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Brief Report

Fludrocortisone Induces Aortic Pathologies in Mice

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Abstract: Background and Objective: In an experiment designed to explore the mechanisms of fludrocortisone-induced high blood pressure, we serendipitously observed aortic aneurysms in mice infused with fludrocortisone. The purpose of this study was to investigate whether fludrocortisone induces aortic pathologies in both normocholesterolemic and hypercholesterolemic mice. **Methods and Results:** Male adult C57BL/6J mice were infused with either vehicle (85% polyethylene glycol 400 (PEG-400) and 15% dimethyl sulfoxide (DMSO); N=5) or fludrocortisone (12 mg/kg/day dissolved in 85% PEG-400 and 15% DMSO; N=15) for 28 days. Fludrocortisone-infused mice had higher systolic blood pressure, compared to mice infused with vehicle. Fludrocortisone induced aortic pathologies in 4 of 15 mice with 3 having pathologies in the ascending and aortic arch regions and 1 having pathology in both the ascending and descending thoracic aorta. No pathologies were noted in abdominal aortas. Subsequently, we infused either vehicle (N=5/group) or fludrocortisone (N=15/group) into male ApoE -/- mice fed a normal laboratory diet or LDL receptor -/- mice fed an either normal or Western diet. Fludrocortisone increased systolic blood pressure, irrespective of mouse strain or diet. In ApoE -/- mice infused with fludrocortisone, 2 of 15 mice had ascending aortic pathologies, but no mice had abdominal aortic pathologies. In LDL receptor -/- mice fed the normal diet, 5 had ascending/arch pathologies, 1 had pathologies in the ascending, arch, and suprarenal aortic regions. In LDL receptor -/- mice fed Western diet, 2 died of aortic rupture in either the descending thoracic or abdominal region, and 2 of the 13 survived mice had ascending/arch aortic pathologies. Aortic pathologies included hemorrhage, wall thickening or thinning, or dilation. Given the low incidence, only ascending aortic diameter in LDLR -/- mice fed Western diet reached statistical significance, compared to their vehicle. **Conclusion:** Fludrocortisone induces aortic pathologies independent of hypercholesterolemia. The findings in mouse studies have the potential to provide caution to people who are taking or have taken fludrocortisone that could have an increased risk of aortic pathologies.

Keywords: aortic aneurysms; aortic dissection; fludrocortisone; angiotensin; hypercholesterolemia; mouse

1. Introduction

Fludrocortisone is a corticosteroid used to improve sodium and water balance under certain conditions such as adrenocortical insufficiency.[1] It is also frequently used in patients with orthostatic hypotension because fludrocortisone can raise blood pressure.[2] However, the mechanism of increased blood pressure is not fully understood.

The renin-angiotensin system regulates blood pressure and water and sodium homeostasis.[3] Angiotensinogen (AGT) is the unique substrate for angiotensin II (AngII), the major bioactive peptide in this hormonal system. Its deletion, e.g. by small interfering RNA (siRNA) targeted to liver AGT, results in lower blood pressure.[4] siRNA-mediated

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blood pressure reduction in case of emergency was reversed by fludrocortisone in rats.[4] Next, we evaluated fludrocortisone in hepatocyte-specific AGT deficient (hepAGT -/-) mice or their wild type (hepAGT +/+) littermates (Dien et al., unpublished data). One mouse infused with fludrocortisone died before termination. Necropsy showed that the mouse had aortic rupture in the suprarenal aortic region, a common location for AngII-induced aortic rupture in mice.[5-8] During termination, we dissected and characterized aortas from both hepAGT +/+ and -/- mice. We found that some mice infused with fludrocortisone exhibited aortic pathologies in either or both ascending/arch and abdominal aortic regions, comparable to what we see in AngII-infused mice.[9]

HepAGT-/- mice and their wild type littermates were developed initially in a mixed 129/C57BL/6N background,[10, 11] then backcrossed to C57BL/6J strain 6 times. Our observations were made in a low-density lipoprotein receptor (LDLR -/-) strain. It is unclear whether the effect of fludrocortisone on aortic pathology is related to hypercholesterolemia or specific strains. AngII-induced abdominal aortic aneurysms (AAA) are augmented under hypercholesterolemic conditions such as LDLR deficiency or apolipoprotein E (ApoE) deficiency, whereas AngII-induced thoracic aortic aneurysms (TAA) are not associated with hypercholesterolemia.[12, 13] Therefore, in this study, we determined whether fludrocortisone induces aortic aneurysms in mice with C57BL/6J background and whether the incidence of aortic pathologies is different between normocholesterolemic and hypercholesterolemic mice.

2. Materials and Methods

2.1. Mice

Male C57BL/6J mice, LDLR -/- mice, and ApoE -/- mice were purchased from The Jackson Laboratory (Table 1). Only male mice were studied because female mice have a low incidence of both AAA and TAA, compared to male mice, as reported in several mouse models.[14] Mice were fed Teklad Irradiated Global 18% Protein Rodent Diet #2918 (Envigo, Indianapolis, IN) and given access to water ad libitum. In one study, LDLR -/- mice were fed a Western diet (Diet # TD.88137, ENVIGO) for 1 week before the vehicle or fludrocortisone was infused, and this special diet was continued for another 4 weeks during vehicle or fludrocortisone infusion.

Table 1. Mouse Strain Information.

Strain	Description	Sex	Persistent ID	URL
C57BL/6J	C57BL/6J	M	000664	https://www.jax.org/strain/000664
ApoE -/-	B6.129P2-*Apoe*^{tm1Unc}/J (>N10 to C57BL/6J)	M	002052	https://www.jax.org/strain/002052
LDLR -/-	B6.129S7-*Ldlr*^{tm1Her}/J (>N10 to C57BL/6J)	M	002207	https://www.jax.org/strain/002207

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Table 2. ARRIVE Guidelines Checklist.

Item	Application
IACUC Protocol	# 2018-2968, approved by the University of Kentucky IACUC.
Sex	This study examined aortic pathologies only in male mice because of the higher prevalence in male mice.[14]
Inclusion criteria	Body weight > 20 g and > 7 weeks of age
Exclusion criteria	1. Body weight < 20 g and < 7 weeks of age 2. Euthanasia or death prior to termination or medical cases reported by a veterinarian were excluded for in situ and ex vivo diameter measurements
Sample size	N=5 for vehicle, and N=15 for fludrocortisone infusion for each experiment
Power analysis (prospective)	Not performed.
Endpoint	1. Death due to aortic rupture 2. Aortic diameters by in situ or ex vivo measurements
Randomization	DLAR staff placed study mice randomly in cages (N=5/group) upon arrival.
Blinding	All experimental data were verified by an independent investigator blinded to the study group information.
Statistical analysis	SigmaPlot version 14.5 (SYSTAT Software Inc., CA)
Statistical method	Continuous variables between groups were analyzed using Mann-Whitney rank sum test.
Data availability	All raw data and analytical methods are available from the corresponding authors upon appropriate request.

All animal experiments reported in this manuscript were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC protocol number 2018-2968) and followed the ARRIVE Guidelines (Table 2).[15]

2.2. Mini osmotic implantation and fludrocortisone infusion

Fludrocortisone (Cat# F6127-1G; MilliporeSigma) was infused subcutaneously via mini osmotic pumps (Alzet Model 2004; Durect Corp.). Polyethylene glycol-400 (PEG-400; 85% vol/vol; Cat# PX1286-B2; MilliporeSigma) and DMSO (15% vol/vol; Cat# D8418-50ML; MilliporeSigma) were used to dissolve fludrocortisone. Therefore, PEG-400 (85% vol/vol) and DMSO (15% vol/vol) are used as vehicle.

Male mice at 8 – 9 weeks of age were infused with either vehicle or fludrocortisone (12 mg/kg/day) for 4 weeks. Mice were sedated with isoflurane and pumps were implanted subcutaneously on the right flank of each mouse in the same procedure described for AngII infusion.[9]

2.3. Systolic blood pressure measurements

Systolic blood pressure was measured daily between days 21-23 of either vehicle or fludrocortisone infusion using a non-invasive tail-cuff system (BP-2000 blood pressure analysis system; Visitech Systems, Inc.) following our standard protocol.[16]

2.4. Measurement of aortic diameters

Immediately after euthanasia, saline was perfused through the left ventricle to remove the blood in the aorta. The organs and tissues surrounding the ascending, aortic arch, and the proximal descending thoracic regions were removed carefully. A thin black plastic sheet was placed behind the heart and thoracic aorta, and in situ images were

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captured using a Nikon dissecting stereoscope.[17, 18] Aortic diameters were measured at the largest width perpendicular to the aortic longitudinal axis at the ascending aorta using Nikon NIS-Elements AR 4.51.

Subsequently, the full length of the aorta from the ascending portion to the iliac bifurcation was dissected and fixed in 10% neutral-buffered formalin for 24 hours. Adventitia were then removed, and aortas were pinned and imaged.[9] Maximal outer width of the suprarenal aortic region was measured using the Nikon NIS-Elements AR 4.51.

2.5. Statistical analyses

Individual data are presented with the median and 25th/75th percentiles. Statistical analyses were performed using SigmaPlot version 14.5 (SYSTAT Software Inc., San Jose, CA). Since N=5 for each vehicle group (small sample size), only Mann-Whitney Rank Sum test was used for data analysis. $P<0.05$ was considered statistically significant.

3. Results

3.1. Fludrocortisone induced aortic pathologies in the thoracic aorta of male C57BL/6J mice

Male C57BL/6J mice were fed a normal laboratory diet and infused with either vehicle or fludrocortisone for 4 weeks. Plasma cholesterol concentrations were below 100 mg/dl for both groups (Figure 1A).

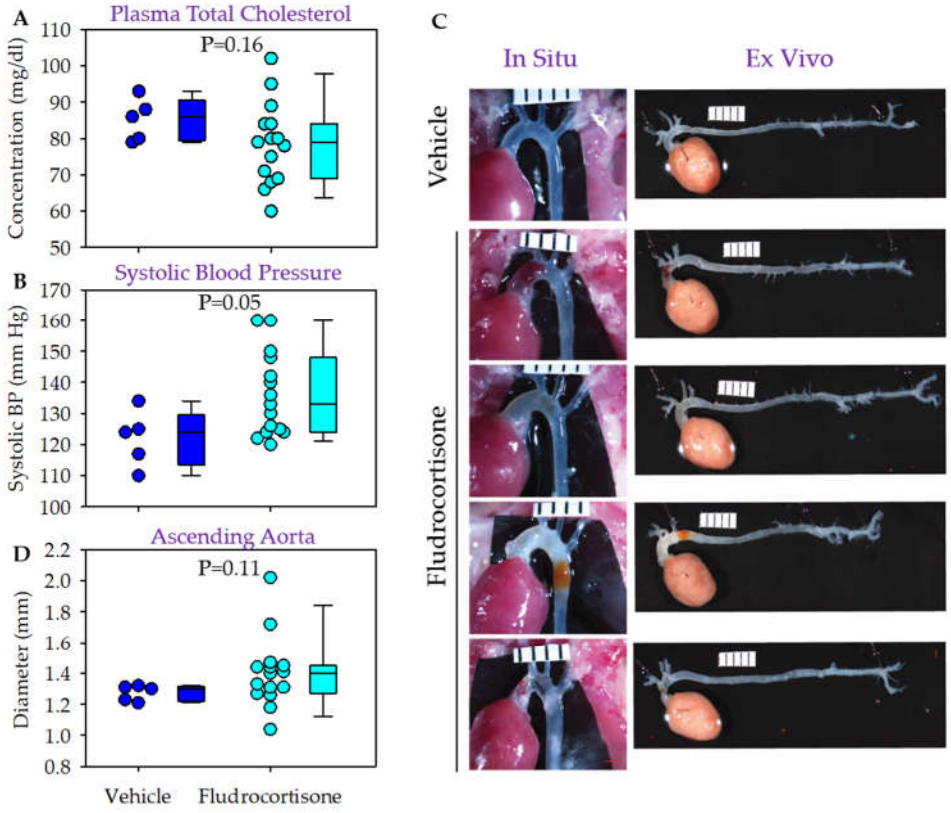

Figure 1. Fludrocortisone induced aortic pathologies in male C57BL/6J mice. (A) plasma total cholesterol concentrations were measured using an enzymatic kit. (B) Systolic blood pressure was measured using a tail-cuff system. (C) In situ and ex vivo images were taken using a Nikon SMZ800 stereoscope. (D) Maximal outer diameter of the ascending aorta was measured using a NIS-Elements AR 4.51 software (Nikon).

Fludrocortisone infusion led to higher systolic blood pressure in male C57BL/6J mice (Figure 1B), but this did not reach statistical significance (P=0.05), compared to mice infused with vehicle. No mice died or was excluded during the study. No pathologies were noted in mice infused with vehicle per in situ and ex vivo imaging analysis. In mice infused with fludrocortisone, 4 of 15 mice had gross pathological changes in the thoracic

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aortic regions, but no pathology was detected in abdominal regions (Figure 1C). One mouse showed extensive hemorrhage and another mouse showed modest hemorrhage in the ascending aortic region. One mouse had profound dilation in the ascending aortic region, and one mouse had dilation, wall thickening, and restricted thinning in the ascending region and discoloration in the descending aorta, possibly due to the hemorrhage resolution.

We measured the maximal diameter of ascending (Figure 1D), descending thoracic (data not shown), and suprarenal abdominal aortic regions (median: 0.8 versus 0.9 mm, P=0.001). No significant differences in diameters of the ascending and descending thoracic aortas, respectively, were detected.

3.2. Fludrocortisone induced aortic pathologies in male ApoE -/- mice and LDLR -/- mice

AAA, but not TAA, is augmented by hypercholesterolemia in AngII-infused male mice.[12, 13] We therefore determined whether hypercholesterolemia augments fludrocortisone-induced aortic pathologies in the two commonly used hypercholesterolemic mouse models, ApoE -/- mice and LDLR -/- mice. ApoE -/- mice become hypercholesterolemic spontaneously even when fed a normal laboratory rodent diet.[19] In male ApoE -/- mice fed a normal laboratory diet, although both groups were modestly hypercholesterolemic, the median plasma cholesterol concentrations in fludrocortisone group was higher than that in the vehicle group (Figure 2A). No death was found and no severe health condition led to withdrawal of any study mouse. Therefore, all study mice were included for blood pressure, imaging, and diameter analysis.

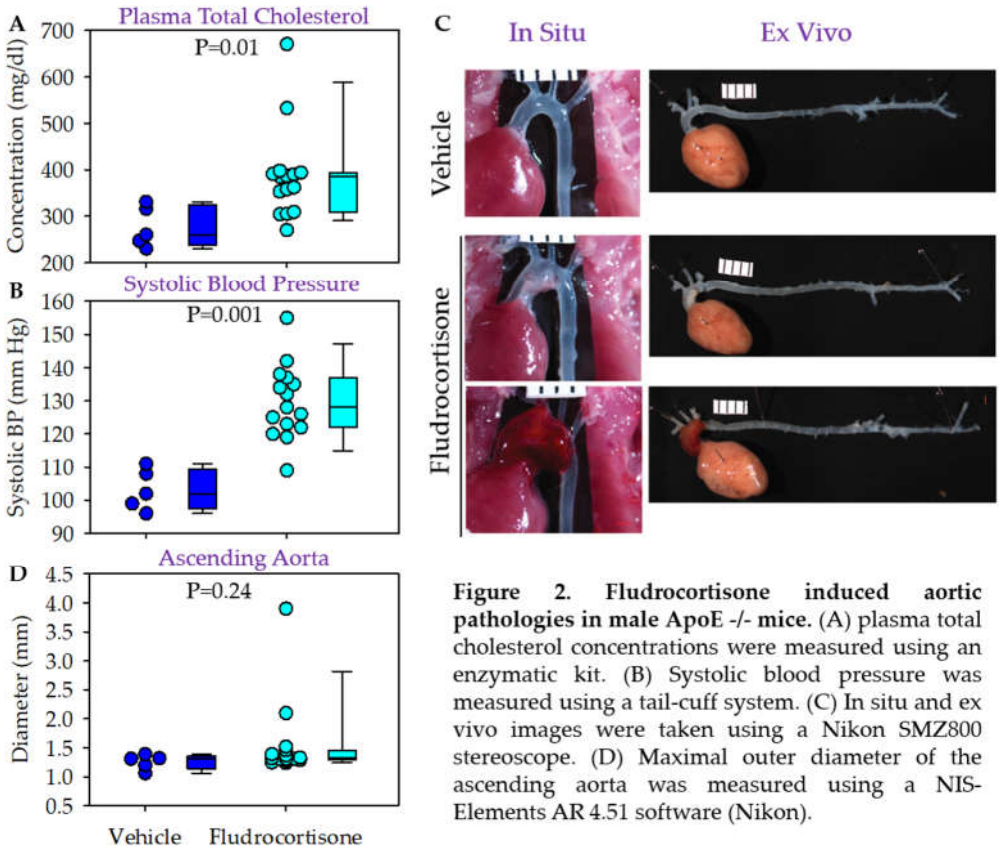

Figure 2. Fludrocortisone induced aortic pathologies in male ApoE -/- mice. (A) plasma total cholesterol concentrations were measured using an enzymatic kit. (B) Systolic blood pressure was measured using a tail-cuff system. (C) In situ and ex vivo images were taken using a Nikon SMZ800 stereoscope. (D) Maximal outer diameter of the ascending aorta was measured using a NIS-Elements AR 4.51 software (Nikon).

Fludrocortisone increased systolic blood pressure (Figure 2B), compared to the vehicle infusion. Among the 15 mice infused with fludrocortisone, one had profound dilation in the ascending and arch regions, and one had a large fresh hemorrhage in the ascending and arch regions (Figure 2C). No mice had apparent abdominal aortic pathologies (Figure 2C). We did not detect significant differences of aortic diameters in the ascending (Figure

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2D), descending thoracic (data not shown), and suprarenal aortic regions (data not shown) between vehicle and fludrocortisone-infused mice.

In contrast to the spontaneous hypercholesterolemia in ApoE -/- mice, LDLR -/- mice have only modestly higher plasma total cholesterol concentrations than C57BL/6J mice when fed normal laboratory diet, but their plasma cholesterol concentrations increase profoundly when fed a saturated fat-enriched diet. We therefore performed two separate studies to determine effects of fludrocortisone on aortic pathologies in male LDLR -/- mice.

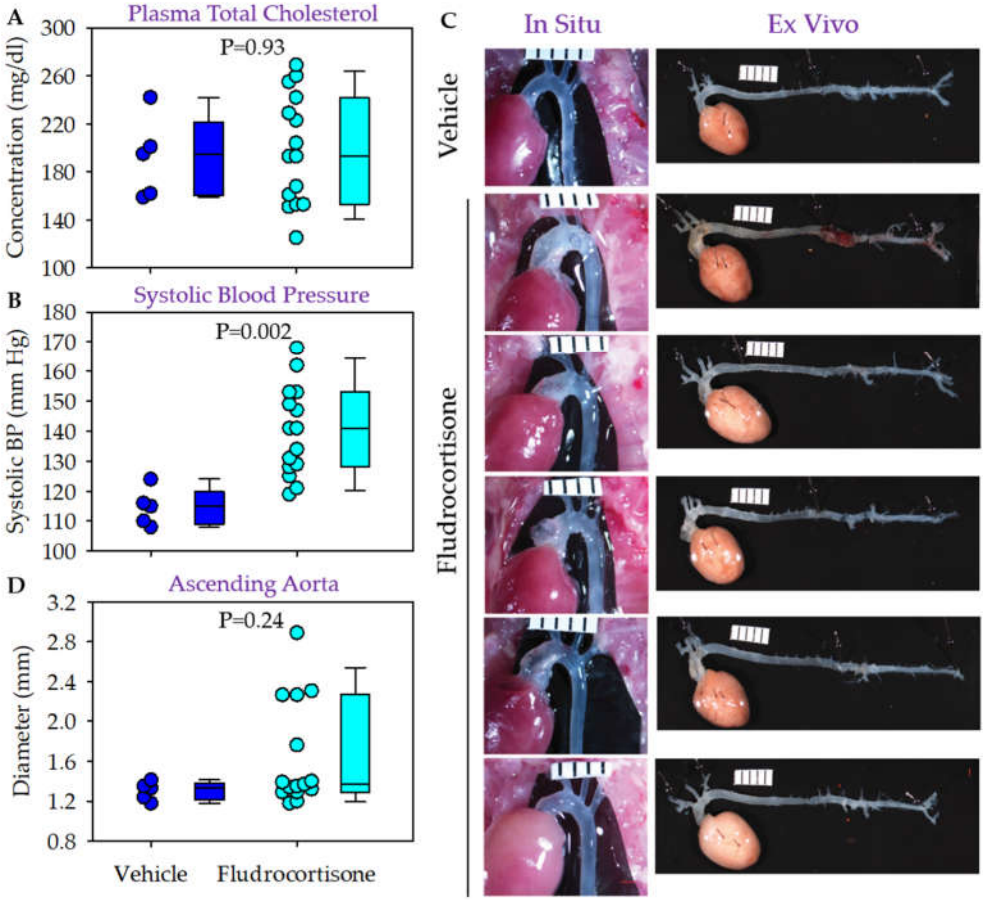

Figure 3. Fludrocortisone induced aortic pathologies in male LDLR -/- mice fed normal diet. (A) plasma total cholesterol concentrations were measured using an enzymatic kit. (B) Systolic blood pressure was measured using a tail-cuff system. (C) In situ and ex vivo images were taken using a Nikon SMZ800 stereoscope. (D) Maximal outer diameter of the ascending aorta was measured using a NIS-Elements AR 4.51 software (Nikon).

In the first study, we fed male LDLR -/- mice normal laboratory rodent diet. Medians of plasma cholesterol concentrations were not different between the two groups (Figure 3A). The systolic blood pressure increase was significant in mice infused with fludrocortisone, compared to mice infused with vehicle (Figure 3B). There was no death prior to termination or exclusion due to other reasons. Among the 15 mice infused with fludrocortisone, 5 had apparent ascending aortic dilation with a grossly thin or thick wall (Figure 3C). Among the 5 mice, one mouse also had aortic pathology with a relatively fresh hemorrhage in the suprarenal region (Figure 3C). We did not find significant differences of aortic diameters in the ascending (Figure 3D), descending thoracic (data not shown), and suprarenal aortic regions (data not shown) between the two groups.

In the subsequent study, we fed male LDLR -/- mice a Western diet containing 42% kcal/kcal from fat for one week. Fludrocortisone or vehicle was then infused for 28 days, while the Western diet feeding was continued. Two of 15 mice died of aortic rupture. These two mice were excluded from plasma cholesterol, blood pressure, and aortic

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diameter data analyses. Although both groups were hypercholesterolemic, plasma cholesterol concentrations were lower in mice infused with fludrocortisone than those infused with vehicle (Figure 4A). Systolic blood pressure was higher in mice infused with fludrocortisone, compared to those infused with vehicle (Figure 4B). Among the surviving 13 mice, 2 had striking dilations and thinned wall in the ascending and aortic arch regions (Figure 4C). For the two mice with aortic rupture, one rupture initiated in the ascending thoracic aortic region, and one occurred in the suprarenal aortic region. No mice had abdominal aortic pathology except the one that died of abdominal aortic rupture. The median of the ascending aortic diameter was larger in fludrocortisone-infused mice than in vehicle-infused mice (Figure 4D). No significant differences in diameters of descending thoracic and abdominal regions were detected between the two groups (data not shown).

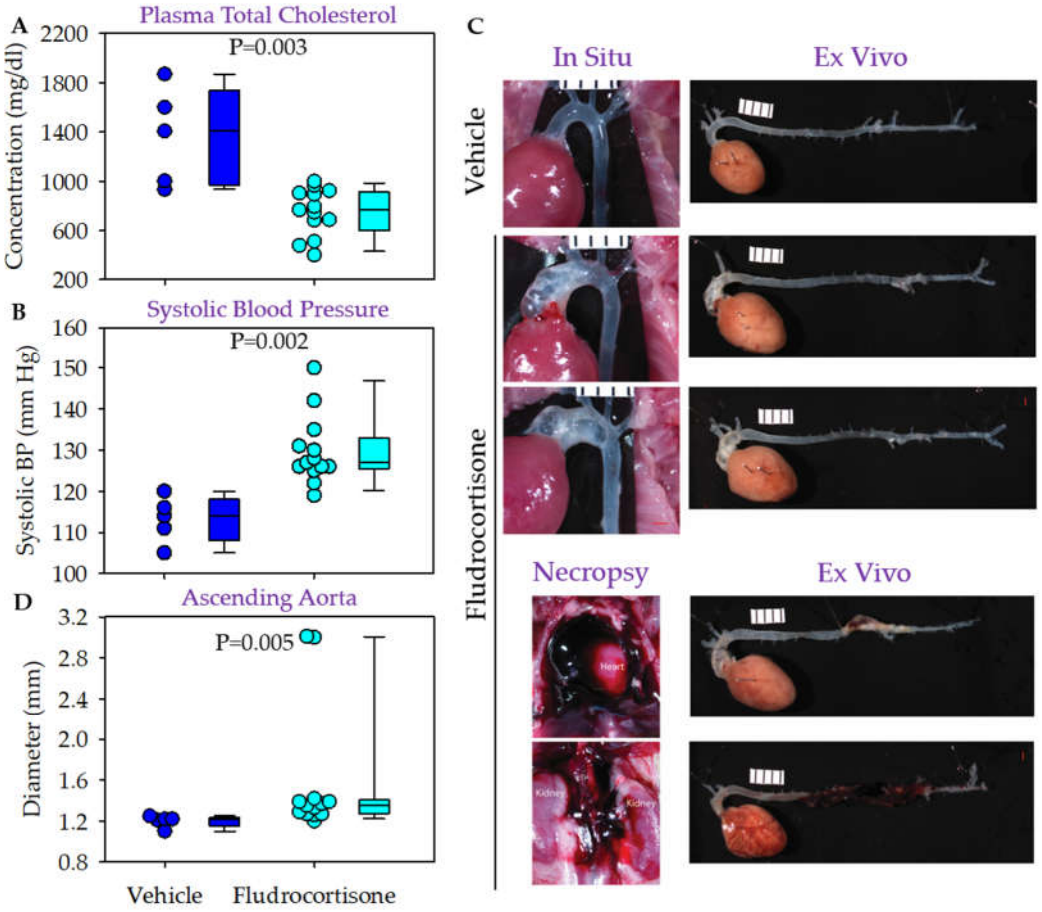

Figure 4. Fludrocortisone induced aortic pathologies in male LDLR -/- mice fed Western diet. (A) plasma total cholesterol concentrations were measured using an enzymatic kit. (B) Systolic blood pressure was measured using a tail-cuff system. (C) In situ and ex vivo images were taken using a Nikon SMZ800 stereoscope. (D) Maximal outer diameter of the ascending aorta was measured using a NIS-Elements AR 4.51 software (Nikon).

Aortic pathologies are summarized in Table 3.

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Table 3. Summary of Aortic Pathologies.

Mouse Strain	Diet	Aortic Pathologies (N of mice)			
		Asc/Arch	Asc/Arch + Desc Thoracic	Asc/Arch + Suprarenal	Desc Thoracic + Suprarenal
C57BL/6J	Normal	3	1	0	0
ApoE -/-	Normal	2	0	0	0
LDLR -/-	Normal	4	0	1	0
LDLR -/-	Western	2	0	1	1

Asc = ascending; Desc = descending

4. Conclusion

The present study determined effects of fludrocortisone in 4 independent experiments using 3 mouse strains (C57BL6/J, ApoE -/-, and LDLR -/- mice fed either normal laboratory or Western diet) on aortic pathologies. Although the incidence of aortic pathology was relatively low (15 of 60 = 25%), it is evident that fludrocortisone contributes to aortic pathologies, irrespective of mouse strain or diet.

Aortic pathologies were found predominantly in the ascending and aortic arch regions. Among the 15 mice with profound aortic pathologies, 14 were involved in the ascending and aortic arch regions, which included 2 having both ascending and descending thoracic aortic pathologies. One mouse died of aortic rupture with descending thoracic and abdominal aortic hemorrhage, and one died of abdominal aortic rupture. Aortic pathologies in the ascending and aortic arch regions include dilatation, thin or thick wall, and hemorrhage. These pathologies are similar to what have been observed in AngII-infused mouse model.[13]

Fludrocortisone-induced abdominal aortic pathology located in the suprarenal aorta, the same location for AngII-induced AAAs,[5, 6, 9] mice with endothelial nitric oxide synthase,[20] mice administered deoxycorticosterone acetate and high salt, and mice administered aldosterone and high salt.[21] The mechanism of this specific location for AAAs in mouse models is unclear.

In C57BL/6J or LDLR -/- mice fed normal laboratory diet, plasma cholesterol concentrations were not different between the two groups. In ApoE -/- mice fed normal diet, plasma cholesterol concentrations were modestly higher in mice infused with fludrocortisone. In contrast, in LDLR -/- mice fed Western diet plasma cholesterol concentrations were much lower in mice infused with fludrocortisone than in mice infused with vehicle. We were unable to find previous reports that fludrocortisone affected plasma cholesterol concentrations. Also, we did not find that plasma cholesterol concentrations correlated with aortic pathologies in fludrocortisone-infused mice. It is well-established that AngII-induced AAA mouse model is augmented by hypercholesterolemia.[12] It is likely that the molecular mechanisms underlying fludrocortisone- and AngII-induced abdominal aortic pathologies are different.

Similar to AngII, fludrocortisone increased systolic blood pressure in mice. It appears that the magnitude of the increases in fludrocortisone infused mice is less than AngII infused mice. However, conclusions cannot be made because we did not perform side-by-side comparisons between these two reagents. There is compelling evidence that AngII-induced aortic pathologies are independent of blood pressure changes.[22-24] Of note, norepinephrine can also increase blood pressure to the same magnitude as that of AngII, but no aortic aneurysms were detected in mice infused with norepinephrine.[23, 24] We compared the blood pressure in mice exhibiting aortic pathologies with those without aortic pathologies. We did not note apparent associations between blood pressure and aortic pathologies (Figure 5). Based on these observations, it seems reasonable to conclude that high blood pressure is not a primary contributing factor to aortic pathologies in fludrocortisone-infused mice.

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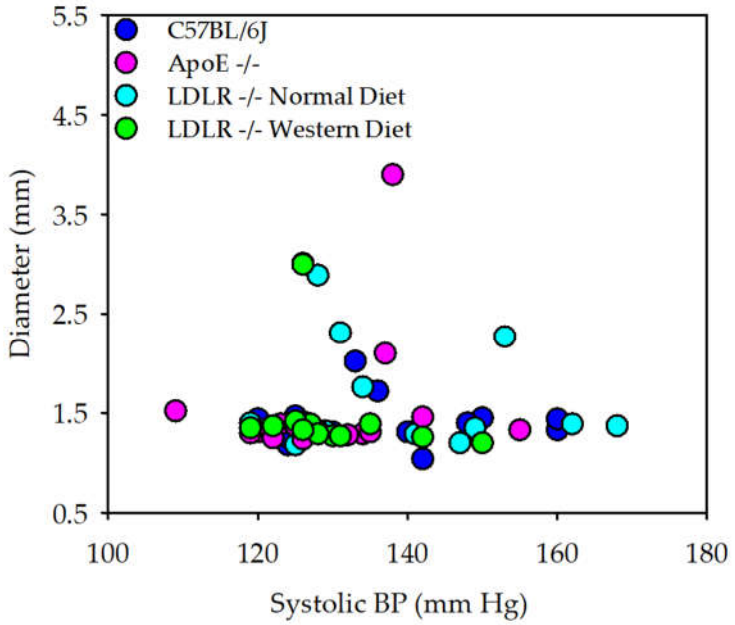

Figure 5. Ascending aortic diameters were not associated with systolic blood pressure in mice infused with fludrocortisone. Linear regression analysis shows P=0.89.

Fludrocortisone is a drug commonly used in patients with adrenocortical insufficiency,[1] orthostatic hypotension,[2] and some other conditions. This drug has many known adverse effects, but most are mild. No studies have reported that fludrocortisone induces aortic pathologies in humans. In fact, there are studies that have reported that fludrocortisone treatment can improve efficiency of hypervolemic therapy in patients with aneurysmal subarachnoid hemorrhage.[25-27] It is unclear whether our findings are species-specific.

Fludrocortisone is a mineralocorticoid receptor agonist. One study reported that deoxycorticosterone acetate (DOCA) or aldosterone, in the presence of high salt in drinking water, induced aortic aneurysms in C57BL/6 mice that mimicked the phenotypes in mice infused with AngII.[21] Aortic aneurysms induced by either DOCA or aldosterone and high salt were attenuated by mineralocorticoid receptor antagonists.[21] A recent prospective human study reported that patients with primary aldosteronism had larger ascending aortas.[28] Together, these findings support the notion that mineralocorticoid receptor may play a role in the development of aortic aneurysms.

Considering human and mouse aortic pathologies have many comparable pathogeneses, it is worth paying attention to possible aortic pathologies in patients who are taking fludrocortisone. It is also important to be cautious when prescribing fludrocortisone to patients with aortic pathologies – our findings suggest that these patients should be at least monitored more rigorously during fludrocortisone administration.

In conclusion, this study provides evidence that fludrocortisone induces aortic pathologies in mice, predominantly in the ascending and arch regions, with risk for rupture in both the thoracic and abdominal aortic regions. Fludrocortisone-induced aortic pathologies are not attributed to mouse strain, hypercholesterolemia, or blood pressure.

Author Contributions: Study design: DY, CW, HSL; Implementation of animal experiments: DY, HC, JCL, DAH, MKF, JJM; Data analyses: DY, CW, HSL; Supervising and data verification: CW, HSL; Writing the draft manuscript: DY, CW, HSL; Editing the manuscript: All authors

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Conflicts of Interest: The authors declare no conflict of interest. No patents are related to the reported studies.

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