RESEARCH ARTICLE

Investigation of oxidant-antioxidant status in patients treated with hirudotherapy: an experimental study

Ismail Sarikan, Hasan Basri Savas

Abstract

Objective: To examine the influence of hirudotherapy on parameters of oxidative stress.

Method: The cross-sectional study was conducted from March 29 to September 29, 2021, at the Alanya Research and Training Hospital's Traditional and Complementary Medicine Application Centre, Turkey, and comprised adult volunteers of either gender. The participants were subjected to two sessions of hirudotherapy 4 weeks apart. Total antioxidant status, total oxidant status, oxidative stress index values, ischaemia-modified albumin level, paraoxonase 1, disulfide, native thiol, total thiol, and arylesterase levels were assessed at baseline and after the second hirudotherapy session. Data was analysed using SPSS 15.

Results: Of the 50 subjects, 30(60%) were females and 20(40%) were males. The overall mean age was 47.10±15.16 years. Oxidative stress, ischaemia-modified albumin and disulfide levels decreased, but not significantly (p>0.05). The reduction in disulfide levels was significant (p=0.021).

Conclusion: Hirudotherapy, within its limitations, could reduce oxidative stress.

Key Words: Antioxidant activity, Oxidative stress, Medicinal leeches, Hirudin.

(JPMA 74: 647; 2024) **DOI: https://doi.org/10.47391/JPMA.9213**

Introduction

Hirudotherapy (HT) is the process of the application of medicinal leeches like Hirudo Medicinalis and Hirudo Verbana to the patient by certified physicians. These leaches are obtained from leech breeding businesses.[1] Leeches are parasitic organisms that feed on blood and have more than 650 species. They live as temporary ectoparasites in mammals and other vertebrates, such as fish and frogs.[2] Leeches have been widely used since ancient times to lower blood pressure. Leeches provide benefits both by sucking blood from the host, and with the bioactive substances they give to the host while they are sucking blood. There are various bioactive substances detected in the saliva of medicinal leeches. Some of these substances are hirudin, tryptase inhibitors and histamine-like substances, eglin, bdellin, hyaluronidase, apyrase, calin, destabilase, hirustatin, acetylcholine, complement inhibitors, and carboxypeptidase-A inhibitors. Such bioactive agents have coagulation inhibition, anti-aggregant, fibrinolytic, antimicrobial, analgesic, and anti-inflammatory effects[3] (Table 1).

The World Health Organisation (WHO) describes traditional medicine as the practice of preventing, treating or diagnosing physical or psychological disorders, depending on beliefs and experiences unique for various cultural characteristics. Between 2014 and 2023, WHO supported member states in developing policies that will strengthen the role traditional medicine plays in keeping people healthy.[4] In 2004, the United States Food and Drug Administration (FDA) permitted the trade of medicinal leeches in the US and their usage for general purposes, in plastic surgery, and in microsurgery applications. Physicians had refrained from using

Table-1: Bioactive substances in leech secretions.

Effect	Bioactive Substance
Analgesic and Anti-Inflammatory Effect	Antistatin, Hirustasin, Ghilantens, Eglin C, LDTI, Complement C1 Inhibitor, Guamerin and Piguamerin, Carboxypeptidase Inhibitor, Bdellins, and Bdellastasin
Extracellular Matrix Destruction	Hyaluronidase and Collagenase
Increasing Blood Flow, Vasodilatation	Acetylcholine, Histamine Like Molecules
Inhibition of Platelet Function	Saratin, Calin, Apyrase, Decorsin
Anticoagulant Effect	Hirudin, Gelin Factor Xa Inhibitor, Destabilase, New Leech Protein-1 Whitide, and Whitmanin
Antimicrobial Effect	Destabilase, Chloromycetin, Theromacin, Theromyzin, and Peptide B
Anaesthetic Effect	Anaesthetic Substance
Dissolves fibrin	Destabilase

LDTI: Leech-derived tryptase inhibitor.

Alaaddin keykubat University, Antalya, Turkey.

Correspondence: Ismail Sarikan. Email: ismail.sarikan@alanya.edu.tr

ORCID ID: 0000-0002-2366-0226

Submission complete: 21-02-2023 **Review began:** 15-04-2023

Acceptance: 29-11-2023 **Review end:** 27-09-2023

traditional and complementary medicine until recently, but nowadays, when its benefits have been shown by scientific research, they have turned to this field again.

Oxidative stress (OS) is defined as a phenomenon resulting out of a discrepancy between the production of free oxygen radicals in cells and tissues, and the capacity of the organism to detoxify these reactive species.[5]

OS refers to a development that emerges during usual metabolic activities. When OS increases, cell, tissue and organ damage can occur. Therefore, an increase in OS should be prevented or balanced with antioxidants. There are many structures that take a role in the antioxidant system and neutralise oxidant substances. Increased OS can lead to undesirable consequences, such as premature aging, atherosclerosis, cancer, Parkinson's disease and Alzheimer's disease.[5,6]

Several techniques have been proposed to determine OS and antioxidant status. The current study was planned to examine the influence of HT on new-generation oxidant and antioxidant parameters.

Materials and Methods

The cross-sectional study was conducted from March 29 to September 29, 2021, at the Alanya Research and Training Hospital's Traditional and Complementary Medicine Application Centre, Turkey, and comprised adult volunteers of either gender.

It has been recommended to include at least 30 participants in studies involving parametric tests, the current study inflated the sample size to account for potential data losses.[7] The subjects included furnished written informed consent. The exclusion criteria were: bleeding diathesis, such as haemophilia, severe anaemia with haemoglobin (Hb) <9.5g/dl), use of anticoagulant drugs, such as warfarin, pregnancy and breastfeeding, history of allergies to leeches, chronic kidney failure and dialysis, presence of pacemaker, age <18 years, chemotherapy, active bleeding, like haemorrhoids, normal menstrual cycle, postoperative period, etc., and glycated Hb (HbA1c) >10, or fasting blood sugar (FBS) >250mg/dl.

Approval was received from Alanya Alaaddin Keykubat University Clinical Research Ethics Committee.

After measuring height, weight and blood pressure of the participants and taking down their blood analyses, they were subjected to 2 HT sessions with an interval of 4 weeks. A total of seven medium-sized leeches weiging approximately 1-1.5g were used per person for HT in each session. The leeches were placed in the lumbosacral region. They were placed on the surface of the skin and separated spontaneously within 1-3 hours. The leeches that separated from the skin were considered medical waste and were thrown into a red medical waste bag. The places where the leeches were separated were cleaned with polyvinylpyrrolidone antiseptic solution and covered with hard cotton. At the end of the second HT session, the baseline blood tests were repeated.

Venous blood samples were taken from the participants for FBG, HbA1C, international normalised ratio (INR), and haemogram. OS status was determined. Around 5cc of blood was collected in a biochemistry tube with ethylene diaminetetraacetic acid (EDTA) and centrifuged for 10 minutes at 1500rpm, and the serum part was taken for analyses. The serum portion was transferred to Eppendorf tubes using Pasteur pipettes. The Eppendorf tubes were divided into 2 groups. The blood taken before HT was labelled as Pre-HT, and the blood taken after the second session of HT was labelled as Post-HT. The samples were kept at -80°C (Thermo Scientific Forma 88000, US) until the day of analysis in the Biochemistry Laboratory of Alkü Medical Faculty.

Biochemical parameters were investigated using fully automated colorimetric methods included total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), paraoxonase activity (PON1), arylesterase (ARES), disulfide, and ischemia-modified albumin (IMA).

Commercial kits were utilised to measure TAS values (REL assay Diagnostics, Turkey). The automated method was based on the disappearance of the characteristic colour of the relatively stable 2,2' Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) cation by antioxidants. The assay provides excellent tender results <3% which are presented in units of mmol Trolox equivalent/L.[8]

Commercial kits were utilised to measure TOS values (REL assay Diagnostics, Turkey). The results are presented in units of micromolar hydrogen peroxide equivalent per liter (µmol H2O2 equivalent/L).[7] The ratio of TOS to TAS was used to calculate OSI values. For sums, the resulting unit of TAS was converted to µmol/L, and the OSI value was figured out based on the equation OSI=TOS/TAS.[8]

PON1 and ARES activities of the sera were identified with a fully automated technique (REL Assay Diagnostics; Mega Tıp, Gaziantep, Turkey). Phenylacetate was utilised as a substrate to estimate ARES activity. As a result of the hydrolysis of phenylacetate, phenol and acetic acid were produced. The phenol joined 4-aminoantipyrine and potassium ferricyanide, and was measured using the colorimetric method. The results are presented as units per liter, which is equal to the hydrolysis of 1 micromole of phenylacetate in 1 litre and 1 minute.[8] PON1 activity results are presented as units per litre, which are equal to the hydrolysis of 1 micromole of the substrate in 1 litre and 1 minute.[8]

The albumin cobalt binding analysis method was employed to determine the presence of IMA. The absorbance of the samples was determined at 470nm with a spectrophotometer. The outcomes are presented as absorbance units (ABSUs).[8]

Thiol/disulfide homeostasis (TDH), which is the sum of thiol-native and thiol/disulfide variations, was measured using a method developed by Erel and Neselioglu[9]. Half of the difference between total thiol and thiol-native amounts provided the quantity of disulfide bonds.[9] TDH analyses were conducted using the automated spectrophotometric technique, with half of the difference between total thiol and thiol-native giving the quantity of the dynamic disulfide.[9]

Data was analysed using SPSS 15. Baseline and post-intervention differences were compared using the parametric independent-samples t-test. Data was expressed as frequencies and percentages as well as mean ± standard deviation, as appropriate. P<0.05 was considered statistically significant.

Results

Of the 50 subjects, 30(60%) were females and 20(40%) were males. The overall mean age was 47.10±15.16 years (range: 18-84 years). There were 42(84%) overweight or obese subjects, 47(94%) were non-smokers, 40(80%) were married and 25(50%) had university degrees) Table 2).

Table-2: Demographic data.

Variable	Participants (N=50)
Age (Mean age in years)	47.10±15.16
Gender (Female/Male; N, %)	Male (20, 40%)
	Female (30, 60%)
Education Level (N, %)	Primary-Secondary School (6, 12%)
	High School (19, 38%)
	University (25, 50%)
Marital Status (Married/Single; N, %)	Married (40, 80%)
	Single (10, 20%)
Smoking Status (N, %)	Smoker (3, 6%)
	Non-smoker (47, 94%)
BMI[1] (Body mass index; N, %)	≥30 (Obese) (16, 32%)
[1]BMI = kg/m^2	≥25 (Overweight) (26, 52%)
	≥18 (Normal Weight) (8, 16%)
	<18 (Underweight) (0, 0%)

BMI: Body mass index.
[1]BMI is calculated using a person's height and weight. The formula is BMI = kg/m^2; kg is a person's weight in kilograms, and m^2 is the square of their height in meters. BMI <18.5: Underweight, BMI 18.5-25: Normal, BMI 25-30: Overweight, BMI >30: Obese.

Biochemical tests showed that there were non-significant differences between baseline and post-intrevention values for TAS, TOS, OSI, PON1, ARES and IMA (p>0.05). The reduction in disulfide levels was significant (p=0.021) (Table 3).

Table-3: Oxidant-antioxidant parameters in serum samples

	Before the 1st session (Pre-HT)	After the 2nd session (Post-HT)	p-value
TAS(µmol Trolox Eq. /L) (mean±SD)	1.1470±0.26109	1.1400±0.31097	0.903
TOS(µmol H2O2 Eq. /L) (mean±SD)	6.0522±0.84049	5.9739±1.19531	0.706
OSI(U/L) (mean±SD)	5.7045±2.46713	5.7448±2.37708	0.934
Disulfide (µmol/L) (mean±SD)	34.0250±16.23943	26.4901±15.98862	0.021*
PON1 (1 nmol 4-nitrophenol/ L serum/min) (mean±SD)	315.8600±187.02886	311.9200±195.92979	0.918
ARES (1 micromole phenol/ mlserum/min) (mean±SD)	369.6600±195.22040	363.2400±206.97092	0.874
IMA(U/mL) (mean±SD)	99.9540±4.88213	99.5460±5.41469	0.454

TAS: Total antioxidant status, TOS: Total oxidant status, OSI: Oxidative stress index, PON1: Paraoxonase1 activity, ARES: Arylesterase, IMA: Ischemia-modified albumin.
* Compliance of numerical variables with normal distribution was determined according to the value of both variables being >30 and p>0.05 as a result of Kolmogorov-Smirnov test. Consequently, one of the parametric statistical methods, independent samples t test, was applied.

Discussion

In the current study, there were 52% participants who were overweight, and 32% were obese. Obesity is a chronic and treatable disease defined as "excessive fat accumulation in the body due to the energy taken with food being more than the energy consumed". According to the WHO data in 2016, there were 39% adults worldwide who were overweight, and 13% were in the obese category.[10] The prevalence of obesity is 39.6% in the US,[11] 30-70% in Europe, and 30.2% in Turkey.[12]

The current study limited itself to 2 sessions 4 weeks apart so that the participants may feel encouraged to complete the study. The waist region (any area between L1 and S2) was chosen for ease of application. OSI, IMA and disulfide levels, which indicate OS, decreased numerically after HT, but only the reduction in disulfide levels was significant (p=0.021).

Despite the finding that reduction in OSI and IMA results was not at the level of statistical significance, the fact that they accompanied a significant decrease in disulfide levels clearly showed that the application of medicinal leeches reduced OS. No relevant study could be found in the current literature investigating the antioxidant effects of leeches. However, in a study by Gröbe A et al., it was reported that leech therapy improved venous obstruction and saved ischemic flaps in 148 patients followed in the plastic surgery service.[13]

Besides, there are studies showing that HT is effective in reducing joint stiffness and pain associated with osteoarthritis.[14] Venous blood collection (phlebotomy) has also been shown to reduce OS.[15,16]

Tagil et al.[17] reported that wet cupping therapy removed oxidants and reduced OS. Another study found that high iron stores in the body increase OS in neurons. Therefore, these levels were found to increase the risk of Alzheimer's disease. It was predicted that phlebotomy performed without causing anaemia can improve brain functions, slow down neurodegeneration, and improve cognitive and behavioural functions in Alzheimer's disease.[18]

The OSI is found elevated in many chronic diseases. For example, a direct relationship was determined between OSI and low-density lipoprotein (LDL) and triglyceride (TG) levels in the serum. However, high-density lipoprotein (HDL) levels were not part of such a relationship.[19] TOS and OSI were determined as significantly higher among patients with schizophrenia in comparison to those without schizophrenia.[20] Therefore, reducing the OSI levels is important.

It is not clear whether the OS-reducing effect noted in the current study was due to the blood sucking of leeches, or due to the bioactive substances in their saliva.[3] It could be both, and there is a need for further research on the topic.

There were limitations to the current study as it did not include a control group, and no dietary restriction was encouraged between the two sessions. For instance, the intake of foods known to have antioxidant effects was not restricted.

Conclusion

HT, with its limitations, could be used as a method to reduce OS in the treatment of chronic diseases, or in the maintenance of one's current health.

Acknowledgement: We are grateful to the staff of Alanya Research and Training Hospital, Traditional and Complementary Medicine Application Centre, and to the authorities that provided permission.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: Alanya Alaaddin Keykubat University (Alkü) Scientific Research Projects Coordination Unit (Project Number: 2021-04-02-MAP10).

References

1. Turkish Ministry of Health. Department of Traditional, Complementary and Functional Medicine. [Online] [cited 2022 Feb 27] Available from: URL: https://shgmgetatdb.saglik.gov.tr/TR-82169/ traditional, complementary medicine practices regulation.html
2. Sağlam N. Internal and external morphological characteristics of the medicinal leech species Hirudo sulukii and Hirudo verbana. Turkish J Parasitol. 2019;43: 204-9.
3. Lemke S, Vilcinskas A. European Medicinal Leeches-New Roles in Modern Medicine. Biomedicines. 2020;8:99. doi: 10.3390 /biomedicines 8050099. PMID: 32349294; PMCID: PMC7277884.
4. WHO: Traditional, Complementary and Integrative Medicine. [online] [cited 2022 October 2022] Available from: URL: https://www.who.int/health-topics/traditional-complementary-and-integrative-medicine#tab=tab_1
5. Misrani A, Tabassum S, Yang L. Mitochondrial Dysfunction and Oxidative Stress in Alzheimer's Disease. Front Aging Neurosci. 2021;13:617588. doi: 10.3389/fnagi.2021.617588. PMID: 33679375; PMCID: PMC7930231.
6. Aslan SN, Karahalil B. Oxidative Stress and Parkinson Disease. J Fac Pharm Ankara / Ankara Ecz Fak Derg. 2019; 43: 94-116. Doi: 10.33483/jfpau.519964
7. Avcu R, Avcu S. The Methodological Quality of Experimental STEM Education Articles Published in Scholarly Journals from 2014 to 2020. Int J Assessment Tools Educ. 2022; 9 :290–318. Doi: 10.21449/ijate.946743.
8. Savas HB, Sayar E. Oxidant-Antioxidant Balance and Trace Elements in Children with Functional Dyspepsia. Turkiye Klinikleri J Med Sci. 2021;41:70-9. doi: 10.5336/medsci.2020-79596.
9. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. Clin biochemistry. 2014; 47:326-332.
10. World Health Organization (WHO). Obesity and overweight. [online] [cited 2022 Oct 23] Available from: URL: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
11. Cantu-Villarreal Y, Herrera D, Dunzz K, Salas J, Villarreal YC, Rodriguez A, et al. The relations between obesity and cardiovascular disease. DHR Proceedings, 2022;2(S2).
12. Turkish Ministry of Health, General Directorate of Public Health. Healthy nutrition and active life department. [online] [cited 2022 Sep 27] Available from: URL:- https:// hsgm.saglik.gov.tr /tr/obesity/ Prevalence of obesity in Turkey.html
13. Gröbe A, Michalsen A, Hanken H, Schmelzle R, Heiland M, Blessmann M. Leech therapy in reconstructive maxillofacial surgery. J Oral Maxillofac Surg. 2012;70:221-7. doi: 10.1016/j.joms.2011.02.083. Epub 2011 May 7. PMID: 21550156.
14. Lauche R, Cramer H, Langhorst J, Dobos G. A systematic review and meta-analysis of medical leech therapy for osteoarthritis of the knee. Clin J Pain. 2014;30: 63-72. DOI: 10.1097/AJP.0b013e31828440ce
15. Holsworth Jr RE, Cho YI, Weidman JJ, Sloop GD, Cyr JS. Cardiovascular benefits of phlebotomy: Relationship to changes in hemorheological variables. Perfusion. 2014;29: 102-116. doi: 10.1177/0267659113505637.
16. Esteban T, Kaito M, Motoh I, Watanabe C, Gabazza AKN. Iron reduction therapy by phlebotomy reduces lipid peroxidation and oxidative stress in patients with chronic hepatitis C. J Gastroenterol. 2006;41:921. doi: 10.1007/s00535-006-1871-5.
17. Tagil SM, Celik HT, Ciftci S, Kazanci FH, Arslan M, Erdamar N, et.al. Wet-cupping removes oxidants and decreases oxidative stress.

Complement Ther Med 2014;22:1032-6. doi: 10.1016/j.ctim.2014.10.008. Epub 2014 Oct 13
18. Dwyer BE, Zacharski LR, Balestra DJ, Lerner AJ, Perry G, Zhu X, et al. Getting the iron out: phlebotomy for Alzheimer's disease? Med Hypotheses. 2009;72:504-9. doi: 10.1016/j.mehy.2008.12.029. Epub 2009 Feb 4. PMID: 19195795; PMCID: PMC2732125.
19. Turkdogan KA, Akpinar O, Karabacak M, Akpinar H, Turkdogan, FT, Karahan O. Association between oxidative stress index and serum lipid levels in healthy young adults. J Pak Med Assoc. 2014; 64: 379-81.
20. Bahceci B, Bagcioglu E, Kokacya MH, Dilek AR, Bahceci I, Selek S. Prolidase activity and oxidative stress in patients with schizophrenia: A preliminary study. J Pak Med Assoc. 2015;65: 131-5.

Author's Contributions
IS, HBS: Conception, design of the work, acquisition, analysis, interpretation of data, drafting, revising critically, final approval.