MODULATION OF MELATONIN SECRETION WITH TRANSCUTANEOUS AURICULAR NERVE STIMULATION: A CASE STUDY

MODULACIJA IZLOČANJA MELATONINA S TRANSKUTANO STIMULACIJO AVRIKULARNEGA ŽIVCA: ŠTUDIJA PRIMERA

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The main study objective was to test the hypothesis that selective electrical transcutaneous auricular nerve stimulation (tANS) under forenoon daylight conditions induces melatonin secretion in a 64-year-old male patient with angina pectoris, hypercholesterolomy and coronary artery disease, assuming that it has beneficial effects on accompanied insomnia (Regensburg Insomnia Scale (RIS) = 22 points, the total score ranges from 0 to 40). Silicone stimulation plugs, with two platinum stimulating cathodes each, were inserted into the left and right external ears. Afterwards, one-second-long pulse trains of cathodic, biphasic and current regulated stimulating pulses at stimulating charge density C_d of 50.88 μC/cm² and frequency of 25 Hz, were delivered for 30 min to selected sites at the upper and lower part of the left and right Cymba Conchae (CC), respectively. The common anode was attached to the neck. The time gap between the pulse trains was measured by the patient using a tactile sensor and was about 250 ms. The results showed that selective tANS under forenoon daylight conditions increased melatonin saliva levels in all the trials accomplished in a patient. Precisely, the lowest increase was obtained in trials with lower right (LR) CC, while the highest increase was obtained in upper-right (UR) CC trials.

Keywords: auricular nerve, stimulation, Cymba Conchae, electrodes, melatonin, insomnia, circadian rhythm

Cilj študije je bil preizkusiti hipotezo, da selektivna transkutana električna stimulacija avrikularne veje živca vagusa (tANS) pri dopoldanski dnevni svetlobi izzove izločanje melatonina pri 64-letnem moškem pacientu z angino pektoris, zvišano ravnijo holesterola in koronarno arterijsko boleznijo ter da ima to pozitivni učinek na nespečnost (Regensburg Insomnia Scale (RIS) = 22 točk, celotno območje lestvice je v mejah od nič do 40). Silikonska stimulacijska čepka, ki sta imela vsak po dve platinasti katodi, sta bila vstavljena izmenoma v levo in desno zunanje uho. Nato so bili, na prednastavljena mesta na zgornjem in spodnjem delu zunanjega ušesa (CC) v času stimulacije dolgem 30 minut, dovedeni eno sekundo dolgi vlaki katodnih, izmeničnih in tokovnih stimulacijskih impulzov z gostoto naboja C_d = 50.88 (μC)/cm² in frekvenco 25 Hz. Skupna anoda je bila nameščena na vrat. Časovni zamik med vlaki impulzov je določal pacient sam z uporabo senzorja na dotik in je znašal približno 250 ms. Pridobljeni rezultati pri vseh opravljenih poskusih so potrdili, da selektivna tANS pri dopoldanski dnevni svetlobi izzove povečano vsebnost melatonina v slini. Bolj natančno, najmanjše povečanje je bilo opaziti pri stimulaciji spodnje desne (LR) CC medtem, ko je bilo največje povečanje opaziti pri stimulaciji zgornje desne (UR) CC.

Ključne besede: avrikularni živec, stimulacija, zunanje uho, elektrode, melatonin, nespečnost, cirkadialni ritem.

1 INTRODUCTION

To treat a number of nervous system disorders, invasive vagus nerve stimulation (VNS)[1–3] and non-invasive transcutaneous vagus nerve stimulation[4,5] used for the diagnosis and treatment of the body's dysfunction through stimulation of specific sites on the ear[6–10] have been proposed.

Compared to other regions, an external ear is the only place on the surface of the human body where there is a large distribution of afferent vagus nerve fibres and a variety receptors such as nociceptors, Golgi-tendon receptors, Meissner corpuscles, Krause's end-bulbs and glomus-bodies that respond to different stimuli by sending signals to the spinal cord and the brain.[11,12]

A recent study showed that tANS of the CC, induces tidal melatonin secretion and has an antidiabetic effect in Zucker Fatty Rats.[13–15]

Melatonin is a hormone secreted mainly by the pineal gland and to a lesser degree by some other tissues and cells.[16,17] Melatonin forms a part of the system that regulates the sleep-wake cycle and controls numerous physiologic processes.[18,19] Normal melatonin patterns vary considerably between individuals during aging and due to the presence of chronic diseases such as coronary artery disease.[20–22] To determine melatonin levels, different methodologies such as a blood test, urine test or saliva test are used.[17,23] The most common methods for the determination of melatonin in blood or saliva are RIAs and ELISAs. Peak secretion of melatonin and its level in the blood, occur in the middle of the night and gradually falls during the second half of the night.[24–29]

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Chronic insomnia clinically presents a patientive perception of dissatisfaction with the amount and/or quality of the sleep.[30–33] It is a frequent disorder that afflicts about 35 % of all adults during the course of a year. About half of these persons, mainly women and older people, experience the problem as serious. In general, prolonged sleep latency and time awake during the night increased with age. Unfortunately, the majority of serious insomniacs (85 %) were untreated by either prescribed or over-the-counter medications.[30,34–36]

It may be proposed that in addition to its natural production by the pineal gland, melatonin production can also be elicited by tANS to restore normal melatonin blood concentration as shown in the animal model.[14] It was suggested that afferent projections from the auricular branch of the VN to the NTS form the anatomical basis for the vagal regulation of auricular acupuncture.[37]

The present study was aimed at investigating the capability of selective tANS of predefined sites at the CC as a potential method to induce melatonin secretion in a 62-year-old patient with stable angina pectoris, hypercholesterolomy, coronary artery disease, subtotal occlusion of the LAD treated by means of a stent, hypothyroidism and mild insomnia, to treat mild insomnia 6 years after an acute myocardial infarction.

2 EXPERIMENTAL PART

The experimental protocol complied with the Helsinki Declaration: recommendations guiding physicians in biomedical research involving human patients. In addition, the protocols of the measurements were approved by the National Medical Ethics Committee, Ministry of Health, Republic of Slovenia (Tel: +386 01 478 69 13, http://www.kme-nmec.si/kontakt/, Unique Identifier No. 0120-297/2018/6).

A written informed consent was obtained from the 64-year-old male patient. The patient was informed about the purpose and the procedures of the research. The patient was diagnosed in January 2014 as having angina pectoris, hypercholesterolomy, coronary artery disease and subtotal occlusion of the LAD. As an immediate intervention, coronar angiography, pecutaneous implantation of the stent and catheterization/canulation of a second vein, were performed.

The patient´s current therapy: Atoris 10 mg in the evening, Prenessa 4 mg in the morning, Concor 2.5 mg in the evening, Aspirin P 100 life time and Eutirox 100 µm (generic name: Levothyroxine) in the morning. The patient has chronic bradycardia with HR significantly below 60, coronary artery disease and insomnia. According to The International Classification of Sleep Disorders, insomnia was classified as mild insomnia that was characterized as waking up during the night, having trouble going back to sleep, waking up early in the morning and having an unrefreshing sleep accompanied by little or no evidence of impairment of social or occupa-

tional functioning. The likely most important causes responsible for mild chronic insomnia are the prescribed beta-blockers, medical disorders and conditioned insomnia.[34,35] To assess insomnia and to evaluate the tANS as therapeutic intervention, the RIS was used.[36]

The stimulating protocol of two-channel selective tANS of the CC belonging to the auricular branch of the VN was developed on the basis of our own experience and published results.[6] In this regard, an upper and lower area of the CC, where supposedly 100 % of innervation belongs to the auricular branch of the VN, were selected for the tANS.[8]

The skin at the CC has four layers with different thickness and conductivity.[38,39] When the voltage drop across the less-conductive layer Stratum Corneum during tANS exceeds approximately 30 V, the conductance across the layers rises significantly.[40–42]

To ensure adequate conditions for selective tANS, the stimuli were a cathodic first, current regulated, biphasic pair with a rectangular cathodic component (i_c) and a rectangular anodic component (i_a).[43] This i_c generates an electric field gradient (driving function) in aforementioned layers under the cathode where a certain population of receptors and nerve endings is located so above a certain threshold, most of them are activated.[44] They send signals via slowly conducting afferent Aδ and C fibres to the spinal cord and the brain. It is proposed that these signals modulate the autonomic and CNS activity.[37] As a result, measurable differences of melatonin secretion can be elicited.

For the tANS, several pieces of equipment, were developed. The most important part was a silicone plug inserted into the external ear with two stimulating cathodes (cathode). For the cathodes, 0.15-mm-thick platinum (99.99 % purity) plates in a shape of partial annulus, having average geometrical surface of approximately
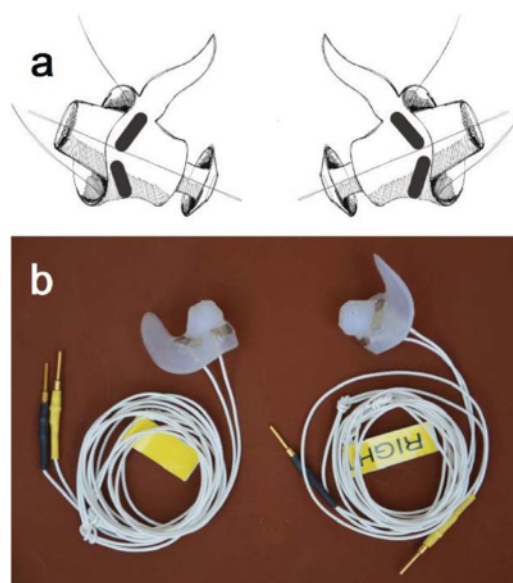

Figure 1: Plugs: a) schematic 3D view of the plugs, b) crafted plugs

0.25 cm^2, made of annealed ribbon, were used. They were spot welded to the insulated lead wires and attached onto the pre-defined sites at the silicone plug. **Figure 1** shows the plugs with upper and lower cathodes. Precisely, **Figure 1a** detail shows the schematic 3D view of the plugs, while **Figure 1b** detail shows the crafted plugs.

A common electrode (anode) with a geometric surface of about 7.5 cm^2 was crafted using a ribbon made of highly water absorptive sponge that was stitched below the stainless-steel mesh and Velcro tape.

For the tANS, two out of four-channel, custom-made, microprocessor-controlled electrical stimulator, were used. The stimulator was triggered by the patient touching a custom-designed tactile sensor based on a piezo sensing element. This sensor was designed to be used also as a respiration sensor so pulse trains could be synchronized with the rhythm of respiration.

Figure 2 shows a schematic diagram and particular pieces of equipment that comprised the setup for the dual-channel tANS.

Before the developed dual-channel set-up was used for the tANS, its performance was assessed by construction of an equivalent circuit model (ECM) of the interface at both the particular cathode and that at the anode. To assess the ensemble ECM elements of an actual load applied to the stimulator output under stimulating conditions, however, the following ensemble ECM elements were measured at two frequencies (1 kHz and at 2.5 kHz): capacitance C_{ep}, resistance R_{ep}, R_{es} and impedance $|Z|_e$.

These two frequencies were predefined based on the power spectral density of the pre-set stimulation pulse obtained using the electrochemical impedance spectroscopy (EIS) technique (not shown in this paper), that exhibits two main peaks, one at about 1 kHz and the other at about 2.5 kHz.

To ensure a high electric conductivity, to enable more uniformly dispersed current paths between the cathode and a particular site on the CC skin, a conductive hypoallergic water-soluble cream (Ca-mi-na, Egna, Italy) was applied to the CC skin.

Dummy headphones shown in **Figure 3** were developed to provide an appropriate pressure onto the plugs and thus to ensure low impedance $|Z|$ of the interface between cathodes and stimulating sites at the CC. The force applicator shown in **Figure 3** (position A), is a latex sponge pad mounted on the vice containing soft spring that is mounted into the dummy headphones (position B). Precisely, during the tANS, each of the plugs is pushed into an external ear at a force of approximately 2.5 N.

tANS trials were performed in a sitting position,[45] the patient being exposed to a daylight illumination measured with an automatic range digital high precision photometer metering instrument (FY1010B, Fook Miriam).

tANS was applied by a 1-s-long pulse trains composed of current regulated biphasic pulses with a rectangular cathodic and anodic phase. Parameters of cathodic phase were: frequency $f = 25$ Hz and width $t_c = 200$ μs. Stimulating cathodic intensity i_c was set by the patient at the level just below minimum discomfort at the particular deployed cathode. The time gap between successive pulse trains was also determined by the patient by touching the tactile sensor. The most comfortable tANS experience was obtained with a time gap of 250 ms. During a 30-min trial, tANS was applied via one of the two cathodes of the left or right ear plug at a time. By doing so, tANS was under the patient´s absolute control.

An example of the waveform of i_c and voltage response, measured at an upper cathode of the plug during stimulation of the right CC, is shown in **Figure 4**. Precisely, **Figure 4a** detail shows the current train waveform with wavelength λ, **Figure 4b** detail shows the voltage
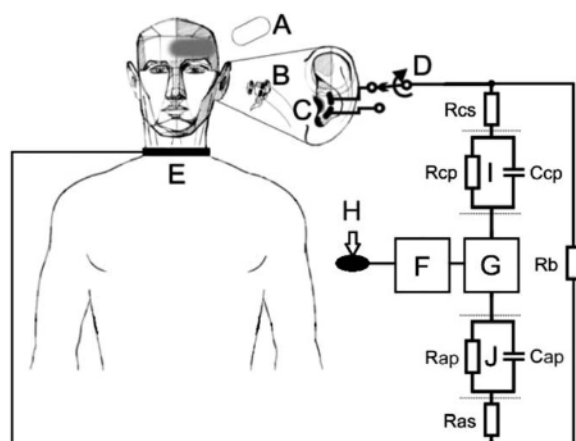

Figure 2: Schematic diagram of the dual channel tANS: A – forehead thermometer, B – ear plug, C – stimulating sites at the CC, D – switching unit, E – anode, F – tactile sensor driver, G – electric stimulator, H – tactile sensor, I – cathodic interface, J – anodic interface, R_{cs} – cathodic serial resistance, R_{cp} – cathodic parallel resistance, C_{cp} – cathodic parallel capacitance, R_{ap} – anodic parallel resistance, C_{ap} – anodic parallel capacitance, R_{as} – anodic serial resistance, R_b – body resistance

Figure 3: Dummy earphones: A – latex sponge pads mounted on the soft springs for arrest of plugs in outer ear, B – dummy headphones

response train waveform, **Figure 4c** detail shows the current biphasic pulse with cathodic phase $i_c = 66$ mA and anodic phase $i_a = 66$ mA, **Figure 4d** detail shows the voltage response with anodic phase $V_{ra} = 180$ V with width $t_a = 200$ μs, cathodic phase $V_{rc} = 180$ V with width $t_c = 200$ μs and time delay between phases $d = 200$ μs.

The stimulating charge Q_c, injected into the specific site at the CC via aforementioned current waveform, was calculated as follows (Equation 1):

$$Q_c = i_c \times t_c \times \text{number of charge squares} =$$
$$= 2 \text{ mA} \times 10 \text{ μs} \times 636 \text{ charge squares} = 12.72 \text{ μC} \quad (1)$$

where the surface under the cathodic part of the biphasic stimulus pulse pair was divided into 636 equal charge squares.

The surface area considered was of $A_c = 0.25$ cm^2. The stimulating charge density C_d was calculated as (Equation 2):

$$C_d = Q_c/A_c = 12.72/0.25 = 50.88 \text{ μC/cm}^2 \quad (2)$$

The anodic charge density A_d was calculated as:

$$A_d = Q_c/A_a = 12.72/75 = 0.17 \text{ μC/cm}^2 \quad (4)$$

To identify the possible influence of experimental conditions on melatonin secretion during tANS, the placebo trials were performed under the same experimental protocol as the tANS trials, except that stimulating pulses were not applied. Placebo trials on the upper and lower CC in the left and right external ear could not be performed separately, so they were performed in the left

or right ear plug at a time. Namely, when the ear plug was inserted in either external ear, it mechanically stimulated both the upper and lower CC. For this reason, effects of tANS in the upper and lower CC of the left and effects of tANS in the upper and lower CC of the right external ear were added together. Thus, the obtained sums were compared to the results of placebo trials on the upper and lower CC in left and right external ears.

To determine the acute effect of tANS on melatonin secretion, saliva samples (sample) were collected just before, and just after a 30-min trial.[46] Altogether, 84 samples were included in the statistical analysis.

Table 1 describes a timing protocol of collecting samples that were included into the statistical analysis.

Table 1: Samples included in the statistical analysis

tANS site	Samples collected during tANS trials	
	before	after
UL	7	7
LL	7	7
UR	7	7
LR	7	7
Placebo Left CC	7	
Entire Left CC	7	
Placebo Right CC	7	
Entire Right CC	7	
Σ	84	

Exclusion criteria observed during sample collection were:

- pitted fruit, bananas and chocolate were avoided 24 h before,
- foods, drinks, chewing, teeth brushing and threading, intensive physical load were avoided 3 h before,
- consumption of prescribed medications within the prior 5 h and 12 h, respectively,
- breakfast taken at least 3 h before,
- foods with sugar, acidity and caffeine taken 3 h before,
- no consumption of biotin-containing multivitamins or supplements within the last 48 h,
- no consumption of alcohol and nicotine within the prior 12 h,
- no presence of oral disease, injury and inflammation during trials,
- mouth rinsed with water 3 h before,
- samples refrigerated for maximum 6 h after,
- samples frozen at –19 °C in household freezer within maximum 6 h after.

Melatonin was determined in 100-μL of saliva using a competitive ELISA (Enzyme-linked immunosorbent assay; IVD, Melatonin direct ELISA, IBL, Germany). Washing was done using automated strip washer (Wellwash Versa Microplate Strip Washer, Thermo Fisher Scientific OyMicroplate Instrumentation Ratastie 2, P.O. Box 100FI-01621 Vantaa Finland, www.thermo.com) while the quantification of the optical
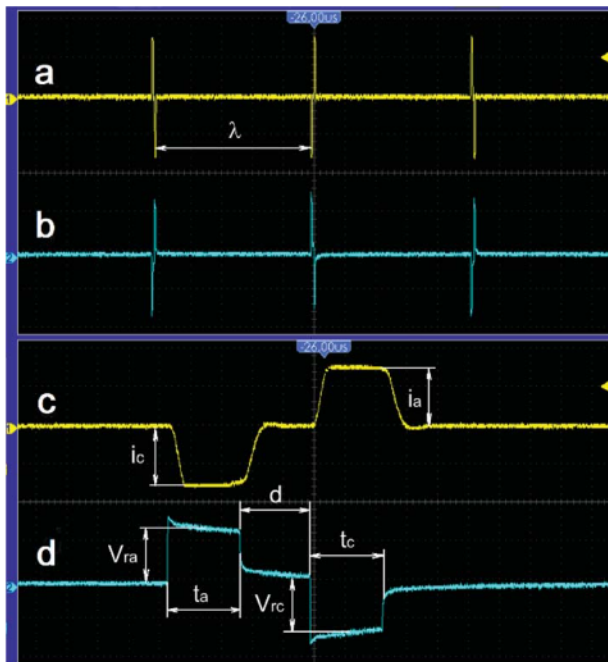

Figure 4: Waveforms of stimulation quantities under stimulating conditions of the upper right CC: a – current train waveform with wavelength λ, b – voltage response train waveform, c – current biphasic pulse with cathodic phase $i_c = 66$ mA and anodic phase $i_a = 66$ mA, d – voltage response with anodic phase $V_{ra} = 180$ V with width $t_a = 200$ μs, cathodic phase $V_{rc} = 180$ V with width $t_c = 200$ μs and time delay between phases $d = 200$ μs

Table 2: ECM elements C_a, R_a and $|Z|_a$

Frequency (kHz)	tANS site	Impedance $|Z|_a$ (kΩ)		Capacitance C_a (nF)		Resistance R_a (kΩ)	
		right	left	right	left	right	left
1	upper	10.54	9.57	21.12	28.00	7.26	7.58
	lower	7.86	6.47	31.20	47.40	5.31	5.51
2.5	upper	6.58	6.17	12.50	15.30	3.94	4.44
	lower	5.32	4.18	18.53	24.70	4.01	3.22

density was performed on a microplate reader BioTek (BioTek instruments, Elx808TM) set at 450 nm and corrected at 630 nm. All samples were assayed in duplicate. All techniques were performed following instructions provided by aforementioned manufacturers.

The patient's core body temperatures were measured by a peripheral non-invasive forehead method using a highly accurate Smart Temporal Thermometer (Model: withings, Withings France SA, Issy-les-Moulineaux, France).[47] The thermometer's specifications: clinical accuracy ± 0.2 °C; display of rectal equivalent temperature; temperature range 35–43.2 °C and resolution 0.1 °C. This thermometer measures the skin temperature above the temporal artery, an ideal place to detect temperature changes, as the blood comes from the core of the body.[47] A temperature measurement is performed by making a fast, effortless gesture, while an automatic sync with the dedicated mobile application allows tracking of the temperature readings on a smartphone.

The trials were performed under the same conditions between 10:15 and 11:15 AM when the body temperature tends to gradually increase from the lowest level in the early morning. The environmental temperature of the patient's room was measured before and during each measurement and remained between 27 °C and 27.2 °C.

Data were statistically analysed using the Matlab R2016a application and presented in graphs.

General design of the study comprised the following steps:
- Development of hypothesis,
- Formulation of protocols,
- Application for Protocol Approval,
- Development of stimulating setup,
- Development of measuring setup,
- Selection of a subject with specific health status,
- Assurance of experimental conditions,
- Performance of tANS trials and collection of saliva samples,
- Analysis of saliva samples,
- Statistical analysis and
- Presentation of results and conclusions.

3 RESULTS

Table 2 represents ECM elements C_a, R_a and $|Z|_a$ of an actual load to the output stage of the stimulator from the interface between a particular upper and lower cath-

ode and the anode when located at the left and right CC at 1 kHz and 2.5 kHz, respectively.

Figure 5 shows the melatonin level within the samples collected before and after the selective tANS of the left and right CC. In **Figure 5**, the axis x represents collected samples, the axis y represents the melatonin levels and zero line represents the pre-stimulation level. Precisely, **Figure 5a** shows differences of melatonin levels within seven samples collected before and seven samples collected after the tANS of an upper left (UL) CC. **Figure 5b** shows differences of melatonin levels within seven samples collected before and seven samples collected after the tANS of a LL CC. **Figure 5c** shows the differences of melatonin levels within seven samples collected before and seven samples collected after the tANS of an UR CC. Finally, **Figure 5d** shows differences of melatonin levels within seven samples collected before and seven samples collected after the tANS of an LR CC. For each difference of the two collected samples (one before and one after the tANS), the corresponding mean ± standard error is presented. The differences are statistically significant at the level of $P = 2.819 \cdot e^{-4}$.

Figure 6, however, compares the melatonin level in samples collected before and after tANS of an entire left and right CC with the melatonin level in samples collected in placebo trials. In **Figure 6**, axis x represents the
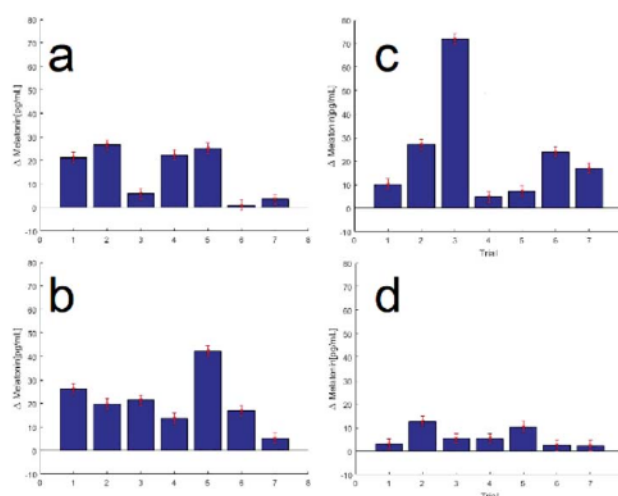

Figure 5: Difference of melatonin level in samples collected before and after selective tANS of the left and right CC and corresponding mean ± standard error (statistical significance $P = 2.819 \cdot e^{-4}$): a) tANS of an UL CC, b) tANS of the LL CC, c) tANS of an UR CC; d) tANS of the LR CC
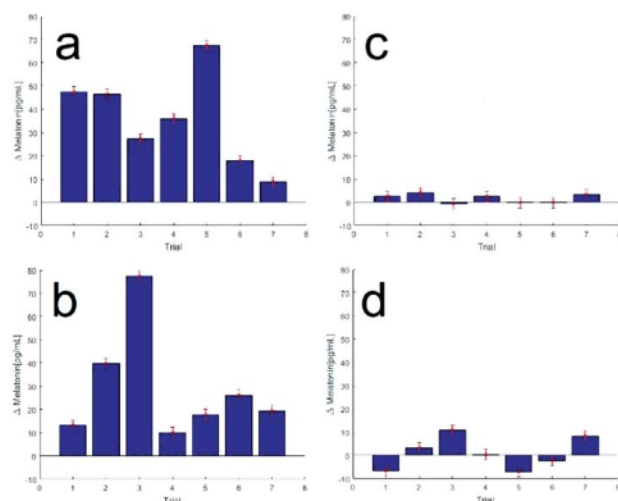
Figure 6: Difference of melatonin level in samples before and after tANS of an entire left and right CC: a) tANS of the left CC, b) tANS of the right CC. Difference of melatonin level in placebo samples of the left and right CC: c) left CC, d) right CC

collected samples, axis y represents the melatonin levels and the zero line represents the pre-stimulation level. Precisely, **Figure 6a** shows the difference of melatonin level within seven samples collected before and seven samples collected after the tANS of an entire left CC, **Figure 6b** shows difference of melatonin level within seven samples collected before and seven samples collected after tANS of an entire right CC, **Figure 6c** shows difference of melatonin level within seven samples collected before and seven samples collected after the placebo trials in an entire left CC and **Figure 6d** shows difference of melatonin level within seven samples collected before and seven samples collected after the placebo in an entire right CC. For each difference of the two collected samples (one before and one after the tANS), corresponding mean ± standard error is presented. The differences are statistically significant at the level of $P = 2.819 \cdot e^{-4}$.

Finally, to confirm the hypothesis that daylight-selective tANS induces the melatonin secretion in a patient with angina pectoris and coronary artery disease and that it had beneficial effects on the accompanied mild insomnia, **Table 3** showing an average value of variables in forenoon daylight tANS and placebo, was constructed.

In the **Table 3**, the melatonin level at each stimulating site is presented as an average of seven melatonin levels before the tANS, seven melatonin levels after the tANS and as corresponding differences.

In **Table 3,** the differences in melatonin level are statistically significant at the level of $P = 2.819 \cdot e^{-4}$.

4 DISCUSSION

In **Table 2**, all the ECM elements have larger values when measured at 1 kHz than when measured at 2.5 kHz.[15] It was assumed that two main peaks within the power spectral density of the pre-set stimulation pulse did not slide much up and down from 1 kHz and 2.5 kHz when slightly above or slightly below 25 Hz tANS was pre-set. More descriptively, tANS with frequencies slightly above or slightly below 25 Hz used in the study, did not affect the interface at the particular cathode and thus, the effect of the tANS efficiency could be neglected.

Selective tANS assumes that the outer ear is the only place on the surface of the human body where afferent vagus nerve distribution can be stimulated so similar effect as classic in VNS may be induced.[6,48] Therefore we assumed that i_c spreading perpendicularly from the cathode may activate a certain population of nerve endings within the particular volume of the CC below the cathode independent of the location of the anode. Electrically, the charge transferred by the cathode is the same as the charge transferred by the anode, while the charge densities differ by orders of magnitude. The geometrical surface of the cathode is approximately 25 mm^2 and is about 300 times smaller than that of the anode, i.e., about 7.5 cm^2. Therefore, the cathodic charge density was sufficiently high to stimulate subcutaneous neural structures at the CC, while anodic charge density applied in all trials was too low to cause any stimulating effect in the muscles of the neck.

Results in **Figure 5** show that daylight-selective tANS induces melatonin secretion in all the trials accomplished in a patient with angina pectoris and coronary artery disease. However, it could be seen in **Figure 5** that significant differences and fluctuations in melatonin levels occurred in all the trials presented in **Figure 5**. This

Table 3: Average value of variables in forenoon daylight tANS and placebo

tANS TRIAL	i_c (mA)	Exposure (lux)	Body temperature (°C)			Ris score			Melatonin level (pg/mL)		
			before	after	Δ	before	after	Δ	before	after	Δ
UL	66	33.85	36.34	36.53	0.19	22.00	18.00	−4.00	19.83	34.93	15.10
LL	66	37.85	36.44	36.91	0.47	18.00	15.00	−3.00	17.58	38.37	20.79
UR	66	35.57	36.53	36.84	0.31	15.00	18.00	3.00	19.97	43.18	23.21
LR	66	32.57	36.78	37.04	0.26	18.00	18.00	0.00	19.17	25.11	5.94
Placebo Left CC	NA	21.28	36.85	37.00	0.15	19.00	19.00	0.00	8.37	9.97	1.60
Placebo Right CC	NA	28.28	36.65	36.95	0.30	22.00	22.00	0.00	15.90	16.95	1.05
Standard Error	± 1mA	± 3 lux	± 0.2 °C	± 0.2 °C	± 0.2 °C	± 0.5	± 0.5	± 0.5	$P = 2.819 \cdot e^{-4}$	$P = 2.819 \cdot e^{-4}$	$P = 2.819 \cdot e^{-4}$

could be explained by the fact that some of the suggested precautions in sample collection could not be strictly followed by the patient who intentionally tried to keep his lifestyle unchanged as much as possible. Melatonin levels and the influence of light can vary considerably also in terms of personal characteristics, a consequence of aging, chronic disease, inflammatory processes and even genetic predispositions.[22,45] Finally, the fluctuations and variations of melatonin level could reflect a mixture of hormonal, immunomodulatory, neuromodulatory, and various types of antioxidant actions.[19]

The results in **Figure 6a** and **6b** show that daylight-selective tANS induces significant melatonin secretion also in all the trials accomplished in an entire left and right CC of a patient. However, it could be seen in **Figure 6a** and **6a** that significant differences and fluctuations in melatonin levels occurred in all the trials.

It could be seen in **Figure 6** that the difference and fluctuation in melatonin level occurred also in the placebo trials.

Results in **Figure 6c** and **6d** show that placebo reduced melatonin secretion in three out of seven trials in the left CC and also in three out of seven trials in the right CC. In the remaining four trials in the left and four trials in the right CC, however, the placebo induced a low level of melatonin secretion. In this regard, the placebo could not be recognized as a promotor of melatonin secretion.

Melatonin levels in saliva are highly dependent on a diurnal pattern of melatonin production. Levels typically rise with dim-light onset in the evening and reach the highest levels in plasma during night-time. Levels are low during the daylight hours but still remain detectable.[27] One important aspect of fluctuation in melatonin secretion could concern also variable light exposure intensities. However, in spite of significant differences in light exposure intensities shown in **Table 3**, this relation could not be discovered in the study. Accordingly, this fluctuation in melatonin secretion was considered as irrelevant and statistically unsignificant.

Table 3 shows that an average body temperature before tANS of the upper as well as lower CC, was slightly lower in all the tANS trials in both external ears than average body temperature after tANS. Since a similar elevation was observed also in the placebo trials, this elevation of body temperature could not be attributed to tANS but to the fact that dummy headphones covered a significant part of the skin, thus preventing dissipation of thermal energy through the skin.

It is shown in **Table 3** that the highest average RIS score (22) was assigned before any tANS trial was accomplished. After the last out of seven trials, with tANS of the the UL CC, a slightly lower average RIS score (18) was assigned. The same average RIS score was assigned also before any tANS trial of the LL CC was accomplished. After the last out of seven trials with tANS of the LL CC however, the lowest average RIS score

(15), was assigned. The same average RIS score was also assigned before any tANS trial of the UR CC was accomplished. After the last out of seven trials with tANS of the UR CC, slightly higher average RIS score (18) was assigned. The same average RIS score was assigned also before any tANS trial of the LR CC was accomplished. Finally, after the last out of seven trials with tANS of the the LR CC, the same average RIS score (18) was assigned again.

It is shown in **Table 3** that the high average RIS score (19) was obtained before any placebo trial was accomplished, remained the same after seven placebo left CC trials and returned back to the highest average RIS score (22) as obtained before any tANS trial was accomplished. More descriptively, before the tANS trials, insomnia was classified as mild insomnia that was characterized as waking up during the night, having trouble going back to sleep, waking up early in the morning and having an unrefreshing sleep accompanied by little or no evidence of impairment of social or occupational functioning. After the completed trials with tANS of the left CC, and particularly LL CC, all items of RIS except "sleep duration", were significantly improved. Significantly less improvement, however, was observed after the completed trials with tANS of both UR and LR CC, respectively. Finally, placebo trials did not elicit any positive effect in the treatment of mild insomnia.

To clearly evaluate the efficiency of tANS to induce melatonin secretion, a difference Δ between average value of melatonin level after and before each of the tANS trial, was calculated. It is shown in the **Table 3** that the lowest efficiency (5.94 pg/mL) was obtained in the LR CC trials, significantly higher efficiency was obtained in UL CC trials (15.1 pg/mL), even higher efficiency was obtained in LL CC trials while the highest efficiency was obtained UR CC trials.

In placebo trials, however, a difference Δ between average value of melatonin level after and before both, placebo left and placebo right trial was small so no effect on melatonin secretion could be identified.

The main difference between our study and the studies of others is the deployment of the two-electrode silicone plug that enables both selective tANS of the CC and repositioning of the stimulated sites without the need to change the physical electrode location.

Limitations of the study were related to the temporal conditions, stimulating conditions via impedance of stimulating electrodes, environmental conditions via ambient lighting and temperature and experimental conditions via collecting and handling saliva samples. The greatest weakness of the system is that a stimulating efficiency is significantly dependent on the pressure applied to the plug and thus on the performance of the platinum cathodes being in close contact with sites of the CC. It was observed that the efficiency increases with increasing pressure. Special attention during the trials was therefore focused on the requirement that good cath-

ode-skin contact is provided, such that no high ic density peaks can occur because of a small contact area. All the aforementioned conditions were kept as steady as possible in all the trials. All the trials were carried out by one researcher, which might have led to some biases.

Since the intention of the research was to develop the system and protocol for tANS not to interfere the quality of life of the patients, the precautions taken in saliva sample collection were not so tight as taken with the pharmacologic management strategies. It should be noted that the results themselves were not the only reason for any therapeutic consequences.

Beside that, melatonin is likely effective for treating circadian rhythm sleep disorders and insomnia associated with various sleep-wake cycle disturbances, melatonin is an effective antioxidant.[29,49] In this relation, Jou et al.[28] demonstrated in human model that melatonin may serve as a therapeutic drug to benefit chronic progressive external ophthalmoplegia (CPEO) that is a disorder characterized by slowly progressive paralysis of the extraocular muscles.

Melatonin is also implicated in the regulation of mood, dreaming, learning and memory, autism spectrum disorders, cognitive impairment,[50] cluster headache and depression,[51] neuro-muscular diseases and neurodegenerative disorders,[52] immune activity, fertility and reproduction.[53,54]

The directions that our further work could take, would be mainly to improve the electrode-skin contact of stimulating electrodes, to modulate the secretion of other hormones and to modulate heart and respiratory function. Other possible uses of tANS potentially include benzodiazepine withdrawal, delayed sleep phase syndrome, jet lag, nicotine withdrawal, preoperative anxiety and sedation, cancer, chemotherapy and other disorders.

5 CONCLUSIONS

The clinical significance of the most important results is that in tANS of the left CC, and particularly the lower left (LL) CC, all items of RIS except "sleep duration" (not presented in this paper), were significantly improved. Significantly less improvement, however, was observed after tANS of both UR and LR CC, respectively. Results of placebo trials showed no effect on the melatonin secretion and thus no positive effect on the status of mild insomnia could be measured.

It could be concluded that that non-invasive selective tANS is recognized as an effective method for the induction of melatonin secretion and was thus appropriate for treatment of mild insomnia in a patient with angina pectoris, coronary artery disease and mild insomnia.

In the case of using more selective stimulation with increased number of channels, this study has the potential to extend the application of tANS for other disorders such as epilepsy, bipolar disorder, morbid, jet lag, insomnia, shift-work disorder, circadian rhythm disorders, and benzodiazepine and nicotine withdrawal.

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