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Effect of mannitol injection by intravenous catheter on ear vein endothelial cell apoptosis and venous thrombus in rabbits

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Abstract. – OBJECTIVE: To evaluate the effect of mannitol injection into the rabbit ear vein by intravenous catheter on endothelial cells apoptosis, thrombus formation, the expression of plasma tissue factor (TF) and von Willebrand factor (vWF).

MATERIALS AND METHODS: Sixty-four Zealand rabbits were randomly divided into experiment and control group and received 20% mannitol or normal saline via ear margin veins, respectively. Both groups were injected daily. On days 1, 3, 5, and 7 after catheterization, rabbits were subjected to intraperitoneal anesthesia and their ear veins were isolated and then subjected to hematoxylin and eosin staining. Cell apoptosis was evaluated using TUNEL (terminal deoxynucleotide transferase mediated d-UTP nick end labeling) staining, and the levels of TF and vWF were analyzed by enzyme-linked immunosorbent assay.

RESULTS: Compared with the control group, the experiment group showed significantly increased thrombus formation ($p < 0.05$), and a significant higher rate of apoptosis in endothelial cells ($p < 0.05$) on days 3, 5, and 7. In addition, the experiment group showed significant elevation of plasma TF and vWF on days 3, 5, and 7 ($p < 0.05$).

CONCLUSIONS: Continuous mannitol injection by intravenous catheterization induces more serious venous thrombus formation and endothelial cells apoptosis and higher TF and vWF levels than normal saline injection. These data suggest that clinical use of hyperosmotic mannitol by intravenous catheter may exert direct deleterious effects on vascular endothelium.

Key Words:
Intravenous catheter, Mannitol, Venous thrombosis, Cell apoptosis.

Introduction

Mannitol, a cell-impermeant, nonmetabolized 6-carbon sugar, is commonly administered intravenously as a therapeutic agent in the treatment of a number of clinical conditions[1-5]. It is used in the clinical settings for reducing brain edema[4], decreasing myocardial reperfusion[6], and improving renal perfusion[7]. In brain edema, mannitol is used to improve cerebral blood flow and to reduce intracranial pressure. A little amount of intravenous mannitol within 1-5 minutes reduces intracranial pressure which affect maximum 20-60 min with residual effect until 1.5-6 hours[4]. Mannitol alleviates tissue edema, reduces the volume and pressure of cerebrospinal fluid to prevent cerebral hernia. Injection with mannitol leads to a rapid increase in plasma osmotic pressure and initiates tissue dehydration by water transferred from tissue to plasma. Therefore, to treat cerebral damage, repeated mannitol injections are normally required.

Venous indwelling trocar has been widely used in clinical practice. The use of intravenous catheters has helped patients reduce the pain of repeated punctures, lower the work load of nurses, and increased operational efficiency. However, up to 80% of patients who received intravenous catheters have shown various degrees of complications including phlebitis, venous thrombus formation, fluid leakage, and tube obstruction[8,9]. Among these, venous thrombus formation is one of the most common and severe complications, while the mechanism is still unclear. Some studies have reported that the vascular endothelium damage caused by catheter may be a critical factor[10,11]. Study has also shown that continuous stimulation by the inserted catheter compromises the endothelial cells, either due to physical perturbation by the catheter itself or due to the characteristics of the injected solution, particularly hyperosmotic chemicals[11]. Recently, hyperosmotic mannitol has been shown to induce apoptosis in bovine endothelial cells and activate coagulation pathways leading to intravascular thrombo-

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sis[12,13]. However, it is not clear if mannitol injection via intravenous catheter also promotes intravascular thrombosis and induces apoptosis in venous endothelial cells *in vivo*.

To investigate the mechanism of mannitol induced venous damage, New Zealand rabbits were used as the animal model in our study and subjected to mannitol injection by intravenous catheter via their ear vein. The effects of continuous mannitol injection on venous endothelial cells apoptosis and thrombus formation were determined for a comprehensive correlative analysis between these two outcomes.

Materials and Methods

Animals

The study was approved by the Animal Ethics Committee of Taizhou University. A total of 64 healthy New Zealand rabbits (weight 2.2 to 3.0 kg, male: female = 1:1) were provided by Zhejiang Medical Laboratory Animal Center.

Experimental Groups and Treatments

We randomly divided the 64 New Zealand rabbits into experiment group and control group, with 32 animals in each group. Each group was subjected to injections using closed intravenous catheters (24G, BD, Franklin Lakes, NJ, USA) and samples were taken on days 1, 3, 5, and 7. The margin hair of the rabbits' right ear was shaved and sterilized. A needle was used to puncture the skin at an angle of 15-30º which was slightly reduced upon bleeding, and then the needle was guided toward the vein ensuring the tip was completely immersed in the blood vessel. After minimal retraction of the needle core, the flexible tube was delivered into the blood vessel, then the needle core was removed. The tube was fixed using a 3 M adhesive. The animals' paws were covered to prevent scratching. Ten milliliters of 20% mannitol was injected into the experimental rabbits and equivalent normal saline was injected into the control rabbits within 15 min. After injection, heparin saline (25 U/ml) was used to seal the introcans in positive pressure. Injections were performed every day under strict aseptic operations.

Sample Preparation

On days 1, 3, 5, 7 of the experiment, all rabbits were subjected to intraperitoneal anesthesia. Blood was collected from the jugular vein into sodium citrate tubes, then plasma was separated for further testing. A rectangular 1 cm × 0.5 cm (length × width) tissue sample spanning the puncture site was truncated along the vein and fixed with 10% formaldehyde. After ethanol dehydration and paraffin embedding, we made serial sectioning whose thickness was 4 μm with ultramicrotome.

Pathology Assays

Ear vein tissue samples were stained by HE (hematoxylin and eosin) staining using the protocol below: the tissue slice was incubated in hematoxylin for 10 min, washed in distilled water for 10 min, detained using 1% acid alcohol, and immersed in eosin for 10 min and then dehydrated, vitrified, and mounted. The change of vessel walls and the formation of thrombus were observed using a light microscope. The sample was believed to have thrombus formation if thrombus was observed in any of three slices. Observed thrombi were classified as "fresh thrombus" or "organized thrombus". Samples in which no thrombus was observed in any of the three slices were defined as no thrombus occurrence.

TUNEL Assay

After paraffin removal, a terminal deoxynucleotide transferase mediated d-UTP nick end labeling (TUNEL) kit (Roche, Basel, Switzerland) was used basing on the manufacturer's instructions. Tissue slices were washed with 3% hydrogen peroxide-methanol solution for 10 to 15 min and incubated with 20 μg/ml proteinase K (Sigma, Dorset, UK) for 15 min. Slices were washed with PBS (three times) and incubated with 50 μl TUNEL reaction mixture containing TdT and fluorescein-dUTP at 37°C for 60 min. Following PBS wash, 50 μl of POD (peroxidase) was added and allowed to incubate at 37°C for 30 min. After PBS wash, 50 μl DBA substrate solution was dropped onto the slice and incubated at room temperature for 10 min. Secondary hematoxylin staining was applied after PBS wash, and the slices were dehydrated using an ethanol series, vitrified with xylene, and mounted with neutral balsam. Slices were then observed and imaged with a light microscope, and positive cells were counted in ten random fields.

Detection of Plasma Levels of Tissue Factor (TF) and von Willebrand factor (vWF)

An ELISA (enzyme-linked immunosorbent) assay was performed to analyze the levels of TF

and vWF in rabbit blood plasma. TF antigen and vWF antigen was quantified using the TF ELISA kit and vWF ELISA kit according to the manufacturer's instructions (ADL, Bloomington, MN, USA). Samples were tested in triplicate.

Statistical Analysis

All data were presented as mean ± SD (standard deviation), and statistical analysis was performed with SPSS 16.0 statistics software (SPSS Inc., Chicago, IL, USA). Comparison of the means was performed by single factor variance analysis, while the percentage comparison was analyzed by χ^2 test. $p < 0.05$ was considered to be statistically significant.

Results

Mannitol Injection by Catheter Increased Intravenous Thrombus Formation

As shown in Figure 1, on day 1 after insertion of the intravenous catheter, both the mannitol and saline group showed intact blood vessel walls without inflammatory cell invasion. However, both groups exhibited aggravated blood vessel wall damage accompanied by endothelial cell dissociation and inflammatory cell invasion over time (Figure 1A). The damage was more serious in the mannitol group compared with the control group. Increased thrombus formation was also observed as catheterization time increased, and the amount of thrombus in the mannitol-treated group was significantly higher on days 3, 5, and 7 than that seen in the control group ($p < 0.05$, Figure 1B).

Mannitol Injection by Catheter Elevated TF and vWF Levels in Rabbits

We found that the plasma level of TF was increased in both mannitol group and the control group over time (Figure 2A). While for vWF, it was significantly increased in mannitol group, and it was maintained at a certain level (< 10 ng/ml) in control group (Figure 2B). Comparing mannitol group with the control group, the mannitol group had significantly higher TF and vWF values on days 3, 5, and 7 ($p < 0.05$).

Hyperosmotic Mannitol Induced Apoptosis in Endothelial Cells

The in situ detection of apoptosis by TUNEL assay in four time points revealed that the number of TUNEL-positive cells in mannitol group was approximately 10% by 3 days after mannitol treatment, increased to approximately 20% by 5 days and decreased to 15% which was still above basal level by 7 days (Figure 3). In contrast, the

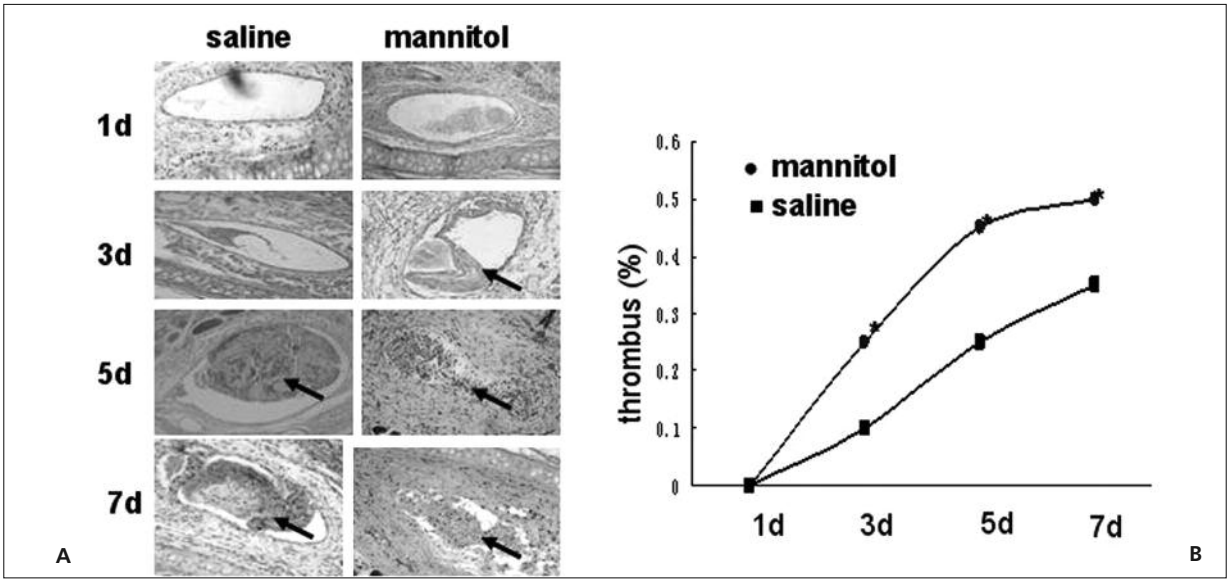

Figure 1. Effect of mannitol injection by intravenous catheter on venous thrombus formation in rabbit ears. Rabbits were subjected to catheterization in ear vein, and 20% mannitol or normal saline was infused through the catheter once daily. At days 1, 3, 5, 7 after catheterization, the pathological changes and thrombus formation in ear vein were determined by HE staining. ***A,*** Representative images of ear vein (HE staining, 200 × magnification). ***B,*** Data are shown as the mean ± SD of each group (n = 5). *$p < 0.05$ versus the saline group. Arrow (→) represents thrombus.

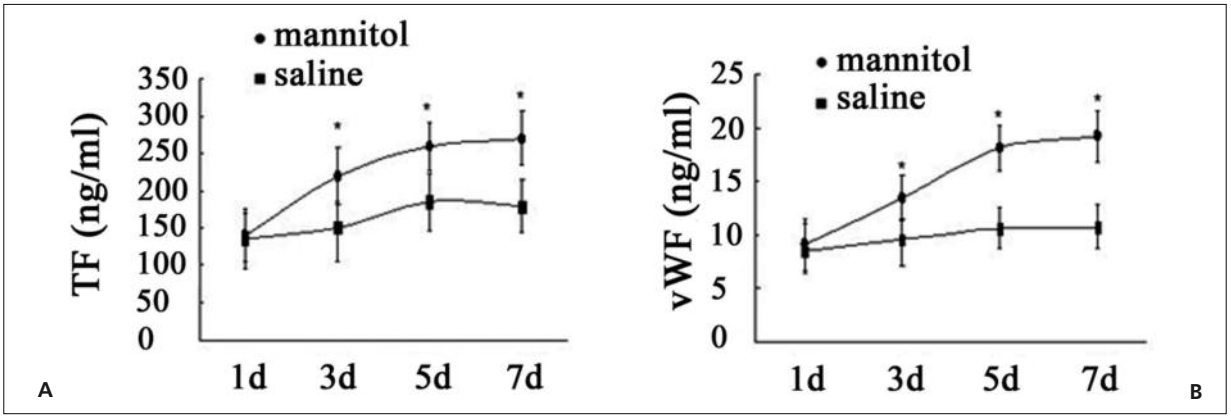

Figure 2. Effect of mannitol injection by intravenous catheter on plasma levels of TF and vWF in rabbits. At days 1, 3, 5, 7 after catheterization, rabbits were sacrificed, and the plasma were prepared. Circulating TF and vWF were measured by ELISA. ***A,*** TF levels (ng/ml) measured in mannitol and saline group. ***B,*** vWF levels (ng/ml) measured in mannitol and saline group. Data are shown as the mean ± SD. *$p < 0.05$ versus the saline group.

number of TUNEL-positive cells in the control group was very low, with a detectable level only at 7 days after injury (5.0%). The number of TUNEL-positive cells in mannitol group was significantly higher than that in the control group ($p < 0.05$).

Discussion

Thrombus formation is a common and severe complication of intravenous catheterization[14]. Fresh thrombus can be easily dissociated, fragmented, dissolved, or absorbed by blood flow, which makes it less detrimental to the organism. However, dissociation of an organized thrombus can lead to embolism or even infarction. It has been suggested that the incidence of thrombus formation is positively correlated with the residence time of intravenous catheters[15-17]. Moreover, a number of studies have indicated that thrombus formation was associated with blood vessel damage induced by catheter, as well as changes in blood flow and in blood composi-

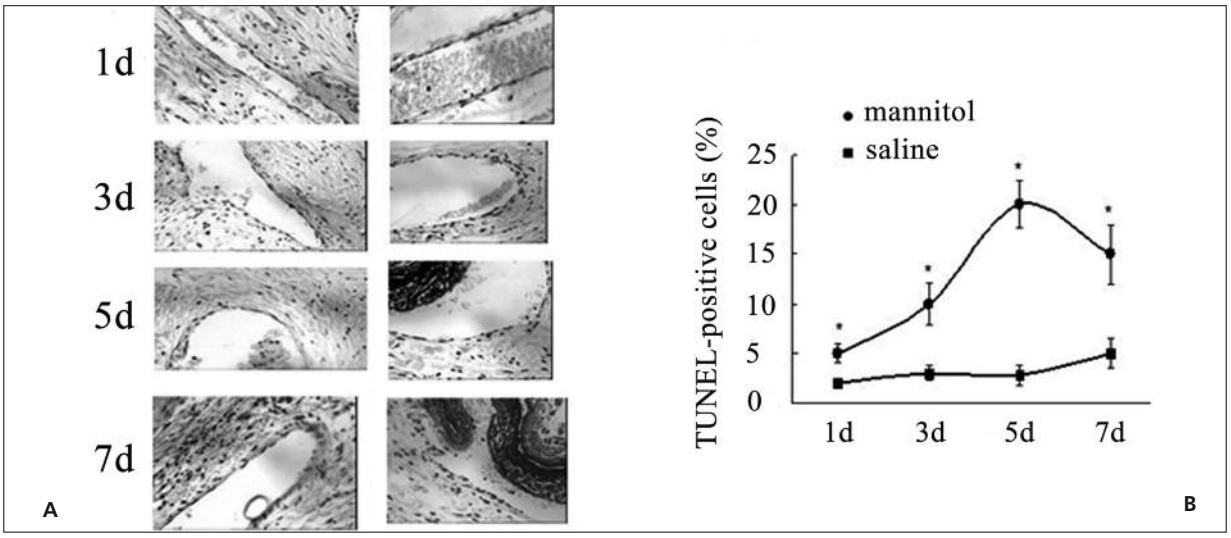

Figure 3. Degree of apoptosis of vascular endothelial cells by TUNEL staining. At days 1, 3, 5, 7 after catheterization, rabbits were sacrificed and the apoptosis of vascular endothelial cells was assayed using TUNEL staining. ***A,*** Representative images of ear vein (TUNEL staining, 400 × magnification). ***B,*** Data are shown as the mean ± SD of each group (n = 5). *$p < 0.05$ versus the saline group.

tion[19,20], while the detailed mechanism driving thrombus formation during intravenous catheterization is still unclear. The study shows that both mannitol and intravenous catheter can promote thrombosis and apoptosis of endothelial cells, which associated with increased levels of coagulation factor TF and vWF. In addition, the deleterious effect induced by mannitol was significantly more serious than intravenous catheter.

The endothelium plays important roles in the control of thrombosis and haemostasis. Healthy endothelial cells are antithrombotic and anticoagulant due to the regulated function of antiplatelet agents including prostacyclin and nitric oxide. While continuous stimulation by the catheter may undermine the integrity of vascular endothelial cells, expose outer endothelial collagen fibrils, and activate platelets. Then the aggregated platelets on the inner blood vessel wall can activate coagulation factors, initiate coagulation, and ultimately lead to thrombus formation. Thus, the degree of vascular endothelial cell damage and consequent thrombus incidence may positively associate with the length of catheterization. Our study has showed that thrombus formation increased from day 3 after intravenous catheter implantation and the prevalence rate was 50% on day 5 when most of the thrombi were organized. It seems that the intravenous catheterization may be relatively safe within 5 days post-catheterization. Since catheterization for more than 5 days may induce local blood vessel obstruction or even tissue infarction, we suggest that intravenous catheter should be replaced every 3-5 days.

Besides, characteristics of the injected solution also play an important role in blood vessel damage and thrombus formation[17]. For example, it has been suggested that resveratrol, a natural polyphenolic compound, can protect human umbilical vein endothelial cells against apoptosis induced by hydrogen peroxide[18]. Drug induced pressure change is one of the most critical factors. Mannitol, as a strong hyperosmotic dehydration solution, can increase plasma osmotic pressure and tissue osmotic pressure during its infusion, which results in the dehydration of vascular endothelial cells and local platelet aggregation. At the same time, prostaglandin leaked from the venous wall can lead to inflammatory responses and white blood cell invasion. Furthermore, it has been demonstrated *in vitro* that mannitol stimulates the expression and release of coagulation factors from blood vessel endothelial cells[13,21], which may be an important mechanism driving thrombus formation. TF released from damaged tissue is a crucial coagulation factor and binds to plasma coagulation factor VII[22]. Then, VII is activated via the VIIa-TF complex, and a cascade of enzymatic reactions is activated through the extrinsic tissue factor coagulation pathway. The vWF protein is a glycoprotein that binds to factor VIII. Over 90% of plasma vWF is synthesized and released by blood vessel endothelial cells and vWF is also the receptor of platelet glycoprotein Ib, IIb, and IIIa. The vWF protein plays an important role in platelet induced thrombus formation by mediating adhesion between platelet and blood vessel endothelial collagen fibrils. Following vessel damage, vWF is released into blood to promote the adhesion and aggregation of platelets to the damaged vessel wall, which increases the risk of thrombus formation[23]. In the present study, an intravenous catheter was put in place and two different injection solutions, hyperosmotic mannitol and normal saline, were used. The results suggest that the mannitol group has more severe blood vessel damage than saline group, with a difference in the amount of thrombus formation starting on day 3. In addition, compared with the saline group, injection of mannitol through an intravenous catheter significantly increased plasma levels of TF and vWF on day 3 to 7 post-catheterization, which may due to the synthesis and release by endothelial cells upon blood vessel wall damage.

Additionally, Malek et al[13] have reported that mannitol can also lead to apoptosis which is associated with the development and onset of several important diseases, including atherosclerosis[24-26] and abdominal aneurysm[27]. Studies[13] *in vitro* suggest that hyperosmotic mannitol stimulation can induce blood vessel endothelial cells apoptosis, but there is no evidence to confirm this conclusion. In our study, TUNEL assay has shown that apoptosis in the mannitol group was significantly higher than that in saline group ($p < 0.05$). Three days after catheter implantation, venous endothelial cells showed apoptosis. This ability of hyperosmotic mannitol stimulation to trigger apoptosis of endothelial cells is potentially important in thrombosis, since apoptosis is associated with large quantity of procoagulant changes on the surface of the cell membrane. These changes include expression and/or release of prothrombotic factors such as TF, vWF, factor V, platelet activating factor and plasmino-

gen, and the loss of antithrombotic components such as heparan sulfate, thrombomodulin and tissue factor pathway inhibitor[21]. In addition, exposure of phosphatidylserine during cell apoptosis provides a template for the assembling of coagulation factor complexes which are essential for the generation of the key enzyme thrombin[28].

Conclusions

Both mannitol and normal saline injected by intravenous catheter in rabbit ears can lead to venous thrombosis, increase the levels of plasma TF and vWF, and induce apoptosis in vascular endothelial cells. However, the mannitol group has more severe effects. These findings suggest that hyperosmotic mannitol may play a major role in thrombus formation and cell apoptosis comparing with intravenous catheterization.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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