

ORIGINAL RESEARCH

# Glucocorticoids Reverse Diluted Hyponatremia Through Inhibiting Arginine Vasopressin Pathway in Heart Failure Rats

Xiaoran Zhu, MD; Yaomeng Huang, BS; Shuyu Li, MD; Ning Ge, MD; Tongxin Li, BS; Yu Wang, BS; Kunshen Liu, MD, PhD; Chao Liu, MD, PhD

**BACKGROUND:** Arginine vasopressin dependent antidiuresis plays a key role in water-sodium retention in heart failure. In recent years, the role of glucocorticoids in the control of body fluid homeostasis has been extensively investigated. Glucocorticoid deficiency can activate V2R (vasopressin receptor 2), increase aquaporins expression, and result in hyponatremia, all of which can be reversed by glucocorticoid supplement.

**METHODS AND RESULTS:** Heart failure was induced by coronary artery ligation for 8 weeks. A total of 32 rats were randomly assigned to 4 groups (n=8/group): sham surgery group, congestive heart failure group, dexamethasone group, and dexamethasone in combination with glucocorticoid receptor antagonist RU486 group. An acute water loading test was administered 6 hours after drug administration. Left ventricular function was measured by a pressure-volume catheter. Protein expressions were determined by immunohistochemistry and immunoblotting. The pressure-volume loop analysis showed that dexamethasone improves cardiac function in rats with heart failure. Western blotting confirmed that dexamethasone remarkably reduces the expressions of V2R, aquaporin 2, and aquaporin 3 in the renal-collecting ducts. As a result of V2R downregulation, the expressions of glucocorticoid regulated kinase 1, apical epithelial sodium channels, and the furosemide-sensitive Na-K-2Cl cotransporter were also downregulated. These favorable effects induced by dexamethasone were mostly abolished by the glucocorticoid receptor inhibitor RU486, indicating that the aforementioned effects are glucocorticoid receptor mediated.

**CONCLUSIONS:** Glucocorticoids can reverse diluted hyponatremia via inhibiting the vasopressin receptor pathway in rats with heart failure.

**Key Words:** aquaporins ■ cardiorenal syndrome ■ glucocorticoids ■ heart failure ■ hyponatremia ■ vasopressin

**H**eat failure (HF) is a pathophysiological state characterized by ventricular dysfunction and water and sodium retention. Patients with HF may present with water retention, increased water intake, or both, together with excessive sodium retention, which may lead to hyponatremia. In patients with HF, arginine vasopressin (AVP) V2R (vasopressin receptor 2) antagonists effectively correct impaired urinary diluting capacity, increasing renal free water excretion and reversing hyponatremia.<sup>1</sup> This suggests that V2R exerts a pivotal role in hyponatremia.

V2R is expressed in the basolateral membrane of distal tubules and collecting ducts. It plays a vital role in the maintenance of water-sodium homeostasis and blood volume regulation.<sup>2</sup> AVP signals through V2R to regulate the expression of AQP2 (aquaporin 2) in the apical membrane and AQP3 (aquaporin 3) in the basolateral membrane.<sup>3</sup> V2R activation increases the shuttling of AQP2 and AQP3 to the apical surface and basolateral membrane, respectively, leading to increased water permeability (ie, water absorption). V2R activation also leads to sodium reabsorption.

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Supplementary Materials for this article are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.014950>

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## CLINICAL PERSPECTIVE

### What Is New?

- In this study, we demonstrate that glucocorticoids can reverse diluted hyponatremia by increasing renal water excretion in rats with heart failure with acute water load.
- The renal diuresis enhancing effects induced by glucocorticoids are mediated by inhibiting the vasopressin receptor 2 pathway.
- Recent clinical studies demonstrated that glucocorticoids could enhance renal water excretion in patients with heart failure.

### What Are the Clinical Implications?

- Our research reveals that glucocorticoids can promote water-sodium excretion and improve cardiac function.
- This provides a theoretical basis for the application of glucocorticoids in the treatment of heart failure with diluted hyponatremia.

## Nonstandard Abbreviations and Acronyms

<b>AQP2</b>	aquaporin 2
<b>AQP3</b>	aquaporin 3
<b>AVP</b>	arginine vasopressin
<b>ENaC</b>	epithelial sodium channels
<b>GR</b>	glucocorticoid receptor
<b>HF</b>	heart failure
<b>NKA</b>	Na <sup>+</sup> /K <sup>+</sup> -ATPase
<b>NKCC2</b>	furosemide-sensitive Na–K–2Cl cotransporter
<b>SGK1</b>	serum and glucocorticoid regulated kinase 1

On the other hand, SGK1 (serum and glucocorticoid regulated kinase 1) regulates the transport of various sodium channels. AVP and SGK1 increase the protein abundance and/or activity of certain ion channels, such as epithelial sodium channels (ENaC),<sup>4,5</sup> NKCC2 (furosemide-sensitive Na–K–2Cl cotransporter), and NKA (Na<sup>+</sup>/K<sup>+</sup>-ATPase). During the past several decades, the causal relationship between hyponatremia and glucocorticoid deficiency has become well established both in the clinical setting and in animal studies. Glucocorticoid deficiency increases AVP release, activates V2R, upregulates AQP2 expression, impairs renal free water excretion, and leads to hyponatremia,<sup>6–8</sup> all of which can be reversed by glucocorticoid supplement therapy. Importantly, recent clinical trials on HF have indicated that glucocorticoid

treatment as an add-on therapy could potentiate renal diuresis, improve renal function, and alleviate hyponatremia.<sup>9–12</sup> The mechanisms underlying the renal protective effects induced by glucocorticoids are not fully understood. Here we test the hypothesis that glucocorticoids potentiate renal excretion of free water, thereby correcting dilutive hyponatremia by inhibiting the V2R pathway.

## METHODS

The data, analytic methods, and study materials will be made available upon request to other researchers for purposes of reproducing the results or replicating the procedure. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Hebei Medical University.

An extended description of the methods can be found in Data S1.

### Chronic HF Model Preparation

The CHF rat model of myocardial infarction was established by coronary artery ligation (left ventricular ejection fraction < 45%) as previously described.<sup>13</sup> An acute water loading test was performed 8 weeks after surgery. A total of 32 rats were randomly distributed into 4 groups (n=8/group): sham surgery, chronic HF (CHF), dexamethasone (1 mg/kg, intramuscular), dexamethasone in combination with the glucocorticoid receptor (GR) antagonist RU486 (mifepristone 100 mg/kg, subcutaneously 1 hour before dexamethasone administration). Exact numbers for each part of the experiment are marked in the annotated section of the Figures. The detailed experimental process is shown in Figure S1.

### Cardiac Hemodynamics Measurement

Cardiac function was assessed 6 hours after water loading using a rat pressure-volume catheter (SPR-869; Millar Instruments, Houston, TX) and an MPVS pressure-volume conductance system (Millar Instruments) coupled to a Powerlab A/D converter (PL3508, AD Instruments, New South Wales, Australia), as previously described.<sup>14</sup>

Hemodynamic parameters were computed according to the protocol published previously.<sup>15</sup> The details can be found in Data S1. The end-systolic pressure-volume relationship describes the maximal pressure developed by the ventricle at different ventricular filling volumes. The end-systolic pressure-volume relationship becomes steeper and shifts to the left as inotropy (contractility) increases. The maximal slope of systolic pressure increment occurs early during isovolumic contraction. It is sensitive to the inotropic state and

correlates with cardiac contractility. However, it is load dependent. Preload recruitable stroke work is determined by the linear regression of stroke work with the end-diastolic volume, which provides a contractility index that is insensitive to preload by definition, but it is also remarkably insensitive to changes in afterload at the expense of lower inotropic sensitivity. The isovolumic relaxation constant ( $\tau$ ) represents the exponential decay of the ventricular pressure during isovolumic relaxation.  $\tau$  is a preload-independent measure of isovolumic relaxation. With an increased  $\tau$  (ie, slowing of relaxation), a higher mean left atrial pressure may be required to achieve normal filling volumes. Preload, also known as the left ventricular end-diastolic pressure, is the amount of ventricular stretch (ie, volume overload) at the end of diastole. The greater the preload, the more pressure is available for the next cardiac contraction.

### AVP Measurement

The EDTA-treated blood samples were centrifuged (3000g, 10 minutes) at 4°C and stored at -80°C. Plasma AVP was determined by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN).

### Western Blot

The inner medulla of the kidneys was quickly removed and placed in liquid nitrogen. Membrane proteins affecting water and sodium excretion, such as V2R, AQP2, AQP3,  $\alpha$ -ENaC, NKA, and SGK1 were assessed by Western blot analysis. Protein bands were visualized and analyzed using an Odyssey system (LI-COR, Lincoln, NE). Band intensities were normalized by total plasma membrane protein quantification (Bio-Rad, Hercules, CA).<sup>16</sup>

### Immunohistochemistry

Membrane proteins, such as V2R and AQP2, were visualized by immunohistochemistry. Three fields from each section were randomly selected using a DP73 microscope (Olympus, Tokyo, Japan).

### Statistical Analysis

All statistical analyses were performed using SPSS software (SPSS version 20.0; IBM Corp, Armonk, NY). The results are expressed as mean $\pm$ SEM. The data in Figures 1 and 2 were statistically analyzed using non-parametric tests (Kruskal-Wallis). For Western blot data with equal variance, 1-way ANOVA was used followed by LSD post hoc comparisons. The Kruskal-Wallis test was used for groups with unequal variance.  $P > 0.05$  means no statistical difference;  $0.05 > P > 0.01$  means a statistical difference;  $P > 0.01$  means the statistical difference is extremely significant.

## RESULTS

### Physiological Data

In the acute water loading test, renal water excretion was dramatically impaired in the CHF rats compared with that in normal controls. As a result, the CHF rats exhibited acute water loading-induced hyponatremia. It is noteworthy that dexamethasone pretreatment dramatically increased renal diuresis during the study period (Figure 1A), thereby successfully preventing or reversing acute water load-induced hyponatremia (Figure 1B and 1C). Moreover, dexamethasone pretreatment restored acute water load-induced plasma osmolality in the HF rats (Figure 1D). It is of note that dexamethasone-induced diuresis was accompanied by increased natriuresis and osmolality (Figure 1E and 1F). In addition, despite the normal plasma osmolality and serum sodium levels, dexamethasone pretreatment inhibited circulating AVP levels in the CHF rats subjected to acute water load (Figure 1G). Except for AVP levels, the beneficial effects of dexamethasone pretreatment were abolished by RU486, a GR antagonist, which indicates that those effects are GR mediated.

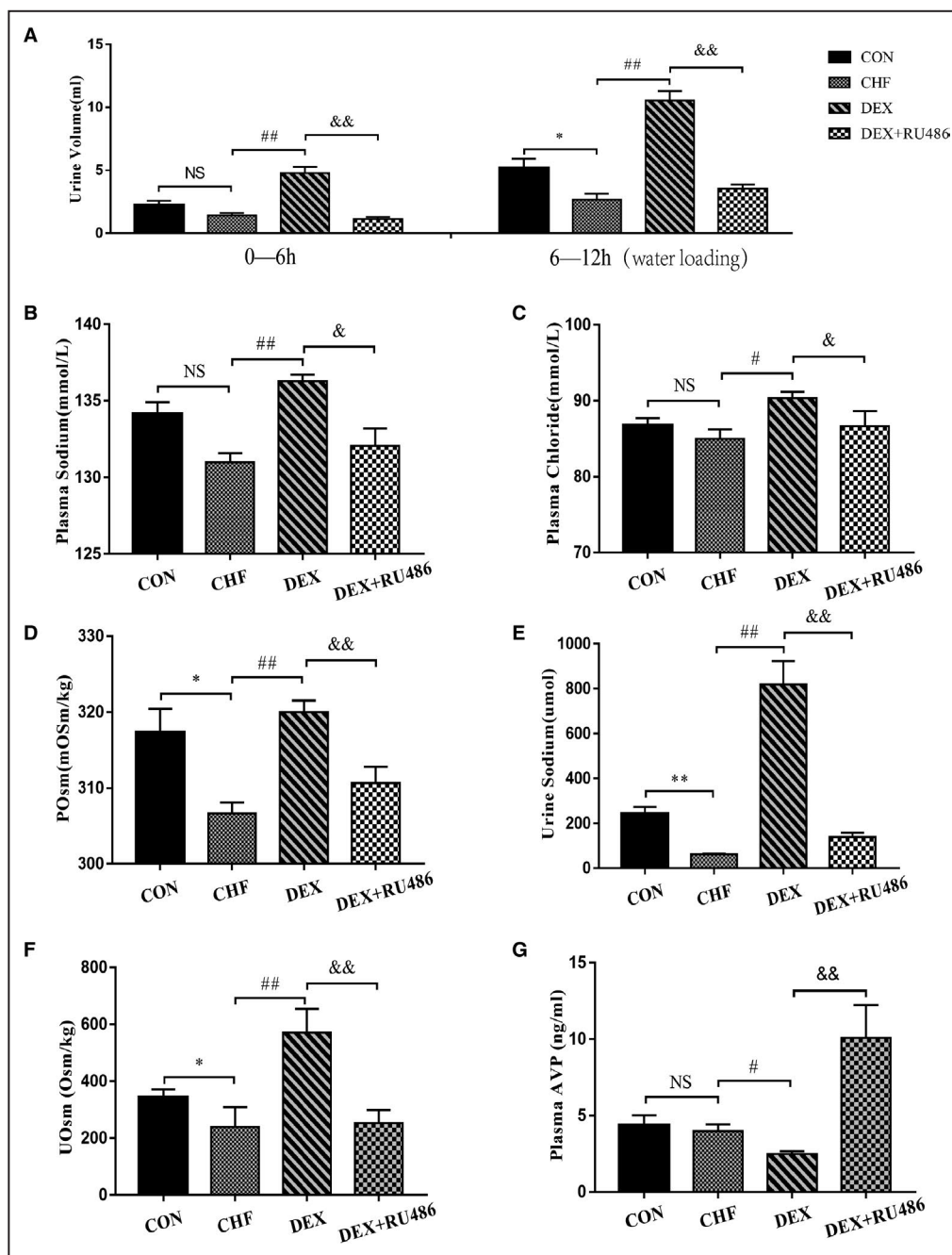
### Cardiac Hemodynamic Measurements and Pressure-Volume Loops

We investigated the effects of dexamethasone on left ventricular function in rats with HF with the Millar pressure-volume catheter.

Figure 2A shows typical changes in the left ventricle pressure-volume loop throughout the cardiac cycle in the 4 groups. The data showed that the rats with CHF had a much lower end-systolic pressure-volume relationship (red line in the loops) than those in the normal controls. Dexamethasone restored end-systolic pressure-volume relationship in the rats with CHF (Figure 2A and 2B). There were no differences in the maximal slope of systolic pressure increment or preload recruitable stroke work between the 4 groups (Figure 2C and 2D). The data also indicated that the rats with CHF had an increased  $\tau$  (ie, slowing of relaxation) compared with those of the normal controls, and dexamethasone could remarkably decrease  $\tau$  (Figure 2E). It is of note that acute water load resulted in a dramatic increase of left ventricular end-diastolic pressure in the rats with CHF, and left ventricular end-diastolic pressure was decreased by dexamethasone (Figure 2F). These favorable effects induced by dexamethasone were abolished by RU486.

### V2R Expression in the Inner Medulla

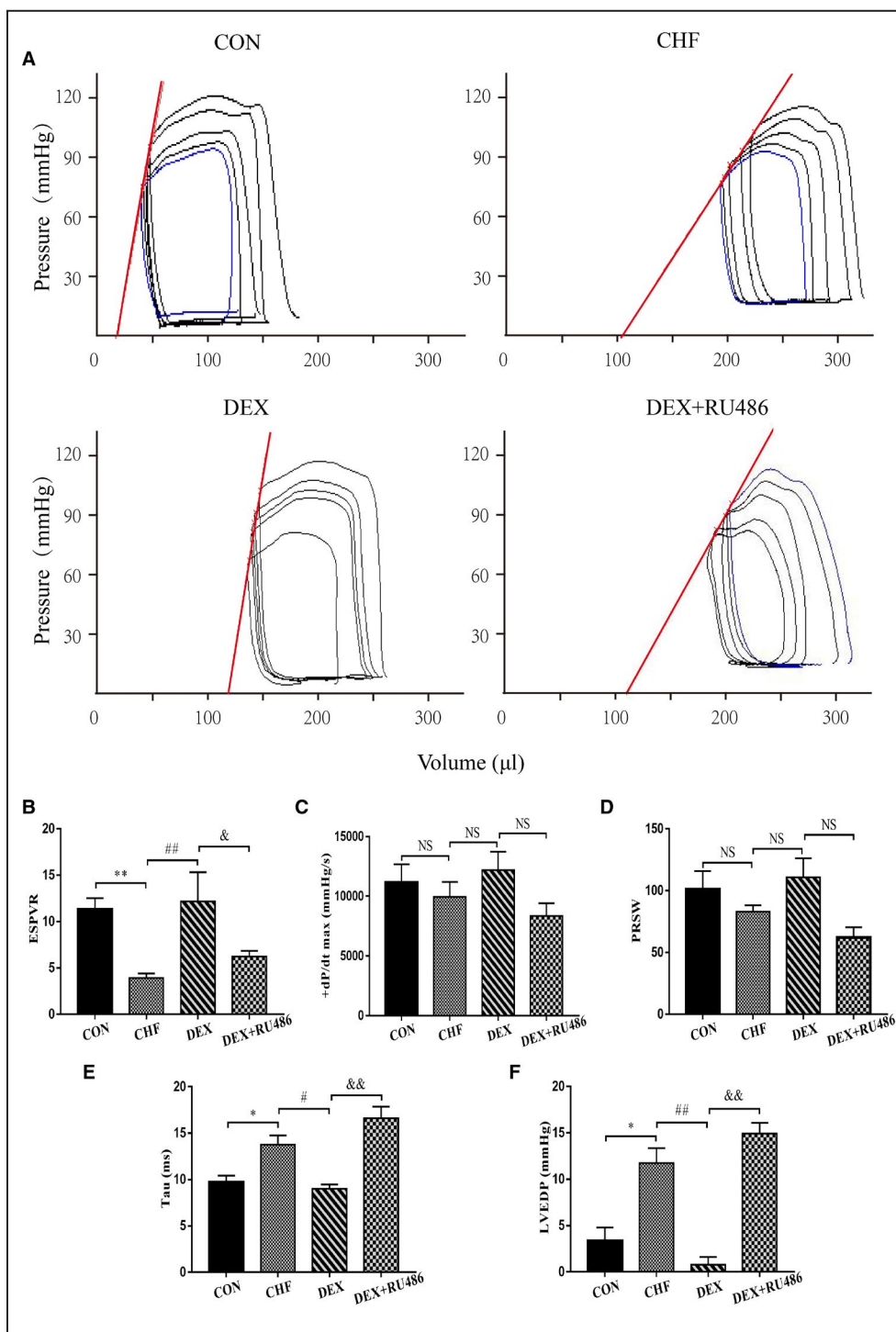
In the kidney, water reabsorption is mainly regulated by AVP binding to V2R, which is mainly expressed in the basolateral membrane of the collecting ducts. To



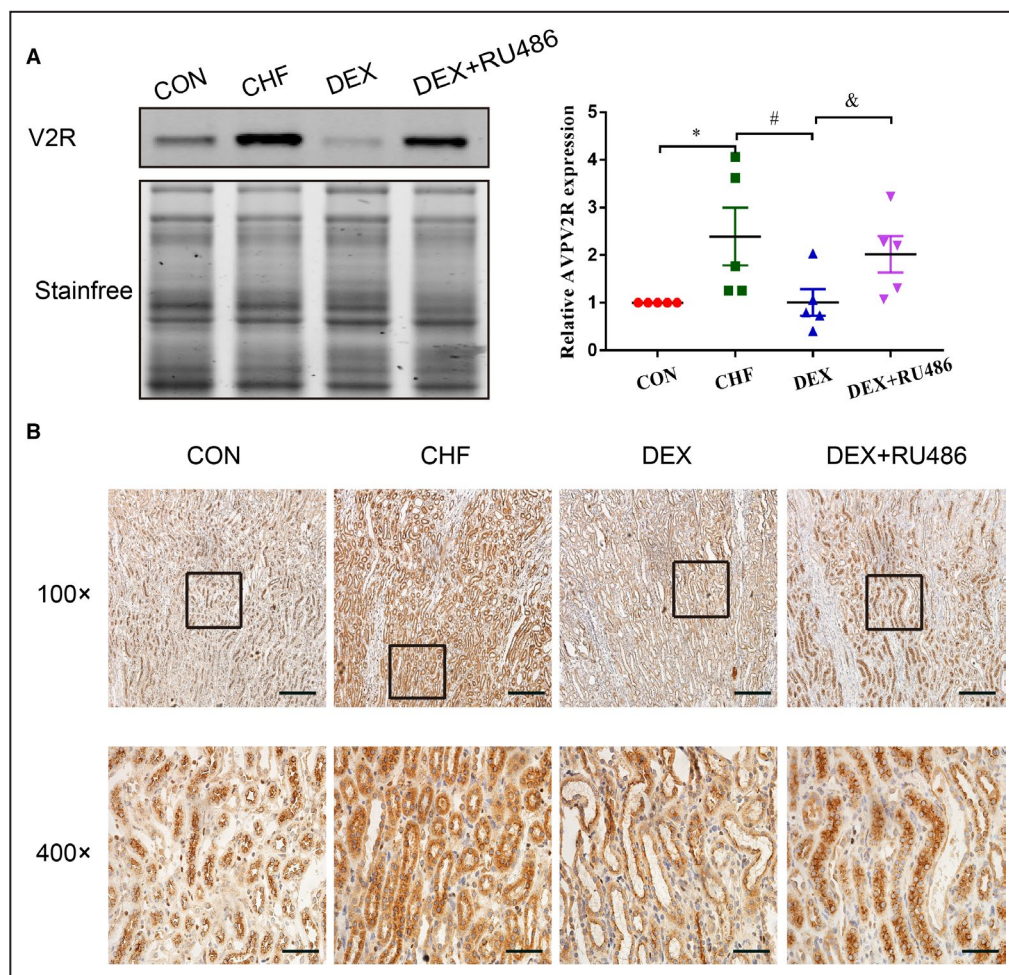
**Figure 1.** The effect of DEX on renal water excretion in rats with heart failure (n=6–8). **A**, Effect of DEX on cumulative urinary volume. **B**, Effect of DEX on plasma sodium concentration. **C**, Effect of DEX on plasma chlorine level. **D**, Effect of DEX on POSm. **E**, Effect of DEX on total urinary sodium excretion. **F**, Effect of DEX on UOsm. **G**, Plasma AVP levels were compared between the 4 groups. AVP indicates arginine vasopressin; CHF, congestive heart failure group; CON, sham surgery group; DEX, dexamethasone group; DEX+RU486, dexamethasone in combination with glucocorticoid receptor antagonist RU486 group; NS, no statistical difference; POSm, plasma osmotic pressure; and UOsm, urinary osmotic pressure. \* $P < 0.05$ , \*\* $P < 0.01$  for CHF vs CON; # $P < 0.05$ , ## $P < 0.01$  for DEX vs CHF; & $P < 0.05$ , && $P < 0.01$  for DEX+RU486 vs DEX.

test the effect of dexamethasone administration on V2R, we measured V2R expression in the collecting ducts. The Western blot result showed that the HF rats had a higher V2R expression than the normal controls.

However, dexamethasone treatment decreased V2R expression in the collecting ducts of the rats with HF. The impact of dexamethasone on V2R expression was blocked by the GR antagonist RU486 (Figure 3A).



**Figure 2. The effect of DEX on cardiac function in rats with heart failure (n=6–8).**  
**A**, Effect of DEX on pressure-volume loops. **B**, Effect of DEX on ESPVR. **C**, Effect of DEX on +dP/dt<sub>max</sub>. **D**, Effect of DEX on PRSW. **E**, Effect of DEX on Tau. **F**, Effect of DEX on LVEDP. Data were obtained with a Millar Instruments (Houston, TX) pressure-volume conductance catheter system. CHF indicates congestive heart failure group; CON, sham surgery group; DEX, dexamethasone group; DEX+RU486, dexamethasone in combination with glucocorticoid receptor antagonist RU486 group; +dP/dt<sub>max</sub>, maximal slope of systolic pressure increment; ESPVR, end-systolic pressure-volume relationship; LVEDP, left ventricular end-diastolic pressure; NS, no statistical difference; PRSW, preload recruitable stroke work; and Tau, isovolumic relaxation constant. \*P<0.05; \*\*P<0.01 for CHF vs CON; #P<0.05; ##P<0.01 for DEX vs CHF; &P<0.05; &&P<0.01 for DEX+RU486 vs DEX.



**Figure 3.** The effect of DEX on V2R in the kidney inner medulla by Western bolt and immunohistochemistry.

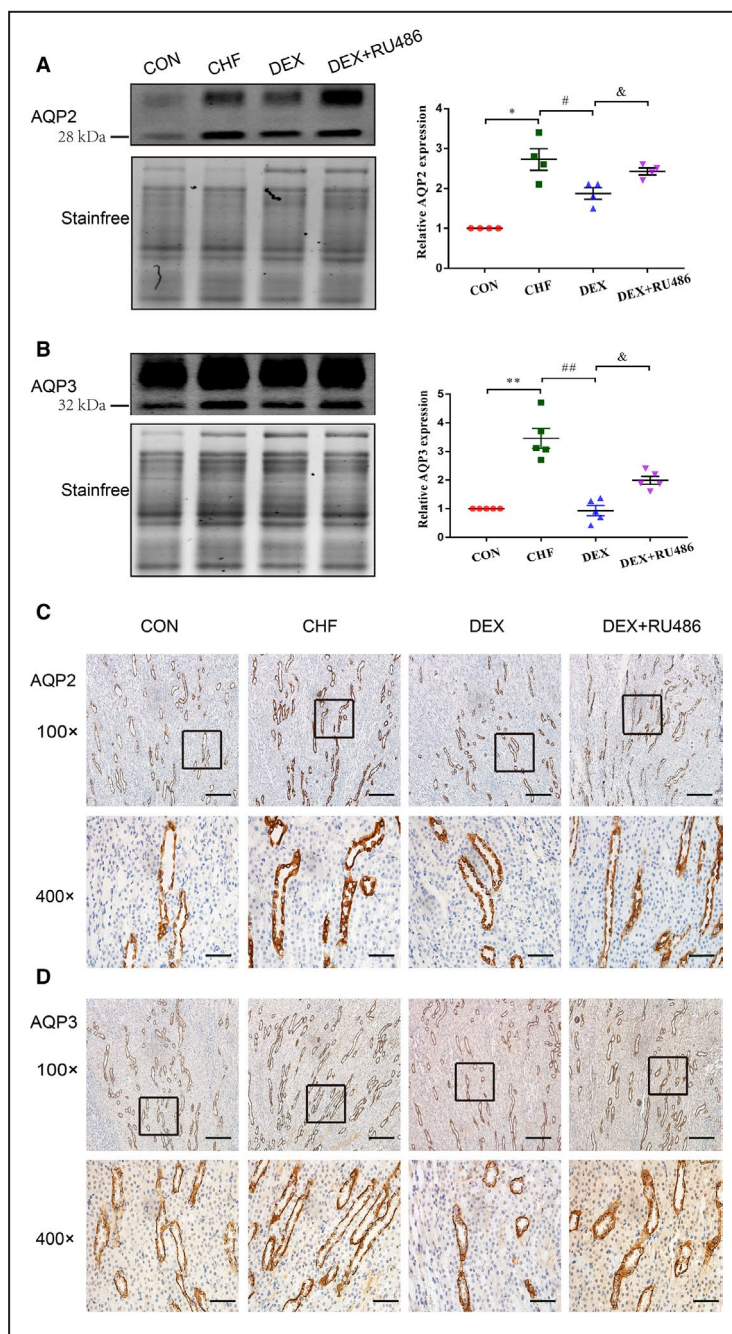
**A**, Western blotting with anti-V2R antibodies was carried out using inner medulla homogenates from 4 groups of rats (40  $\mu$ g/lane, n=5). Densitometric analysis of V2R bands ( $\approx$ 42 kDa) were normalized for the stain-free image of total plasma membrane protein abundance in each fraction. The relative expression analysis was much stronger in the rats with CHF and weaker in the DEX rats. **B**, Immunohistochemistry localization of V2R in the kidney inner medulla. V2R was expressed in the basolateral membrane of the collecting tube. DEX significantly inhibited the expression of V2R. Representative images are shown. AVP indicates arginine vasopressin; CHF, congestive heart failure group; CON, sham surgery group; DEX, dexamethasone group; DEX+RU486, dexamethasone in combination with glucocorticoid receptor antagonist RU486 group; and V2R, vasopressin receptor 2. \* $P$ <0.05 for CHF vs CON; # $P$ <0.05 for DEX vs CHF; & $P$ <0.05 for DEX+RU486 vs DEX. (Magnification  $\times$ 100, bar=200  $\mu$ m; magnification  $\times$ 400, bar=50  $\mu$ m.)

Immunohistochemistry also confirmed the downregulating effect of dexamethasone on V2R expression in the inner medullary collecting duct basolateral membrane in the rats with HF (Figure 3B and 3C).

### Expression of Aquaporins in the Inner Medulla

Activation of V2R increases AQP2 and AQP3 shuttling to the apical surface and basolateral membrane, respectively, thereby leading to water absorption. To determine the effect of dexamethasone on AQP2

and AQP3, we measured their expression using Western blotting and immunohistochemistry. We found that both AQP2 (28 kDa) and AQP3 (32 kDa) were increased in the collecting ducts of the rats with HF compared with the levels in normal controls and that these increases were attenuated by dexamethasone administration (Figure 4A and 4B).<sup>17</sup> Immunohistochemistry confirmed the downregulating effect of dexamethasone on AQP2 in the inner medullary collecting duct apical membrane and on AQP3 in the basolateral membrane in the rats with HF (Figure 4C and 4D).



**Figure 4.** The effect of DEX on the expression of aquaporins in CHF rats.

**A**, AQP2 expression in the membrane protein from inner medulla homogenates (30  $\mu$ g/lane, n=4). Immunoblots showed a band at 28 kDa and a glycosylated protein band between 35 to 45 kDa. A stain-free image of total membrane protein imaging was visualized on Criterion TGX Stain-Free. Relative expression analyses of the data are demonstrated in the 4 different groups. **B**, AQP3 expression in the membrane protein using total protein normalization (30  $\mu$ g/lane, n=5). Immunoblots showed a band at 32 kDa and a glycosylated protein band between 40 and 55 kDa. **C**, Immunohistochemistry for AQP2 expression in the inner medullary collecting duct apical membrane. **D**, Immunohistochemistry for AQP3 expression in the inner medullary collecting duct basolateral membrane. AQP2 indicates aquaporin 2; AQP3, aquaporin 3; CHF, congestive heart failure group; CON, sham surgery group; DEX, dexamethasone group; and DEX+RU486, dexamethasone in combination with glucocorticoid receptor antagonist RU486 group. \* $P$ <0.05, \*\* $P$ <0.01 for CHF vs CON; # $P$ <0.05, ## $P$ <0.01 for DEX vs CHF; & $P$ <0.05 for DEX+RU486 vs DEX. (Magnification  $\times$ 100, bar=200  $\mu$ m; magnification  $\times$ 400, bar=50  $\mu$ m).

## SGK1 and Sodium Channel Expression in the Inner Medulla

In HF, V2R activation increases the protein abundance and/or activity of ENaC and NKCC2 via the SGK1 pathway, thereby increasing sodium reabsorption. We found that the HF rats had higher SGK1 expressions than the normal controls, which was inhibited by dexamethasone (Figure S2). We also investigated the effect of dexamethasone pretreatment on ENaC and NKCC2, which express in the collecting ducts. We found that the HF rats had higher protein expressions of  $\alpha$ -ENaC (Figure 5A) and NKCC2 (Figure 5B) than normal controls. Dexamethasone treatment down-regulated the expression of both sodium channels. Immunohistochemistry confirmed our findings and the localization of these sodium channels (Figure 5C and 5D). However, dexamethasone had no impact on NKA in the inner medullary collecting duct of rats with HF (Figure S3).

## DISCUSSION

Our study showed that pretreating HF rats with glucocorticoids significantly increased the ability of the kidneys to excrete free water, producing a strong diuretic effect and reversing acute water load-induced dilutive hyponatremia. This protective effect of glucocorticoids is associated with the inhibition of the V2R pathway.

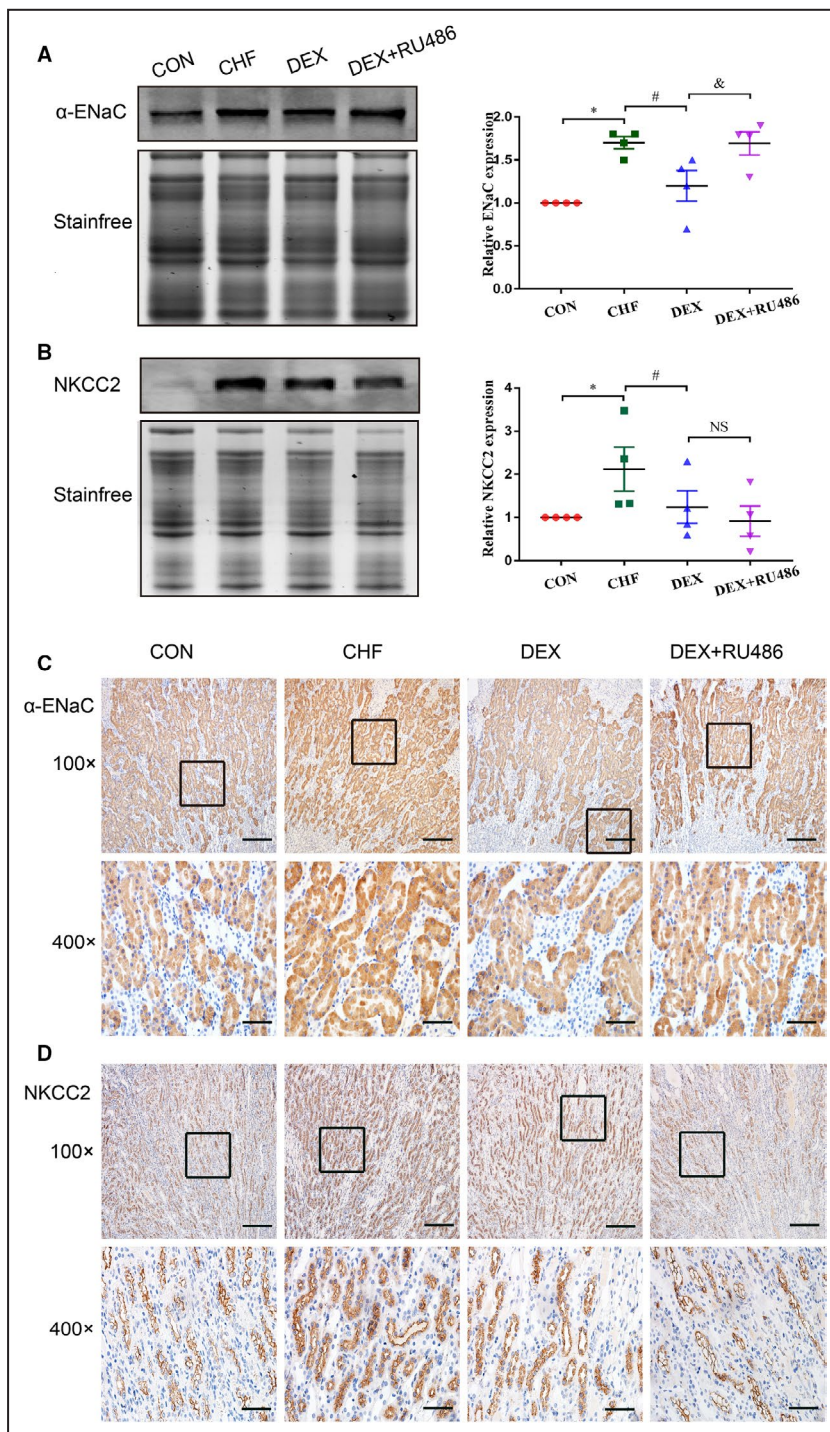
In the kidney, AVP and its primary receptor, V2R, play a critical role in body water homeostasis by influencing renal water excretion.<sup>18</sup> Its release is triggered by low effective blood volume and hypernatremia.<sup>19</sup> AVP causes free water absorption by activating V2R on the basolateral surface of the principal cells in the collecting duct. AQP2 water channels are critical for this cascade by allowing water molecules to cross the apical membrane of the principal cells in response to the osmotic gradient caused by the countercurrent urine-concentrating mechanism.<sup>20</sup> Simultaneously, AQP3 expression is increased in the basal membrane, allowing the absorbed water to cross from the basal membrane into the vessel.<sup>21,22</sup> V2R activation in the basolateral membrane increases water permeability through aquaporin water channels and stimulates sodium reabsorption through SGK1, which modulates a wide variety of transporters, such as the ENaC and NKCC2.<sup>23,24</sup> Therefore, V2R activation is an important water and sodium retention mechanism in HF. In systolic HF, reduced stroke volume accompanied by decreased systemic arterial pressure and renal perfusion activates the sympathetic nervous system, the renin-angiotensin-aldosterone system, and the AVP system. The water-handling ability of the kidney is subsequently reduced. Consistently, our investigation showed that

rats with systolic HF exhibited increased V2R expression on the basolateral surface of the principal cells in the collecting duct as well as SGK1 overexpression. Consequently, renal water and sodium excretion would increase dramatically, thereby reversing acute water load-induced hyponatremia. Our findings indicate that, in HF, the V2R pathway is inhibited by dexamethasone administration in the acute water loading test. The benefits of dexamethasone administration are abolished by the GR antagonist RU486, suggesting that such effects are mediated by GR.

The effect of glucocorticoids on AQP2 in renal cells has been widely studied both in vitro and in vivo. A paradoxical phenomenon has been observed in which glucocorticoids consistently increase AQP2 expression in renal cells in vitro,<sup>25,26</sup> whereas they decrease AQP2 expression in renal cells in vivo.<sup>27,28</sup> In our study, dexamethasone treatment was associated with a reduction in V2R expression, a decrease in AQP2 and AQP3, and an increase in renal water excretion. These findings are consistent with previous studies in vivo.<sup>27,28</sup> Discrepancies between in vitro and in vivo results may occur because molecular biological studies in vitro may overlook the importance of competing or compensatory events occurring in vivo. Our previous findings support this view. We found that glucocorticoids can upregulate the expression of NPR-A (natriuretic peptide receptor A) in the inner medullary collecting duct, both in vitro and in vivo, thereby potentiating renal responsiveness to natriuretic peptides.<sup>29</sup> Activation of NPR-A in collecting tubule cells could inhibit V2R activity.<sup>13</sup> In addition, the effect of glucocorticoids on AQP2 was analyzed in vitro in nonpolarized cells and in vivo in polarized renal cells.<sup>30</sup> The different cell context may have also contributed to the discrepancy. Attempts to resolve these actions (sometimes through countervailing actions) into specific tubular components are confounded by the following 2 phenomena: the pleiotropic effects of glucocorticoids and the promiscuity of steroid receptor-ligand interactions.

The GR and mineralocorticoid receptor share high in vitro affinity for both steroid classes. However, the in vivo specificity of the mineralocorticoid receptor for its cognate ligand is conferred by the prereceptor metabolism of glucocorticoids by 11 $\beta$ HSD2 (11 $\beta$ -hydroxysteroid dehydrogenase 2). 11 $\beta$ HSD2 converts glucocorticoids into physiologically inactive 11-keto glucocorticoid derivatives. Thus, 11 $\beta$ HSD2 confers aldosterone specificity to the mineralocorticoid receptor. In vivo mineralocorticoid receptor activation by glucocorticoids depends on low 11 $\beta$ HSD2 activity. Moreover, glucocorticoids exert important renal hemodynamic actions; they increase renal blood flow and the glomerular filtration rate by





**Figure 5. The effect of DEX on the expression of sodium channels in CHF rats.** **A**,  $\alpha$ -ENaC (85 kDa) expression from the inner medullary collecting duct (50  $\mu$ g/lane, n=4). Total protein normalization was used to quantify the results. **B**, NKCC2 (120 kDa) expression in the membrane protein using total protein normalization (30  $\mu$ g/lane, n=4). **C**, Representative immunostaining image for ENaC expressed in the apical membrane of the inner medullary collecting duct. **D**, Representative immunostaining image for NKCC2 expressed in the apical membrane of the inner medullary collecting duct.  $\alpha$ -ENaC indicates  $\alpha$ -epithelial sodium channels; CHF, congestive heart failure group; CON, sham surgery group; DEX, dexamethasone group; and DEX+RU486, dexamethasone in combination with glucocorticoid receptor antagonist RU486 group. NKCC2, furosemide-sensitive Na-K-2Cl cotransporter; and NS, no statistical difference. \* $P$ <0.05 for CHF vs CON; # $P$ <0.05 for DEX vs CHF; & $P$ <0.05 for DEX+RU486 vs DEX. (Magnification  $\times$ 100, bar=200  $\mu$ m; magnification  $\times$ 400, bar=50  $\mu$ m).

increasing renal production of prostaglandins, nitric oxide, and dopamine.<sup>31–33</sup> Finally, glucocorticoids upregulate the gene expression of natriuretic peptides and increase their levels in the circulation.<sup>34,35</sup> These data indicate a dual effect of glucocorticoids on renal tubules: direct action and indirect action secondary to hemodynamic changes. Nevertheless, the integrated response of the kidneys to the systemic administration of glucocorticoids (ie, increased glomerular filtration rate, enhanced renal diuresis, decrease serum creatinine, increased creatinine clearance, and induced natriuresis or antinatriuresis) is well established and has been demonstrated by clinical trials and our studies (Figure S4). This phenomenon is generally explained by the hemodynamic actions of glucocorticoids, which override their direct actions on renal tubules, increasing the net urinary Na<sup>+</sup> excretion. Our experimental data indicate that the direct action of glucocorticoids on renal tubules may also play an important role in the maintenance of body water homeostasis.

We also observed that dexamethasone might increase cardiac contractility and alleviate ventricular diastolic dysfunction, leading to HF improvement. These favorable cardiac effects may partly contribute to renal hemodynamic changes, thereby improving renal function (Figure S4). Conversely, it could be a consequence of preload reduction attributed to glucocorticoid-induced diuresis. We also found that dexamethasone treatment is associated with an inhibition on myocardial fibrosis and inflammation in HF (Figures S5 and S6), which may also play a role in cardiorenal protection.<sup>36–38</sup> Furthermore, Nishimura et al<sup>39</sup> reported an association between methylprednisolone treatment and an increase in cardiac output and  $\beta$ -adrenergic receptor density in an animal model of HF, which suggests the existence of another mechanism.

Although in our study RU486 abolished most of the biomolecular changes induced by dexamethasone, some changes remained unaffected or were even positively affected by RU486 coadministration. First, RU486 had no impact on dexamethasone-induced NKCC2 downregulation, probably because NKCC2-induced sodium retention is not mediated by the classical GR.<sup>40</sup> Second, RU486 significantly increased AVP levels, consistent with previous reports,<sup>41</sup> probably reflecting a direct signaling effect via the progesterone receptor because RU486 is a progesterone receptor antagonist. In addition, RU486 is capable of crossing the blood–brain barrier and could cause a central hypocortisolemic state, finally leading to increased AVP production.<sup>42</sup>

NKA is a key protein of sodium excretion regulation located in the basement membrane. In the present study, there was no NKA overexpression in the

CHF group, and dexamethasone did not affect NKA expression in the HF rats. One possible explanation is that NKA is relatively stable in the cell plasma membrane, and external stimulation affects its activity rather than its protein abundance.<sup>43</sup>

This study provides experimental evidence supporting the clinical application of glucocorticoids to relieve water and sodium retention. However, it has several limitations. First, the effect of glucocorticoids was only studied on acute water load, and further studies are required to investigate their long-term effects. Second, the experiment was performed using a rat model of HF. Further research in cytology is needed to confirm our findings. Finally, we tested the renal effects of glucocorticoids in the context of acute water load–induced dilutive hyponatremia. The renal effects of glucocorticoids in other contexts, such as acute salt load, remain unclear.

In summary, glucocorticoids downregulate the expression of V2R in the inner medullary collecting duct, inhibit V2R activity, increase the ability of the kidneys to secrete water and sodium, and reverse dilutive hyponatremia in rats (Figure S7). Future studies are warranted to confirm whether glucocorticoids have the same effects on humans.

## CONCLUSIONS

Glucocorticoids increase renal water sodium excretion and reverse diluted hyponatremia by inhibiting the V2R pathway in an acute water loading test in rats with chronic heart failure.

## ARTICLE INFORMATION

Received October 18, 2019; accepted April 2, 2020.

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### Acknowledgments

The authors thank Dr Cowling at the University of California San Diego for his critical suggestion, comments, and extensive editing. The authors thank Dr Yin at the First Hospital of Hebei Medical University for her technical assistance.

### Sources of Funding

This work was supported by the National Natural Science Foundation of China (No. 81670357).

### Disclosures

None.

### Supplementary Materials

Data S1

Figures S1–S7

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# **Supplemental Material**

## **Data S1.**

### **Supplemental Methods**

#### **Surgical Procedure**

Healthy male Wistar rats with a body weight of 180–220 g were provided by the Hebei Medical University. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Hebei Medical University. During the experiments, the vivarium was kept under a temperature of 20–24°C and a relative humidity of 45–65%. The room was controlled by automatic lighting from 6:00 A.M. to 6:00 P.M., and commercial, standard rat chow and water were provided *ad libitum*. The rats were anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively, ip). After endotracheal intubation using a mini otoscope, mechanical ventilation was maintained with a small animal ventilator, respiratory rate at 70 breaths/min, tidal volume at 3 ml. After disinfection, the intercostal muscles were separated along the 3,4 intercostal spaces, the pericardium was separated, and the heart was exposed. The left anterior descending artery (LAD) was ligated with a 6-0 suture needle 2-3 mm below the pulmonary outflow tract and the left atrium. Close the chest in layers, restore positive pressure ventilation and spontaneous breathing. The sham group rats underwent the same procedure but the LAD was not ligated. After surgery, the rats were given penicillin (30,000 U, im) to prevent infection.

#### **Acute water loading test and sample collection**

After 8 weeks, 8 rats without LAD ligation were set as CON group; the surviving 24 rats were randomly assigned into the following groups: chronic heart failure (CHF), DEX (1 mg/kg, im), RU486 + DEX (mifepristone 100 mg/kg, sc 1 h before DEX administration). The rats were placed in metabolic cages for environmental acclimatization 3 days prior to the start of the experiment. The rats were deprived of food and water during experiment period. The acute water loading test was performed

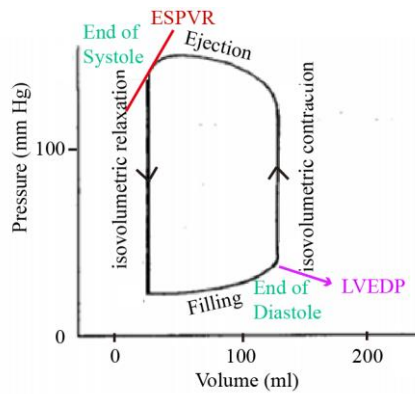
6 h after DEX administration, through intraperitoneal injection of 6% (volume/body weight; ml/100g) deionized water (Figure S1). Six hours after acute water loading, blood samples and kidneys were collected and processed for downstream studies.

### **Hemodynamic Recordings and Calculated Parameters**

Real-time left ventricular (LV) pressure-volume loops measures the pressure and volume of the left ventricle in real time to obtain some physiologically related hemodynamic parameters, which provides a framework for understanding cardiac mechanics in experimental animals and humans. At multiple points in a single cardiac cycle, the relationship between LV pressure and LV volume is plotted as multiple points in time, resulting in a visual PV loop.

Left ventricular function was analyzed in rats anesthetized with uratan (1.5 g/kg, ip). The rats were placed on a 37°C controlled heating pad and the PV catheter (SPR-869, Millar Instruments, Houston, TX) was inserted into the left ventricle through the right carotid artery. After stabilization for 20 min, the signals were continuously recorded using the MPVS pressure–volume conductance system (Millar Instruments) coupled to a Powerlab A/D converter (PL3508, AD Instruments, Australia) as described previously. All pressure–volume data were analyzed using the cardiac pressure–volume analysis module, PV loop (Labchart 8, AD Instruments, Australia). The end-systolic pressure-volume relationship (ESPVR), maximal slope of systolic pressure increment occurs early during isovolumic contraction ( $dp/dt_{max}$ ), preload recruitable stroke work (PRSW), isovolumic relaxation constant (Tau), left ventricular end-diastolic pressure (LVEDP) were collected or calculated.

The following diagram is a schematic diagram of the PV loop during a single cardiac cycle. Proceeding anticlockwise, recording the pressure-volume relationship of a cardiac cycle.



The left ventricular end-diastolic pressure (LVEDP), i.e. the preload of the heart, is the load of the cardiac muscle fibers before the heart contracts, i.e. the blood flow to the ventricles at the end of the diastolic period. LVEDP is the most important marker of heart failure. LVEDP increases when heart failure occurs and goes up when heart failure becomes worse or decompensated. An increase in LVEDP means occurrence of volume overload or fluid retention. The greater the preload, the more pressure is available for the next cardiac contraction.

End-systolic pressure volume relationship (ESPVR) describes the maximal pressure that can be developed by the ventricle at different LV filling volumes. ESPVR is considered to be the most reliable index of ventricular contractility. In the experiment, we changed the preload by blocking the inferior vena cava of rats, and plotted the pressure at the end point of ventricular contraction and the corresponding volume value under different loads, so as to get a straight line with a certain slope. ESPVR flattens and shifts to the right during heart failure, representing a decrease in myocardial contractility. Because ESPVR is independent of changes in preload, afterload, and heart rate, it is a better indicator of systolic function than other hemodynamic parameters such as ejection fraction, cardiac output,  $dP/dt_{max}$ , and stroke volume.

The stroke work, i.e. cardiac contractility, not only varies with afterload but also with preload. Therefore, the linear relationship between stroke work and end-diastolic volume is used as the index to measure cardiac contractility, which is called preload recruitable stroke work (PRSW). It is insensitive to preload by definition, but it is also

remarkably insensitive to changes in afterload at the expense of lower inotropic sensitivity. During heart failure, myocardial contractility is reduced, and the slope of the PRSW becomes shallow.

The maximal slope of systolic pressure increment ( $dP/dt_{max}$ ) occurs in the early stage of isovolumetric contraction and is one of the parameters associated with cardiac contractility. However,  $dP/dt_{max}$  is affected by preload, afterload, heart rate, and cardiac hypertrophy, makes it a poor contractility index.

Isovolumic relaxation constant (Tau) represents the exponential decay of the ventricular pressure during isovolumic relaxation. Several studies have shown that Tau is a preload-independent measure of isovolumic relaxation. With an increased tau (i.e., slowing of relaxation), a higher mean left atrial pressure is required to achieve normal filling volumes. In heart failure, there is an increase of LVEDP and right atrial pressure. Isovolumic relaxation is impaired and Tau is prolonged.

### **Western Blots**

Tissues were cut into small fragments and fully homogenized with a glass homogenizer. Plasma membrane fractions were purified using a plasma membrane isolation Kit (Minute, Invent Biotechnologies, USA). The purity of the plasma membrane fractions was assessed with NKA (Abcam, USA, ab76020) and GAPDH (Proteintech, Wuhan, China) antibody reactivity. Protein concentration was measured using the BCA protein assay reagent kit (Solarbio, Beijing, China). Equal loading of protein was further confirmed by quantification of total protein staining using Bio-Rad stain-free gels. Plasma membrane proteins with 5×loading buffer were denatured for 10 min at 65°C, separated on 4–10% Criterion TGX Stain Free gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% milk in TBST for 2 h at room temperature and then incubated overnight at 4°C with rabbit primary antibodies against AVPV2R (diluted 1:1000; Affinity Biosciences, USA, DF5171), AQP2 (diluted 1:1000; Abcam, USA, ab199975), AQP3 (diluted 1:1000; abclonal, China, A2838),  $\alpha$ -ENaC (diluted 1:500; StressMarq, Canada, SPC-403D), NKCC2 (diluted



1:1000; Abcam, USA, ab191315), Collagen I (diluted 1:1000; Servicebio, China, GB11022-2), Collagen III (diluted 1:1000; Servicebio, China, GB11023), IL6 (diluted 1:500; Huaan, China, AH21-63) or GAPDH (diluted 1:10000; proteintech, China, 10494-1-AP). The membranes were washed, incubated with IRDye 800CW goat anti-rabbit secondary antibody (Abcam, USA, ab216773) for 1 h at room temperature and rewashed.

Protein bands were visualized and analyzed using an Odyssey system (LI-COR, NE, USA). Band intensities were normalized by total plasma membrane protein quantification (Bio-Rad, Hercules, CA).

### **Immunohistochemistry**

Left kidneys were fixed with 4% paraformaldehyde for 48 h at room temperature and embedded in paraffin. The sections (5  $\mu\text{m}$ ) were deparaffinized, rehydrated, and heated in citrate buffer (Solarbio, Beijing, China) at 100°C for 5 min in order to expose antigens. Endogenous peroxidase activity was blocked with 3%  $\text{H}_2\text{O}_2$  for 20 min at room temperature. The sections were incubated overnight at 4°C with primary antibodies against AVPV2R (diluted 1:50; Affinity Biosciences, USA, DF5171), AQP2 (diluted 1:4000; Abcam, USA, ab199975), AQP3 (diluted 1:50; abclonal, China, A2838),  $\alpha$ -ENaC (diluted 1:50; StressMarq, Canada, SPC-403D), or NKCC2 (diluted 1:500; Abcam, USA, ab191315). The next day, the sections were washed three times with PBS and then incubated with secondary antibody for 20 min at room temperature. Subsequently, the sections were washed again in PBS and incubated with DAB under a microscope. Finally, the sections were counterstained with hematoxylin for 30 s and dehydrated with graded concentrations of ethanol and dimethylbenzene.

### **Masson staining**

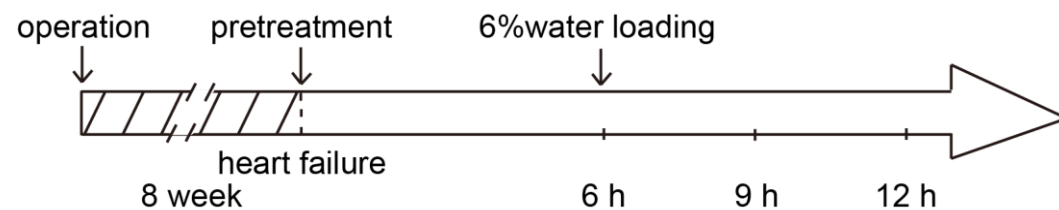
Masson staining was determined by enzyme-linked immunosorbent assay kits (Solarbio, Beijing, China). Wiegert's iron haematoxylin solution was used to dye the cell nucleus for 5 min. Following rinsing with distilled water 1 min, the sections were stained with Masson-Ponceau-acid fuchsin staining solution for 10 min. Sections were

rinsed in 1% glacial acetic acid, differentiated in phosphomolybdic acid for 5 min and stained with aniline blue dye solution for 10 s. Following dehydrating with ethanol series and clearing with xylene.

## Results

**Figure S1. All 4 group rats were given drug pretreatment at 8 weeks after surgery.**

1. The experiment protocol

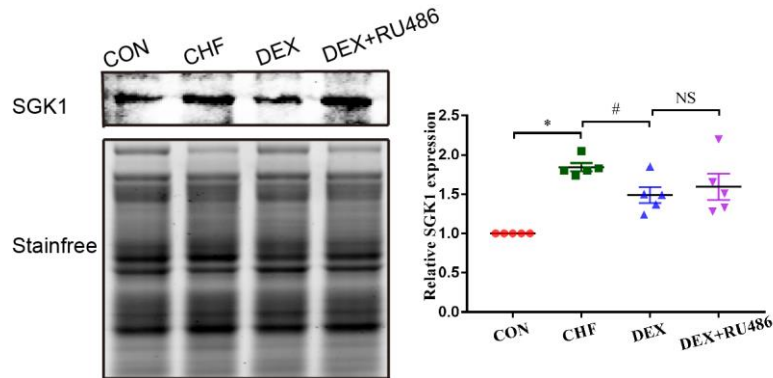


After 6 hours of drug treatment, 6% (volume/body weight, ml/100g) deionized water was injected into abdomen, and the samples were sacrificed at 12 hours for follow-up experiments.

2. The effect of DEX on SGK1 expression in heart failure rats.

In HF, V2R activation increases the protein abundance and/or activity of ENaC and NKCC2, via the SGK1 pathway, thereby increasing sodium reabsorption. We found that HF rats had higher SGK1 expression than normal controls, which was inhibited by DEX (Fig. S2)

**Figure S2. The effect of DEX on SGK1 expression in heart failure rats.**

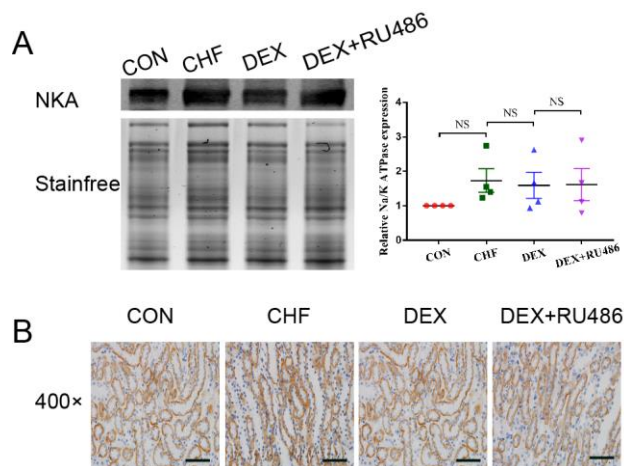


WB analysis of membrane protein SGK1 (60kDa) expression from IMCD (50 $\mu$ g/lane, n=5). Total protein normalization was used to quantify the results. \*  $P < 0.05$  CHF versus CON; #  $P < 0.05$  DEX versus CHF. NS stands for no statistical difference.

### 3. The effect of DEX on NKA in heart failure rats.

NKA is a key protein of sodium excretion regulation, located in the basement membrane. In the present study, There was no NKA overexpression in CHF group and DEX did not affect NKA expression in the HF rats (Fig. S3).

**Figure S3. The effect of DEX on NKA in heart failure rats.**



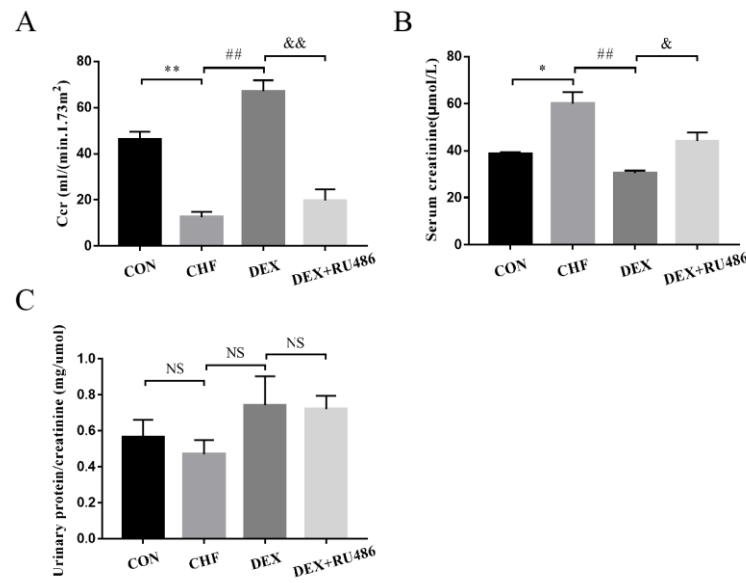
(A) WB analysis of membrane protein NKA (113kDa) expression from IMCD (20 $\mu$ g/lane, n=4). Total protein normalization was used to quantify the results; NS stands for no statistical difference; (B) Representative immunostaining image for

NKA expressed in the basolateral membrane of IMCD.  $100\times$ ,  $Bar = 200\mu m$ ;  $400\times$ ,  $Bar = 50\mu m$ .

#### 4. The effect of DEX on renal function.

DEX improved renal function in HF rats via increasing creatinine clearance (Ccr) and decreasing serum creatinine, but had little impact on urinary protein/creatinine (Fig. S4).

**Figure S4. The effect of DEX on renal function.**



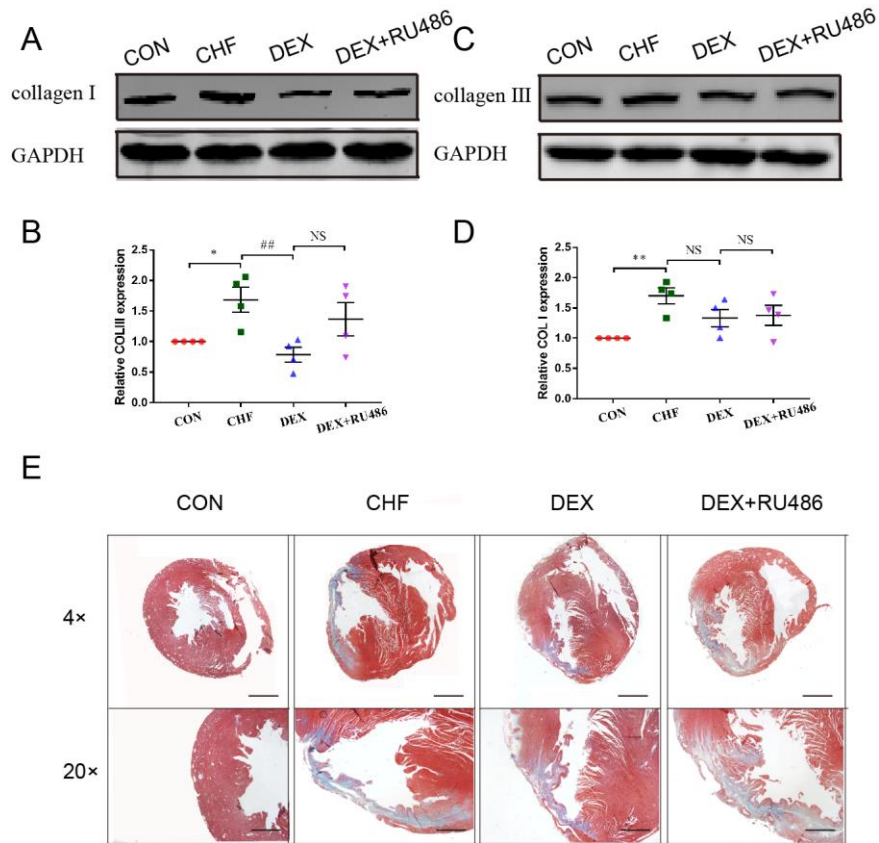
(A) Effect of DEX on creatinine clearance. (B) Effect of DEX on serum creatinine. (C) Effect of DEX on urine protein/creatinine. \*  $P < 0.05$ , \*\*  $P < 0.01$  for CHF versus CON; ##  $P < 0.01$  for DEX versus CHF; &  $P < 0.05$ , &&  $P < 0.01$  for DEX+RU486 versus DEX. NS stands for no statistical difference. Results are mean  $\pm$  SEM of the mean of 6 to 8 experiments in each group. Creatinine clearance = Total urinary creatinine / (Serum creatinine \* Urine collection time)

#### 5. The effect of DEX on collagen in heart failure rats.

We found that dexamethasone down-regulated the expression of collagen I, but did not affect the expression of collagen III (Fig. S5A-D). This was confirmed by masson

staining (Fig. S5E).

**Figure S5. The effect of DEX on collagen in heart failure rats.**

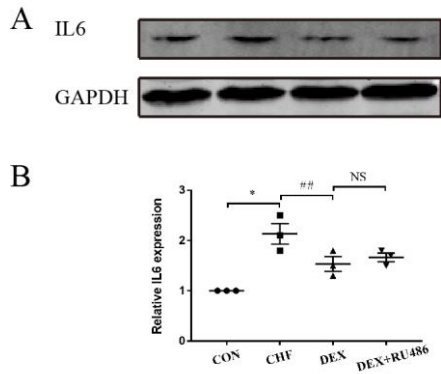


(A) (B) WB analysis of protein collagen I (140kDa) expression from cardiac (100 $\mu$ g/lane, n=4). (C) (D) WB analysis of protein collagen III (120kDa) expression from cardiac (100 $\mu$ g/lane, n=4). \*  $P < 0.05$ , \*\*  $P < 0.01$  for CHF versus CON; ##  $P < 0.01$  for DEX versus CHF; NS stands for no statistical difference. (E) Dexamethasone reduced myocardial collagen by masson staining. 4 $\times$ , Bar = 5mm; 20 $\times$ , Bar = 1mm.

6. The effect of DEX on IL6 in heart failure rats.

We found DEX treatment was associated with an inhibition on IL6 (Fig. S6).

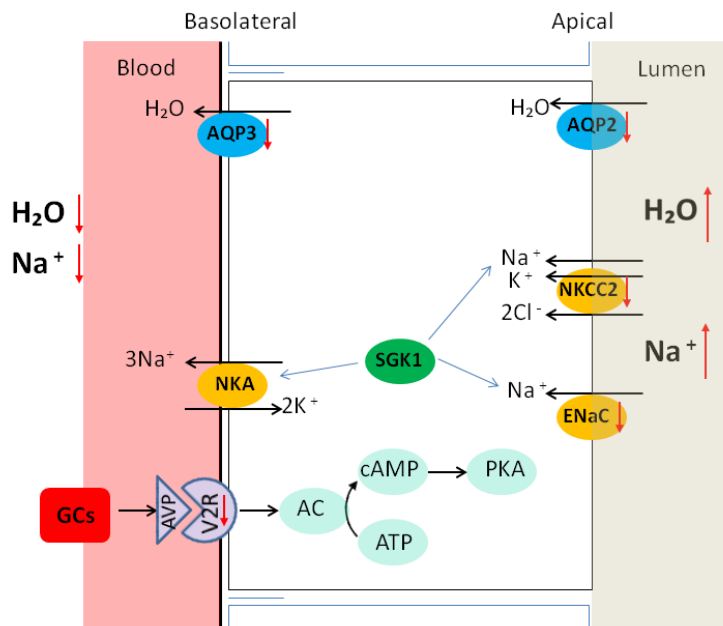
**Figure S6. The effect of DEX on IL6 in heart failure rats (100µg/lane, n=3).**



\*  $P < 0.05$  for CHF versus CON; ##  $P < 0.01$  for DEX versus CHF. NS stands for no statistical difference.

7. The molecular mechanism of DEX for improving water and sodium excretion in rats with heart failure.

**Figure S7. Molecular mechanism of diuretic of glucocorticoid: AVP causes water- sodium absorption by activating V2R on the basolateral surface of the principal cells in the collecting duct.**



V2R activation in the basolateral membrane increases water permeability through

upregulating aquaporin water channels (i.e. AQP2 and AQP3) and stimulates sodium reabsorption through SGK1, which modulates a wide variety of transporters, such as the ENaC and NKCC2. AQP2 water channels are critical for this cascade by allowing water molecules to cross the apical membrane of the principal cells. AQP3 is expressed in the basolateral membrane, allowing the absorbed water to cross from the basal membrane into the vessel. V2R activation is also an important sodium retention mechanism through ENaC and NKCC2 on the basolateral surface and NKA in the basolateral membrane.

Glucocorticoid treatment could lead to decreased expression of AQP2, AQP3 and sodium channel proteins through the down-regulation of V2R, which ultimately leads to decreased renal water and sodium reabsorption and increased renal water and sodium excretion.