Moreover, the activation of calpain and apoptosis-inducing factor induced renal cell apoptosis [7,8] and myocardial apoptosis [9,10]. A calpain-dependent mechanism was involved in the ALD-induced apoptosis of cardiomyocytes. Accumulating experimental data indicates that calci-
knowledge, this is the first demonstration about the protective effect of OMT against ALD-mediated cardiomycytes injury.

**Materials and Methods**

**Chemicals and Animals**

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise. Between 1 and 3 days old Sprague-Dawley (SD) rats were provided by the experimental animal center ofGuiyang Medical University. All animal procedures and experiments were performed following the approval of the Bioethics Committee of Guiyang Medical University.

**Cell Culture and Treatments**

Primary cultured new born rats' cardiomycytes were prepared from SD rats between 1 and 3 days old according to previous methods [10]. The purity of cultured cardiomycytes was ≥97% as evaluated by immunocytochemical staining using cardiac muscle sarcomeric α-actinin antibody (Boster Biotechnology, China). Cardiomycytes treated with 0.2% dimethylsulfoxide (DMSO) (Amresco, USA) was served as a vehicle, and for other groups, treatment of either 25 μg/mL OMT (Green Valley Pharmaceuticals, Co. Ltd., Shanghai, China, with a purity of 98%), or 10 μM Spiro (National Institute for the control of pharmaceutical and biological products, China, purity ≥98%), or 10 μM ALD (Fluka, Switzerland, with a purity of 98%) for 24 h. All drugs were freshly dissolved in DMSO, and there was not significant effect on cardiomycytes (data not shown).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

At the designated experimental time point, the supernatant of each well was removed, and 20 μL of 5 mg/mL MTT (Amresco, USA) solution was added into and incubated at 37°C for 4 h. Formazan salt crystals were then dissolved with 150 μL dimethylsulfoxide for each well. The mixtures are determined at 570 nm using a microplate reader (ELX800, GE, USA).

Lactate Dehydrogenase (LDH) Leakage Ratio

LDH activity was measured using the relative LDH activity assay kit (KeyGEN, Nanjing, China). LDH leakage rate was expressed as the percentage of the total LDH activity (the extracellular LDH activity plus the intracellular LDH activity), according to the following equation: % LDH release rate = (LDH activity in medium/total LDH activity) ×100.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling (TUNEL) Assay

TUNEL (KeyGEN, Nanjing, China) assays were performed in accordance with the manufacturer’s protocol. Apoptotic cells were stained brown and normal cells were stained purple-blue. The percentage of TUNEL-positive cells was determined by counting at least 200 cells in 5 randomly selected fields.

Annexin-V/PI Staining

The annexin-V fluorescein isothiocyanate (FTTC) apoptosis detection kit (KeyGEN, Nanjing, China) was used according to the manufacturer’s instruction. Observation through a fluorescence microscope (BX.51, OLYPUS, Japan), normal cells could only give off a weak green fluorescence. In the early stages of apoptosis, the cell membranes were stained with annexin V and gave off a vibrant green fluorescence, while the nucleolus was not stained with PI. The cells in the media and at late stages of apoptosis were highly stained by both annexin V and PI, therefore the cell membranes gave off green fluorescence and the nucleolus gave off a red fluorescence. The apoptotic cell counts were expressed as a percentage of the total number of cells giving off fluorescence.

Caspase-3 Activity

The caspase-3 colorimetric assay kit (KeyGEN, Nanjing, China) was used to determine caspase-3 activity following the instruction of the manufacturer’s protocol. Cardiomycytes lysates were incubated with 1 M DTT and the labeled caspase-3 substrate DEVD-p-nitroanilide (DEVD-pNA) for 4 h at 37°C. Cleavage of a substrate was quantified by measuring the absorbance at 405 nm using a microplate reader (ELX800, GE, USA).

Western Blot Analysis

The primary cultured cells were washed once in PBS and lysed on ice in lysis buffer (Beyotime, Jiangsu, China) after treatments were completed. The protein concentration was determined using a BCA protein assay kit (Beyotime, Jiangsu, China). Equal amounts of proteins were subjected to 8–12% SDS-polyacrylamide gel. After being electrophoresed, the proteins were transferred onto a PVDF membrane by using a Bio-Rad western blot analysis apparatus. The membrane was incubated with blocking buffer for 1.5 h at room temperature and then incubated overnight at 4°C with the primary polyclonal antibodies against calpain, BID and AIF (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with corresponding secondary antibodies. Specific protein bands were visualized with an ECL advanced western blot analysis detection kit (Beyotime, Jiangsu, China).

**Statistical Analysis**

Data were expressed as mean ± standard error of the mean (S.E.M.) of at least 3 independent experiments. Between-group comparisons were performed by using t-test, statistical significance was set at P<0.05, P<0.01 was considered extraordinarily significant.

**Results**

OMT Protected Cardiomycytes from ALD-induced Cell Injury

Previous study showed that ALD caused cardiomycytes injury was in the time- and dose-dependent manners and at the dose of 10 μM for 24 h caused 33.33% cells death in MTT assay [10]. At present study, the MTT assay showed that cardiomycyte viability was increased from 67.61% to 77.09% by being pre-treated with 25 μg/mL OMT 1 h, the cell damage was also attenuated by Spiro pre-treatment (Figure 1(A)). LDH leakage ratio is served as a biomaker of cellular membrane injury. There was a significant increase in LDH leakage ratio (P<0.01 compared with the vehicle group) in cells exposed to 10 μM ALD alone for 24 h. However, the high level of LDH leakage ratio was significantly reduced in cells pre-treated with OMT, as well as with Spiro (Figure 1(B)), and there was no difference between two pre-treated groups (P> 0.05).
OMT Protected Against Cardiomyocytes from ALD-induced Cell Apoptosis

The apoptotic cells were observed and calculated after annexin-V/PI staining. The pro-apoptotic impact of ALD on cardiomyocytes is obvious (P<0.01 compared to the vehicle group). The percentage of apoptotic cells reached to 16.43% in the ALD alone treatment group, but it was remarkably reduced to 11.43% in the group pre-treated with OMT and to 10.00% in the Spiro pre-treatment group (Figure 2(A)).

The activated caspase-3 is a key executioner in apoptotic pathological process. The level of caspase-3 activity in the cells exposed to ALD alone was significantly higher than that in the vehicle group (P<0.01). However, when pre-incubation with OMT, the activation of caspase-3 was reduced from (0.73±0.06) to (0.58±0.10) (P<0.05 compared with the ALD group). The caspase-3 activity was decreased to (0.50±0.06) in the Spiro pre-treatment group (P<0.05 compared with the ALD group) (Figure 2(B)).

The further confirmation of anti-apoptotic effect of OMT was exhibited via TUNEL assay. The representative morphological views of the vehicle and ALD-treated groups were shown in Figure 3(A). The TUNEL positive cells account for 12.09% in the OMT group and 9.82% in the Spiro group, compared to 15.03% in the ALD group (P<0.05) (Figure 3(B)).

Relative Signal Molecular Expression of OMT Protected Against ALD-induced Cardiomyocyte Apoptosis

Calpain, cysteine protease, which is activated by sustained elevation of intracellular Ca\textsuperscript{2+}, was confirmed to be involved in ALD-induced cardiomyocytes apoptosis. At present, the expression of calpain decreased to (158.33±39.99)% in the OMT group, and also decreased to (156.17±31.25)% in the Spiro group,
compared with that in ALD group (235.67 ± 38.03)% (P < 0.05) (Figure 4).

Bid, a pro-apoptotic BH3-only member of the Bcl-2 family, is a classic substrate for calpain. The active truncated form, tBid, can promote release of cytochrome C. The present data showed that OMT could inhibit the transformation of Bid induced by ALD, and the level of tBid was (223.54 ± 36.26)% in the OMT group, (210.59 ± 31.91)% in the Spiro group, which were significantly less than that in the ALD group (306.15 ± 31.49)% (P < 0.05) (Figure 4).

AIF is thought to promote cell death by binding to DNA and inducing large scale fragmentation, and by increasing mitochondrial permeability. After administration with either OMT or Spiro, the AIF expression in cytosol decreased to (252.73 ± 45.21)% and 216.25 ± 40.84)%, respectively, compared with the ALD group (374.38 ± 60.52)% (P < 0.05) (Figure 4).

**Discussion**

Evidences from present study indicated that ALD-mediated primary cultured cardiomyocytes damage could be significantly ameliorated by OMT. Meanwhile, the results showed that Spiro could attenuate significantly cardiomyocytes apoptosis due to ALD stimulation. Our previous studies suggested that two different
substrates of calcium, calpain- and AIF-mediated pro-apoptotic pathways were involved in the ALD-induced apoptosis [10]. In the present study, the over-expression of pro-apoptotic proteins, including calpain, tBid (calpain target), and AIF, were significantly inhibited because of OMT intervention, and the actions were similar to Spiro.

The cardiomyocytes are terminally differentiated muscle cells with no longer proliferation. Therefore, cardiomyocytes lose with the absence of regenerative capacity may cause irreversible cardiac dysfunction [22], such as heart failure. It is well known that the genesis and development of heart failure are characterized by a number of neurohormonal abnormalities [23,24]. These include activation of the ren-angiotensin-ALD system (RAAS), which could increase plasma levels of ALD. Besides, ALD can be synthesized and excreted locally in the cardiac tissue, and the ALD system will be activated in heart failure entirely pathological progress. In addition to angiotensin II, elevated potassium concentration, and corticotrophin, etc., the supposed to be short-lived corticotrophin can gradually increase in plasma of patients with heart failure resulting in increase of ALD secretion. The dysregulation of circulating catecholamines, endothelins, and arginine vaspressin in patients with heart failure are also contributing to the high level of ALD in plasma. Moreover, decreased metabolic clearance of ALD by the reduced hepatic perfusion in patients with HF accounts for a several fold increase plasma concentration of ALD in patient with HF [25]. All together, these abnormal increased ALD could produce lots of serious adverse effects on cardiac tissue including cardiac hypertrophy, apoptosis, necrosis and fibrosis, etc.

The [Ca\(^{2+}\)] overload is regarded as a pivotal point for lots of signaling pathways leading to pathological status. The role of calcium in ALD signaling-mediated heart failure is a field that has recently been highlighted. For example, the production of reactive oxygen species (ROS) caused by [Ca\(^{2+}\)] overload, as a by-product of ALD metabolism, is considered to be one of mechanisms related to ALD-induced cardiotoxicity [26,27,28]. In our study, we established the in vitro cardiomyocytes injury model by ALD stimulation to induce calcium-related adverse events, which was confirmed by our previous study and other’s observation [3,9,10]. ALD co-incubation promoted LDH leakage and decreased the viability of cardiomyocytes, whereas pre-treatment with OMT significantly alleviated the LDH leakage and preserved the viability of cardiomyocytes. These results indicated that OMT could prevent cardiomyocytes from ALD-induced cytotoxicity. Moreover, the annexin V/PI and TUNEL staining, and the activity of caspase-3-based evidences suggested that OMT could significantly decrease the apoptosis. Our previous study indicated that ALD-induced cardiomyocytes apoptosis might be mediated by two independent pathways, calpain- and AIF-mediated pro-apoptotic pathways. It is well accepted that over-activated calpains, calcium-dependent cysteine proteases, can result in mitochondrial dysfunction and lead to cell apoptosis [29,30,31,32]. Bid, as calpain target, mediates reactions of mitochondrial proteins, consequently leading to cell death both in in vivo and in vitro models [33,34,35]. Cleavage of Bid results in formation of the active truncated form, tBid, which could promote the release of cytochrome C from mitochondria to cytosol. Several pathways have been proposed for the action of tBid, which may contribute to cytochrome C release followed by caspase-3 activation [36,37,38,39]. AIF, a flavoprotein with NADH oxidase activity anchored to the mitochondrial inner membrane, is known to be relevant to complex I maintenance. During apoptosis, AIF can be released from mitochondria to the cytosol, then to the nucleus, in which it participates in chromatin condensation and large-scale DNA fragmentation. Recent studies on the investigation of the impacts of over-loaded [Ca\(^{2+}\)]

Conclusions

The present study for the first time demonstrates that OMT could protect cardiomyocytes against ALD-mediated injury, and the beneficial effect was not inferior to Spiro. The mechanism probably related to suppressing the calcium-related calpain and AIF signalings. OMT as a promising potent drug needs further basic and clinical observations in the coming years.

Author Contributions

Conceived and designed the experiments: TTX XCS. Performed the experiments: TTX YYW YZ CHB. Analyzed the data: TTX YYW. Contributed reagents/materials/analysis tools: TTX YYW. Wrote the paper: TTX XCS.
References

1. Leal J, Luengo-Fernandez R, Gray A (2012) European Cardiovascular Disease Statistics 2012. 2012 ed. Oxford: The European Heart Network.

2. Dooley R, Harvey BJ, Thomas W (2011) The regulation of cell growth and survival by aldosterone. Front Biosci 16: 440–457.

3. Mano A, Tatsus T, Shiraishi J, Keira N, Nomura T, et al. (2004) Aldosterone Directly Induces Myocyte Apoptosis Through Calcineurin-Dependent Pathways. Circulation 110: 317–323.

4. Velez Rueda JO, Palomeque J, Mattiazzi A (2012) Early apoptosis in different models of cardiac hypertrophy induced by high renin-angiotensin system activity involves CaMKII. Journal of applied physiology 112: 2110–2120.

5. Ferro L, Ruchon Y, Renaud JF, Cappiano Y (2011) T-type Ca(2+) signalling regulates aldosterone-induced CREB activation and cell death through PP2A activation in neonatal cardiomyocytes. Cardiovascular research 90: 105–112.

6. Shababz AU, Kamalov G, Zhao W, Zhao T, Johnson PL, et al. (2011) Mitochondria-targeted cardioprotection in aldosteronism. Journal of cardiovascular pharmacology 57: 37–43.

7. Hui H, Sanchez-Nino MD, Delles C, Muller W, Vlahous E, et al. (2013) A combinatorial approach of Proteomics and Systems Biology in unravelling the mechanisms of acute kidney injury (AKI): involvement of NMDA receptor GRIN1 in murine AKI. BMC systems biology 7: 110.

8. Yogi A, Callera GE, O’Connor S, Antunes TT, Valinsky W, et al. (2013) Aldosterone signaling through transient receptor potential melastatin 7 cation channel (TRPM7) and its alpha-kappa domain. Cellular signalling 25: 2163–2175.

9. Zhao J, Li L, Li W, Li Y, Shan H, et al. (2010) Effects of spironolactone on atrial structural remodelling in a canine model of atrial fibrillation produced by prolonged atrial pacing. British journal of pharmacology 159: 1364–1394.

10. Xiao T, Zhang Y, Wang Y, Xu Y, Yu Z, et al. (2013). Activation of an apoptosis signal transduction pathway involved in the upregulation of calpain and apoptosis-inducing factor in aldosterone-induced primary cultured cardiomyocytes. Food and chemical toxicology 53: 364–370.

11. Mihaliadou AS, Loan Le TY, Mardini M, Funder JW (2009) Glucocorticoids of rats. Phytother Res 22: 985–989.

12. Burniston JG, Saini A, Tan LB, Goldspink DF (2005) Aldosterone induces myocyte apoptosis in the heart and skeletal muscles of rats in vivo. Journal of molecular and cellular cardiology 39: 395–399.

13. Soberman J, Chafin CC, Weber KT (2002) Aldosterone antagonists in molecular and cellular cardiology 39: 395–399.

14. Armstrong PW (2011) Aldosterone antagonists–last man standing? The New England journal of medicine 351: 526–528.

15. McMurray JJ, O’Meara E (2004) Treatment of heart failure with spironolactone–trial and tribulations. The New England journal of medicine 364: 79–80.

16. Yang YP, Shen XC, Yang YP, Xiao TT, Peng J, Liu XD (2011) Protective effect of Oxymatrine, the Main Alkaloid Component of Sophora Roots, Protects Heart against Arrhythmias in rats. Planta Med 77: 226–230.

17. Cao YG, Jing S, Li L, Gao JQ, Shen ZY, et al. (2010) Antiarrhythmic effects and ionic mechanisms of oxymatrine from Sophora flavescens. Phytother Res 24: 1844–1849.

18. Gun RT, Dong G, Yu JB, Wang X, Yang SS (2011) Oxymatrine, the Main Alkaloid Component of Sophora Roots, Protects Heart against Arrhythmias in Rats. Planta Med 77: 226–230.

19. Shen XC, Yang YP, Xiao TT, Peng J, Liu XD (2011) Protective effect of oxymatrine on myocardial fibrosis induced by acute myocardial infarction in rats involved in TGF-beta1-Smad5 signal pathway. J Asian Nat Prod Res 13: 213–224.

20. Sun HH, Li L, Shang L, Zhao D, Dong DL, et al. (2008) Cardioprotective effects and underlying mechanisms of oxymatrine against Ischemic myocardial injuries of rats. Phytother Res 22: 983–989.

21. Zhao J, Yu S, Tong L, Zhang F, Jiang X, et al. (2008) Oxymatrine attenuates intestinal ischemia/reperfusion injury in rats. Surg Today 38: 931–937.

22. Tokarska-Schlattner M, Zaug M, Zuppinger C, Wallimann T, Schlattner U (2006) New insights into doxorubicin-induced cardiotoxicity: the critical role of cellular energetics. J Mol Cell Cardiol 41: 389–405.

23. Alves AJ, Eynon N, Oliveira J, Goldhammer E (2010) RAAS and adrenergic genes in heart failure: Function, predisposition and survival implications. World J Cardiol 2: 187–197.

24. Vijayaraghavan K, Deerdwania P (2011) Renin-angiotensin-aldosterone blockade for cardiovascular disease prevention. Cardiol Clin 29: 137–156.

25. Weber KT (2001) Aldosterone in congestive heart failure. The New England journal of medicine 345: 1689–1697.

26. Hayashi H, Kobara M, Abe M, Tanaka N, Gouda E, et al. (2008) Aldosterone nonselectively produces NADPH oxidase-dependent reactive oxygen species and induces myocyte apoptosis. Hypertens Res 31: 363–375.

27. Manrique C, Lastra G, Gardner M, Sowers JR (2009) The renin angiotensin aldosterone system in hypertension: roles of insulin resistance and oxidative stress. Med Clin North Am 93: 569–582.

28. Manrique C, Lastra G, Halabi J, Wei Y, Morris EM, et al. (2007) Methods in the evaluation of cardiovascular renin angiotensin aldosterone activation and oxidative stress. Methods Mol Med 139: 163–179.

29. Ajiro K, Bornert CD, Westmoreland J, Cidlowski JA (2008) An endogenous calcium-dependent, caspase-independent intranuclear degradation pathway in thymocyte nuclei: antagonism by physiological concentrations of K+ ions. Exp Cell Res 314: 1237–1249.

30. Movsesyan VA, Stoeica BA, Yakovlev AG, Knoblauch SM, Lea PM, et al. (2004) Anandamide-induced cell death in primary neuronal cultures: role of calpain and caspase pathways. Cell Death Differ 11: 1112–1132.

31. Rizzuto R, Ponti P, Ferrari D, Chami M, Scabalati G, et al. (2003) Calcium and apoptosis: facts and hypotheses. Oncogene 22: 8619–8627.

32. Vindis C, Elbaz M, Escargueil-Blanc I, Augé N, Heniquèza E, et al. (2005) Two Distinct Calcium-Dependent Mitochondrial Pathways Are Involved in Oxidized LDL-Induced Apoptosis. Arterioscler Thromb Vasc Biol 25: 639–645.

33. Lee WK, Abouhamad M, Thevenet F (2006) Caspase-dependent and -independent pathways for cadmium-induced apoptosis in cultured kidney proximal tubule cells. Am J Physiol Renal Physiol 291: 8293–832.

34. Mandie A, Viktorsson K, Strandberg L, Heiden T, Hanson J, et al. (2002) Calpain-mediated Bid cleavage and caspase-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. Mol Cell Biol 22: 3003–3013.

35. Zhang YM, Bluvnani BR (2006) Glutamate-induced apoptosis in neuronal cells is mediated via caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens. BMC NEUROSCI 7: 49–71.

36. Henry-Mowatt J, Dave C, Martinez JC, James D (2004) Role of mitochondrial membrane permeabilization in apoptosis and cancer. Oncogene 23: 2850–2860.

37. Kroemer G, Reed JC (2000) Mitochondrial control of cell death. Nat Med 6: 513–519.

38. Orenurra S, Zhirovitsky B, Nicotera P (2003) Regulation of cell death: the calcium-aptosis link. Nat Rev Mol Cell Biol 4: 552–565.

39. Zhao Y, Ding WX, Qian T, Watkins S, Lammers J, et al. (2003) Bid activates multiple mitochondrial apoptotic mechanisms in primary hepatocytes after death receptor engagement. Gastroenterology 125: 854–867.

40. Callera GE, Yogi A, Briones AM, Montezano AC, He Y, et al. (2011) Vascular proinflammatory responses by aldosterone are mediated via c-Src trafficking to cholesterol-rich microdomains: role of PDGF. Cardiovasc Res 91: 720–731.

41. Huang LL, Nikolae-Paterson HJ, Ma PY, Toshi GH (2012) Aldosterone Induces Kidney Fibroblast Proliferation via Activation of Growth Factor Receptors and P13K/MAPK Signalling. Nephron Exp Nephrol 120: c114–c121.

42. Tirard M, Jashinek J, Almeida OF, Machadinho TM (2004) The manifold actions of the protein inhibitor of activated STAT proteins on the transcriptional activity of mineralocorticoid and glucocorticoid receptors in neural cells. J Mol Endocrinol 32: 825–841.