Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Inhibitive effects of anti-oxidative vitamins on mannitol-induced apoptosis of vascular endothelial cells

PAN Kai-yu[†1,2], SHEN Mei-ping[1], YE Zhi-hong[3], DAI Xiao-na[3], SHANG Shi-qiang[†‡1]

([1]*Affiliated Children's Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

([2]*The People's No. 1 Hospital of Xiaoshan, Hangzhou 311200, China*)

([3]*Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China*)

[†]E-mail: beyondmy2002@163.com; shangsq33@mail.hz.zj.cn

Received Nov. 6, 2005; revision accepted Apr. 14, 2006

Abstract: Objective: Study blood vessel injury and gene expression indicating vascular endothelial cell apoptosis induced by mannitol with and without administration of anti-oxidative vitamins. Methods: Healthy rabbits were randomly divided into four groups. Mannitol was injected into the vein of the rabbit ear in each animal. Pre-treatment prior to mannitol injection was performed with normal saline (group B), vitamin C (group C) and vitamin E (group D). Blood vessel injury was assessed under electron and light microscopy. In a second experiment, cell culture specimen of human umbilical vein endothelial cells were treated with mannitol. Pre-treatment was done with normal saline (sample B), vitamin C (sample C) and vitamin E (sample D). Total RNA was extracted with the original single step procedure, followed by hybridisation and analysis of gene expression. Results: In the animal experiment, serious blood vessel injury was seen in group A and group B. Group D showed light injury only, and normal tissue without pathological changes was seen in group C. Of all 330 apoptosis-related genes analysed in human cell culture specimen, no significant difference was seen after pre-treatment with normal saline, compared with the gene chip without pre-treatment. On the gene chip pre-treated with vitamin C, 45 apoptosis genes were down-regulated and 34 anti-apoptosis genes were up-regulated. Pre-treatment with vitamin E resulted in the down-regulation of 3 apoptosis genes. Conclusion: Vitamin C can protect vascular endothelial cells from mannitol-induced injury.

Key words: Anti-oxidative vitamins, Inhibitive effects, Apoptosis, Vascular endothelial cells, Mannitol
doi:10.1631/jzus.2006.B0825 **Document code:** A **CLC number:** R54

INTRODUCTION

Hyperosmotic mannitol therapy is widely used in the treatment of brain edema. Phlebitis is known as a common side effect, which may result from apoptosis among vascular endothelial cells induced by mannitol and mediated by inflammatory mediators and mitogen-activated protein kinases (MAPKs) (Malek *et al*., 1998). In the first part of this study, we observed vascular impairment induced by mannitol in rabbit vein endothelium with and without pre-treatment with potentially protective substances. In human endothelial cell cultures, we then analysed apoptosis gene expression after incubation with mannitol with and without pre-treatment with anti-oxidative vitamins.

MATERIALS AND METHODS

Animal experiment

Sixteen healthy New Zealand rabbits were randomly divided into four groups. Group A served as the control group. Mannitol was injected into the vein of the rabbit ear in each animal. Pre-treatment prior to mannitol injection was performed in group B (normal saline), group C (vitamin C), and group D (vitamin E). Blood vessel injury was assessed under electron and

[‡] Corresponding author

light microscopy.

Gene chip

1. Cell culture

Human endothelial cells from the umbilical vein were cultured and randomized into four groups named samples A, B, C and D. Culture medium was supplemented to 3 ml. We added 0.5 ml of normal saline to sample B, 0.5 ml of vitamin C (final concentration 100 μmol/L) to sample C and 0.15 ml of vitamin E emulsion (final concentration 50 μmol/L) to sample D. After 30 min, we injected 1 ml of mannitol with a final osmolar concentration of 300 mosm (Kwak *et al*., 2000) to each of the four incubators and maintained 5% CO_2, saturated humidity and 37 °C for 12 h.

2. Preparation of the probes

Total RNA was extracted with the one-step method of Trizol Reagents (Kwak *et al*., 2000). In short, the cells were washed with cold PBS twice and broken with Trizol. Total RNA was extracted with chloroform, sedimented with isopropyl alcohol one time and with 75% alcohol for two times, and was used to prepare probes hybridized with the microarray. The control group specimen was labelled with Cy3-dUTP and the other groups were labelled with Cy5-dUTP.

3. Hybridization with DNA chips

DNA chips and probes labelled with Cy3 or Cy5 were denatured for 5 min at 95 °C. The probes were then added to DNA chips and sealed with a slide. Hybridization took place at 60 °C for 15 to 17 h. After hybridization, the slide was taken off and the chips were washed. Finally, the chips were dried at room temperature and were then ready for use. Four thousand and ninty-six target cDNA clones were used on the cDNA chip. Of all 4096 genes, 224 apoptosis genes and 106 anti-apoptosis genes were included.

4. Scanning of chips and analysis of results

The DNA chips were scanned with an ScanArray 5000 scanner and analyzed with GenePix 3.0 to obtain the intensity of two kinds of fluorescence, Cy3 intensity in the control group and Cy5 intensity in the groups with pre-treatment. The PCR results were satisfactory (Fig.1). Cy5/Cy3 ratio was calculated for all genes and adjusted with the normalization coefficient. A ratio of more than 2.0 or less than 0.5 was considered as significant.

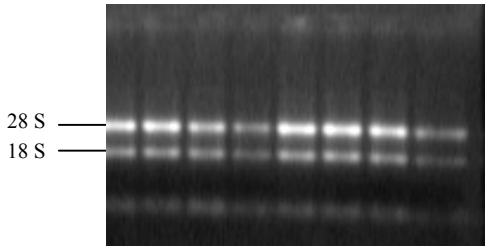


Fig.1 The gelose-electrophoresis of the PCR results

RESULTS

Animal experiment

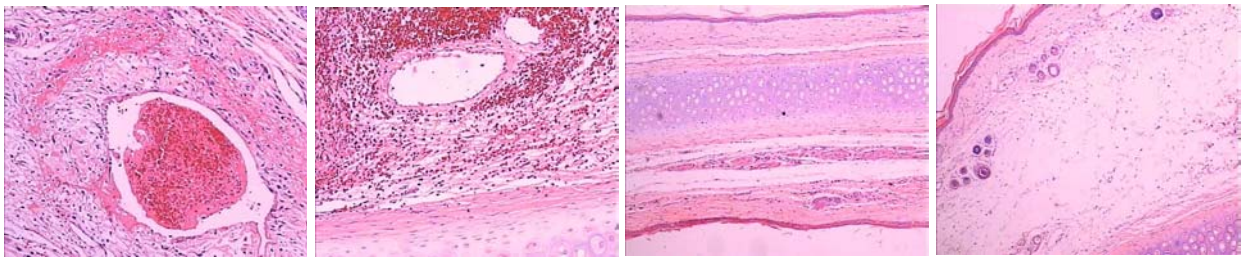
Blood vessel injury was found to be serious in group A and moderate to serious in group B. Light injury only was seen in group D, while group C showed normal tissue without pathological changes on light microscopy (Fig.2) and electron microscopy (Fig.3).

Gene chip

The hybridized probes were labelled with Cy3 (control group) and Cy5 (three pre-treated groups) fluorescence. The intensity of Cy3 and Cy5 fluorescence at the corresponding positions on the chip were added together (Figs.4a~4c). Green spots denote down-regulation of gene expression, red spots indicate up-regulation of gene expression and yellow spots represent insignificant changes in gene expression.

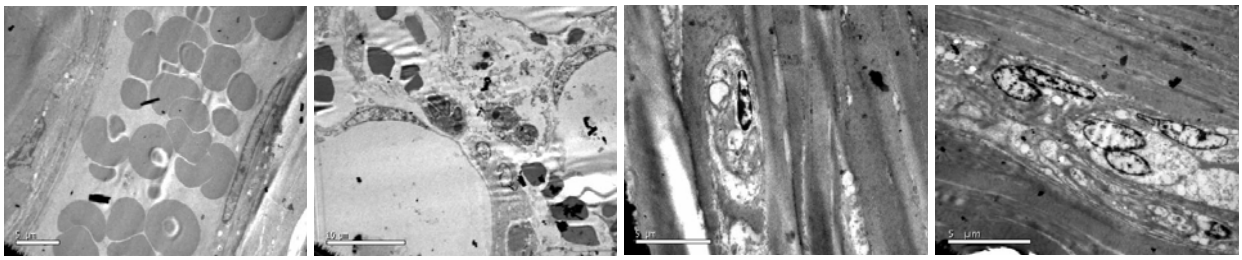
Figs.4d~4f show a scatter plot of Cy3 fluorescence intensity (*x*-axis) and Cy5 fluorescence intensity (*y*-axis) for apoptosis genes with significant changes in gene expression. In this figure, red spots stand for Cy5/Cy3 ratios between 0.5 and 2.0 showing no significantly different gene expression, while yellow spots represent Cy5/Cy3 ratios of less than 0.5 or more than 2.0, thus indicating significant differences in gene expression.

None of the 330 apoptosis-related genes showed significant differences on the chip pre-treated with normal saline, compared with the chip without pre-treatment. With vitamin C pre-treatment, 45 apoptosis genes were down-regulated and 34 anti-apoptosis genes were up-regulated. Nine important genes are listed in Table 1. Three apoptosis genes only were down-regulated on the chip with vitamin E pre-treatment.



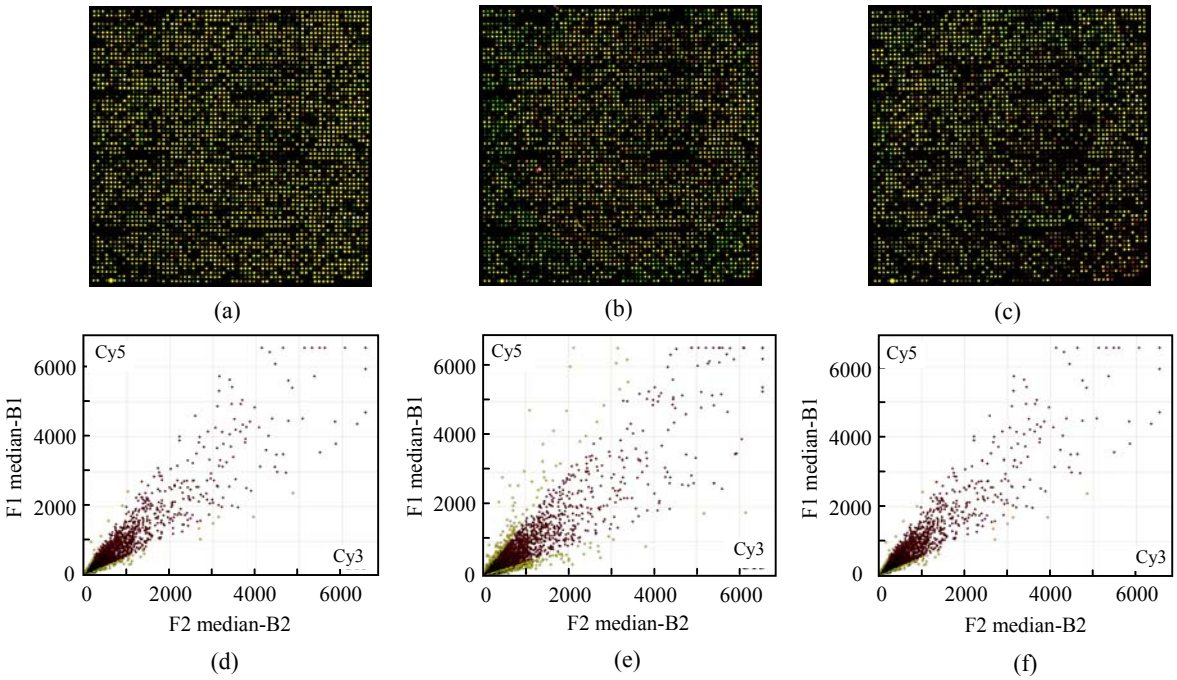
(a) (b) (c) (d)

Fig.2 Light microscopic pictures of blood vessel injury after mannitol injection with and without pre-treatment. (a) Vascular wall damage with thrombus formation and necrosis in the mannitol group (no pre-treatment), ×100; (b) Vascular wall damage with inflammatory cell infiltration in the group pre-treated with normal saline, ×100; (c) Normal vascular wall structure in the group pre-treated with vitamin C, ×40; (d) Subcutaneous edema in the group pre-treated with vitamin E, ×40



(a) (b) (c) (d)

Fig.3 Electron microscopic pictures of blood vessel injury after mannitol injection with and without pre-treatment. (a) Vascular wall damage and severe RBC exudation visible in the mannitol group, ×2250; (b) Vascular wall damage with vessel dilatation and infiltration of inflammatory cells after pre-treatment with normal saline, ×1850; (c) Normal vascular wall structure after pre-treatment with vitamin C, ×3700; (d) Vascular wall damage with slight RBC exudation in the vitamin E-pre-treated group, ×2550



(a) (b) (c)

(d) (e) (f)

Fig.4 (a)~(c) Summation picture of Cy3 and Cy5 fluorescence for the same position point on the microarray; (d)~(f) Scatter plot of hybridizing signal intensity on the gene chip. (a) & (d) Number 1 gene chip (Cy3 control group, Cy5 normal saline pre-treated group); (b) & (e) Number 2 gene chip (Cy3 control group, Cy5 vitamin C pre-treated group); (c) & (f) Number 3 gene chip (Cy3 control group, Cy5 vitamin E pre-treated group)

Table 1 Genes with significant expression difference in vitamin C pre-treated gene chip

Genbank-ID	UniGene	Definition	Ratio
BC003636	Hs. 112278	Homo sapiens arrestin, beta 1	0.170
NM_005228	Hs. 77432	Homo sapiens epidermal growth factor receptor	0.204
AF023917	Hs. 14611	Homo sapiens protein tyrosine phosphatase	0.243
NM_003447	Hs. 55481	Homo sapiens zinc finger protein 165	0.244
NM_001753	Hs. 74034	Homo sapiens caveolin 1, caveolae protein	0.275
NM_005124	Hs. 146449	Homo sapiens nucleoporin 153 kDa	0.286
NM_002154	Hs. 90093	Homo sapiens heat shock 70 kDa protein 4	0.344
NM_000584	Hs. 624	Homo sapiens interleukin 8 (IL8), mRNA	6.482
NM005019	Hs. 41606	Homo sapiens phosphodiesterase 1A, calmodulin-dependent, mRNA	6.818

Fourteen apoptosis genes induceable by mannitol are considered most important: arrestin, epidermal growth factor receptor, protein tyrosine phosphatase, caveolin 1, ATP-binding cassette, heat shock 70 kDa protein, nuclear factor of activated T-cells, laminin, tubulin, tumor necrosis factor, mitochondrial ribosomal protein, integrin, N-cadherin and calcium channel. All these 14 genes were down-regulated after vitamin C pre-treatment, whereas only one (arrestin) was found down-regulated after vitamin E pre-treatment.

Our results suggest that vitamin C pre-treatment has strong protective effect against mannitol-induced apoptosis. Vitamin E was found to exert similar effect, although to a much lower degree.

DISCUSSION

Advanced DNA microarray technique allows to monitor the expression of tens of thousands of genes simultaneously in one hybridization experiment. DNA segments are transfered to one slide in close arrangement. cDNA is retro-transcribed from mRNA derived from control group and treated groups, labelled with Cy3 and Cy5 fluorescence and hybridized in the microarray slide. Through this technique, detection of differentially expressed genes and the construction of differential gene expression profiles is greatly facilitated.

As an important clinical drug, mannitol causes phlebitis as a common side effect, which at this point cannot be prevented. In this study, we found that vitamin C could effectively inhibit phlebitis induced by mannitol. Vitamin E had a slight protective effect in the animal experiment. Findings from the gene chip technology supported these findings, as vitamin C and vitamin E appeared as inhibitors of mannitol-induced apoptosis.

The 45 down-regulated genes in the vitamin C group were all demonstrated to be apoptosis genes, including 14 genes that are known to play a central role in apoptotic cell death. The epidermal growth factor receptor gene (0.204) shows three peaks of activation when exposed to hypertonic environment. These peaks coincide with the up-regulation of Jun NH_2-terminal kinase (JNK), extracellular signal-regulated protein kinases, ERK1 and ERK2, and p38 MAP kinase (Berl *et al*., 1997). Epidermal growth factor receptor triggers apoptosis by activating the PI3K-AKT and MEK-ERK pathway. Continuous activation and up-regulation of protein tyrosine phosphatase (0.243) when exposed to high hyperosmolarity, such as mannitol, was reported from earlier studies (Droillard *et al*., 2002). PTP participated in the non-specific apoptosis signal transduction pathway by phosphorylation and dephosphorylation. During the early stages of osmotic shock, accumulation of hsp70 mRNA suggests the preferential up-regulation of the hsp70 gene and the synthesis of heat shock protein (0.344) (Smith *et al*., 1999).

The 14 up-regulated genes were all shown to be anti-apoptosis genes. Mitogen-activate kinase (2.046) appears to exert anti-apoptotic effects by suppressing the activation of caspase 3 (Lee and Shukla, 2005). Interleukin 8 (6.482) plays an anti-apoptotic role by increasing cell proliferation and by mediating anti-apoptotic signaling (Ryningen *et al*., 2005). Phosphodiesterase (6.816) inhibits apoptosis via a mechanism involving the suppression of cGMP concentrations (Zhu *et al*., 2005).

ACKNOWLEDGEMENT

We thank the Chinese Shanghai Biostar, Inc. for excellent technical and scientific support and helpful discussion and critical review of the manuscript.

References

Berl, T., Siriwardana, G., Ao, L., Butterfield, L.M., Heasley, L.E., 1997. Multiple mitogen-activated protein kinases are regulated by hyperosmolality in mouse IMCD cells. *Am. J. Physiol.*, **272**(3 Pt 2):F305-311.

Droillard, M., Boudsocq, M., Barbier-Brygoo, H., 2002. Different protein kinase families are activated by osmotic stresses in Arabidopsis thaliana cell suspensions. Involvement of the MAP kinases AtMPK3 and AtMPK6. *FEBS Lett.*, **527**(1-3):43-50. [doi:10.1016/S0014-5793(02)03162-9]

Kwak, H.J., Lee, S.J., Lee, Y.H., Ryu, C.H., Koh, K.N., Choi, H.Y., Koh, G.Y., 2000. Angiopoietin-1 inhibits irradiation- and mannitol-induced apoptosis in endothelial cells. *Circulation*, **101**(19):2317-2324.

Lee, Y.J., Shukla, S.D., 2005. Pro- and anti-apoptotic roles of c-Jun N-terminal kinase (JNK) in ethanol and acetaldehyde exposed rat hepatocytes. *Eur. J. Pharmacol.*, **508**(1-3):31-45. [doi:10.1016/j.ejphar.2004.12.006]

Malek, A.M., Goss, G.G., Jiang, L., Lzumo, S., Alper, S.L., 1998. Mannitol at clinical concentrations activates multiple signaling pathways and induces apoptosis in endothelial cells. *Stroke*, **29**(12):2631-2640.

Ryningen, A., Wergeland, L., Glenjen, N., Gjertsen, B.T., Bruserud, O., 2005. In vitro crosstalk between fibroblasts and native human acute myelogenous leukemia (AML) blasts via local cytokine networks results in increased proliferation and decreased apoptosis of AML cells as well as increased levels of proangiogenic Interleukin 8. *Leuk. Res.*, **29**(2):185-196. [doi:10.1016/j.leukres.2004.06.008]

Smith, T.R., Tremblay, G.C., Bradley, T.M., 1999. Hsp70 and a 54 kDa protein (Osp54) are induced in salmon (*Salmo salar*) in response to hyperosmotic stress. *J. Exp. Zool.*, **284**(3):286-298. [doi:10.1002/(SICI)1097-010X(19990801)284:3<286::AID-JEZ6>3.0.CO;2-J]

Zhu, B., Vemavarapu, L., Thompson, W.J., Strada, S.J., 2005. Suppression of cyclic GMP-specific phosphodiesterase 5 promotes apoptosis and inhibits growth in HT29 cells. *J. Cell Biochem.*, **94**(2):336-350. [doi:10.1002/jcb.20286]