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# Effects of zinc and manganese on advanced glycation end products (AGEs) formation and AGEs-mediated endothelial cell dysfunction

Xiuyuan Zhuang, Xiufeng Pang, Wen Zhang, Wenbin Wu, Jingjing Zhao, Huangjian Yang, Weijing Qu *

*School of Life Science, East China Normal University, Shanghai 200062, People's Republic of China*

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ABSTRACT

*Aims:* The present study investigated the effects of $ZnCl_2$ and $MnCl_2$ supplementations on advanced glycation end products (AGEs) formation and AGEs-mediated endothelial cell dysfunction.
*Main methods:* Fluorescence detection was used to monitor the Maillard reaction. Inductively coupled plasma optical emission spectroscopy was used to test cellular zinc and manganese levels. Real-time reverse transcription polymerase chain reaction and western blot were used to analyze the expression of endothelial nitric oxide synthase (eNOS), nuclear transcription factor kappa B (NF-κB), and receptor for AGEs (RAGE). Intracellular reactive oxygen species (ROS) and nitric oxide (NO) production, NOS activity were determined by fluorescent probe assay, superoxide dismutase (SOD) activity was determined by water soluble tetrazolium salt assay.
*Key findings:* $MnCl_2$ showed excellent inhibitory effect on AGEs formation. Primary cultured bovine aortic endothelial cells (BAECs) were exposed to AGEs for 30 min, followed by trace element treatments. Cell viability and the zinc levels declined due to AGEs exposure, which were improved with the supplementations of $ZnCl_2$ and $MnCl_2$. Furthermore, $ZnCl_2$ supplementation effectively enhanced intracellular NO production, elevated eNOS expression and enzymatic activity, and down-regulated NF-κB activation and RAGE expression. $MnCl_2$ dose-dependently impaired ROS formation, down-regulated NF-κB protein expression and nuclear translocation, as well as restored Mn-SOD enzymatic capability.
*Significance:* Our findings suggested that trace elements relevant to diabetic, such as zinc and manganese played different roles in the formation of AGEs. Both the elements benefited the AGEs-injured BAECs through different mechanisms.



## Introduction

Increased accumulation of advanced glycation end products (AGEs) is considered one of the essential factors in the pathogenesis of diabetes and various macro- or micro-vascular complications, including atherosclerosis, diabetic retinopathy, cataracts, diabetic renal disease, and Alzheimer's disease (Ahmed, 2005). The formation of AGEs, also called the Maillard reaction, is not a simple reaction but a series of complicated biochemical steps that take place between reducing sugar and free amino groups, resulting in the unstable Schiff bases (Lapolla et al., 2005). After spontaneous oxidation or rearrangement, a more stable Amadori product is generated. Then the Amadori product is oxidized and degraded into highly reactive dicarbonyls that react again with the amino groups to form the irreversible AGEs, possibly through intricate oxidation, dehydration, cyclization, rearrangement, and covalent bonding (Basta et al., 2004; Lapolla et al., 2005). Aside from dicarbonyls, increased reactive oxygen species (ROS, another Maillard reaction side-product) is also a risk factor in the evolution of diabetic complications (Mullarkey et al., 1990).

However, the focused mechanism of glycation correlated with diabetic complications is the engagement of AGEs with their major cell surface receptor, receptor for AGEs (RAGE). Once AGEs interact with RAGE, through the participation of NADPH oxidase (Guimarães et al., 2010), sudden increase in intracellular ROS is exhibited, leading to the activation of nuclear transcription factor kappa B (NF-κB) (Liu et al., 2010). Activated NF-κB subunits then translocate into the nucleus, followed by the enhanced expression of genes regulated by NF-κB. In the case of macro-vascular complications, endothelial cell dysfunction is associated with the accumulation of AGEs, causing impaired nitric oxide (NO) release and endothelium-dependent relaxation, elevated ROS production, NF-κB activation, and RAGE expression (Orasanu and Plutzky, 2009).

The abundance of glucose in diabetes provides a great source for glycation (Rojas and Morales, 2004) and accelerates AGEs-modification of functional proteins, nucleic acids, and other biologic molecules (Lapolla et al., 2005). A large amount of evidence has confirmed that transition metal ions play important roles in the Maillard

* Corresponding author at: School of Life Science, East China Normal University, 3663 North Zhongshan Road, Shanghai 200062, People's Republic of China. Tel.: +86 21 62232019; fax: +86 21 62233754.
*E-mail address:* wjqu@bio.ecnu.edu.cn (W. Qu).

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reaction. Metal ions, especially copper (II) ion and iron (III) ion, together with oxygen, catalyze both glucose and Amadori products into ROS and dicarbonyls (Ahmed, 2005), contributing to tissue damage and AGEs-modified molecule generation. In addition, other authors observed impaired trace elements metabolism in diabetes, with elevated levels of copper and diminished levels of zinc and manganese in the blood (Kazi et al., 2008). These alterations coexist with glycation and oxidative stress in vivo (Abou-Seif and Youssef, 2004; Viktorínová et al., 2009).

Considering that copper, zinc, iron, and manganese are period 4 transition metals, they exhibit different status in pathological processes in vivo. Except for copper and iron, the effects of the other two elements on the formation of AGEs are rarely reported. The present study investigated the effects of zinc and manganese supplementations on protein glycation and AGEs-mediated endothelial cell dysfunction.

## Materials and methods

### *Chemicals and materials*

Bovine serum albumin (BSA, fraction V) was from Sigma (St. Louis, MO, USA), Dulbecco's modified Eagle medium (DMEM, low glucose) was from Invitrogen's GIBCO (Grand Island, NY, USA). 3-(4,5-Methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). RNAiso plus, and SYBR PrimeScript reverse transcription polymerase chain reaction (RT-PCR) kit (Perfect Real Time) were from TaKaRa (Dalian, China). Rabbit anti-NF-κB p65 antibody, mouse anti-actin antibody, Cy5-labeled goat anti-mouse IgG (H + L), $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME), S-methylisothiourea sulfate (SMT), 3-amino,4-aminomethyl-2′,7′-difluorescein diacetate (DAF-FM DA), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), nitric oxide synthase assay (NOS) kit, Cu/Zn-superoxide dismutase (SOD) and Mn-SOD assay kit with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (water soluble tetrazolium salt, WST-1), and NF-κB activation nuclear translocation assay kit were from Beyotime (Haimen, China). Rabbit anti-RAGE antibody was from AnaSpec (San Jose, CA, USA), rabbit anti-endothelial NO synthase (eNOS) antibody was from Cell Signaling Technology (Boston, MA, USA), and IRDye 800CW conjugated goat (polyclonal) anti-rabbit IgG (H + L) was from Li-Cor Biosciences (Lincoln, NE, USA).

Bovine aortas were obtained from newborn oxen at a local slaughterhouse. The primary bovine aortic endothelial cells (BAECs) were harvested using trypsin–EDTA solution. After centrifugation, the cells were resuspended in DMEM supplemented with 10% newborn bovine serum (NBS) and 1% penicillin/streptomycin. The cells were identified by morphology and immunofluorescence staining for factor VIII-related antigen (Wang et al., 2006). BAECs (passaged 3–8 times) were redistributed into plates at $2\times10^5$ cells/ml. After starved in 2% NBS DMEM overnight, each well was filled with 2% NBS DMEM or 100 μg/ml AGEs solution. Half-hour later, trace element solutions were added in, and the cells were cultured in this mixture at 37 °C for 48 h for cell viability and cellular trace elements detection, 2 h for real-time RT-PCR, and 3 h for other tests.

### *Preparation and determination of AGEs*

AGEs were prepared by incubating 10 mg/ml BSA with 0.5 M glucose in 20 mM phosphate-buffered saline (PBS, pH 7.4) as a glycation mode solution. The AGEs used as stimuli on BAECs were obtained by keeping the mode solution at 37 °C for 12 month. The effects of trace elements on AGEs formation were determined by adding $ZnCl_2$ and $MnCl_2$ to the mode solutions, and incubated at 37 °C for 2, 4, and 8 wk. BSA was similarly incubated in the absence of glucose and trace elements. After incubating, the solutions were extensively dialyzed and diluted to 1 mg/ml protein. All the samples were examined using microplate reader (infinite M200, Tecan, Australia) at excitation/emission wavelengths of 370/440 nm and 335/385 nm (Hamada et al., 1996), and the fluorescence intensity (FI) values of each group were obtained by normalizing fluorescence values to those of their relevant BSA controls.

### *Cell viability assay*

MTT assay was used to detect cell viability. The treated BAECs were rinsed and incubated with 0.5 mg/ml MTT in serum-free DMEM at 37 °C for 4 h. The formazan was dissolved in 150 μl dimethyl sulfoxide with vibration. Absorbance values were detected at 570 nm (Wang and Zheng, 2002), and all the values were expressed as percentages of the controls.

### *Cellular trace elements detection*

The treated BAECs were collected and washed thrice. Then the cell suspensions were counted and evaporated, followed by a nitric acid digestion (Sun et al., 2002). Cellular zinc and manganese levels were tested by inductively coupled plasma optical emission spectroscopy (ICP-OES, IRIS Intrepid II XSP, Thermo Electron Corporation, USA). Results were expressed as fg per cell. Several doubtful values were deleted according to Grubbs' rule and Chauvenet's criterion.

### *SYBR green real-time RT-PCR*

Total RNA was isolated from the treated BAECs, followed by RT reaction using PrimeScript RT reagent kit. SYBR green real-time PCR was monitored on real-time PCR detection system (CFX96, Bio-Rad, USA). All results were normalized to those of GAPDH. Primers were synthesized by TaKaRa (Dalian, China) as follows:

Endothelial NOS forward primer: 5′-GTTTGTCTGCGGCGATGT-3′;
Endothelial NOS reverse primer: 5′-AAATGTCCTCGTGATAGCGTTG-3′;
Mn-SOD forward primer: 5′-AAGTTGACTGCTGTATCTGTTGGTG-3′;
Mn-SOD reverse primer: 5′-TGTAAGCGTCCCTGCTCCTT-3′;
NF-κB forward primer: 5′-AGTTTGATACCGATGAAGAC-3′;
NF-κB reverse primer: 5′-TCACCAGGCGAGTTATAG-3′;
RAGE forward primer: 5′-CGAGTCCGAGTCTATCAG-3′;
RAGE reverse primer: 5′-TCTCTTGGTCTCTTCCTTC-3′;
GAPDH forward primer: 5′-TTGTGATGGGCGTGAACC-3′;
GAPDH reverse primer: 5′-CCCTCCACGATGCCAAA-3′.

### *Protein detection by western blot analysis*

The lysates of treated BAECs were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. After blocked with 5% powdered non-fat milk (Wang et al., 2006), the membranes were incubated with primary antibodies (1:1000 diluted) at 4 °C overnight, and then with fluorescence-labeled secondary antibodies (1:10 000 diluted) at room temperature in darkness for 40 min. Each step above was followed by washing four times for 5 min each. Finally, the membranes were scanned using Infrared Imaging System (Li-Cor, Odyssey, USA), and analyzed by Odyssey Application Version 3.0.X. All results were normalized to those of β-actin.

### *Intracellular ROS, NO, and NOS determination by fluorescent probe assay*

BAECs cultured in black 96-well plates (flat clear bottom, Corning, USA) were supplemented with 20 μl DCFH-DA (10 μM) or DAF-FM DA (5 μM) followed by incubation at 37 °C for 20 min. After washing, the cells were exposed to DMEM or AGE solutions for 30 min, and treated