Demethylation treatment restores erectile function in a rat model of hyperhomocysteinemia

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INTRODUCTION

Homocysteine (hcy) has gained arising attention for its association with increased risk of myocardial infarction, stroke and venous thromboembolism. Hyperhomocysteinemia (hhcy), is defined as a pathological condition characterized by elevations of total plasma hcy. Erectile dysfunction (ED), also known as impotence, is a sexual dysfunction characterized by the inability to develop or maintain an erection of the penis during sexual activity. ED, especially for vasculogenic ED, is a vascular disorder of cavernosal vascular bed. Several clinical trials and animal experiments conducted on ED have demonstrated its close correlation with hhcy.

The underlying mechanisms with which hcy induced vascular injury focused on: increased oxidative stress, decreased bioavailability of endothelium-derived nitric oxide (NO), promoting endothelial inflammation, endoplasmic reticulum stress and the unfolded protein response. Studies both in vitro and in vivo have demonstrated hcy induced endothelial dysfunction. Khan et al. reported hcy had an inhibitory effect on endothelium-dependent relaxation and NO formation in rabbit corpus cavernosum and this effect was augmented by copper and reversed by superoxide dismutase or catalase. Demir et al. showed that the presence of elevated levels of hcy (>12.1 μmol l⁻¹) strongly predicted the occurrence of ED while Al-Hunayan et al. demonstrated that 1 μmol l⁻¹ increase of plasma hcy concentration was associated with 2.95 times the odds of ED when compared to control subjects. Wang concluded in a review that hhcy to be “a novel risk factor of ED” and the measurement of plasma hcy might be “added to the ED diagnostic procedure.” However, the underlying mechanisms of hcy-related endothelial dysfunction were not fully understood. Protein dimethylarginine dimethylaminohydrolase (DDAH) acts as a pivotal role of endothelium function. DDAH metabolizes asymmetric dimethylarginine (ADMA), which serves as an endogenous inhibitor of nitric oxide synthase (NOS). There are two types of DDAH identified: DDAH-1 and DDAH-2, which is associated with neuronal NOS and endothelial NOS, respectively. On the other hand, recent studies found that DDAH-2 promoter methylation was associated with endothelial dysfunction. DNA methylation modification is an important cellular mechanism of gene expression regulation. Based on all the previous findings, in this study, we investigated the hypothesis that DDAH-2 promoter hypermethylation associated with ED pathogenesis and we observed the possible effect of 5-aza-deoxycytidine (5-aza), a demethylation agent, for the treatment of ED in a rat model of hhcy.

MATERIALS AND METHODS

Animal groups and treatment protocol

This study complied with the guidelines of animal experiments of Nanjing University, and was approved by the Laboratory Animal Users Committee at Drum Tower Hospital, Nanjing, China. Forty male 12-week-old Sprague-Dawley rats weighing 220–280 g were purchased from Shanghai Slac Laboratory Animal Co., Ltd., (Shanghai, China). Twelve rats fed a standard diet were regarded as a control group (Con). Twenty-eight rats were fed a standard diet and L-methionine supplemented diet (2 g kg⁻¹ body weight). These rats were randomly divided into a methionine group (Met) (n = 14) that were administered...
with intraperitoneally injection of normal saline and an experimental group that were administered with intraperitoneally injection of 5-aza (Sigma-Aldrich, USA) at 1 mg kg⁻¹ (Met + 5-aza) (n = 14) at day 0 and every 3 days of the treatment procedure. After the treatment for 6 weeks, blood sample was collected from the ear vein. Plasma hcy levels, evaluated with enzymatic cycling assay (Boster, Wuhan, China) and body weights were measured at day 0 and prior to euthanasia.

**In vivo assessment of erectile function and tissue procurement**

After 6 weeks, all rats underwent the measurement of erectile function through the stimulation of cavernous nerve. The nerve stimulation was conducted at a frequency of 20 Hz with a pulse width of 5 ms. Stimulations were performed at 5 V for 60 s with resting periods of 5 min between subsequent stimulations. The mean arterial pressure (MAP) and intracavernosal pressure (ICP) were measured as described previously.¹⁰ At the end of the experiment, all animals were sacrificed. The penis was excised, and the cavernous tissues were dissected from skin and divided into two fragments: one fragment was for target proteins and mRNA and NO detection, and the remaining part was then frozen in liquid nitrogen and then stored at −80°C for future use.

**Measurement of plasma ADMA levels**

Plasma ADMA levels were measured using high-performance liquid chromatography (HPLC) as described previously in detail.³¹,³²

**Measurement of NO**

The NO and cyclic guanosine monophosphate (cGMP) concentration in corpus cavernosum were evaluated with spectrophotometry method, using a commercial kit (Boster, Wuhan, China), following the protocols provided by the manufacturer.

**DDAH-2 protein level**

DDAH-2 protein expression of the three groups were detected by Western blot. The penile tissue strips were homogenized in a buffer containing 50 mM Tris-HCl PH 7.2, 0.1 M NaCl, 5 mM EDTA, 0.5% Triton X-100 and protease inhibitor. After homogenization, samples were centrifuged at 12 000 g for 15 min at 4°C. The supernatants were collected for protein concentration measurement using bicinchoninic acid protein assay (Keygen, China). Equal amounts of proteins (50 μg) were loaded and run on 10% sodium dodecyl sulfate-polyacrylamide gels and then electrotransferred to nitrocellulose membranes, which were incubated with primary antibody against DDAH-2 (Abcam Inc., China; 1:1000) or β-actin (Abcam Inc., China; 1:1000) at room temperature for 4 h. This was subsequently followed by secondary antibodies (Maibio Inc., China; 1:5000). Detection was performed by chemiluminescence (Boster, Wuhan, China) with exposure to X-ray film.

**DDAH-2 mRNA expression**

DDAH-2 mRNA levels were analyzed using real-time PCR methods. Total RNA was extracted from the penile strips using Trizol regent (Invitrogen, China) according to the manufacturer’s protocol. 1 μg RNA was reversely transcribed into cDNA respectively. The total quantitative real-time PCR volume is 25 μl containing SYBR Green PCR mix (Takara, Japan) and oligonucleotide primers. Primer sequences and PCR reaction conditions were shown in **Table 1**. The thermal cycling conditions for the real-time PCR was 95°C for 30 s (denaturation), followed by 50 cycles of 95°C for 5 s, 60°C for 30 s (40 cycles). Relative expression levels for DDAH-2 were normalized to the expression of housekeeping gene β-actin by the 2⁻ΔΔCT method: ΔCT = (CTDDAH-2 - CTβ-actin); −ΔΔCT = ΔCT (Met group or Met+5-aza group) − ΔCT (Con group); RQ (Relative Quantitation) = 2⁻ΔΔCT, RQ (Con group) = 1.

**Detection of DDAH-2 promoter methylation levels**

Genomic DNA was extracted from blood samples using TIANamp Blood DNA kit (Tiangen, China) and fresh frozen cavernosum tissues using a DNA isolation kit (Qiagen AB, Solna, Sweden), as per the manufacturer’s instructions. Bisulfite modification was undertaken with 500 ng samples of DNA using DNA Methylation Kit™ (Zymo Research, Hiss Diagnostics, CA, USA), as per the manufacturer’s instructions. The samples were then eluted in 15 μl of elution buffer. The pyrosequencing assay was used to assess methylation status of the DDAH-2 promoter (Chr 20-NC_005119.4 (5049265-5050613)). This assay detected the level of methylation in a region (Chr 20-NC_005119.4 (5049522-5049598)) of the DDAH-2 gene (Chr 20-NC_005119.4 (5049265-5052573)) (Figure 1). PCR and pyrosequencing primers were designed using PyroMark Assay Design 2.0 (Qiagen, Germany). The PCR volume was 20 μl, and incorporated 0.5 μM forward and reverse primers, 10 μl ZymoTaq Premix (Zymo Research, Hiss Diagnostics, CA, USA) and 2 μl bisulfite-modified DNA. The forward and reverse primer sequences were 5'-AGTTGGGGGTGTGGTATAGT-3' and 5'-biotin-CCCTCAACTACCTAACATATC-3', respectively. PCR testing was carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30s, and 72°C for 1 min, with a final extension of 72°C for 10 min. PCR product quality was confirmed on 1% agarose gels with

| Gene   | Primer sequence (5'→3') | Products size (bp) | Ta (°C) |
|--------|-------------------------|--------------------|---------|
| DDAH-2 | GGCTCTAGAATTGTTGAGAT   | 97                 | 60      |
|        | ACTGAGAGGGCCTAGAAA      |                    |         |
| β-actin| CACCGCGAGTACAACCTCTC   | 207                | 60      |
|        | CCCATACCCACATCAACC      |                    |         |

**Table 1:** Primer sequences, annealing temperature (Ta) and product size

**Figure 1:** The tested position of DDAH-2 promoter regions.
ethidium bromide staining. Pyrosequencing was subsequently carried out on the PyroMark™ Q24 pyrosequencer (Qiagen, Germany), with the sequencing primer 5'-GGGTTTGTATATGAG-3. The degree of methylation of all six CpG sites was automatically analyzed by the PyroMark™ Q24 software. Briefly, methylation frequency of CpG site was measured in accordance with the ratio of light units between the sum of methylated alleles and the unmethylated alleles.

**Statistical analysis**

All data were expressed as mean ± standard error. Differences among the groups were assessed using one-way analysis of variance for multiple comparisons, followed by post hoc comparisons using least significant difference test. \( P < 0.05 \) was regarded significant.

**RESULTS**

**The body weights and plasma hcy levels**

The body weights and plasma hcy levels of the experimental rats were shown in Table 2. The body weights of Met group were lower than controls after 6 weeks \( (P < 0.05) \). The hcy levels of Met group were statistically higher compared to Con group \( (P < 0.05) \). However, 5-aza did not change these values.

**Plasma ADMA and cavernosum NO-cGMP levels**

As shown in Table 3, decreased cavernosum NO and cGMP levels were observed in Met group compared to the Con group \( (P < 0.05) \). Six weeks of 5-aza administration increased NO and cGMP levels significantly compared with methionine-fed rats \( (P < 0.05) \). However, comparisons of ADMA yielded nonsignificant results \( (P > 0.05) \).

**Erectile function of the three groups**

The ICP/MAP ratio was presented in Figure 2. The mean ICP/MAP ratio was decreased in Met group compared to Con group \( (P < 0.01) \). However, after 5-aza administration, the mean ICP/MAP ratio increased as compared with Met group \( (P < 0.05) \).

**Protein and mRNA levels of DDAH-2 in cavernous tissue**

As indicated in Figure 3, the expression of DDAH-2 protein and mRNA decreased significantly in the Met group compared to Con group \( (P < 0.01) \). However, after 5-aza treatment, the expression of DDAH-2 protein and mRNA improved when compared to the Met group \( (P < 0.05) \).

**DISCUSSION**

Recent studies have confirmed a positive link between hcy and endothelial dysfunction. However, existing mechanisms, including increased oxidative stress, decreased bioavailability of endothelium-derived NO and promoting endothelial inflammation were not exclusively enough for the explanation of hcy-related endothelial dysfunction. For example, it failed to explain that cysteine, which has a similar molecular structure with hcy, was not regarded as a risk factor for endothelial dysfunction.

ADMA is an endogenous inhibitor of NO synthase, and serves as a possible mediator of hcy and endothelial dysfunction. The major pathway of ADMA clearance is DDAH, a key enzyme responsible for ADMA elimination. Liu et al. found that declined DDAH-2 activity was associated with an accumulation of ADMA and decreased NO production in endothelial cells. Additionally, Rodionov et al. concluded DDAH-1 overexpression was a protective manner from hcy-induced alternations in cerebral arteriolar structure and vascular muscle function, and this protective effect was independent from the elevation of serum ADMA. DDAH is recognized as a protective factor of endothelial function and plays an important role of endothelium function.

Some studies have suggested elevation of ADMA is an inner mechanism of endothelial dysfunction in hcy. In the present study, the plasma ADMA levels were of no significance among the three groups. These results were consistent with previous observational studies that hcy was not always accompanied with ADMA elevation. A possible explanation is that there is a ADMA-independent pathway of DDAH-mediated endothelium...
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Figure 3: The protein and mRNA levels of DDAH-2 in cavernosum tissues. (a) DDAH-2 protein decreased in Met group compared with Con and increased in Met+5-aza group compared with Met group. (b) The DDAH-2 mRNA decreased in Met group compared with Con group while increased in Met+5-aza group in comparison with Met group. (c) Western blot results of DDAH-2 protein and β-actin. *P < 0.05 versus Con group; #P < 0.05 versus Met group; Con: control group; Met: methionine group; Met+5-aza: 5-aza treated group.

Figure 4: The methylation levels of DDAH-2 promoter region in blood and cavernosum tissues. (a) Pyrosequencing graphic results of Con group in cavernosum tissues. (b) Pyrosequencing graphic results of Met group in cavernosum tissues. (c) Pyrosequencing graphic results of Met+5-aza group in cavernosum tissues. (d) DDAH-2 promoter methylation levels increased in Met group compared to Con group and Met+5-aza group. *P < 0.05 versus Con and Met+5-aza group. Con: control group; Met: methionine group; Met+5-aza: 5-aza treated group.
methyl-CpG binding proteins (MBDs), which in turn establishes a transcriptional inactive chromatin environment.\textsuperscript{52}

Recent researches demonstrated that the methylation changes of DDAH-2 gene were associated with hhcy-induced endothelial dysfunction.\textsuperscript{46} Niu \textit{et al.} concluded in a clinical study that hypermethylation of DDAH-2 promoter contributed to the impaired function of endothelial progenitor cells in coronary artery disease patients.\textsuperscript{29} Jia \textit{et al.} found hcy induced apoptosis of endothelial cells via elevating the methylation level of DDAH-2 promoter regions in cultured human umbilical vein endothelial cells.\textsuperscript{53} Similar results were further confirmed in some other studies \textit{in vivo}.\textsuperscript{6,46} These observations were connected with each other and possibly provided a novel mechanism (DDAH-2 promoter methylation) of hcy-related endothelium impairments.

In the present study, the CpG islands were predicted in the promoter region of DDAH-2 using an online program (http://www.urogene.org/cgi-bin/methprimer). A specific CpG-rich sequence in the promoter region (Chr 20-NC_005119.4 (5049522-5049598)) with six CpG dinucleotide sites included were selected. Tomikawa \textit{et al.} previously demonstrated that methylation in this region associated with the silencing of DDAH-2 expression.\textsuperscript{54} These results showed a similar trend with the current experiments.

In the present study, the results indicated a lower-methylation patterns in Con group and Met+5-aza (a demethylation agent) group, as compared with Met group. Otherwise, we detected DDAH-2 protein expression and mRNA levels. The results showed that DDAH-2 protein and mRNA expression were markedly attenuated in the Met group, but elevated in demethylation treatment group and showed a consistent trend with the methylation patterns of DDAH-2 promoter regions. Hence, we successfully demonstrated that down-regulation of DDAH-2 protein in parallel with DDAH-2 promoter region hypermethylation resulted in impaired endothelial function and ED subsequently.

However, there were several limitations of the present study that should take into consideration. First, we did not examine vascular functional studies using endothelium-dependent or endothelium-independent agent in rats cavernosal strips treated with 5-aza. Jones \textit{et al.} reported an impaired cavernosal relaxation response to carbachol (an endothelium-dependent agent) in isolated hhcy rabbit corpus cavernosum. However, when stimulated with sodium nitroprusside, an endothelium-independent or using nonadrenergic noncholinergic (NANC)-mediated electrical-field stimulation, this relaxation response was unaffected.\textsuperscript{7} Shukla \textit{et al.} showed that carbachol-induced relaxation and NANC-mediated electrical-field stimulation-induced relaxation were significantly attenuated in cavernosal strips of diabetes mellitus rabbit. However, this reduced response was reversed by in rats administrated folic acid, which decreased hcy levels.\textsuperscript{13} The main neurotransmitter released by NANC neurons and endothelium of corpus cavernosum is NO, which is a key molecular that mediates penile erection. In this study, in 5-aza treated rats, elevated DDAH-2 protein level and NO content in cavernous tissues compared with the methionine-fed rats were observed, which possibly showed an elevated response to endothelium-dependent agent or NANC-mediated relaxation in 5-aza treated rats cavernosal strips. This hypothesis, however, is necessary and needs further investigation. Second, DDAH-2 is associated with endothelial NOS while DDAH-1 neuronal NOS and hcy is widely regarded as a risk factor for cardiovascular diseases. Therefore, in the current experiment, the methylation level, protein expression, and mRNA level of DDAH-2 were evaluated, while DDAH-1 remains unknown. Dayal \textit{et al.} demonstrated both \textit{in vitro} and \textit{in vivo}, hhcy caused decreased DDAH-2 mRNA level in the liver.\textsuperscript{66} Furthermore, Imamura \textit{et al.} reported that DDAH-1 protein was down-regulated in rabbit cavernous tissue following cigarette smoke extract treatment.\textsuperscript{57} These results indicated a possibility of DDAH-1-related role in the pathogenesis of hhcy ED. Additionally, using the online program (http://www.urogene.org/cgi-bin/methprimer), CpG islands (Chr 20-NC_005101.4 (251634306-251634769)) were also identified in the promoter region of DDAH-1. This offers a new direction for future studies and enriched the theory of hcy-related ED and clinically beneficial subsequently. Third, in this study, we did not detect the protein levels of eNOS and nNOS in correspondence with DDAH subtypes or eNOS and nNOS activities in rats cavernosal tissues. NO production depends on NOS activity and NOS protein expression. Although studies demonstrated that hcy could inhibit eNOS activity and NO production \textit{in vivo}\textsuperscript{46} or promote uncoupling eNOS,\textsuperscript{58} a condition that eNOS produces superoxide rather than NO, further studies are warranted to illustrate the possible mechanisms of hcy-induced endothelial dysfunction and protective effect of demethylation treatment.

**CONCLUSION**

For the first time, we provided the evidence that methylation mechanisms involved in ED pathogenesis and demethylation therapy with 5-aza restored erectile function in a rat model of hhcy. This offers new insight into the pathogenesis of ED and thus provides novel therapeutic methods.

**AUTHOR CONTRIBUTIONS**

ZZ and LLZ carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. HSJ carried out the immunoassays. ZZ and HC participated in the sequence alignment. YC participated in the design of the study and performed the statistical analysis. YTD conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

**COMPETING INTERESTS**

All authors declare no competing interests.

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