

Wet cupping therapy removes oxidative stress related miRNAs

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Abstract

Background: Wet cupping therapy is commonly used in conditions such as hypertension, diabetes, and inflammatory and infectious diseases. The mechanism of action of wet cupping therapy is not yet precise; however, several studies have demonstrated that it has a role in limiting oxidative stress. This study aimed to investigate the effect of wet cupping therapy on oxidative stress-related miRNAs miRNA-34a, miRNA-200a, miRNA-21 levels and oxidant-antioxidant status markers malondialdehyde (MDA) and glutathione (GSH) levels.

Methods: 60 healthy volunteer women aged 20-75 years (n=30, 20-45 years; n=30, 45-75 years) who had wet cupping at Necmettin Erbakan University Meram Medical Faculty Traditional and Complementary Medicine Center were included in this study. Plasma miRNA-34a, miRNA-200a, and miRNA-21 levels were analyzed using real-time quantitative PCR (RT-PCR) analysis. MDA and GSH levels were measured using commercial ELISA kits.

Results: MDA levels [2003.10 (1810.17-7392.15) vs. 1884.04 (1800.10-4488.05), p=0.027] of wet cupping blood samples were higher than venous blood samples, while GSH levels [125.97 (90.65-219.43) vs. 131.77 (88.77-246.42), p=0.037] were lower. miRNA-34a [(0.94±0.74) vs. (0.53±0.28), p<0.001], miRNA-21 [0.058 (0.01-0.54) vs. 0.033 (0.01±0.18), p=0.001] and miRNA-200a [24.42 (7.46-103.97) vs. 20.32 (4.32-56.49), p=0.037] levels of wet cupping blood samples were significantly higher than venous blood samples.

Conclusions: Wet cupping therapy eliminates oxidative stress-related miRNAs from the body. Therefore, it is seen as a promising method for the welfare of reactive oxygen species (ROS)-related diseases by limiting oxidative damage.

Keywords: Cupping Therapy, miRNAs, Oxidative Stress, Reactive Oxygen Species.

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INTRODUCTION

Cupping therapy is a traditional therapy method that has been practiced for nearly 2000 years (1). Eber's papyrus (1550 BC) is one of the earliest medical texts to mention cupping therapy (2). Cupping therapy is applied by creating localized pressure through a glass, plastic, or bamboo cup on patients' skin over acupuncture points, painful areas, or a reflex zone (3). In general, there are two main classes of cupping therapy, wet cupping (bleeding cupping) and dry cupping (4). However, the former is one of the most widely preferred types of cupping therapy (5). Other types include needle, retained, flash, and medicinal (herbal) cupping (6). Dry cupping is performed by stimulating the skin through the application of cups with a vacuum pressure without allowing blood to flow (7). In wet cupping therapy, also known as Hijama, the skin is incised with a needle, and then the blood is removed from the body by creating a vacuum with a cup (8). The reported effects of cupping therapy include increasing cutaneous blood flow, altering the biomechanical characteristics of the skin, raising the pain threshold, improving local anaerobic metabolism, restriction of inflammation, and regulating the immune system (9). Cupping therapy is applied for preventive and therapeutic purposes in various disorders such as back, neck, shoulder, and knee pain, migraine and headache, acne, asthma, inflammatory and infectious diseases, immune system disorders, diabetes mellitus, hypertension, anxiety, depression, and sleep disorders (10). Wet cupping is believed to remove toxins, heavy metals, metabolic wastes, and free radicals from the body (8). An imbalance between the generation of reactive oxygen species (ROS) and the enzymatic or non-enzymatic antioxidant defense system leads to oxidative stress (11). Excess free radicals attack cellular proteins, lipids, and DNA, inhibiting everyday functions (12). Therefore, oxidative stress is involved in the pathogenesis of various diseases, including cancer, diabetes, cardiovascular diseases, neurodegenerative diseases, and aging (13). miRNAs are 21-23 nucleotides long, small non-coding RNAs that affect gene expression at the translational or posttranslational level through mRNA degradation or repression (14). Although evaluated separately, microRNA (miRNA) networks and oxidative stress are tightly linked. Oxidative stress affects the expression level of various miRNAs, while miRNAs affect the expression levels of essential genes that play a vital role in the oxidative stress response (15). Abnormal expression of miRNAs has been related to various diseases, including cancer, cardiovascular, and

neurodegenerative diseases, diabetes, and viral infections (16). Although various studies investigated the impact of cupping therapy on heavy metal concentrations, oxidative stress, and antioxidant status markers, no studies have been reached to determine the effects on miRNA levels (5, 17-19). The study aimed to investigate the effect of wet cupping therapy on oxidative stress-related miRNAs miRNA-34a, miRNA-200a, miRNA-21 levels and oxidant-antioxidant status markers malondialdehyde (MDA) and glutathione (GSH) levels.

MATERIALS AND METHODS

Study design

Subjects

This study included a total of 60 healthy volunteer women aged 20-75 years (n=30, 20-45 years; n=30, 45-75 years) who had wet cupping at Necmettin Erbakan University Meram Medical Faculty Traditional and Complementary Medicine Center. Enrollment criteria of the study were hemoglobin levels above 9.5 mg / dL, being over 18 years old and healthy, and attending Traditional and Complementary Medicine Center of Necmettin Erbakan University Meram Medical Faculty for wet cupping therapy to preserve health. Exclusion criteria were antioxidant, vitamin, mineral supplements, diabetes, cardiovascular diseases, chronic liver or kidney disease, infectious diseases, use of any blood thinning drug (antiaggregant, salicylic acid, coumadin), pregnancy, alcohol, or smoking. Within the scope of the study, 107 volunteers who wanted to have wet cupping therapy could be reached. Of these, 15 were smokers, 18 individuals had comorbidities such as diabetes and hypertension, 7 individuals were taking supplements, and 7 participants were men. Males were excluded from the study due to the limited number of male participants, and only female participants were included. Thus, the study was carried out with 60 volunteer participants. The participants' 5 mL venous blood samples were drawn into serum separator gel tubes after 12 hours of fasting and just before the first wet cup application. 2 mL wet cupping blood samples were transferred from the cup to serum separator gel tubes during application. Serum samples were separated at 2000 g for 10 min; then, serum samples were aliquoted and kept at -80 °C until analysis. For miRNA, blood samples were drawn into EDTA tubes and centrifuged at 1300 g for 10 min, and plasma samples were separated and stored at -80°C until analysis. The Necmettin Erbakan University Faculty of Medicine ethics committee approved the study (Number: 2017/1110, Date: 01/12/2017).

Wet Cupping

Wet-cupping therapy was carried out using sterile vacuum cups on acupuncture point locations; Du-14(Dazhui) point on the posterior median line, in the depression below the processus spinosus of the 7th cervical vertebra; Ub-42 (Pohu) points bilaterally on the back 3.0 cun lateral to the lower border of spinosus process of the 3rd thoracic vertebra; Ub-46(Geguan) points bilaterally on the back, 3.0 cun lateral to the lower border of spinosus process of the 7th thoracic vertebra, and interscapular region; on the posterior median line between 3rd and 7th thoracic vertebra. BL-12-15 is near the interscapular site for wet cupping. Furthermore, according to Chinese medicine, several important back-shu points, including the heart back-shu point, are known to stimulate the whole body in the area. Points BL-13 through 15 are associated with the lungs, pericardium, and heart, respectively, and are used in acupuncture theory to support these organs (20).

Laboratory tests

The serum levels of urea, creatinine, total cholesterol, triglycerides, high-density lipoprotein-cholesterol (HDL-C), very low-density lipoprotein (VLDL-C), glucose, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed with an Architect C 8000 Auto Analyzer (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. Low-density lipoprotein (LDL-C) was calculated using Friedewald's formula.

Serum MDA (Cat.No E-BC-K025-S) and GSH (Cat.No E-EL-0026) levels were analyzed using commercial human ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's instructions. The absorbance of all wells was measured at 450 nm in an ELx800 Absorbance Microplate Reader (Biotek, Winooski, VT, USA).

miRNA expression profiling

Total RNA was extracted from plasma samples via High Pure miRNA Isolation Kit (Roche Life Science, Mannheim, Germany). Total RNA was reversely transcribed to cDNA using miScript II RT Kit (Qiagen, Hilden, Germany). Obtained cDNA samples are PreAmplified via miScript Microfluidics PreAMP Kit (Qiagen, Hilden, Germany). Quantitative real-time PCR (qRT-PCR) analysis was carried out using miScript miRNA Assays (Qiagen, Hilden, Germany) with Dynamic Array (Fluidigm, South San Francisco, CA, USA) on BioMark System (Fluidigm, South San Francisco, CA, USA) according to manufacturer's protocol. miRNA expressions were normalized to β -actin.

β -actin (forward, 5'-GGCACCCAGCACAATGAAG-3', and reverse, 5'-CGTCATACTCCTGCTTGCTG-3') was used as the internal control. The relative gene expression was calculated by comparing cycle times for target PCR using this equation: relative gene expression = $2^{-(\Delta C_{t\text{sample}} - \Delta C_{t\text{control}})}$.

Statistical analysis

Statistical evaluation was carried out using SPSS statistical software package version 21.0. One-Sample Kolmogorov-Smirnov test was used to determine the distribution of data. The mean and median values between the two groups were compared using Student's t and Mann-Whitney U tests, respectively. Kruskal – Wallis test (post-hoc analysis Mann-Whitney U) also compared multiple groups. Correlation analyses were performed using Spearman's correlation test. $p < 0.05$ was considered to be statistically significant.

RESULTS

The study was completed with 60 volunteer women aged 20 to 75 years. The mean age of the participants was 45.5 years, and the mean BMI was 26.91 ± 4.20 kg/m². All participants were female. The demographic properties of the participants are expressed in Table 1.

Table 1. Demographic characteristics of the participants.

Variable	Subjects (n=60)
Age (years)	45.5 (20-75)
Gender (n, %)	Female (60, %100)
Education (n, %)	Primary (6, 10%)
	Secondary (42, 70%)
	University (12, 20%)
Length (m)	1.62±0.57
Weight (kg)	71.59±11.71
BMI (kg/m ²)	26.91±4.20
BMI: body mass index.	

As a result of our study, it was shown that there was a significant difference between the cupping blood samples and venous blood samples of the participants in terms of MDA, GSH, and circulating miRNA levels. MDA levels [2003.10 (1810.17-7392.15) vs. 1884.04 (1800.10-4488.05) ng/mL, $p=0.027$] were higher in wet cupping blood samples compared to venous blood samples, while GSH levels [125.97 (90.65-219.43) vs. 131.77 (88.77-246.42) $\mu\text{g/mL}$, $p=0.037$] were lower. Circulating miRNA-34a [(0.94±0.74) vs. (0.53±0.28), $p<0.001$], miRNA-21 [0.058 (0.01-0.54)

vs. 0.033 (0.01±0.18), p=0.001] and miRNA-200a [24.42 (7.46-103.97) vs. 20.32 (4.32-56.49), p=0.037] levels of wet cupping samples were significantly higher than venous

blood samples. The miRNA-34a, miRNA-21, miRNA-200a, MDA, and GSH levels of the blood samples are expressed in Table 2.

Table 2. The miRNA-34a, miRNA-21, miRNA-200a, MDA and GSH levels of the blood samples.

Parameters	Venous blood (n=60)	Wet cupping blood (n=60)	p
miRNA-34a	0.53±0.28	0.94±0.74	<0.001
miRNA-21	0.033 (0.01±0.18)	0.058 (0.01-0.54)	0.001
miRNA-200a	20.32 (4.32-56.49)	24.42 (7.46-103.97)	0.037
MDA (ng/mL)	1884.0 (1800.1-4488.1)	2003.1 (1810.2-7392.2)	0.027
GSH (µg/mL)	131.77 (88.77-246.42)	125.97 (90.65-219.43)	0.037

Moreover, the participants were divided into groups according to age and biochemical findings; miRNA-34a, miRNA-21, miRNA-200a, MDA, and GSH levels were

compared. The biochemical findings of the participants were summarized in Table 3.

Table 3. Biochemical findings of the participants.

Biochemical Parameters	Group 1 (n=30)	Group 2 (n=30)	p
Glucose (mg/dL)	94.48 ± 11.15	106.74 ± 16.23	0.002
Cholesterol (mg/dL)	202.23 ± 38.10	206.77 ± 33.33	0.626
LDL (mg/dL)	115.57 ± 32.58	119.97 ± 25.26	0.561
HDL (mg/dL)	53.99 ± 11.49	51.18 ± 11.10	0.340
VLDL (mg/dL)	28.83 ± 12.64	30.83 ± 14.66	0.574
TG (mg/dL)	146.23 ± 62.85	156.07 ± 73.36	0.579
Urea (mg/dL)	26.87 ± 6.94	30.71 ± 10.03	0.091
Creatinine (mg/dL)	0.79 ± 0.08	0.78 ± 0.08	0.610
ALT (IU/L)	16.36 ± 6.26	19.50 ± 10.12	0.156
AST (IU/L)	16.73 ± 5.05	18.34 ± 4.21	0.189
Hemoglobin (mg/dL)	13.08 ± 0.92	13.15 ± 1.17	0.818

Group 1: women between the ages of 20-45, n = 30; Group 2: women between the ages of 45-75, n = 30.

When the blood samples of the participants are classified according to their age, they are divided into the following four groups: Group 1, venous blood samples of women between the ages of 20-45; Group 2, venous blood samples of women between the ages of 45-75; Group 3, wet cupping blood samples of women between the ages of 20-45; Group 4, wet cupping blood samples of women aged 45-75. In addition, the comparison of Group 1 and Group 3 showed that miRNA-200a, miRNA-34a, miRNA-21, and MDA levels were higher, and GSH levels were lower in Group 3 compared to Group 1. However,

only the difference between miRNA-200a, miRNA-34a, and miRNA-21 levels was statistically significant (p<0.05). Similarly, the statistical comparison of Group 2 and Group 4 revealed that miRNA-200a, miRNA-34a, miRNA-21, and MDA levels were higher, and GSH levels were lower in Group 4 compared to Group 2. However, only the difference between MDA and GSH levels was statistically significant (p<0.05). The miRNA-34a, miRNA-21, miRNA-200a, MDA, and GSH levels of blood samples according to age groups are expressed in Table 4.

Table 4. The miRNA-34a, miRNA-21, miRNA-200a, MDA and GSH levels of blood samples according to age groups.

Parameters	Group 1 (n=30)	Group 2 (n=30)	Group 3 (n=30)	Group 4 (n=30)	p
miRNA-34a	0.54±0.33	0.52±0.23	0.70±0.31	1.17±0.95	a:0.789 b:0.015 c:0.049 d:0.001
miRNA-21	0.022 (0.01-0.12)	0.053 (0.01-0.54)	0.062 (0.01-0.18)	0.055 (0.02-0.18)	a:0.193 b:0.003 c:<0.001 d:0.900
miRNA-200a	17.04 (4.32-49.52)	25.63 (4.79-56.49)	24.42 (7.46-84.45)	27.99 (9.13-103.97)	a:0.001 b:0.905 c:0.005 d:0.888
MDA (ng/mL)	1908.7 (1800.1-4069.1)	1861.6 (1800.2-4488.1)	2350 (1810.2-6432.9)	1924.0 (1826.5-7392.2)	a:0.650 b:0.905 c:0.334 d:0.030
GSH (µg/mL)	132.89 (103.34-246.42)	131.76 (88.77-236.52)	131.37 (94-188.49)	117.92 (90.65-219.43)	a:0.633 b:0.078 c:0.279 d:0.047

Group 1: venous blood samples of women between the ages of 20-45; Group 2: venous blood samples of women between the ages of 45-75; Group 3: wet cupping blood samples of women between the ages of 20-45; Group 4: wet cupping blood samples of women aged 45-75. a: Comparison of Group 1 and Group 2, b: Comparison of Group 3 and Group 4, c: Comparison of Group 1 and Group 3, d: Comparison of Group 2 and Group 4.

Comparison of venous blood samples according to age groups showed that there was no significant difference between miRNA-34a ($p=0.789$), miRNA-21 ($p=0.193$), MDA ($p=0.650$), and GSH ($p=0.701$) levels of Group 1 and Group 2 while miRNA-200a ($p=0.001$) levels of Group 1 were lower than the Group 2. A comparison of cupping blood samples showed that miRNA-34a ($p=0.031$) and miRNA-21 ($p=0.003$) levels of Group 3 were lower than Group 4.

DISCUSSION

Wet cupping therapy is one of the traditional therapies used for centuries. Although it is commonly used to treat inflammatory, infectious, and immune disorders, its mechanism of action is still unknown (10). Previous studies have reported that wet cupping therapy affects oxidative balance (18, 19). In this study, we hypothesized that wet cupping therapy might also affect oxidative stress-related-miRNA levels and therefore be involved in limiting oxidative stress via the non-coding transcriptome. Tagil

et al. implemented wet cupping therapy on 31 healthy volunteers. Wet cupping blood and venous blood samples were collected simultaneously from the participants. The nitric oxide (NO), MDA levels, superoxide dismutase (SOD), and myeloperoxidase (MPO) activities of participants were evaluated. As a result of the study MPO activity, MDA, and NO levels were higher, and SOD activity was lower in wet cup blood compared to venous blood (18). Twenty-four participants were enrolled in the study conducted by Ersoy et al., and wet cupping therapy was carried out on participants every month for 3 months. Venous blood samples of the participants were taken before the application (Venous 1) and after the last application (Venous 2). Wet cupping blood samples were taken during the first and last applications. The oxidant status markers MDA, total oxidant status (TOS), and antioxidant status markers GSH, SOD, total antioxidant status (TAS), and catalase (CAT) activities were measured in the collected blood samples. As a result of the study, it was reported that the highest MDA and TOS levels

and the lowest TAS, GSH, SOD, and CAT levels were detected in the initial cup of blood. At the same time, these parameters improved in the second cupping blood samples compared to the first cupping blood samples. In addition, MDA and TOS levels, as well as TAS, GSH, SOD, and CAT, have been shown to change favorably in Venous 2 compared to Venous 1 (19). Our findings were consistent with previous studies. Similarly, MDA levels, which is an indicator of oxidative damage, were higher ($p=0.027$) in wet cupping samples compared to venous blood. In comparison, GSH levels, one of the antioxidant indicators, were lower ($p=0.037$) (Table 2). Unlike other studies, our study investigated the influence of wet cupping therapy on oxidative stress-related miRNAs. To our best knowledge, this is the first study to investigate the effect of wet cupping therapy on oxidative stress-related miRNAs. Studies have shown that ROS generated under oxidative stress is involved in the pathogenesis of various diseases such as cancer, cardiovascular diseases, and neurodegenerative diseases and modulates miRNAs' expression (20-22). These alterations in miRNA expression levels mainly occur through the modulation of nuclear factor erythroid 2-related factor 2 (Nrf2), sirtuins (SIRT1), calcineurin / nuclear factor of activated T cell (NFAT), or nuclear factor kappa B (NF- κ B). Therefore, various miRNAs can be identified as potential biomarkers for ROS-related diseases. Increasing evidence suggests that SIRT1 is involved in cellular responses to oxidative stress. SIRT1 is the target of a variety of redox-sensitive mechanisms. Various miRNAs have been revealed to affect oxidative stress via SIRT1. One of these is miRNA-34a. Downregulation of SIRT1 by miRNA-34a has been shown to promote aging and inflammation of vascular smooth muscle cells in aged mouse aortas by mechanisms mediated by oxidative stress (23). NADPH oxidases (NOXs) are a family of membrane-bound enzymes that oxidize NADPH to generate ROS through the catalytic metabolism of oxygen during host defense. Increased expression of the NOX2 isoform has been demonstrated due to the overexpression of miR-34a in glioma cells (24). In addition, miRNA-34a has been shown to regulate various genes and pathways involved in cancer initiation, progression, and metastasis (25). miRNA-21 is one of the miRNAs reported to be a potential biomarker for oxidative stress related-cardiovascular diseases, similar to miRNA-34a (23). NF- κ B transcriptionally modulates various miRNAs. NF- κ B is an important modulator of pro-inflammatory / stress-like responses that play important roles in DNA damage response and apoptosis in various cell types. ROS-induced NF- κ B upregulates miRNA-21

and promotes cancer progression and fibrogenesis (24). Recently, miRNA-21 has been shown to play a role in the pathogenesis of diabetes, and studies aimed at elucidating the role of miRNA-21 in diabetes pathogenesis have drawn attention to miRNA's role in ROS homeostasis (26). Sala et al. demonstrated that miRNA-21 could support the suppression of homeostatic signaling, which limits ROS damage (27). Their subsequent study showed that circulating miRNA-21 could be an early marker of ROS-mediated damage in people at high risk of developing diabetes and in type 2 diabetic individuals who were not on medication (28). Under oxidative stress conditions, Nrf2 / Keap1 complexes disrupt, and Nrf2 transfers to the nucleus. This change increases the expression and activity of several antioxidant genes that inhibit cell apoptosis and support the survival and tumorigenesis of cancer cells. Previous studies have reported that one of the miRNAs targeting the Nrf2 signaling pathway is miRNA-200a (29). Eades et al. and Yang et al. revealed that miR-200a and miR-28 might modulate Nrf2 expression levels by directly targeting Keap1 mRNA in breast cancer cells (30, 31). Mateescu et al. reported that miRNA-200a modulates ROS production by targeting p38a under oxidative stress and potentiates tumor growth and progression (32). The primary aim of our study was to demonstrate that wet cupping therapy provides excretion of markers associated with oxidative stress. As a result of our study, the presence of these markers in wet cup blood has been demonstrated. The second important finding of our study was that the levels of miRNA-200a, miRNA-34a, and miRNA-21, which are markers associated with oxidative stress, were higher in wet cupping blood samples compared to venous blood samples when both age groups were evaluated among themselves and when a general comparison was performed. GSH levels were higher in the venous blood sample than in the wet cup blood sample. These findings show that wet cupping therapy may significantly remove oxidant markers from the body while contributing to an increase in the antioxidant defense. The mechanism of action of wet cupping therapy is not fully known despite its common use. Wet cupping therapy might act through a lot of different mechanisms. We hypothesize that one of the mechanisms of action of wet cupping may be through oxidative balance, and our findings support our hypothesis. This study showed that venous blood and wet cupping blood did not have the same characteristics. Wet cupping blood had higher oxidants compared to venous samples. The therapeutic effects of wet cupping in various conditions might be due to the excretion of these oxidants from the body.

In conclusion, our findings show that miRNA-34a, miRNA-21, and miRNA-200a, which are involved in the pathogenesis of diabetes, cardiovascular diseases, or cancer through oxidative stress-mediated mechanisms in previous studies, are significantly removed from the body by cupping therapy. In addition, our findings indicate that MDA levels, which can cause oxidant damage, are reduced by cupping therapy, and antioxidant defense is improved. However, to prove the effects of cupping treatment on oxidant damage and these markers, further studies are needed in a larger population, especially involving the measurement of these markers before and after cupping therapy. We hope that the relationships between wet cupping and other harmful substances in the body will be investigated in the future and this preliminary study would be a guide for these studies.

Declarations

The authors have no conflicts of interest to declare.

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