The Controversial Role of Folic Acid on Diabetic Auditory Neuropathy

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Research Article

Keywords: Auditory brainstem response, Auditory neuropathy, Diabetes mellitus, Folic acid, Stereology

Posted Date: October 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-884001/v1>

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Abstract

Purpose

Diabetic auditory neuropathy(DAN) is a common complication of diabetes that seriously affects the quality of life in patients. In this study, we investigate the role of folic acid in the treatment of DAN in an experimental rat model.

Methods

Thirty-two Sprague-Dawley rats were equally divided into 4 groups: group 1, normal; group 2, diabetic rats; group 3 and 4, rats treated with folic acid (40 and 80 mg/kg, respectively). The tools we used in this study to investigate the effect of folic acid on DAN were auditory brain stem response, stereology method for estimation of the volume and number of spiral ganglion, volume of stria vascularis, and spiral ligament, and measurement of homocysteine (HCY), malondialdehyde(MDA) and superoxide dismutase.

Results

Our study showed that folic acid treatment was not significantly effective in improving structural and functional disorders in DAN, despite its effect in reducing HCY and MDA as oxidative biomarkers.

Conclusion

Folic acid is not effective in relieving morphological and functional disorders in DAN.

Introduction

Diabetic neuropathy is a common complication of diabetes mellitus(DM), which affects the sensory, motor and autonomic nerves and in some cases the central nervous system[1-3]. Ischemia caused by damage to the feeding vessels of the nerves in diabetic patients causes neural fibers demyelination, degeneration and the resulting neuropathy[2,4].

The major metabolic disorders that cause vascular damage in diabetic patients, followed by neuropathy, are mainly due to polyol pathway and oxidative stress, which increase free radicals, especially reactive oxygen species in the tissues[4-6].

Impaired neural microcirculation in the auditory system can cause morphological and functional disorders. Diabetic auditory neuropathy (DAN) is mainly accompanied by debilitating symptoms such as hearing loss, tinnitus and dizziness[7,8].

Due to the significant occurrence of neuropathy, as well as its auditory type in diabetic patients, various agents have been evaluated for prevention and treatment of neural damage in experimental and clinical studies. Among the drugs that have been investigated for diabetic neuropathy, rutin[9], folic

acid[10,11],alpha-lipoic acid[12] and benfotiamine(a vitamin B1 derivative) can be mentioned[13]. The results of these investigations are not sufficient for clinically acceptable applications and results.

In a study conducted by the authors of this article on rutin in the prevention and treatment of diabetic auditory neuropathy in rats, the useful effect of this drug in terms of morphological and functional improvement was defined to some extent[9].

One of the drugs under investigation which is relatively more clinically used than others in diabetic neuropathy is folic acid. Due to the discrepancies in animal and clinical studies and clinical observations that in some cases show the ineffectiveness of this drug, we set out to do an investigation about folic acid effectiveness against diabetic auditory neuropathy in rats, following our previous study on rutin.

In this study, auditory brain stem response(ABR), stereology of spiral ganglion, homocysteine (HCY), superoxide dismutase(SOD), and malondialdehyde(MDA) levels were used as defining criteria to investigate the effect of folic acid treatment in DAN.

Folic acid (vitamin B9) is categorized as a water-soluble vitamin which is involved in the synthesis of genetic materials, the production of red blood cells and fetal health. Folic acid is a synthetic form of folate that is better absorbed than folate. The main sources of folate include leafy vegetables, citrus fruits, mushrooms, grains and liver[14,15].

Folate, as a donor of single carbon units, is involved in the synthesis of serine from glycine, nucleotides from purine precursors, and in the formation of deoxy thymidylate monophosphate. In addition to its role in the synthesis of DNA and messenger RNA, it acts as a methyl donor to produce methyl cobalamin, which is used in the remethylation of HCY to methionine[16,17].

Folic acid seems to have the potential ability for growth and differentiation of neural cells and also has a neuroprotective property due to its effects on HCY metabolism as well as decreasing malondialdehyde levels and increasing the expression of nerve growth factors[18,19].

ABR is one of the best tools for evaluating auditory neuropathy functionally. In this method, the evoked electrical activities in the auditory nerve and its brainstem connections are measured. Diabetes mellitus can cause changes in ABR potentials latency and waveforms by disrupting the microcirculation of the auditory pathway[7,20].

Impaired microcirculation in DAN and subsequent cochlear ischemia can cause morphological changes in the hair cells, spiral ganglion, spiral ligament and stria vascularis[7]. In this study, we used the stereology method in the spiral ganglion, spiral ligament, and stria vascularis to examine morphological changes in the DAN and the response to treatment by measuring the volume and number of cells.

HCY is a non-proteinogenic amino acid made from the amino acid methionine following a transmethylation reaction. Many studies have shown an association between high levels of HCY in diabetic patients and its vascular and neurological complications[21-23].

SOD is an antioxidant enzyme that catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide. This enzyme acts as a biomarker in antioxidant activity in controlling oxidative stress [24].

MDA is a product of polyunsaturated fatty acids peroxidation. MDA is considered as a reliable biomarker in oxidative stress measurement levels in many experimental and clinical studies [25,26].

In this study, ABR was used to evaluate the hearing function, the stereology method for morphological evaluation in the spiral ganglion and oxidative biomarkers of HCY, MDA, and SOD levels as tools to evaluate the efficacy of folic acid treatment on DAN.

Materials And Methods

Materials

Folic acid and Streptozotocin (STZ) were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany).

Animals preparation

Thirty-two male *Sprague-Dawley* rats (250-300 g) were obtained from the Center of Comparative and Experimental Medicine (Shiraz, Iran). The rats were kept under standard lighting (12 hour-light/dark cycles), humidity (25–35 %), temperature (22-26 °C) per day and had free access to food and water. The rats were randomly allocated to four groups of eight rats, including non-diabetic normal rats with distilled water (Normal group), type 1 diabetic rats with distilled water without folic acid treatment (DM group), type 1 diabetic rats that received folic acid (40 mg/kg/day) (DM+FA40 group), and type 1 diabetic rats that received folic acid (80 mg/kg/day) (DM+FA80 group).

The experimental procedures were approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethical code: IR.SUMS.REC.1397.459).

Induction of type 1 diabetes mellitus

To induce type 1 diabetes mellitus, a single intraperitoneal administration of STZ (60 mg/kg) was dissolved in cold citrate buffer and injected into animals. Three days after induction, fasting blood sugar (FBS) levels were checked using a Glucometer (Accu-check®, Germany), and rats with FBS levels greater than 300 mg/dL were considered as type 1 diabetic animals [27]. The confirmation day was considered 3 days after STZ injection.

Treatment

After the confirmation of diabetes, two groups received an oral solution of folic acid (40 mg/kg/day, DM+FA40 group) and (80 mg/kg/day, DM+FA80 group) by gavage once a day for 8 weeks. Normal and Diabetic groups received an equal amount of distilled water.

After 8 weeks of treatment, rats were prepared for auditory assessment by ABR and histological studies.

ABR assessment

At the end of treatments, Xylazine (10 mg/kg) and Ketamine (75–100 mg/kg) were administered to the rats via intraperitoneal injection[9]. Thermal blanket and heat lamp were used to avoid hypothermia during anesthesia. In the ABR recording, ground electrode (ECG Ambu Blue Sensor, Penang, Malaysia) was fixed on the dorsal neck of rat. Also, active electrode (+) and the reference electrode (–) were attached to the forehead and post auricular area of right and left ears, respectively. Evoked potentials were achieved by intra acoustic EP25 (Copenhagen, Denmark) system with OtoAccess software (Middelfart, Denmark) after releasing clicks to the right ear via embedded earphones. Minimum 700 stimuli at a rate of 11.1 Hz were distributed to right ear and electrode impedance was below 3 K Ω . ABR evaluations were accomplished using band pass filter of 100–3,000 Hz and time window of 10 ms[28]. Evoked potentials were estimated according to the following parameters: absolute latencies and wave morphology of wave's II and V and hearing threshold. Hearing threshold was detected in 5 dB steps declining from maximum stimulus of 70 dB until wave patterns morphology disappeared.

Tissue preparation & stereological analysis

Preparation of inner ear in rat

At the end of study, all of the animals were sacrificed after induction of deep anesthesia by intraperitoneal injection of 100 mg/kg Ketamine and 10 mg/kg xylazine[9]. The temporal bone was carefully dissected and separated from skull bones, *decalcification* of temporal bone was performed by use of 8% HCL and 8% formic acid for 3 days[29]. All temporal bones underwent tissue processing and after that were embedded in paraffin wax. The paraffin blocks were cut into 25 μ m thickness serial section together with the external acoustic meatus axis by a microtome (Microm, Germany). All of the serial sections of cochlea were collected and separated from apical to basal cochlear.

Thirty-two to thirty-six sections of cochlear tissue per rat were obtained and all slides were stained with Hematoxylin and eosin. Afterward, 8-12 sections of the 32-36 sections of cochlea in a systematic uniform random sampling were selected. The first section was randomly selected and the next sections were chosen at equal intervals.

Stereological techniques were applied to estimate the volumes of the spiral ganglion, spiral ligament and stria vascularis and the number of neurons in spiral ganglion. The cochlear sections evaluation was done by an examiner who was blind to the animal groups.

Estimation of the spiral ganglion, spiral ligament and stria vascularis volumes

The live figure of each cochlea section was evaluated by a video microscopy system and the stereology software (Stereolite, SUMS, Shiraz, Iran).

Regions of the selected structure (spiral ganglion, spiral ligament, and stria vascularis) were recognized in each cochlealsection(Fig. 1a). The volume of the selected structure was analyzed by the point-counting methods based on “Cavalieri's principle” at the final magnification of 20 on 8–12 sections per animal[30-32].

The probe of stereology (a grid of points) was overlaid on the cochlear images(Fig. 1b), the cross-sectional area of the selected structure “ $\sum A$ ” was calculated by the stereology software. Then the cross-sectional area of the selected structure “ $\sum A$ ” was multiplied by the distance between the *sections*(T). The volume of selected structure was estimated by the following formula:

$$V = T \cdot a/p \cdot \sum P$$

where V is the volume of selected structure; T is distance between two selected sections; a/p is area per point; and $\sum P$ is total points hitting selected structure. The area per point (a/p) was 722500 (850×850) μm^2 and an average of 121 points were counted per animal.

Estimation of the neuron numbers in the spiral ganglion

The spiral ganglion is a collection of bipolar neuron cell bodies in the modiolus of inner ear, the lemon-like shaped central axis of the cochlea whose fibers innervate the corti organ[30]. One of the stereological techniques for spiral ganglion neurons counting in a *histological section* thickness is the optical dissector[32-34].

The site of the microscopic field was chosen by moving the stage in directions (x and y) at the same distances pursuant to systematic uniform random sampling order. Objective lens (40×, NA 1.30) was employed by oil immersion.

The optical disector is made of several parts. An Eclipse microscope (E200, Nikon, Tokyo, Japan) with a large numerical aperture (NA=1.30) ×40 oil-immersion objective was linked to a video camera, which conveyed the live image of microscope to a computer monitor, and an electronic microcator with digital readout (MT12, Heidnehain, Traunreut, Germany) for estimating the movements in the Z-axis with a precision of 0.5 μm .

One of the stereological probes is unbiased counting frame that is employed to count the number of select cells, by a stereology software system (Stereolite, SUMS, Shiraz, Iran). The unbiased counting frame, composed of two exclusion lines (the lower and left borders and their extensions) and two inclusion lines (the upper and right borders), was overlaid on the live image at final magnification of 1500× (Fig. 2a).

The guard areas are zones at the superior and inferior part the histological sections. These regions were applied to avoid cutting tissue artifacts that occur throughout tissue processing on these areas of the sections. Any counting incident in focus within the up (the first 3.5 μm) or down guard zones was deleted.

Each nucleolus cell that came into focus within the guard areas was not counted. The “height of disector” was the distance between the guard areas which was 18µm here. Every nucleolus cell appearing in the focus inside the later focal sampling plane was selected if it was located completely or partially inside the counting frame (Fig. 2b) and did not contact the forbidden lines [32-34].

The numerical density (NV) of the spiral ganglion neurons were evaluated using the following formula:

$$N_v = [\sum \bar{Q} / [\sum P \times a(f) \times h]] \times [t/BA]$$

Where “ $\sum \bar{Q}$ ” is the number of the spiral ganglion neurons coming into high quality focus in disector height, “ $\sum P$ ” is the total of the counting unbiased frames in all fields of microscopic, and “h” is the height disector. Where “a/f” is the area of counting frame, “t” was the true section thickness calculated with the microcator, and “BA” is the *microtome block advance* set. The thickness of the internal ear section was calculated in the whole microscopic fields of view with uniform random sampling order from each section.

On average, 100-200 neurons were counted in 26 disectors in 8-12 sections per animal. The area of the counting frame (a/f) was 1082.41 (32.9×32.9) µm². The real average thickness of the sections was 22 µm (t) and “BA” was 25 µm. The total number of the neurons was estimated by multiplying the numerical density (Nv) by V (submucosa or muscularis layers).

Biochemical assays

At the end of treatments, SOD activity, MDA serum levels, and serum activity of HCY were measured in all groups, using the SOD ELISA kit (Cayman, Ann Arbor, MI, USA), Rat MDA ELISA kit (Cusabio Biotech, China), and HCY ELISA kit (Axis Homocysteine Enzyme Immunoassay, IBL, Germany), respectively.

Statistical analysis

Data were presented as mean for stereological results and mean ± SEM for other results. Statistical analysis was determined using one-way analysis of variance (ANOVA) for parametric data such as latency of wave II, hearing threshold, volume of spiral ganglion, spiral ligament and striavascularis, number of spiral ganglion neurons, SOD, MDA, and HCY. Also, Kruskal-Wallis test was applied for nonparametric data such as different forms of wave V. GraphPad Prism software (Version 6) was used to compare data. If a significant difference was obtained, the source of difference was located by Tukey or Dunn post-hoc test. $P < 0.05$ was considered as statistically significant.

Table 1. Wave V types in the normal, diabetic, DM+FA40, and DM+FA80 groups

Group	Wave V Normal (%)	Wave V with delay (%)	Wave V Absent (%)	P-value
Normal	100	0	0	<0.0001
Diabetic	0	0	100	-
DM+FA40	12.5	0	87.5	0.99
DM+FA80	0	37.5	62.5	0.99

Differences were tested by Kruskal-Wallis and Dunn's post hoc test. All data is in comparison to diabetic group.

Results

Outcome of folic acid on the function of ABR

After 8 weeks of DM induction, the effect of diabetes on ABR wave forms was observed. The negative changes were significantly on the latency of wave II, hearing threshold, and the presence of wave V ($P < 0.001$). The latency of wave II, increased hearing threshold, and the rate of presence of wave V are shown in Fig. 3 and Table 1, respectively. Folic acid (40 and 80 mg/kg) had no significant recovery effect on ABR recordings at the end of study (about 8 weeks after the induction of diabetes) (Fig. 3a, 3b and Table 1).

Stereological studies

Evaluation of spiral ganglion, spiral ligament, stria vascularis volume, and Spiral ganglion neuron count

There is a significant decline in the total volume of the spiral ganglion, spiral ligament, stria vascularis and the count of spiral ganglion neurons in the rats with DM compared to the normal animals ($P < 0.05$) (Fig. 4a, 4c, 4d, and 4b).

The total volume of spiral ganglion, spiral ligament, stria vascularis and the number of spiral ganglion neurons did not change significantly in groups of DM+FA40, and DM+FA80 (Fig. 4a, 4c, 4d, and 4b).

Biochemical assays

Erythrocyte level of SOD of diabetic group was significantly lower than normal group ($P < 0.001$). On the other hand, these values for folic acid groups (DM+FA40 and DM+FA80) were not higher than the diabetic group (Fig. 5a).

The MDA serum level in the diabetic group was significantly higher than the normal group ($P < 0.01$) but, serum levels of MDA for folic acid-treated groups (40 and 80 mg/kg/day) were significantly lower than

that of the diabetic group ($P<0.05$) after the intervention (Fig. 5b).

Serum level of HCY was increased significantly in the diabetic group compared to the normal group ($P<0.001$; Fig. 5c). Administration of folic acid 40 and 80 mg/kg/day showed a significant decrease in serum level of HCY ($P<0.001$, and $P<0.0001$, respectively; Fig. 5c).

Discussion

This study revealed that folic acid does not have a significant effect on prevention and treatment of functional and structural deficits in DAN despite reduced MDA and HCY levels.

In this study, folic acid was administered in two doses of 40mg/kg and 80mg/kg, but neither of them showed a significant response in ABR recordings and spiral ganglion stereology.

In our opinion, spiral ganglion stereology and ABR recordings are indicators that are more closely related to clinical situation than MDA and HCY levels.

The advantage of our study is the histological examination through stereological method and its compliance with functional outcomes in diabetic neuropathies treated with folic acid.

ABR is an important tool for assessing hearing function. Numerous clinical and experimental studies have shown the effect of diabetes on ABR recordings. These changes include an elevation in the hearing threshold and an increase in waves latency as well as a decrease in the amplitude with changes in waveforms[35,36].

Our study showed that 8 weeks' treatment of diabetic rats with folic acid at different doses(40mg/kg,80mg/kg) did not significantly improve hearing threshold and also waves II and V latency as compared to control diabetic rats. There are some differences in rat ABR compared to humans that we have considered in this study. It seems wave II that was generated in the cochlear complex nucleus, is the most prominent in rats ABR recordings[37].

Morphological changes in diabetic auditory neuropathy mainly include atrophy of stria vascularis, thickening of the basilar membrane in the stria vascularis, and a decrease in the number of spiral ganglion neurons[7,38].

Similar to other studies, the histological changes shown in our study following the induction of diabetes in rats included a decrease in the volume of the stria vascularis, spiral ganglion, spiral ligament and a decrease in spiral ganglion neurons. The stereological method we used in this study allows us to more quantitatively and more accurately examine structural and cellular changes in DAN. The structural deficits in DAN are consistent with the functional findings of ABR. In this study, the preventive effect of folic acid on structural changes at different doses(40mg/kg,80mg/kg) was not shown.

Our study revealed a significant decrease in HCY levels following folic acid administration in diabetic rats. At higher doses of folic acid(80mg/kg), this reduction is more significant. Its role and underlying mechanism in development of vascular complications in diabetes and reducing its levels on complication incidence following treatment are not exactly defined[39,22].

The increased oxidative degradation of nitric oxide(a regulator of endothelial homeostasis) during the process of oxidative stress, is consideredthe presumptive mechanism of endothelial vascular injury in elevated HCY levels[40,41]. Folic acid through its bioactive form, 5-methyltetrahydrofolate, donates a methyl- group forHCY remethylation to methionine [17,42].

Our study showed that plasma MDA levels were significantly increased following folic acid treatment in diabetic rats. This finding is consistent with other findings in experimental studies on the folic acid treatment of neuropathy [10]. Another important finding in this study was the lack of SOD increase following folic acid treatment. These antioxidant enzymes control and eliminate reactive oxygen species (ROS)during oxidative stress in cells [43]. Based on the findings of this study, folic acid lowered plasma HCY and MDA levels significantly while it had no significant effect on SOD. It may imply that oxidative stress is reduced via a mechanism other than an increase in the amount or action of SOD.

Another perception that can be drawn from these findings is that, due to the lack of improvement in structural and functional disorders in DAN and the lack of significant change in SOD levels, SOD may be a better measure of oxidative stress than HCY and MDA.

It can be deduced that the selected doses of folic acids were not high enough to produce meaningful action on diabetic neuropathy. However, based on our scientific bibliography the selected doses werehigher thanthose applied in similar studies.

Conclusion

Diabetic auditory neuropathy is one of the serious complications of diabetes that has a serious impact on quality of life. Our study showed that folic acid is not effective in relieving structural and functional disorders in DAN, despite its effect in reducing HCY and MDA. Due to this issue, more studies are needed to find potent drugs for preventing and treating this complication in diabetic patients.

Declarations

Acknowledgments

The present article was financially supported by Shiraz University of Medical Sciences (grant No. 16690). The authors would like to thank Shiraz University of Medical Sciences, Shiraz, Iran, and also Ms. Sheryl Thomas-Nikpoor, English language editor, for her invaluable comments in editing this manuscript.

Funding

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and materials

All data is presented within the manuscript file.

Authors' contributions

A. Doostkam, H. Mirkhani, and K. Iravani conceived and designed research. A. Doostkam, K. Iravani, S. Karbalay-Doust, A. Doosti, and E. Nadimi conducted experiments. A. Doostkam, S. Karbalay-Doust, A. Doosti and E. Nadimi contributed new reagents or analytical tools. A. Doostkam, H. Mirkhani and E. Nadimi analyzed data. A. Doostkam and K. Iravani wrote the manuscript. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

Figure 1

Stereological method estimation of the stria vascularis, spiral ligament and spiral ganglion volumes. The location of stria vascularis, spiral ligament and spiral ganglion of cochlea displayed on the histological section (a). The volume of the stria vascularis, spiral ligament and spiral ganglion was evaluated by Cavalieri's principle and point-counting technique (b).

Figure 2

Stereological technique estimation of the numbers of spiral ganglion cells. Estimated number of ganglion cells assessed using optical dissector method. The cells nuclei that appeared in the high focus during scanning of the height of the disector were counted (the arrow) (a&b).

Figure 3

Latency of wave II (a), and Hearing threshold (b) in the normal, DM, DM+FA40, and DM+FA80 groups. *** indicates $P < 0.001$ vs. diabetic group.

Figure 4

The aligned dot plot of the volumes of the spiral ganglion(a), spiral ligament(c), striavascularis(d), and number of spiral ganglion neurons(b)in the normal, DM, DM+FA40, and DM+FA80 groups. Each dot represented an animal and the horizontal bar is the mean of the listed parameters. *indicates $P < 0.05$ vs. diabetic group.

Figure 5

Serum level of SOD(a), MDA(b), and HCY (c) in the normal, DM, DM+FA40, and DM+FA80 groups.

*indicates $P < 0.05$ vs. diabetic group. **indicates $P < 0.01$ vs. diabetic group. ***indicates $P < 0.001$ vs. diabetic group. ****indicates $P < 0.0001$ vs. diabetic group.

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