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# Endothelial relaxation mechanisms and nitrative stress are partly restored by Vitamin $D_3$ therapy in a rat model of polycystic ovary syndrome

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ABSTRACT

*Aims:* In polycystic ovary syndrome (PCOS), metabolic and cardiovascular dysfunction is related to hyperandrogenic status and insulin resistance, however, Vitamin D3 has a beneficial effect partly due to its antioxidant capacity. Nitrative stress is a major factor in the development of cardiovascular dysfunction and insulin resistance in various diseases. Our aim was to determine the effects of vitamin D3 in a rat model of PCOS, particularly the pathogenic role of nitrative stress.
*Main methods:* Female Wistar rats weighing 100–140 g were administered vehicle (C), dihydrotestosterone (DHT) or dihydrotestosterone plus vitamin D3 (DHT + D) (n = 10 per group). On the 10th week, acetylcholine (Ach) induced relaxation ability of the isolated thoracic aorta rings was determined. In order to examine the possible role of endothelial nitric oxide synthase (eNOS) and cyclooxygenase-2 (COX-2) pathways in the impaired endothelial function, immunohistochemical labeling of aortas with anti-eNOS and anti-COX-2 antibodies was performed. Leukocyte smears, aorta and ovary tissue sections were also immunostained with anti-nitrotyrosine antibody to determine nitrative stress.
*Key findings:* Relaxation ability of aorta was reduced in group DHT, and vitamin D3 partly restored Ach induced relaxation. eNOS labeling was significantly lower in DHT rats compared to the other two groups, however COX-2 staining showed an increment. Nitrative stress showed a significant increase in response to dihydrotestosterone, while vitamin D3 treatment, in case of the ovaries, was able to reverse this effect.
*Significance:* Nitrative stress may play a role in the pathogenesis of PCOS and in the development of the therapeutic effect of vitamin D3.


## Introduction

The diagnostic criteria for polycystic ovary syndrome (PCOS), besides polycystic ovary morphology, are hyperandrogenic status and oligo-amenorrhea. Insulin resistance is a common metabolic sign in PCOS, it occurs in 50–80% of all patients (Diamanti-Kandarakis, 2012). The long-term consequences of PCOS are metabolic disorders and cardiovascular diseases. PCOS affects 5%–8% of women in reproductive age, and it is a leading cause of female infertility. Earlier studies demonstrated the presence of vascular dysfunction in PCOS; it was previously described that aortic relaxation ability is decreased (Keller et al., 2011; Lakhani et al., 2006), and this phenomenon is probably not genetically encoded (Walch et al., 2008). Although PCOS is characterized by insulin resistance, and in prediabetic conditions endothelial function is deteriorating along with decreasing NO bioavailability (Sydow et al., 2005), earlier studies did not examine the possible changes in eNOS and COX-2 expressions in PCOS.

Nitric oxide (NO) is a ubiquitous transmitter, modulating for example local blood flow, platelet aggregation and neural activity, and it also plays a crucial role in the immune response. Peroxynitrite is a reactive free radical that is produced from the diffusion-controlled reaction between NO and superoxide anion (Pacher et al., 2007). Neither superoxide nor NO is highly toxic in vivo, because there are numerous mechanisms to minimize their accumulation. On the other hand when they react and form peroxynitrite in a non-enzymatic manner, it becomes a very potent oxidant agent. Peroxynitrite is able to damage proteins and nucleic acid, leading to loss of function and DNA breaks (Beckman et al., 1993). It is also known that peroxynitrite formation is elevated

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in subacute inflammation, and in prediabetic states (Fallarino et al., 2004; Grohmann et al., 2003; Zappulla, 2008).

Earlier data show that in PCOS the expression of both eNOS and COX-2 is decreased (Elia et al., 2006) in the ovary. Our hypothesis was that NO and vasodilator prostaglandins are produced independently in the endothelium, and along with decreased NO bioavailability, COX-2 expression can either increase as a counterregulatory mechanism, or decrease as part of the hampered endothelial function.

The physiological level of vitamin $D_3$ is 30–60 ng/L (Bloomgarden, 2011). About 40% of adult European population lives with $D_3$ hypovitaminosis. As vitamin $D_3$-need depends on body mass index, and women with PCOS are usually overweight, $D_3$ hypovitaminosis affects about 80% of women suffering from PCOS (Li et al., 2011). Vitamin $D_3$ concentrations also show connection to estrogen levels in PCOS (Lerchbaum and Obermayer-Pietsch, 2012). Vitamin $D_3$ may be also used as adjuvant therapy in PCOS, however earlier studies mainly focused on calcium homeostasis (Thys-Jacobs et al., 1999). On the other hand, the positive effects of vitamin D on cardiovascular function (Yiu et al., 2011), inflammation (Mabley et al., 2007) and carbohydrate metabolism (Kotsa et al., 2009) are well-known.

Our aim was also to determine systemic and tissue-specific nitrative stress (estimated by tyrosine nitration), besides the abundance of endothelial relaxant producing enzymes: endothelial NO synthase (eNOS) and cyclooxygenase-2 (COX-2) in a rat model of PCOS. We hypothesized as well that nitrative stress is linked to vascular dysfunction in PCOS, and vitamin D therapy is able to alter this effect.

## Materials and methods

### *Animals*

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the institutional Animal Care Commission (IRB approval: 22.1/2960/003/2009). Our study was designed after Manneras et al. (2007). As previously reported (Masszi et al., 2012; Sara et al., 2012a) thirty 21–28 day-old female Wistar rats (Semmelweis University Animal Colony, Budapest, Hungary which originated from Charles River Ltd.), weighing 100–140 g were randomized into 3 treatment groups. Under anesthesia (by Nembutal 45 mg/kg) continuous-release pellets containing 7.5 mg DHT (Innovative Research of America, Sarasota, FL, USA) were applied subcutaneously for 20 rats. Releases of 83 mg/d for 90 days are guaranteed by the manufacturer. The effectiveness of the DHT treatment was validated using Diasource DHT-RIA Kit (Diasource, Gmb[illegible]ssels, Belgium). Ten animals underwent sham operations receiving [illegible]llets that did not contain DHT (control group). After the surgical intervention, a mixture of 20 mg amoxicillin and 4 mg clavulanic acid (Augmentin; Glaxo Smith Kline, Brentford, UK) in 0.2 mL saline solution was administered intramuscularly to prevent infection. Ten DHT animals received 120 ng/100 g body weight/week 1,25 (OH)2 D3 vitamin (Injectable Calcijex, 2 mg/mL; Abbott Laboratories, Abbot Park, IL; USA DHT + D3 group) subcutaneously. No medical or surgical complications were observed. Conventional rat chow and tap water were provided ad libitum. On the 8th week of the experiment oral glucose tolerance test (OGTT) was performed in short ether narcosis in order to assess insulin response. Blood glucose and plasma insulin levels were measured 120 min after the administration of 20 mg glucose/100 g body weight, in gauge. Insulin response was calculated as insulin concentration divided by the glucose concentration 120 min after glucose load. After 10 weeks of DHT treatment, heparinized venous blood was collected from v. cava caudalis (10 IU/6 mL blood) of the anesthetized animals, then the rats were perfused transcardially with 10 mL heparinized (10 IU/mL) Krebs' solution ($CaCl_2$ 2.5 mM; $MgSO_4$ 1.17 mM; EDTA 0.027 mM; NaCl 119 mM; $NaHCO_3$ 20 mM; KCl 4.7 mM; $KH_2PO_4$ 1.18 mM; glucose 11 mM; Sigma Aldrich, Saint Louis, MO, USA). Thoracic aorta (TA) segments of 3 mm length from each experimental group were placed in warmed (37 °C) oxygenated (95% $O_2$ balanced with 5% $CO_2$, Lindegas, Répcelak, Hungary) Krebs' solution. Isometric tension was measured with isometric transducers (610-M Multi Myograph System; Danish Myo Technology, Aarhus, Denmark), digitized using BioPac A/D converter, stored and displayed on computer. A tension of 1.5 g was applied and the rings were equilibrated for 60 min. Concentration-dependent relaxation to acetylcholine ($10^{-8}$ to $10^{-5}$ M) was measured after precontraction with norepinephrine ($5 \times 10^{-8}$ M).

### *Immunohistochemistry*

Ovaries and aorta rings of the animals were freshly fixed for histological examinations. Circulating mononuclear cells were isolated from whole blood using Histopaque-1083 according to the users' manual (Sigma Aldrich). To demonstrate polycystic morphology, hematoxylin–eosin staining was performed on tissue sections of the ovaries. Circulating leukocyte smears and paraffin-embedded sections of aortas and ovaries – after deparaffinization and antigen retrieval (0.1 mmol/L citrate buffer, pH 3, heating in microwave oven for 15 min) -–were immunostained with polyclonal rabbit anti-nitrotyrosine antibody (1:200; overnight, 4 °C, Millipore, Billerica, MA, USA). After antigen retrieval aorta segments were also stained for endothelial NO synthase (polyclonal rabbit anti-eNOS antibody, 1:60, 70 min, 37 °C, Abcam, Cambridge, MA, USA) and cyclooxygenase-2 (polyclonal rabbit anti-COX-2 antibody, 1:200, 70 min, 37 °C, Abcam, Cambridge, MA, USA). Secondary labeling was achieved by using biotinylated anti-rabbit goat antibody (Vector Laboratories, Burlingame, CA, USA; 30 min, RT). Horseradish peroxidase conjugated avidin (Vectastain ABC kit, Vector Laboratories, 30 min, RT) and black colored nickel-cobalt enhanced diaminobenzidine (Vector Laboratories, 6 min, RT) were used to visualize the labeling (Vector Laboratories). Sections and smears were counterstained by the red colored Nuclear Fast Red (NFR, Sigma Aldrich). Data collections were made by Zeiss AxioImager.A1 microscope coupled with Zeiss AxioCAm MRc5 CCD camera. Staining intensity was determined by MBF ImageJ for microscopy (McMaster Biophotonics Facility, Ontario, Canada). In case of nitrotyrosine labeling the percentage of positively stained tissue area to total area of the section was calculated. In case of eNOS and COX-2 antibodies the endothelial layer of aortas was evaluated. To estimate the nitrotyrosine positivity of circulating leukocytes, staining intensity was scored with a mark between 1 and 10 by a blinded experimenter.

### *Statistical analysis*

Results are reported as mean ± SEM. Variance analysis of data was performed using one- and two-way ANOVAs, where appropriate. Statistical significance between groups was determined by Tukey's (immunohistochemistry) or Bonferroni's (in case of vascular function) post hoc tests. Probability values of $P \leq 0.05$ were considered significant.

## Results

Dihydrotestosterone levels of DHT and DHT + D treated rats were significantly higher than in the control animals (C: 267.3 ± 14.1; DHT: 370.9 ± 35.0; DHT + D: 438.4 ± 24.1 pg/mL; mean ± SEM, $p \leq 0.05$ C vs. DHT and DHT + D). As our team already confirmed, ovarian structure became polycystic, glucose metabolism was compromised in dihydrotestosterone-challenged rats, and vitamin $D_3$ treatment prevented these negative effects of hyperandrogenic status (Fig. 2, left column) (Sara et al., 2012a). In the 120th minute of OGTT, insulin response (insulin/glucose) was significantly elevated in the DHT group, which was reduced by vitamin $D_3$ (C: 93.3 ± 15.1; DHT: 232.4 ± 57; DHT + D: 57.5 ± 2.7 μg/mol; mean ± SEM, $p \leq 0.05$ DHT vs. C and DHT + D). Norepinephrine induced contraction of aortas was not influenced by any treatment (Fig. 1, Panel A)



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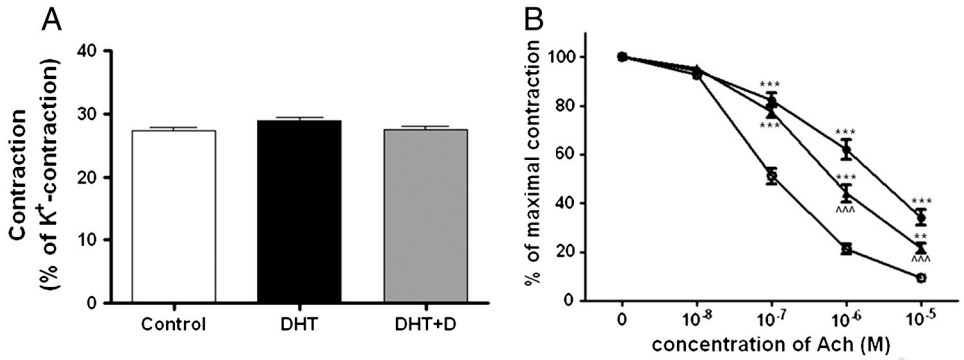


**Fig. 1.** Vasoactivity of aortic segments. Panel A: norepinephrine induced contraction. Contractions caused by $10^{-5}$ M norepinephrine was unaffected by DHT and vitamin $D_3$ treatments. Data are presented in the percentage of $K^+$-induced contraction in the 3rd minute of hyperkalemic environment. (Each data point represents N = 8 rats. Data represented as mean ± SEM.) Panel B: acetylcholine dose–response curve. Concentration-dependent relaxation to acetylcholine ($10^{-8}$ to $10^{-5}$ M) after precontraction with norepinephrine ($5 \times 10^{-8}$ M) was significantly lower in DHT group (black circles) compared to controls (open circles). Vitamin $D_3$ (open triangles) improved acetylcholine induced relaxation. (Each data point represents N = 8 rats. Data represented as mean ± SEM, **: $p \leq 0.01$ vs. Control; ***: $p \leq 0.001$ vs. Control; ###: $p \leq 0.001$ vs. DHT).

(Masszi et al., 2012). The acetylcholine induced relaxation ability of the aorta was deteriorated in the DHT group compared to controls, and vitamin $D_3$ treatment significantly ameliorated this result (Fig. 1, Panel B). In the DHT group, nitrative stress estimated by nitrotyrosine formation was significantly higher in the ovarian (C:4.42 ± 0.6, DHT: 21.47 ± 3.4 area%, Fig. 2, right column and Fig. 4, Panel B) and aortic (C:0.39 ± 0.06, DHT: 1.41 ± 0.23 area%, Fig. 3, left column and Fig. 5, Panel A) tissues as well as in circulating leukocytes (Fig. 4, Panel A). Expression of endothelial NO synthase was lower than the control level (Fig. 3, intermediate column and Fig. 5, Panel B), and – probably due to a compensatory mechanism – cyclooxygenase-2 staining intensity showed antagonistic tendency in DHT animals (Fig. 3, right column and Fig. 5, Panel C). Vitamin $D_3$ treatment had a mosaic effect: in the ovaries of the rats nitrotyrosine formation was reduced (DHT + D: 10.45 ± 1.5 area%) in parallel with the morphological protection (Fig. 2, right column and Fig. 4, Panel B). Although glucose metabolism returned to the control level, general nitrative stress (that was investigated by leukocyte nitrotyrosine levels) was rather stabilized than altered by vitamin $D_3$ therapy (Fig. 4, Panel A). In case of the aorta, nitrotyrosine grade was still elevated (DHT + D: 0.78 ± 0.17 area%) (Fig. 3, left column and Fig. 5, Panel A), but the quantity of eNOS was reversed to the control level (Fig. 3, intermediate column and Fig. 5, Panel B). At the same time

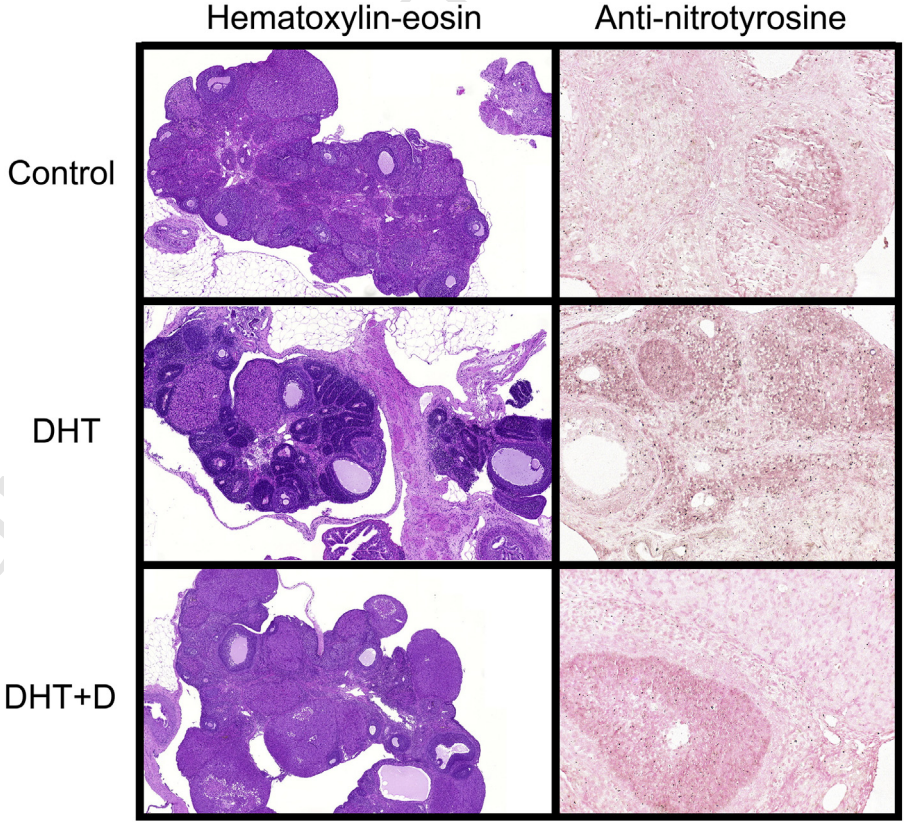


**Fig. 2.** Representative images of ovarian structure and nitrotyrosine staining. Left column: hematoxylin–eosin staining of ovarian sections (10×). Right column: anti-nitrotyrosine immunohistochemistry; gray colored diffuse staining over the red NFR counterstaining shows nitrated proteins (20×). In hyperandrogenic rats (DHT group, intermediate row) ovarian structure became polycystic, and nitrative stress indicated by nitrotyrosine staining was significantly higher than in controls. Vitamin $D_3$ inhibited nitrotyrosine formation parallel with the development of polycystic morphology (DHT + D, bottom row).

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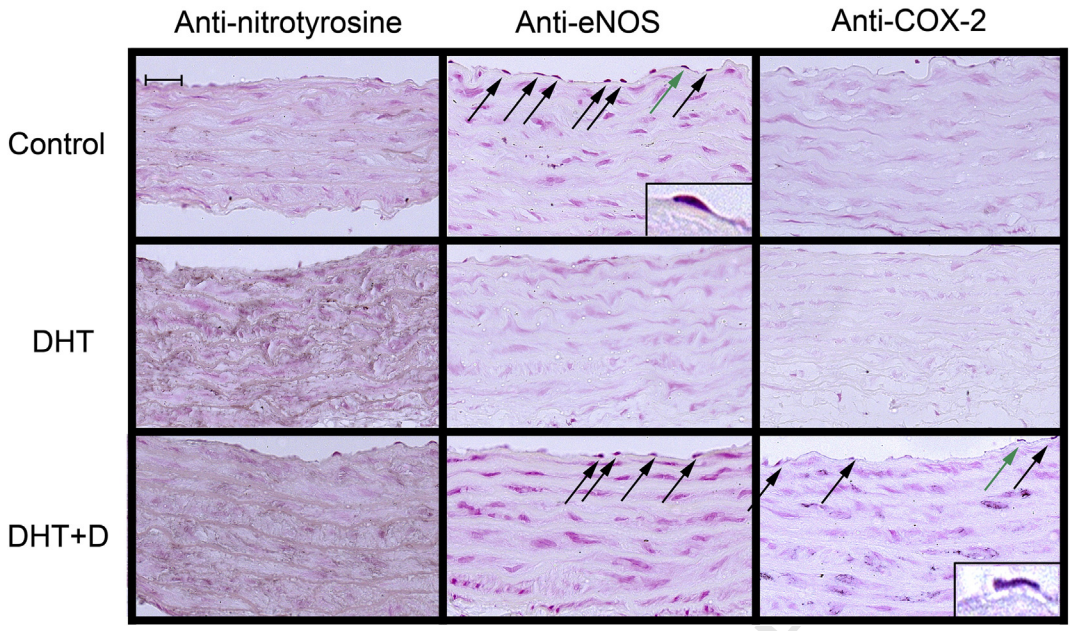


**Fig. 3.** Representative immunohistochemical images of aortas stained against nitrotyrosine, eNOS and COX-2. Aortic nitrotyrosine staining was significantly higher in both DHT-receiver groups. Vitamin $D_3$ treatment did not change this trend (left column: gray colored diffuse staining over the red NFR counterstaining shows nitrated proteins). The presence of endothelial NO synthase was lower in DHT group (middle column) and COX-2 expression showed a tendency for increment (right column). As a response to vitamin $D_3$ dosing, eNOS expression rose to the control level, but COX-2 presence was elevated. On eNOS and COX-2 stained vessels arrows show positively labeled endothelial cells. Positive staining is represented in black; red colored NFR was used as counterstain. Cells indicated with the green arrow are magnified in the bottom right corner of appropriate blocks. (Scale bar, 50 μm.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

COX-2 expression was significantly elevated compared to the controls (Fig. 3, right column, Fig. 5. Panel C).

## Discussion

Low-grade chronic inflammation and consequent leukocytosis are present in women with PCOS, reviewed in Duleba (2012) and Gonzalez (2012). As a consequence, or as a cause, insulin resistance is also a characteristic symptom of PCOS. Decrement of ovarian NO and prostaglandin E (PGE) production (Elia et al., 2006) and endothelial dysfunction due to diminished NO bioavailability are already described (Keller et al., 2011; Lakhani et al., 2006). Our group recently published, that arteriolar NO metabolism and NO-mediated vasorelaxation are severely hampered in this model of polycystic ovary syndrome (Sara et al., 2012a, 2012b, 2012c). However, on the level of resistance vessels, vitamin D3 treatment failed to improve acetylcholine sensitivity. In the present study we demonstrated that acetylcholine-induced relaxation ability of the thoracic aorta is impaired ex vivo in our model of PCOS.

Inflammation leads to increased oxidative–nitrative stress. To estimate systemic nitrative stress, our team measured tyrosine nitration in circulating leukocytes. The isolation method allowed us to accumulate lymphocytes in the samples. As lymphocytes have long half-life, they have stronger defense mechanisms against oxidative–nitrative stress compared to macrophages and polymorphonuclear cells. Our results are consistent with the theory of chronic inflammation, showing stronger nitrotyrosine staining in circulating mononuclear cells. Also, in the ovaries and aortic tissue (both endothelial cells and smooth muscle layers) we found stronger tyrosine nitration in rats. Parallel with our work, Abdollahi's research group published, that lipid peroxidation and peroxynitrite levels are elevated after 21 days of hyperandrogenous state. They also found that the production of inflammatory cytokines is elevated in the rat model of polycystic ovary syndrome (Rezvanfar et al., 2012a; Rezvanfar et al., 2012b). Our results together with their

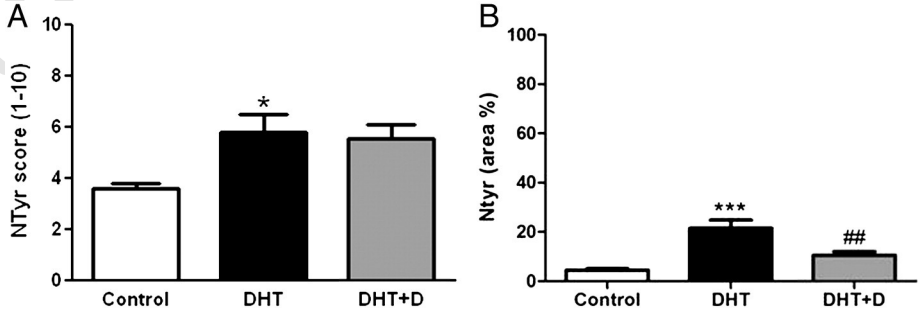


**Fig. 4.** Nitrative stress in circulating leukocytes and ovaries. Panel A. Level of nitrotyrosine staining of circulating leukocytes. In dihydrotestosterone-receiving rats (DHT group) overall nitrative stress estimated by nitrotyrosine staining of circulating leukocytes was significantly higher than in controls. Vitamin $D_3$ treatment (DHT + D) only diminished significance. Panel B. Nitrotyrosine labeling of ovaries. On the contrary, D-vitamin therapy inhibited nitrotyrosine formation in the ovaries. (Each data point represents N = 9 (C, DHT) or N = 10 (DHT + D) rats. Data represented as mean ± SEM, *: $p \leq 0.05$ vs. Control, ***: $p \leq 0.001$ vs. Control, #: $p \leq 0.05$ vs. DHT.)



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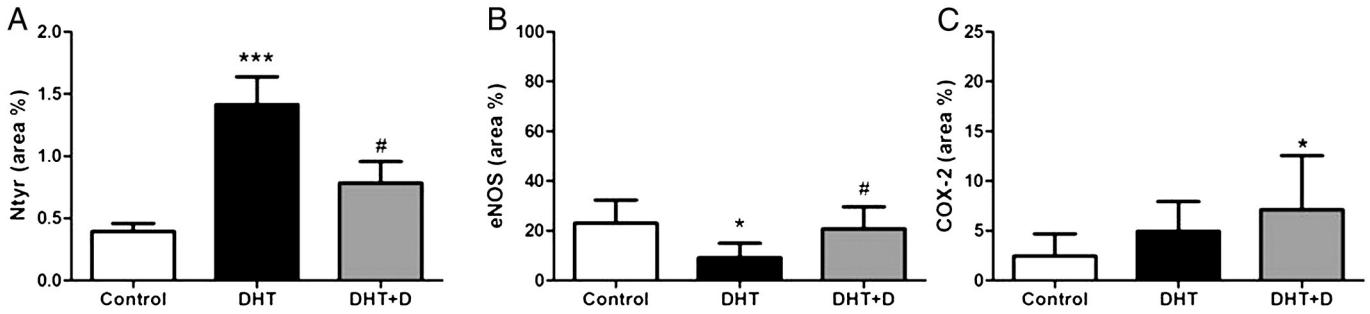


**Fig. 5.** Evaluation of the immunohistochemical staining of the aortas. Panel A. Nitrotyrosine labeling of the vessel wall. Panel B. Endothelial eNOS staining. Panel C. Endothelial COX-2 labeling. In control group, nitrotyrosine and inducible COX levels were low, and eNOS staining was strong. Elevation of DHT resulted in a three-fold increase in nitrotyrosine and a two-fold increase in COX-2 staining intensity, while eNOS levels significantly decreased. Vitamin $D_3$ dosage inhibited the elevation in nitrotyrosine formation and decrement of eNOS transcription, although it also caused a threefold increase in COX-2 staining compared to controls. (Each data point represents N = 10 (C, DHT) or N = 9 (DHT + D) rats. Data represented as mean ± SEM, *: $p \leq 0.05$ vs. Control, ***: $p \leq 0.001$ vs. Control, #: $p \leq 0.05$ vs. DHT.)

observations, which state that nitrative stress is significantly higher in PCOS, underlines the theory that oxidative–nitrative stress and inflammatory responses may have an important role in the pathogenesis of PCOS. They also found that ovarian estrogen and progesterone levels were reduced, while concentration of prostaglandin E was elevated in the ovaries of rats with PCOS.

In the reproductive tract vitamin $D_3$ is an important agent for estrogen biosynthesis (Kinuta et al., 2000). Hyperandrogenic environment, combined with low vitamin $D_3$ level, leads to explicit ovarian hypofunction. Vitamin $D_3$ supplementation in our model controlled ovarian morphology (Masszi et al., 2012; Sara et al., 2012a) and nitrotyrosine formation. There is strong evidence that vitamin $D_3$ is involved in both insulin secretion and insulin receptor expression (Maestro et al., 2003; Oh and Barrett-Connor, 2002; Ortlepp et al., 2001; Zeitz et al., 2003). Whereas according to Ardabili et al. (2012), 3 months of vitamin $D_3$ supplementation failed to improve insulin resistance (but insulin secretion) in women with PCOS, in our model insulin sensitivity was normalized (Sara et al., 2012a). The major differences between the two studies – besides the subjects – were the onset (young adult women vs. adolescent rats) and the dosage of vitamin $D_3$ supplementation. In their study the target value for vitamin $D_3$ was over 20 ng/mL (average: 23.4 ng/mL), so in their study they supplemented vitamin D from a severe to a mild vitamin D deficient state. Hipponen et al. and Pittas et al. found a protective effect of vitamin D intake against the development of both type 1 and type 2 diabetes mellitus (Hipponen et al., 2001; Pittas et al., 2006). Vitamin $D_3$ also lowers low-density lipoprotein and decreases cardiovascular risk in women with PCOS (Thomson et al., 2012). Vitamin $D_3$ also shows an anti-inflammatory effect (Capri et al., 2006; Krishnan and Feldman, 2010; Mabley et al., 2007) and a cardiovascular protection (Zittermann et al., 2005). As previous research showed, vitamin $D_3$ was able to trigger estrogen synthesis and also, downregulated aromatase expression, followed by a decrease of proinflammatory cytokines in macrophages (Villaggio et al., 2012). As we proved, vitamin $D_3$ therapy significantly enhanced aortic response to acetylcholine, therefore improved endothelial function. However, this influence of vitamin $D_3$ exerted limited results, for acetylcholine induced relaxation was below control level. As vitamin $D_3$ has complex and diverse effects, further studies would be required to clarify the involved mechanisms and pathways.

Although in our PCOS rat model, vitamin $D_3$ treatment failed to decrease the systemic nitrative stress significantly, a statistical trend was discernible. These data suggest that low-grade inflammation was still present. As thoracic aorta endures the highest mechanical stress in the vascular system, and systemic nitrotyrosine formation was still elevated in vitamin $D_3$ treated group, higher level of nitrotyrosine staining is comprehensible in the aorta.

Walch et al. demonstrated in a human study that eNOS genotype is probably independent of PCOS (Walch et al., 2008). Endothelial NO synthase level was significantly lower in the DHT group, explaining why NO-dependent relaxation ability was below the control groups. Besides, Skogastierna et al., recently published, that testosterone in supraphysiological doses inhibits eNOS expression but also increases NO production (Skogastierna et al., 2013). This allows us to conclude that the source of the elevated peroxynitrite levels is more likely the inducible, and not the endothelial isoform of NO synthase (Elia et al., 2006; Perreault and Marette, 2001; Shimabukuro et al., 1997). This phenomenon partly explains the altered effectiveness of blood pressure control mechanisms in women with PCOS. Also, lowered eNOS expression increases the risk of ischemia–reperfusion injury, as a consequence of elevated risk of thrombosis. Probably as a compensatory mechanism, COX-2 staining was twofold stronger in the DHT rats compared to the baseline. The present study demonstrates the reverse changes in eNOS and COX-2 levels in PCOS in the aortic endothelium for the first time. As a result of vitamin $D_3$ treatment, eNOS expression was significantly higher than in the DHT group, and did not differ from the controls. The fact that quasi normal eNOS translation did not result in control level relaxation, and as systemic as well as local aortic nitrotyrosine levels were not lowered by vitamin $D_3$ supplementation, allows us to draw the conclusion that low-grade inflammation might be still present during vitamin $D_3$ therapy. COX-2 expression in vitamin $D_3$ group remained elevated compared to the control group, probably to compensate challenged NO-bioavailability. We can hypothesize that the cause is the decrement of eNOS expression and the effect is the elevation of COX-2 translation. At the molecular level, vitamin $D_3$ treatment increases eNOS expression (Xiang, 2011), possibly due to receptor-mediated mechanisms, leading to improved responsiveness to acetylcholine and relaxation ability.

## Conclusion

This is the first study to demonstrate the linked changes in eNOS and COX-2 levels in PCOS in the aortic endothelium. This phenomenon might partly explain – besides elevated cardiovascular risk – the altered effectiveness of blood pressure control mechanisms in women with PCOS.

Our results also confirm the advantageous effects of adjuvant vitamin $D_3$ therapy in PCOS. On the level of ovaries and carbohydrate metabolism we could detect an almost complete reversal of the damages caused by DHT. As previous research showed, vitamin $D_3$ is able to trigger estrogen synthesis too. The beneficial effect of vitamin $D_3$ supplementation in controlling the vascular and systemic nitrative stress is not clear, but at the molecular level, vitamin $D_3$ treatment increased eNOS expression, leading to an improved responsiveness to acetylcholine and relaxation ability.



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**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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