Influence of Vitamin D status on the vasoactive effects of aorta rings in a rat model of polycystic ovary syndrome

PhD thesis

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1. Introduction

The prevalence of PCOS is 8 – 13% in women of reproductive age, and 6% in adolescent girls.

Diagnosis of PCOS relies on the Rotterdam criteria: two of the following three should be observed: clinical or biochemical hyperandrogenism, ovulatory dysfunction and polycystic morphology of the ovaries. The early complications are menstrual irregularity, infertility, hirsutism, and acne. Later co-morbidities present as part of metabolic syndrome including type 2 diabetes, hypertension, and other cardiovascular diseases (CVD).

The early appearance of vascular dysfunction in the pathogenesis of the disease may contribute to the increased CV risk. Deterioration of endothelium-dependent vasorelaxation can be the first sign of vascular dysfunction. The lower prevalence of hypertension in women of reproductive age might be related to the vasorelaxant effect of estrogen, and the higher risk of hypertension in women with PCOS could be caused by the impairment of this mechanism.

2. Objectives

Our aim is to examine how aorta vessel function changes in these different groups:

1. the hypothetical damage of the relative contractile ability of the aorta in female rats was tested with norepinephrine
2. vessel relaxation and hypothetical damage was examined using acetylcholine and estradiol, both in cumulative concentrations
3. to observe the different effects of NO and prostanoids on vasorelaxation

L – NAME, and COX-2 inhibitor were used

4. we would like to quantify the adequate receptor and enzyme amount, which could mean the underlying reason for the

detected vessel function changes seen in the hyperandrogenic state, thus ERα (estradiol receptor α), eNOS, and COX-2 enzymes were measured with immunohistochemical staining.

3. Methods

3.1. Animals and chronic treatment of the rats

We created a model of chronic hyperandrogenism and VDD in adolescent (21–28 day-old), female Wistar rats (N=46). The animals were randomly assigned to four experimental groups; : hyperandrogenic vitamin D deficient group (T + D-, N = 12), hyperandrogenic, vitamin D supplemented group (T + D+, N = 12), vitamin D deficient group (T-D-, N = 12), and vitamin D supplemented group (T-D+, N=12).

Hyperandrogenism was induced by an 8-week-long transdermal testosterone treatment applying 5 times a week on a previously shaved area on the back of the animals. VDD was generated by reduced vitamin D intake the animals being fed with Vitamin D Free chow. Supplemented animals had access to a regular chow containing 1000 IU/kg of vitamin D_3. Vitamin D-supplemented animals received additional oral vitamin D supplementation as the following: 500 IU cholecalciferol on the 2nd week and a weekly dose of 140 IU/100 g on the 5th, 6^{th}, and 7th. According to the human vitamin D supplementation guidelines, the target serum 25-hydroxy-cholecalciferol levels were optimized to 30–50 ng/ml.

3.2. Wire myograph protocol

The carefully prepared aortic segment was cut into 9 (3 mm long) equal pieces. 8 of these were placed on a conventional wire myograph setup.

After achieving stable pretension, 124 mmol/L K+ was administered for 3 minutes to confirm the vessels' contractile ability and establish a maximal value for contraction force. Then, K+ was washed out with KR solution, and the contraction

force after cumulative concentrations of norepinephrine (NE) (10-9, 10-8, 10-7, and 10-6 mol/L) was measured. After maximal contraction, we investigated the vasorelaxant potential of raising concentrations of acetylcholine (Ach) (10-8, 10-7, 10-6, and 10-5 mol/L). After another equilibration with KR, norepinephrine newly generated precontraction (5 × 10-8 mol/L). The relaxation of estradiol was evaluated at three distinct doses (10-7 – 10-5 mol/L) after 5x10-8 norepinephrine precontraction. To identify possible endothelium-dependent relaxing pathways the following inhibitors were used before the 2nd and 3rd precontraction endothelial nitric oxide synthase (eNOS) inhibitor, L-NAME 10^{-4} M and cyclooxygenase 2 (COX-2) inhibitor, NS398, 10^{-5} M).

3.3. Immunohistochemistry

Paraffin-embedded tissue sections were stained against estrogen receptor alpha (ERα), eNOS, COX-2. The positively stained area as a percentage of total tissue area or non-calibrated optical density of specific staining was measured in the intimal and medial layers of the vessel walls using the ImageJ software.

3.4. Statistics

Two-way ANOVA with Tukey's post-hoc test in Prism 8 (GraphPad Software, USA) was used to assess the impact of testosterone therapy and vitamin D status. Using repeated-measures two-way ANOVA with Bonferroni's post hoc test, we studied vascular function curves. The critical value of p0.05 was agreed upon by all researchers.

4. Results

4.1. Vascular function

Norepinephrine concentration had no effect on the degree of vasoconstriction that was induced in any of the treatment groups

(Figure 1). This indicates that the aortic wall's smooth muscle cell function is well preserved.

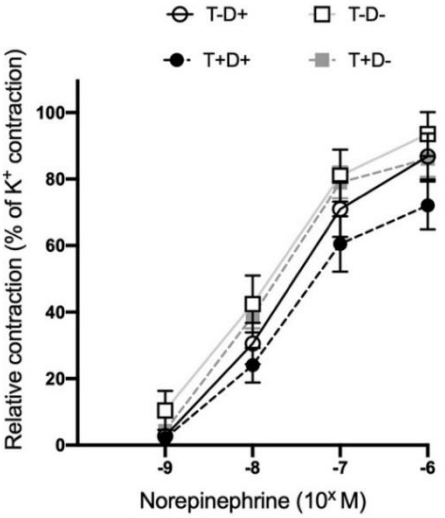

Figure 1. Norepinephrine-induced vasoconstriction

N = 10 − 11 in each group. Two-way ANOVA, Tukey's post hoc test. Data are presented as mean ± SEM.

In vitamin D-deficient groups, however, impaired endothelial function could be detected as early as the measurement of attenuated Ach-mediated vasorelaxation. The T-D- group demonstrated substantially less dilatation than the D+ groups. At concentrations of 10-7 M Ach, the difference reached statistical significance, while it remained constant at higher concentrations. The T+D- group was substantially less relaxed than the T+D+ group at 10-5 M **(Figure 2)**.

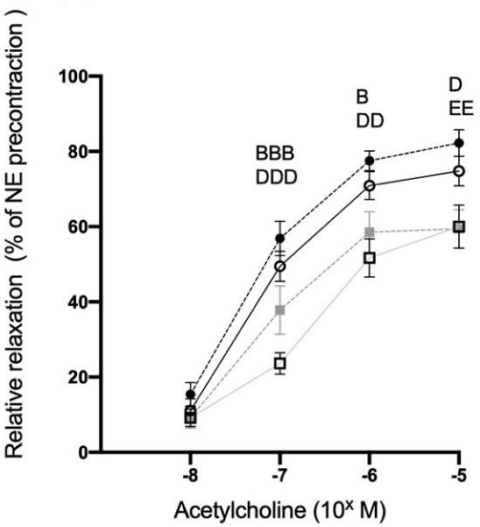

Figure 2. Acetylcholine-mediated vasorelaxation

N = 9 − 11 in each group. Two-way ANOVA, Tukey's post hoc test. BBB: T-D- vs. T-D+ p<0:001, B: T-D- vs. T-D+ p<0:05, DDD: T-D- vs. T+D+ p<0:001, DD: T-D- vs. T+D+ p<0:01, D: T-D- vs. T+D+ p<0:05, EE: T+D- vs. T + D+ p < 0:01. Data are presented as mean ± SEM.

The increasing concentration of estradiol (10-7 − 10-5 M) lead to the relaxation of the pre-contracted (5x10-8 norepinephrine) thoracic aorta segments in all experimental groups. Regardless of their androgenic status, the aortic segments of VDD rats revealed considerably reduced estradiol-induced relaxation **(Figure 3)**.

The inhibition of cyclooxygenase 2 (COX-2) further decreased the reduced estradiol-dependent relaxation of vitamin D-deficient aortas, whereas COX-2 inhibition had no effect on the vessels of vitamin D-supplemented rats. The estradiol-induced relaxation was eliminated across all groups when eNOS was inhibited by pretreatment with L-NG-Nitro arginine methyl ester (L-NAME) **(Figure 4)**.

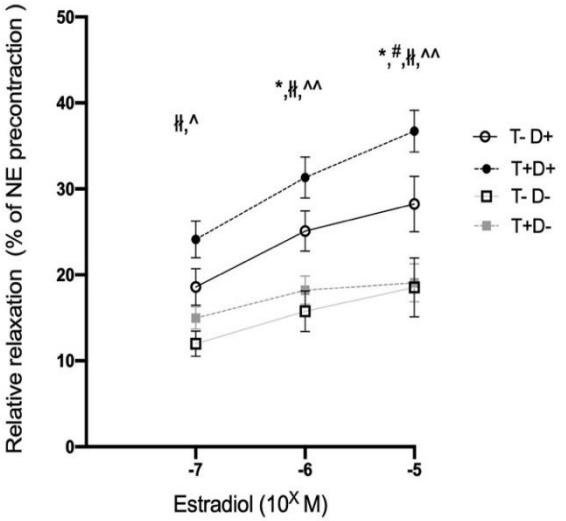

Figure 3. Estradiol-dependent vasorelaxation

*Repeated measures two-way ANOVA using Bonferroni's post hoc test: Data are presented as mean ± SEM. *: p<0.05 T-D+ vs. T-D-, #: p<0.05 T-D+ vs. T+D-, ǂ: p<0.01 T+D+ vs. T-D-, ^: p<0.05 T+D+ vs. T+D-, ^^: p<0.01 T+D+ vs. T+D-.*

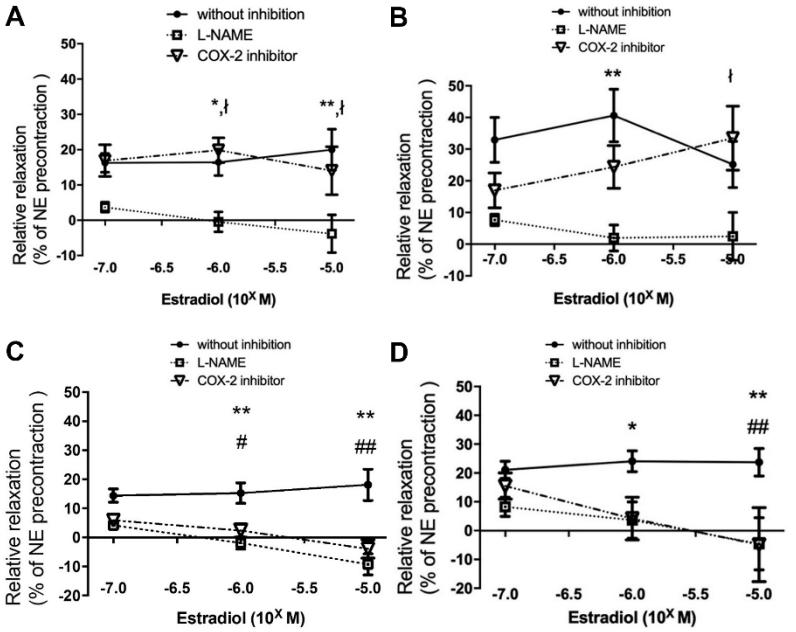

Figure 4. Estradiol-induced vasorelaxation in the presence of eNOS (L-NAME) or COX-2 (NS398) inhibitors (A) in T-D+ group; (B) in T+D+ group; (C) in T-D- group and (D) in T+D- group

*Data are presented as mean ± SEM. Repeated measures two-way ANOVA using Bonferroni's post hoc test: *: p<0.05 L-NAME vs. without inhibition, **: p<0.01 L-NAME vs. without inhibition, ł: p<0.05 L-NAME vs. COX-2 inhibitor; , ł: p<0.05 L-NAME vs. COX-2 inhibitor; #: p<0.05 COX-2 inhibitor vs. without inhibition, ##: p<0.01 COX-2 inhibitor vs. without inhibition.*

4.2. Immunohistochemistry

In the aortic intima, ER specific labeling was found to be higher after testosterone treatment and lower after VDD. The T-D- group had the lowest ER staining when the post hoc test was performed compared to the other groups **(Figure 5)**.

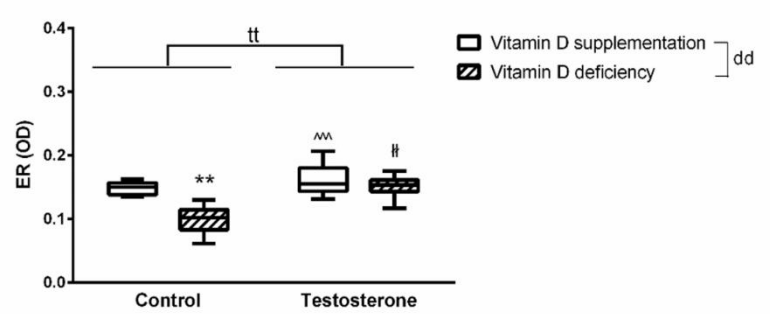

Figure 5. Immunohistochemical staining of the aortic wall with ERα antibody

*Non-calibrated optical density was measured. Data are presented as mean ± SEM. Two way (testosterone treatment and vitamin D status) ANOVA; Tukey's post hoc test, **: p<0.01 T-D+ vs. T-D+ group, ^^: p<0.005 T-D+ vs. T+D+ group, łł: p<0.01 T-D- vs T+D- group; tt: p<0,01 T-vs T+, dd: p<0,01 D+ vs D-. N=5-6 in each group.*

Two-way ANOVA results showed that VDD considerably decreased eNOS specific staining intensity in the analyzed vessels. However, therapy with testosterone had little effect. There were no obvious differences between the groups, according to a post hoc analysis **(Figure 6)**.

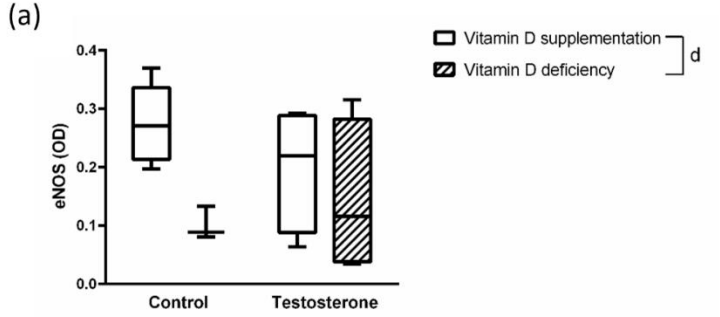

Figure 6. Immunohistochemical staining of the aortic wall using eNOS antibody

Data are presented as mean ± SEM. Two-way (testos-terone treatment and vitamin D status) ANOVA; d: p<0,05 D+ vs D-. N=4-6 in each group.

Testosterone treatment significantly increased COX-2 staining in our experiment. Greater amounts of staining were seen in the T+D+ group than in the control group **(Figure 7)**.

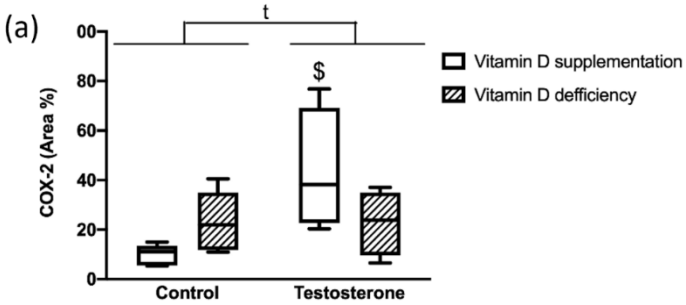

Figure 6. Immunohistochemical staining of the aortic wall with ERα antibody

Data are presented as mean ± SEM. Two-way (testosterone treatment and vitamin D status) ANOVA; Tukey's post hoc test, $: p<0.05 T-D+ vs. T+D+ group, t: p<0.05 D+ vs D-. N=4-5 in each group.

5. Conclusions

In our rodent model of PCOS we examined the possible early vascular changes of the disease, and the possible interplay of hyperandrogenism and vitamin D deficiency.

1.There was no difference in the developed vasoconstriction amongst the treatment groups;
2.As the effect of VDD (in both VDD groups), significantly reduced relaxation was measured;
3.Following L-NAME incubation, estradiol-dependent vasorelaxation significantly decreased in each group. Following incubation with COX-2 inhibitor, vasorelaxation decreased only in the vitamin D deficient groups which was not modified by testosterone treatment;
4.Vitamin D deficiency significantly decreased, while testosterone treatment increased ERα expression by immunohistochemistry. T-D- group had significantly lighter staining compared to all other experimental groups. Vitamin D deficiency significantly decreased eNOS specific staining while testosterone treatment did not alter this result. Testosterone treatment significantly increased COX-2 staining in our experiment. T+D+ group demonstrated significantly larger stained areas compared to control.

Vascular dysfunction was significantly exacerbated by VDD, a typical co-morbidity of PCOS. Reduced ER and eNOS immunostaining paralleled with endothelial dysfunction.
Beside short-term testosterone treatment, endothel dysfunction did not evolved, however, other initial alterations were observed causing endothelial dysfunction and play key roles in target organ damages observed in PCOS, eg. increased COX activation. Testosterone increased estradiol receptor expression,

via this mechanism reached similar level of aortic estradiol relaxation comparing to testosterone free animals.

Further investigation needed to determine the positive and the less favorable effect of testosterone to vascular function and to determine the ideal interventions involved.

The development of endothelial dysfunction and the subsequent rise in cardiovascular risk may be postponed with treatment of VDD. Our findings suggest that, in addition to regulating the menstrual cycle, sufficient vitamin D supplementation may be fundamental in relieving the abnormal vascular adaption in early PCOS.

6. Bibliography of the candidate's publications

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