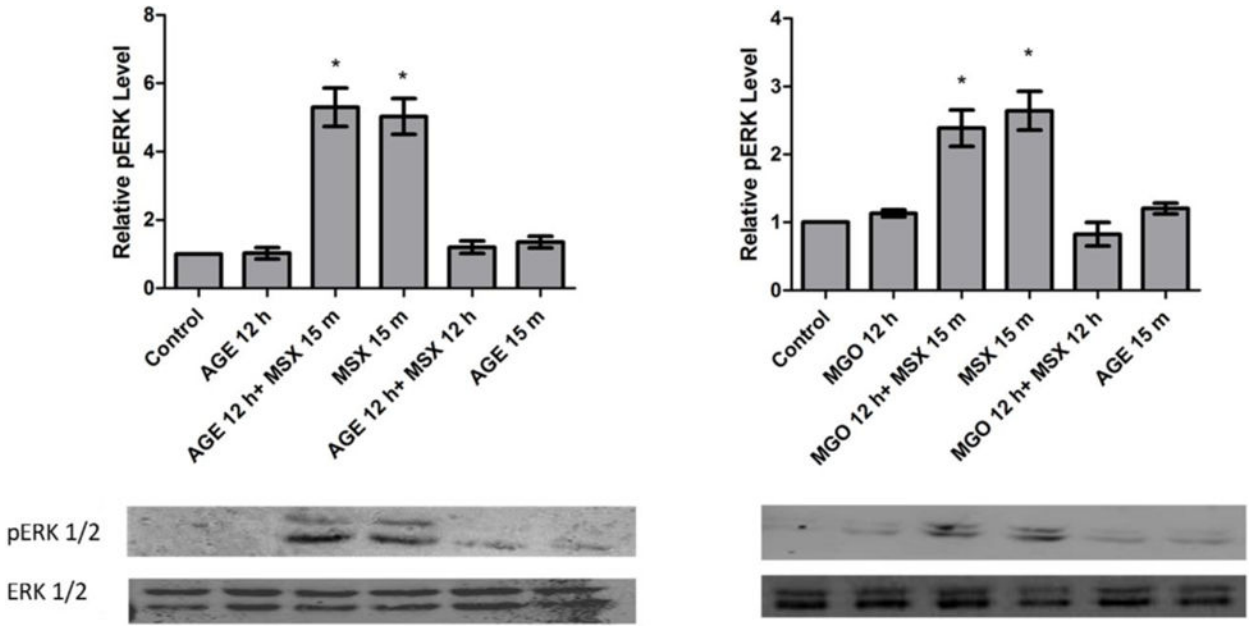

Figure 8.

The effects of MSX on ERK1/2 phosphorylation. The normal human colon (CCD-18Co) cells were treated with AGE (200 μM) or MGO (50 μM) in the absence or presence of 250 μg/mL MSX for 15 mins or for 12 h. The cell lysates were analyzed for pERK1/2 and ERK1/2. Band intensity relative to the untreated control was plotted as a bar graph showing mean ± SE.