

## Full-length article

# Recombinant adeno-associated virus-mediated human kallikrein gene therapy prevents high-salt diet-induced hypertension without effect on basal blood pressure<sup>1</sup>

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## Key words

recombinant adeno-associated virus vector; human tissue kallikrein; high-salt diet; hypertension

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## Abstract

**Aim:** To investigate the effects of the expression of human kallikrein (HK) on basal level blood pressure and high-salt diet-induced hypertension. **Methods:** We delivered the recombinant adeno-associated viral (rAAV)-mediated HK (rAAV–HK) gene and rAAV–LacZ (as the control) to normal, adult Sprague–Dawley rats. The animals were administered a normal diet in the first 4 weeks, followed by a high-salt diet. The expression of HK in the rats was assessed by ELISA and RT–PCR. Blood pressure and Na<sup>+</sup> and K<sup>+</sup> urinary excretion were monitored. **Results:** Under the normal diet, no obvious changes in blood pressure and Na<sup>+</sup> and K<sup>+</sup> urinary excretion were observed. When the high-salt diet was administered, systolic blood pressure in the control animals receiving rAAV–LacZ increased from 122.3±1.13 mmHg to a stable 142.4±1.77 mmHg 8 weeks after the high-salt diet. In contrast, there was no significant increase in the blood pressure in the rAAV–HK-treated group, in which the blood pressure remained at 121.9±1.73 mmHg. In the rAAV–HK-treated group, Na<sup>+</sup> and K<sup>+</sup> urinary excretion were higher compared to those of the control group. The morphological analysis showed that HK delivery remarkably protected against renal damage induced by a high-salt intake. **Conclusion:** Our study indicates that rAAV-mediated human tissue kallikrein gene delivery is a potentially safe method for the long-term treatment of hypertension. More importantly, it could be applied in the salt-sensitive population to prevent the occurrence of hypertension.

## Introduction

Hypertension is a chronic disease, with a high rate of morbidity and low rate of treatment and control, which affects approximately 1 billion individuals worldwide<sup>[1]</sup>. Hypertension is the major risk factor of cardiovascular diseases, such as coronary heart diseases, heart failure, stroke, and kidney diseases. Clinical trials have demonstrated that anti-hypertensive therapy has been associated with 35%–40% reduction in stroke incidence, 20%–25% in infarction, and more than 50% in heart failure<sup>[2]</sup>.

The low control rate of hypertension is partly attributed to the current drugs, which are expensive and short lasting.

Gene therapy may provide an effective and stable control of blood pressure for hypertensive patients through long-term-expressing target genes<sup>[3]</sup>. The recombinant adeno-associated viral (rAAV) vector is an attractive vector for gene therapy protocols<sup>[4–6]</sup>, which can mediate stable transduction and long-term expression of target genes *in vivo*, but without any known adverse effects. These features of rAAV make it possible that a single injection of the rAAV-mediated human kallikrein (rAAV–HK) target gene could control blood pressure of hypertensive patients.

The tissue kallikrein belongs to the serine proteinase family that cleaves a low molecular weight kininogen substrate

to produce a vasodilator kinin peptide<sup>[7]</sup>. The binding of kinins to the bradykinin B2 receptor activates second messengers that trigger a broad spectrum of biological effects, such as vasodilation, smooth muscle contraction and relaxation, inflammation, pain, and cell proliferation<sup>[8]</sup>. The vasodilatory action of the kallikrein–kinin system (KKS) counterbalances the vasoconstrictive actions of the renin–angiotensin system. Our previous studies have shown that the overexpression of HK mediated by rAAV decreases blood pressure and ameliorates the secondary injuries of organs associated with hypertension in spontaneous hypertensive rats in the long term<sup>[9]</sup>. However, the rAAV–HK gene delivery lacks expression regulation. Therefore, whether the overexpression of the HK gene can cause hypotension, a dangerous condition for hypertensive patients, needs to be examined. To identify the effects of the rAAV–HK gene on normal blood pressure is critical and necessary before any potential clinical applications.

The positive relationship between sodium intake and blood pressure has been well established in ecological, epidemiological, and experimental human studies<sup>[10–14]</sup>. Renal kallikrein is synthesized in the connecting tubule cells in the distal nephron, and is involved in the homeostasis of sodium and water balance in the kidney. Its attenuation is suggested as a possible etiological factor in salt-sensitive hypertension<sup>[15,16]</sup>. Our previous studies have demonstrated that rAAV–HK delivery attenuates the increased blood pressure induced by fructose or streptozotocin in conjunction with a high-fat diet and ameliorates organ injury<sup>[17]</sup>. However, its effect on high-salt diet-induced hypertension in rats with normal blood pressure remains unknown.

In the present study, we delivered the rAAV–HK gene to normal, adult Sprague–Dawley (SD) rats and determined the effects of overexpression of human tissue kallikrein on the basal level of blood pressure and high-salt diet-induced hypertension.

## Material and methods

**Construction plasmid** A rAAV vector plasmid, pXXUF1, and rAAV plasmid containing a report gene, pdxII–LacZ as the control, packaging plasmid pXX2, and adenovirus helper plasmid pXX6, were described previously<sup>[18]</sup>. The pXXUF1 contains 2 inverse terminal repeats, cytomegalovirus promoter (CMV) and a poly A tail. A 860 base pair HK fragment (*NotI/NotI*) containing an open reading frame was subcloned into pXXUF1 downstream of CMV to the resultant plasmid pUF1–HK.

**Production and measurement of titers of rAAV vectors** rAAV–HK and rAAV–LacZ were produced as described pre-

viously<sup>[9]</sup>. Human 293 cells (adenovirus 5-sheared DNA transferred embryonic kidney epithelial cells) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics in a 5% CO<sub>2</sub> atmosphere at 37 °C. Immediately before transfection, 15 mL fresh DMEM containing 10% FBS was added to each 15 cm-diameter plate of the cells (70%–80% confluency). A total of 85 µg plasmid DNA (ratio of pUF1–HK or pdxII–LacZ:pXX2:pXX6 was 1:1:1 in molar) was dissolved in 2.5 mol/L CaCl<sub>2</sub> and then quickly mixed with 1× BES-buffered saline and added to the cells. After 8–12 h of transfection, the medium was replaced with fresh DMEM containing 10% FBS, and the cells were harvested at 48–72 h post-transfection. After low-speed centrifugation on a tabletop centrifuge, the cell pellets were resuspended in 1–2 mL of 100 mmol/L NaCl and 10 mmol/L Tris–HCl (pH 8.0) and subjected to 3 cycles of freeze–thaw; cell debris was removed by centrifuge. For the purification of rAAV, a single-step gravity-flow column purification method was carried out as described by Auricchio *et al*<sup>[19]</sup>. The eluted rAAV was aliquoted and stored at –80 °C for the experiments.

The titer of rAAV–LacZ and rAAV–HK was determined by dot blot hybridization.

**Animal treatment and gene delivery** A total 12 male SD rats (3 months old), weighing 250–280 g, were randomly divided into 2 groups ( $n=6$  each group). The rats were housed at room temperature with a 12 h light/dark cycle and fed normal rat chow and tap water. All animal experimental protocols were approved by the Institutional Animal Research Committee of Tongji Medical College (Wuhan, China), and were carried out according to the guidelines of the National Institutes of Health.

For gene delivery, the animals were anesthetized by an intraperitoneal injection of pentobarbital at a dose of 50 mg/kg body weight. The SD rats were administered a single tail vein injection of rAAV–HK or rAAV–LacZ (approximately  $1 \times 10^{11}$  transducing units in 1 mL saline solution), respectively. After the injection, the rats were kept warm by an infrared lamp until they regained consciousness.

At week 5 after the rAAV injection, the animals were fed a standard rodent diet containing 8% NaCl for 8 weeks. All the animals were killed at 13 weeks after rAAV delivery under pentobarbital anesthesia (50 mg/kg body wt), and each rat's heart, lung, kidney, muscle, and liver were collected in liquid nitrogen and then stored at –80 °C until further experiments were performed.

**Systolic blood pressure measurement** From the beginning of the experiment, the systolic blood pressure of the rats was measured weekly with a manometer-tachometer (Rat

Tail NIBP System; ADI Instruments) by cuffing the tails of the animals. This device requires minimal warming of rats (usually 15 min) before blood pressure determination and a brief period of restraint in a plastic cage. For each animal, the systolic blood pressure was represented as the mean of at least 5 recordings.

**Collection of urine** The urinary volume of each animal was measured weekly. The 24 h urine samples were collected in metabolic cages with 500  $\mu$ L toluene to prevent the decay of urine and stored at  $-80^{\circ}\text{C}$  until used for the measurements of the kallikrein levels, urinary osmotic pressure, and urinary excretion of  $\text{Na}^{+}$  and  $\text{K}^{+}$ .

**RT-PCR analysis of human tissue kallikrein mRNA** An RT-PCR analysis specific for HK mRNA was carried out using the RT-PCR kit (TAKARA Biotechnology). Total RNA was extracted from rat tissues by TRIZol reagent (Gibco), and was used for the synthesis of cDNA with AMV reverse transcriptase. The resulting cDNA sample was PCR amplified with HK-specific primers (forward 5'-CCACCATGGGGTTCCTGGTT-3' and reverse 5'-CGCGGATCCACATTTGATT-3') and the  $\beta$ -actin primer (forward, 5'-GGAGAAGATGACCCAGATC-3' and reverse, 5'-GATCTTCATGAGGTAGT CAG-3') as the control, respectively, under the following conditions: a denaturing phase of 1 min at  $94^{\circ}\text{C}$ , annealing phase of 40 s at  $65^{\circ}\text{C}$ , and extension phase of 1 min at  $72^{\circ}\text{C}$  for 20 cycles.

**ELISA specific for human tissue kallikrein in urine** ELISA reagents specific for human tissue kallikrein were supplied by Dr Lee CHAO (Department of Biochemistry and Molecular Biology, Medical University of South Carolina, SC, USA). The ELISA analysis for human tissue kallikrein in the animal urine samples was performed according to the method described previously<sup>[9]</sup>. The plates were read at 405 nm on an ELX 800 ELISA reader (Bio-Tek Instruments).

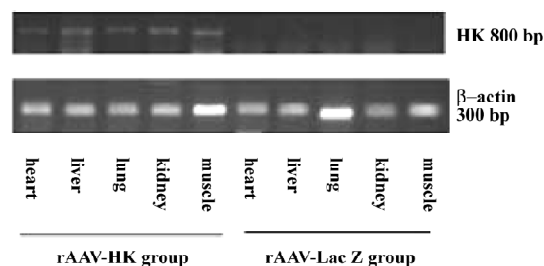
**Analysis of morphology and collagen deposition** A part of the kidney for each animal was preserved in 4% phosphate-buffered saline-buffered formaldehyde solution and embedded in paraffin. Four micrometer-thick sections were cut and stained with Sirius red (collagen stains red with Sirius red staining) using the method previously described<sup>[20]</sup>.

**Statistical analysis** The results were expressed as mean  $\pm$  SEM. The statistical significance of the difference in systolic blood pressure between SD rats receiving rAAV-LacZ and rAAV-HK was determined by ANOVA. In addition, we used an unpaired Student's *t*-test to assess the differences of the HK levels in urine, body weight, urinary volume, and urinary osmotic pressure between the rAAV-LacZ and rAAV-HK groups after gene delivery.

## Results

### Expression of human tissue kallikrein mRNA in SD rats

The mRNA levels of human tissue kallikrein in the heart, renal, lung, liver, and muscle tissues in SD rats were examined by RT-PCR at 13 weeks after an intravenous injection of rAAV. The results showed that human tissue kallikrein mRNA was expressed in all tested tissues in rAAV-HK-treated rats, but not in rAAV-LacZ-treated rats (Figure 1).



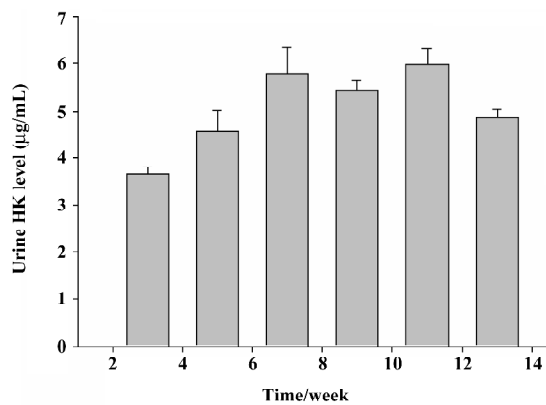
**Figure 1.** Transcription of human tissue kallikrein in SD rats injected intravenously with rAAV-HK and rAAV-LacZ was detected by RT-PCR. HK mRNA was detected in all of the main tissues in the rAAV-HK-treated rats, but not in the rAAV-LacZ-treated rats.

### Immunoreactive human tissue kallikrein levels in the urine of SD rats

The ELISA specific for human tissue kallikrein was used to detect the immunoreactive human tissue kallikrein levels in the urine of SD rats. No immunoreactive human tissue kallikrein was detected in the urine of rats with rAAV-LacZ delivery. The immunoreactive human tissue kallikrein levels in the urine in SD rats reached  $3.67 \pm 0.135 \mu\text{g/mL}$  at 2 weeks after rAAV-HK injection, and maintained at approximately  $4.32 \pm 0.268 \mu\text{g/mL}$  until the end of the experiment. These results demonstrated that rAAV-HK transfer can drive the HK gene to express the target protein stably *in vivo* in the long term (Figure 2).

### Blood pressure regulation effects of rAAV-HK in SD rats

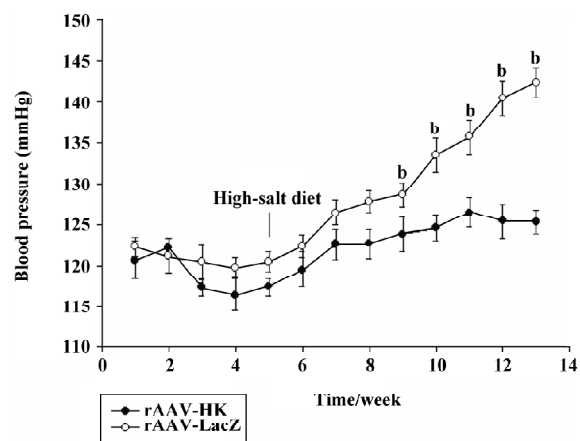
Under the normal diet, no change in blood pressure was observed in all SD rats after the injection of rAAV-HK or rAAV-LacZ. When a high-salt diet was given, the systolic blood pressure of the rats receiving rAAV-LacZ increased from  $122.3 \pm 1.13 \text{ mmHg}$  to a stable  $142.4 \pm 1.77 \text{ mmHg}$  in 8 weeks after being fed the high-salt diet. However, the blood pressure in the rAAV-HK-treated SD rats had no significant changes on the condition of different diet intake, which remained at approximately  $121.9 \pm 1.73 \text{ mmHg}$ . After the high-salt diet was given, the systolic blood pressure in rats receiving rAAV-HK was significantly lower than that in rAAV-LacZ-injected rats ( $P < 0.05$ ,  $n = 6$ , ANOVA), and the antihypertensive effect of rAAV-HK lasted until the end of the experiment (Figure 3).



**Figure 2.** Time-course of human tissue kallikrein expression in SD rats. Urine was collected biweekly and human tissue kallikrein concentrations were measured by ELISA. Immunoreactive HK was detected in the urine from the second week after injection, and the urinary HK level was at  $4.32 \pm 0.268 \mu\text{g/mL}$ . No immunoreactive HK was detected in the urine of control rats receiving rAAV-LacZ (data not shown).

**Physiological parameters in SD rats injected with rAAV-HK or rAAV-LacZ** Under the normal diet, no changes in urinary volume, urinary osmotic pressure, and urinary excretion of  $\text{Na}^+$  and  $\text{K}^+$  were observed in all SD rats after rAAV delivery. When the high-salt diet was given, urine volume, urinary osmotic pressure, and urinary excretion of  $\text{Na}^+$  and  $\text{K}^+$  increased in the rAAV-HK-treated animals significantly more than the rAAV-LacZ-treated animals (Table 1).

**Protective effect of rAAV-HK injection on the kidney** Sirius red staining was used to analyze the collagen deposition in the kidney of rats given a high-salt diet for 8 weeks. As shown in Figure 4, there was much more intensive collagen deposition in the glomerulus in the control group than that in the rAAV-HK-treated animals. Sirius red-stained sections were further analyzed by using HAIPS Pathologic Imagic Analysis System for the percentage of extracellular matrix (ECM)/collagen area surface. ECM was markedly reduced in the rAAV-HK-treated animals compared with the rAAV-LacZ-treated control group ( $19.36\% \pm 5.11\%$  vs



**Figure 3.** Regulation of blood pressure in SD rats injected intravenously with rAAV-HK and rAAV-LacZ. Under the normal diet, no change in the blood pressure was observed. When the high-salt diet was given, the systolic blood pressure in the control animals receiving rAAV-LacZ increased from  $122.3 \pm 1.13 \text{ mmHg}$  to  $142.4 \pm 1.77 \text{ mmHg}$  in 8 weeks. In contrast, the blood pressure in the rAAV-HK-treated group had no obvious increase (approximately  $121.9 \pm 1.73 \text{ mmHg}$ ), and was lower significantly than that of the rAAV-LacZ-treated group ( $^bP < 0.05$ ,  $n = 6$ , ANOVA). The antihypertensive effect of rAAV-HK lasted until the end of the experiment.

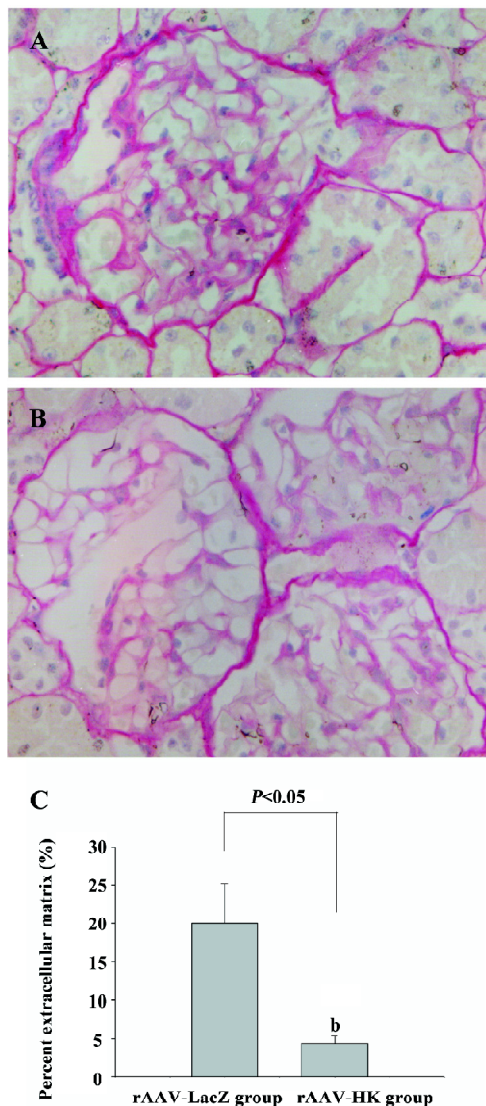
$3.36\% \pm 1.97\%$ ,  $n = 5$ ,  $P < 0.05$ ). These findings indicated that HK gene treatment significantly prevented collagen deposition in the kidney of high salt-induced hypertensive rats (Figure 4).

### Discussion

Our study demonstrated that a single intravenous injection of rAAV-HK could prevent high-salt diet-induced hypertension in SD rats. It also showed that the antihypertensive effect lasted throughout the duration of the experiment without any effect on the basal blood pressure in SD rats fed a normal diet. Furthermore, human tissue kallikrein delivery significantly increased the urinary osmotic pressure and urinary excretion of  $\text{Na}^+$  and  $\text{K}^+$ , as well as reduced collagen deposition in the kidney in rats fed a high-salt diet. These

**Table 1.** Physiological parameters in SD rats injected with rAAV-HK and rAAV-LacZ and fed a normal diet or high-salt diet. Data are mean  $\pm$  SEM.  $n = 6$ .  $^bP < 0.05$  compared to rAAV-LacZ group,  $t$ -test.

	rAAV-LacZ group		rAAV-HK group	
	Normal diet	High-salt diet	Normal diet	High-salt diet
Urine volume (mL/day)	$2.3 \pm 0.11$	$15.6 \pm 1.12$	$2.4 \pm 0.23$	$21.8 \pm 2.13^b$
Urinary osmotic pressure (mOsm/kgH <sub>2</sub> O)	$897 \pm 22.5$	$913 \pm 31.9$	$779 \pm 23.5$	$1178 \pm 35.9^b$
Urine $\text{Na}^+$ (mmol/day)	$2.26 \pm 0.128$	$18.9 \pm 2.43$	$2.37 \pm 0.229$	$25.6 \pm 2.75^b$
Urine $\text{K}^+$ (mmol/day)	$1.36 \pm 0.135$	$2.06 \pm 0.211$	$1.42 \pm 0.147$	$2.65 \pm 0.183^b$



**Figure 4.** Fibrosis and collagen deposition in SD rat kidneys fed a high-salt diet. Sirius red staining showed that in the untreated control SD rats (A) Sirius staining (representing the collagen deposition) was more intense in the glomerulus than the rAAV-HK-treated SD rats (B). Sirius red-stained sections were analyzed by using HAIPS Pathologic Imagic Analysis System for the percentage of ECM/collagen area surface. ECM was markedly reduced in rAAV-HK-treated animals compared with the rAAV-LacZ-treated control group (19.36%±5.11% vs 3.36%±1.97%,  $n=5$ ,  $^bP<0.05$ ) (C).

findings indicated that rAAV-HK delivery resulted in a long-term and stable expression of human tissue kallikrein and had a significant protective effect against high-salt diet-induced hypertension and kidney injury in SD rats. In addition, rAAV-HK delivery did not lower the normal blood pressure on rats fed a normal diet, which suggested that rAAV-HK gene therapy might be the ideal treatment for hypertension.

In the gene therapy research area of hypertension, there

are many problems that need to be resolved, such as, how to control the reduction extent of blood pressure and potential risk of over-reduction of blood pressure in hypertensive patients. Therefore, the choice of vectors for gene delivery and the selection of the targeting gene are critical for the successful gene therapy strategy. The rAAV vector is an attractive vector for gene therapy protocols. It has the ability to remain expressed throughout the full life cycle of a cell by integrating into host cellular DNA or remaining episomal inside the cell. This unique feature can render a long-term and stable expression of the target gene<sup>[4-6]</sup>. Previous studies have shown that a single injection of rAAV-HK gene delivery causes a prolonged reduction of systolic blood pressure in different hypertensive animal models, which makes it possible to treat hypertension through gene therapy<sup>[9,17]</sup>. However, it remains unknown whether the overexpression of the HK gene can induce hypotension in normotensive rats. Our study demonstrated that the expression of human tissue kallikrein delivered by rAAV would not cause hypotension in SD rats with normotensive SD rats. Endogenous bradykinin is suggested to be involved in the vasodilation or the hypotensive effects of ACE inhibitors using short-acting peptide B2 receptor antagonists. However, endogenous bradykinin is found not to contribute to the regulation of blood pressure under basal conditions<sup>[21-24]</sup>, which is consistent with our results. Our data are further supported by a recent study of the bradykinin B2 receptor in null mice. In these mice, the lack of B2 receptor from early development did not alter the maturation of blood pressure under conditions of normal sodium intake<sup>[25]</sup>.

Interestingly, our results showed that human tissue kallikrein gene delivery in normal SD rats had no effect on the excretion of natrium from the kidney under normal sodium intake, although it has been known that renal KKS is involved in the excretion of natrium in the kidney<sup>[15]</sup>. The renal KKS is composed of renal kallikrein, its inhibitor (kallistatin), low molecular weight kininogen, 2 tubular-specific kinases, and kinin receptors. All components of the renal KKS are localized along the renal distal tubules, particularly from the connecting tubules to collecting ducts. However, it is also well known that 90%-95% of sodium is reabsorbed in the proximal portion and particularly in the loop of Henle before it reaches the distal nephron in the kidney, which could explain that human tissue kallikrein gene delivery does not increase the excretion of natrium from the kidney with normal sodium intake.

In our study, we found that a high-salt diet induces hypertension in normal SD rats; however, rAAV-human tissue kallikrein delivery could protect against the increase of blood

pressure with a high-salt diet. Research results from a normotensive population demonstrate that sodium loading induces a change of blood pressure that is associated with the basal urinary excretion of renal kallikrein, although sodium loading decreases the urinary excretion of renal kallikrein<sup>[26]</sup>. Human tissue kallikrein gene overexpression decreases the sensitivity to a high-salt diet. Our study indicated that the SD rats with human tissue kallikrein gene delivery had a higher excretion of urinary natrium compared to the control rats fed a high-salt diet. These data suggested that rAAV-mediated kallikrein delivery counteracts the high-salt diet-induced renal kallikrein decrease, increases the urinary natrium excretion, and consequently protects against blood pressure increase.

The renal KKS may be involved in the renal vasodilation during high natrium intake, and hence a functionally normal KKS is important to prevent salt-sensitive hypertension and secondary renal injuries. As shown in our study, human tissue kallikrein gene treatment significantly prevented the collagen deposition in the kidney of high-salt diet-induced hypertensive rats.

In summary, we report that rAAV-mediated human tissue kallikrein gene delivery does not decrease the basal blood pressure in normotensive rats fed a normal diet. However, it significantly protects against hypertension induced by a high-salt diet and secondary renal injuries. Our study suggested that rAAV-mediated human tissue kallikrein gene delivery is a safe way to treat hypertension, and could have potential when applied to the salt-sensitive population to prevent the occurrence of hypertension.

### Author contribution

Dao-wen WANG designed research; Jiang-tao YAN, Tao WANG performed research; Juan LI, Xiao XIAO contributed analytical reagents and tools; Jiang-tao YAN, Tao WANG analyzed data; Jiang-tao YAN wrote the paper.

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