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PRELIMINARY INVESTIGATION INTO COMPARATIVE TRANSCRANIAL BRAIN TARGETED AND TRANSDERMAL SYSTEMIC EFFECTS OF DIAZEPAM ON SLEEP LATENCY

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ABSTRACT

Emissary veins that drain blood from the scalp into sinuses of the brain are a potential route for targeted central nervous system drug delivery. Structure of the scalp, cranial bones and cerebrospinal fluid flow indicate that they can facilitate transcranial drug delivery. A cascade for the transcranial drug diffusion is proposed. Diazepam 2 mg/3 ml of sesame oil was administered transcranially and transdermally in order to investigate brain targeting in human volunteers. Sleep latencies were monitored with Multiple Sleep Latency Tests involving 5 Naps employing standard electroencephalography, electrooccullography and electromyography electrodes. The six volunteers were subjected to four days of electroencephalography screenings for the base line, placebo oil, diazepam on scalp and diazepam on forearms. The mean sleep latencies for the Nap 3 with peak responses were 13.8, 8.7, 5.9 and 7.7 minutes respectively indicating that the transcranial brain targeting of diazepam is possible.

Key Words: Mean Sleep Latency Test, transcranial, skin appendages, emissary veins, transmeningeal drug diffusion, diazepam.

INTRODUCTION

The study aims at investigating if the ability of diazepam in shortening the sleep latency is due to a general non targeted transdermal systemic absorption from the skin of the scalp or due to a specific more pronounced transcranial brain targeted effect mediated through emissary veins that drain venous blood from the scalp into the brain sinuses.

***Background*:** Transcranial brain targeted studies on rat models with diazepam and methadone base in sesame oil yielded positive results. Centrally mediated muscle relaxant effect of diazepam[1] and antinociceptive effect of methadone[2] were tested. Two more studies involving human volunteers also yielded encouraging results with shortening of sleep latencies compared to the controls. Application of

transcranial diazepam for three days at 24 hour intervals yielded drastically reduced sleep latencies [3, 4]. The basis for selecting electroencephalography (EEG) studies is the ability of diazepam to bring about diffuse β activity and to reduce the sleep latency [5, 6].

The investigations were based on the *Ayurvedic* system of medicine where transcranial administration of drugs have been performed for centuries in the form of *Shirodara, Shiropichu, Shiroabyanga, Shirovasthi, and Shiropralepa* [7].

***Transcranial drug delivery prospects*:** Transcranial drug delivery has eluded pharmaceutical researches in spite of the fact that brain is the only vital organ located nearest to the external surface. Scientific information is not widely available on drug absorption by the transcranial route. The absorption process possibly involves the functions of the scalp skin appendages, the emissary veins, Haversian canal ridden cranial bones, the meninges with their spaces filled with cerebro- spinal fluid (CSF) and the microcirculation of these structures.

Following can be cited in support of the transcranial brain targeting. Miniscule doses of eye drops lead to disproportionately intolerable systemic side effects while a similar dose given orally is unlikely to have any effect at all. A single drop of timolol eye drops 0.5% contains 0.25 mg of timolol (counting 20 drops per ml) whereas oral tablet strengths are in the range 5-20 mg. A similar argument is presented in an article on central nervous system (CNS) toxicity following timolol eye drops 0.5% in an infant after inadvertent administration through nose. Plasma concentration of timolol following 5 mg oral dose was 10-20 ng/ml as against one drop on each eye of a 0.5% solution was found to be 0.3-0.5 ng/ml, a difference of 30-40 times[8].

The skull bones are thin and extremely porous. In addition to lacunae of the diploe, a large number of blood vessels crisscross these bones through Haversian canals [9]. A simple demonstration is to see gentian violet in ethanol migrating from one side of a skull bone into the opposite side.

***Convection and diffusion transcranial absorption pathways*:** In the transcranial drug delivery cascade first pathway is the faster 'vascular convection pathway' via emissary veins which take the drug molecules right down to the sinuses of the brain. The second is the slower 'solid tissue diffusion pathway' in which the drug molecules diffuse towards the brain through the solid cellular tissues of the scalp skin, the Haversian canals of the cranial bones and finally across the meninges (Figure 1). The transcranial drug diffusion is aided by the presence of skin appendages of which the sebaceous glands alone in the scalp is 400-900/cm^2 compared to rest of the skin averaging 100/cm^2 [10]. On reaching the cerebrospinal fluid in the subarachnoid space, the drug molecules will diffuse around the periphery of the brain lined with the pia mater that dips in to gaps of numerous sulci, fissures and also into Virchow – Robin Spaces (Figures 1, 2).

There are several foramina in the skull bones such as parietal, mastoid and the supraorbital, through which thirteen pairs of emissary veins from outside the skull gain access into the sinuses of the brain [11]. Part of the nasal and ocular drainage also connects the sinuses.There are seven interconnecting sinuses in all. The arteries supplying the scalp send numerous small twigs in to the under lying bones of the skull [12]. Therefore in all probability both the veins and the arteries that carry blood from the scalp across the cranial bones are responsible for transcranial brain targeting of drugs.

***Anatomical features favoring transcranial drug diffusion*:** Transcranial blood flow across the bones of the scalp had been demonstrated on two fresh cadavers. Massaging of the scalp produced drops of blood on the inner surface of the bone demonstrating that cutaneous blood can flow inward through the bone [13]. Ability of drugs to diffuse through the bone has been supported in a study in which diazepam has been detected in femoral bone tissue following the administration of the drug in rats by intraperitonial route [14]. It has also been reported that there is a decrease in the resistivity to electrical conductance at the sutures of the skull indicating movement of ions through the sutures [15].

In another *in vitro* study, drug diffusion in the direction of dura mater to pia mater on monkey spinal meninges had been established. This study which included common drugs such as morphine, fentanyl, tetracycline and haloperidol, their membrane crossings have been confirmed [16]. In an *in vivo* rat study that is relevant to transmeningeal drug diffusion, it has been shown that intracranial CSF uptake of insulin following ocular administration was probably through the meninges that surround the optic nerve. Further the same study has shown that insulin has diffused into the brain from the CSF [17]. In another *in vivo* study diffusion of methylene blue and N-methyl-D-aspartate (NMDA) across rat cerebral meninges and CSF using implanted epidural cups on ambulatory animals have been demonstrated

[18]. Methylene blue was found diffused into the rat cortex within 15 minutes.

MATERIALS AND METHODS

***Selection of subjects*:** Healthy male and female volunteers 20 – 40 years were invited for the study. Drug dependants, smokers, alcoholics and those under any medication were not considered. Informed written consent was obtained prior to enrollment. The volunteers were screened for illnesses with emphasis on cardiovascular, respiratory, neurological, excessive daytime sleepiness and nocturnal sleep disturbances. They underwent history taking, clinical examination and evaluation with Epworth Sleepiness Score with standard questionnaire [19]. The selected volunteers were required to undergo basic blood and urine tests. Volunteers who had normal findings in the above screening process were selected for Day 1 baseline study.

***Four day test plan*:** The experiments were carried out on four separate days. The volunteers were provided with shampoo base sodium laureth sulfate samples without additives to wash the scalp prior to tests. The head must be thoroughly dry before application of the oil. Following single application of the blank oil or medicated oil as the case may be at the beginning of the day, Mean Sleep Latency Tests (MSLT) were carried out employing five Naps with an interval of two hours between the Naps.

In the case of female subjects, as a part of blinding process the sequence of application sites in Day 3 and 4 were reversed. The sleep latency values for the four days are given in table 1.

***Day 1 baseline electroencephalography (EEG) study and multiple sleep latency test (MSLT)*:**
On day 1, EEG and MSLT procedures were carried out using the standard methods that were employed in our previous study [4]. The EEG was combined with electrooccu1ography (EOG), electromyography (EMG), electrocardiogram (ECG) and synchronized video monitoring. The baseline EEG study was performed without any application to determine the acceptability of background EEG wave patterns by ensuring that the baseline EEG has no β activity and there is well defined α activity in the posterior background of the EEG at rest. MSLT was performed to establish the baseline mean sleep latency (MSL) and ensure that it is about 10 minutes or more. The subjects were to follow standard instructions for MSLT [20, 21,]. Those who had no β activity in the posterior background of the baseline EEG and acceptable MSL around 10 minutes or more in MSLT were selected for next stages of the study.

***Day 2 placebo oil study*:** The placebo stage was to study the effect of sesame oil and the rubbing action of scalp on sleep latency. MSLT was performed after blank sesame oil was applied in two locations at the same time, 3 ml on the scalp and 1.5 ml each on the two forearms since 3 ml was too much for one forearm leading to spillage of the oil.

For the rest of the study, the male subjects were treated as follows.

***Day 3 diazepam transcranial application*:** MSLT was performed to assess the effect of 2 mg diazepam in 3 ml sesame oil application on the scalp and 1.5 ml blank oil each on the two forearms as a requirement of the blinding process.

***Day 4 diazepam transdermal application*:** Day 4 study was undertaken after a wash out period of over three weeks from Day 3 since the effects of diazepam on the EEG is known to persist up to two weeks [22]. MSLT was performed to assess the transdermal effect of 2 mg diazepam in 3 ml sesame oil application. It was divided in to 1.5 ml portions and each applied on the two forearms and blank oil 3 ml applied on the scalp.

All the EEG analyses and sleep scoring were done by one investigator. The sleep scoring was carried out at the end of all three treatment days in order to avoid bias. EEG background activities were visually examined using standard methods. Sleep was scored in 30 second epochs according American Academy of Sleep Medicine Guidelines. Sleep onset was identified as the first epoch of any stage of sleep (Stage N1, N2, N3 or R). Sleep latency for each Nap was obtained. If sleep did not start, sleep latency was counted as 20 minutes. Mean Sleep Latency values of each volunteer for the 4 Days and average for a given Nap were also calculated by averaging the sleep latencies of six volunteers.

***Preparation of diazepam stock solution 2 mg/ml*:** On the day before the test, a stock solution was prepared by dissolving 50 mg of diazepam B.P. 250 mesh powder in 25 ml of sesame oil. It dissolves in half an hour when reduced to 250 mesh and dried at 45^0 for 15 minutes. Assay of diazepam in oil solution was carried out by adopting the assay methods for Diazepam Injection and Diazepam Rectal Solution B.P. 2007[4].

***Diazepam oil solution administration procedure*:** The medicated and placebo oil portions were prepared by two investigators who applied the oil so that the subject, EEG technician and the sleep scorer were blinded as to which oil was applied. The medicated oil was applied, either on the scalp for transcranial effect or on two forearms for transdermal effect while blank oil was applied on the other site. Medicated oil sample was diluted to yield 2 mg/ 3 ml dose at the time of the experiment since 1 ml is insufficient for proper spread over the scalp. When placebo was needed 3 ml or alternatively 2 X 1.5 ml of non medicated sesame oil was used for the scalp and two forearms respectively.

One investigator was attending to scalp application. The hair was parted manually within the electrode attachment area and about 2/3 of the 3 ml dose was poured on to the top of the scalp of the volunteer in sitting position. The 'rubbing in' of the oil was carried out immediately for 5 minutes with the convex side of a spoon-end of a stainless steel spatula, first within the electrode attachment points and then beyond to the periphery as the oil spread. The volunteers were rested for 10 minutes in sitting position. Then the balance 1/3 was administered under the same scheme following which the MSLT commenced. This allows a total of 30 minutes from starting the application up to the commencement of MSLT at about 08:00 h.

At the same time as the scalp application was made, a second investigator applied 1 ml of blank oil on each forearm held horizontally, streaking the palmer side, elbow to wrist. The oil was immediately 'rubbed in' for 5 minutes on the arms between elbow and wrist, while promoting the oil spread around the forearm covering the entire skin. The volunteer was rested for 10 minutes. Same scheme was followed with two balance 0.5 ml portions when it is time to commence the MSLT. Unlike the scalp there was no hair to soak the oil in the forearms resulting in a wider area of oil spread. The simultaneous treatment of the scalp and two forearms were to comply with the MSLT time schedule.

'Rubbing in' with a degree of friction is the practice in applying *Ayurvedic* head oils and was adopted for the tests. It dispels interfering air pockets and bring oil right on to the pore outlets of the skin appendages. Rubbing may help the oil to bypass the epidermis reaching the inner layers of dermis through the tubules of these appendages and improve the blood flow to the scalp (Figure 2).

***Equipment and sleep lab*:** Dimensions of the spatula for application of oil were as follows. Overall length 120 mm, long axis of elliptical spoon 25 mm, short axis 20 mm, radius of curvature long axis wise 22 mm, short axis wise 17 mm. All the EEG and MSLT procedures were performed by the same technician in the same sleep lab which was maintained at ambient temperature between 20º- 25ºC. The equipments used for EEG and MSLTs were, NEUROFAX 9200K Digital Video EEG machine (NIHON KOHDEN, Tokyo, Japan) with Ag/AgCl electrodes. Skin preparation for electrode placement was done using abrasive skin prepping gel ('NUPREP', D. D. Weaver and Co, USA) and the electrodes were applied using electrode paste ('ELIFEX' Paste for EEG, Z-401CE F510, Japan.) The electrode attachments were secured with hypoallergic surgical tape (3M PLASTER, Micropore). Diazepam B.P raw material was obtained from MSJ Industries (Ceylon) Limited, Colombo 15, Sri Lanka.

Standard emergency medicine and equipment package including flumazenil intramuscular injection was made available bedside and an ICU bed was kept in readiness for any emergency.

Ethics clearance for the study was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka (reference number EC/07/001). The project was registered with the International Clinical Trials Registry under No: SLCTR/2007/003 maintained at the Sri Lanka Medical Association, No. 6 Wijerama Mawatha, Colombo 07, Sri Lanka.

RESULTS

Out of seven volunteers, subject M2 had distinctly low MSL in baseline study and was excluded. Remaining 4 males and 2 females were recruited for the study. All of them had α activity as the posterior basic rhythm in the baseline EEG and MSL around 10 minutes or more in accordance with the selection criteria. Sleep latency values for the complete study are presented in table 1.

There is drastic reduction of MSL after transcranial diazepam treatment in the male subject M5. The same male subject M5 and female subject F1 show reduction of MSL after Day 4 transdermal diazepam application in arms. The subject M3 show reduction of sleep latency 7 minutes or less for at least 2 Naps and M4 has 3 Naps with sleep latencies less than 7 minutes after transcranial diazepam. The baseline MSLs for subjects M3, M4 and M5 are normal (> 10 min) on that day, the median sleep latency 6.5

minutes of subject M4 is less than 7 minutes. Day 3 transcranial Nap 3 values show that 5 out of 6 volunteers had significantly low sleep latency values of less than 7 minutes, the minimum being 1.5 minutes.

When the Nap wise average sleep latency values of six volunteers were plotted against five Naps for the 4 days, maximum inflection among the curves can be clearly seen for Day 3 transcranial treatment (Figure 3). In Days 2, 3 and 4, there is a reducing trend of average sleep latency from Nap 1 towards Nap 3 and return towards starting level by Nap 5. Here, the lowest mean value 5.9 minutes reached for Nap 3 on transcranial Day 3 is an unequivocally abnormal range (< 7 min). Day 4 transdermal application of diazepam leads to a lowest value of 7.7 minutes for Nap 3. However this figure is only slightly less than 8.7 minutes for the placebo application.

***EEG activities*:** No diffuse β activity was observed following diazepam in any of the recordings in any subject in the present study. This finding is similar to that of the previous study [4].

DISCUSSION

***Interpretation of the results*:** The results demonstrate the reduction of sleep latency after transcranial as well as transdermal application of diazepam. The study provides new information supporting absorption of diazepam through scalp as well as skin exerting its sedative property. According to the brain targeting hypothesis, the scalp application of diazepam should be more effective leading to a greater reduction of sleep latency than skin application elsewhere such as in the forearms. The findings support for this fact since Day 3 transcranial application led to the lowest among the average sleep latency values for the six subjects at Nap 3 (5.9 min) which is considerably less than the lowest values for Day 4 transdermal diazepam (7.7 min) and Day 2 placebo (8.7) treatments (Table 1). A similar curve patterns to that of figure 3 was observed in an earlier study for antinociceptive effect of transcranial methadone on rats [2].

Several possibilities were identified as the reasons for not showing a distinct superiority for the transcranial route against transdermal route. The most important reason being the smaller surface area of the scalp compared to that of the two forearms. The following calculation demonstrates this difference. Under the test plan the same dose 2 mg/ 3 ml were to be applied on each of the two test sites. It takes the surface areas of both the forearms to accommodate the dose as it spreads in a thin layer. The difference in surface areas of the two sites can be expressed as follows. Representing scalp application area by a flat circle with a diameter of 20 cm, the area πr^2 = 22/7 x 10 X 10 = 314.2 cm^2. In the case of two forearms, considering a circumference of 15 cm and 25 cm at the wrist and the elbow ends respectively and a distance of 25 cm between them and by applying these values to a trapezoid, the surface area of the skin of the two forearms is {(15 +25)/2} x 25} = 500 cm^2. Therefore it can be seen that area for transdermal application is 560/314.2 ~ 1.6 times greater than that of the scalp. The smaller scalp area may have hindered the expected amount of drug absorption reducing the results that may have been in favor of transcranial route.

An equally important reason is that a substantial portion of the applied dose not being available for transcranial effect as a result of soaking in to hair particularly in the females. Other possible reasons are the insufficient number of volunteers and carrying out only a single application at the beginning of the test day instead of repeat applications [4]. The single dose applied should have been at a higher strength of 3 mg/ml.

The lipophilic sebum secreted by the sebaceous glands is an attractive medium for the oil solution of a drug to form a continuous lipid medium straight into the glandular invaginations of the skin. The continuous fatty medium facilitates the migration of oil soluble compounds such as diazepam towards the interior of the glands and hair follicle epithelia for absorption. In order to retain lipophilicity, the original drug molecules as such must be employed without converting in to salt forms.

It is evident that in the Days 3 and 4, Nap 5 values have returned to near normal values and does not represent any action due to diazepam as the activity has worn out by then (Figure 3).

***Practical applications*:** Transcranial brain targeted dosage design if successfully adopted will have wide implications in the way diseases located in the CNS are treated. A large number of pharmacological groups of drugs can be potential candidates for an equally large number of diseases. These include drugs acting on CNS proper, centrally mediated autonomic nervous system drugs, anticoagulants, vasoconstrictors and dilators for cerebral vascular system, endocrine glands located in the brain, antivirals, antibiotics, antiprotozoals, drugs for the special senses as in diseases of the labyrinth and antidotes for CNS side effects of other drugs. The muscle relaxant, tranquilizer and antinociceptive

effects of diazepam and methadone by transctanial route had been demonstrated in previous studies [1, 2 and 4]. Drugs affecting the liver during long term CNS disease therapy may be inter-currently administered by the transcranial route by-passing the liver allowing the organ to rejuvenate. There is a remote possibility of new chemical entities for CNS therapy failing exclusively on liver toxicity being tried anew through the transcranial route. Transcranial delivery of anesthetics is another possibility.

In a previous study, compared to base line values of 20 minutes the dramatic shortening of sleep latencies between 2.4 - 4.4 minutes resulted following three repeat once daily applications of diazepam 2 mg/3 ml at 21: 00 h, followed by the test dose in the morning of the experiments[4]. The results of the present study indicate that one more loading dose administered about 4-6 h prior to the dose on the test day could have yielded greater reduction of sleep latencies. On the other hand the shape of the curves in figure 3 indicates that 1 mg/ ml application after Nap 3 test could possibly bring down coordinates of Naps 4 and 5. Such a scheme should be repeated for both transcranial and transdermal routes. It is advisable to avoid substantial dose increases until the action of the drug by the new route is fully understood.

***The way forward*:** Application of diazepam solution on two forearms covering an area 1.6 times larger than the scalp area need to be addressed in the future. On the two forearms 1 ml each of diazepam solution 2 mg/2 ml may be applied making absorption areas of the two different sites nearly similar to each other. However this is at a higher strength to that of transcranial dose 2 mg/3 ml. Trimming hair can promote availability of the drug for transcranial absorption.

Useful information related to transcranial drug diffusion pathways is available from a quick reference pictorial and graphic based monograph on 'Physics of cerebrospinal fluid (CSF) circulation in brain' that could be accessed typing the same on search bar. Transcranial drug delivery is also useful in veterinary medicine in all higher animals in which emissary veins are present starting from Aves [23].

CONCLUSION

Despite the apparent variations in the Nap wise sleep latency values natural for a biological event such as falling asleep, data show that transcranial route has yielded the shortest sleep latencies. Transcranial Day 3, Nap 3 has yielded sleep latency values 1.5 to 6.5 minutes for all volunteers except F2. These are below the significant 7 minutes cut off indicating enforced drug effect. This is despite the disadvantage of a smaller area of the scalp available for drug absorption and a smaller dose available as a result of soaking of hair in the transcranial route compared to the transdermal route. Transdermal Day 4, Nap 3 has three sleep latencies 4- 5.5 minutes indicating that diazepam could be administered by this route as well. The pharmacokinetics of diazepam favors repeated doses for better sleep latency results since the active metabolite desmethyldiazepam tend to accumulate in blood.

The use of a non aqueous oil vehicle in transcranial dosage design is to promote a continuous medium with sebum and to retain product on the skin for prolonged periods. More details are desirable about the intricate anatomy, histology and physiological functions related to the scalp, calvaria, the meninges, blood and cerebrospinal fluid flow in the light of transcranial drug delivery. Since a single cycle of CSF flow through the brain takes 6 -8 hours, for all intent of drug diffusion this fluid can be considered to be static.

One of the hypnotics directly affecting sleep latency may be a better candidate drug in order to yield uniform sleep latency results unlike a sedative such as diazepam that brings about sleep secondary to sedation.

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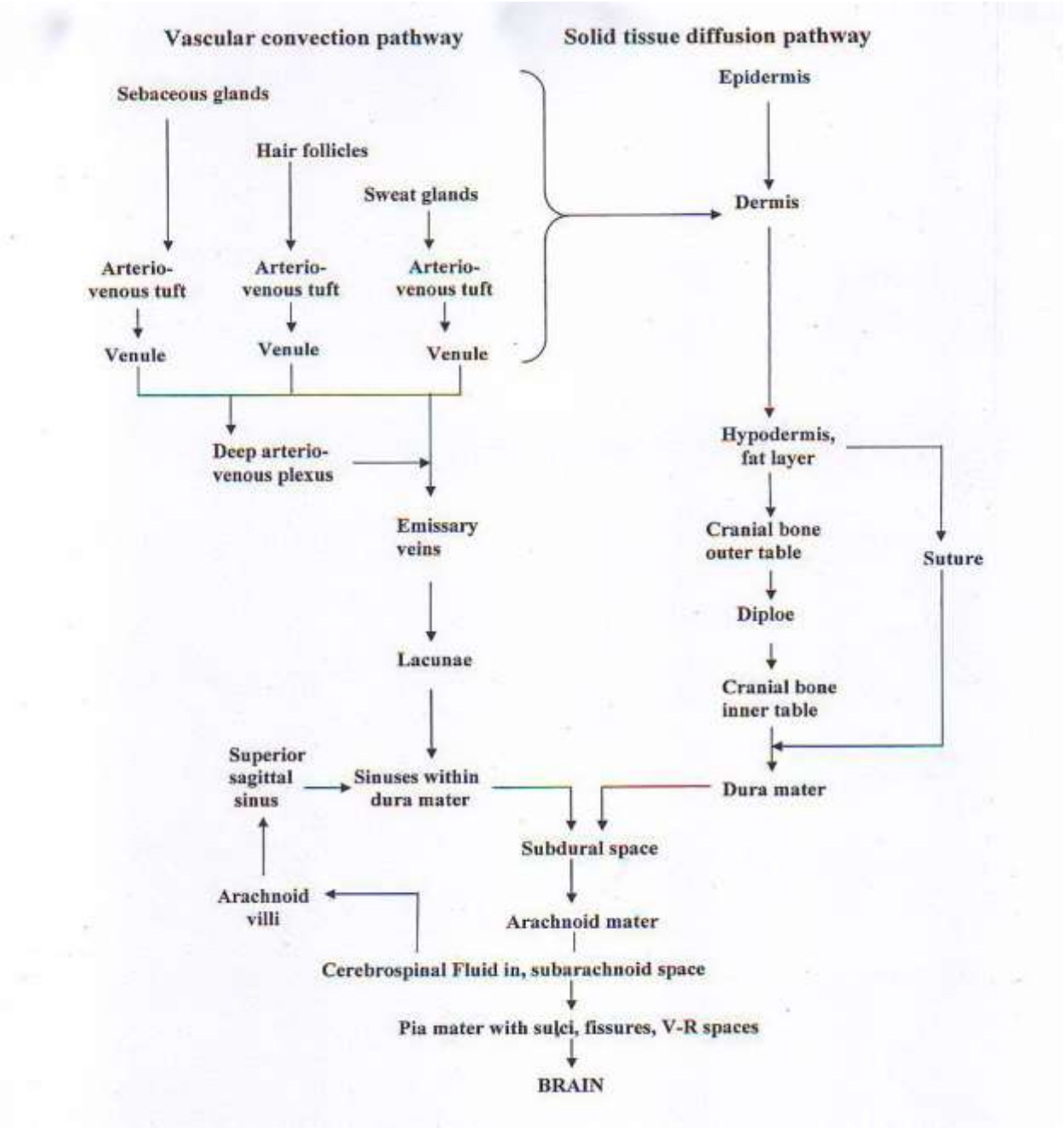

Figur1: Cascade for the possible pathways of vascular convection and solid tissue diffusion of the drug molecules from the scalp in transcranial brain targeting.

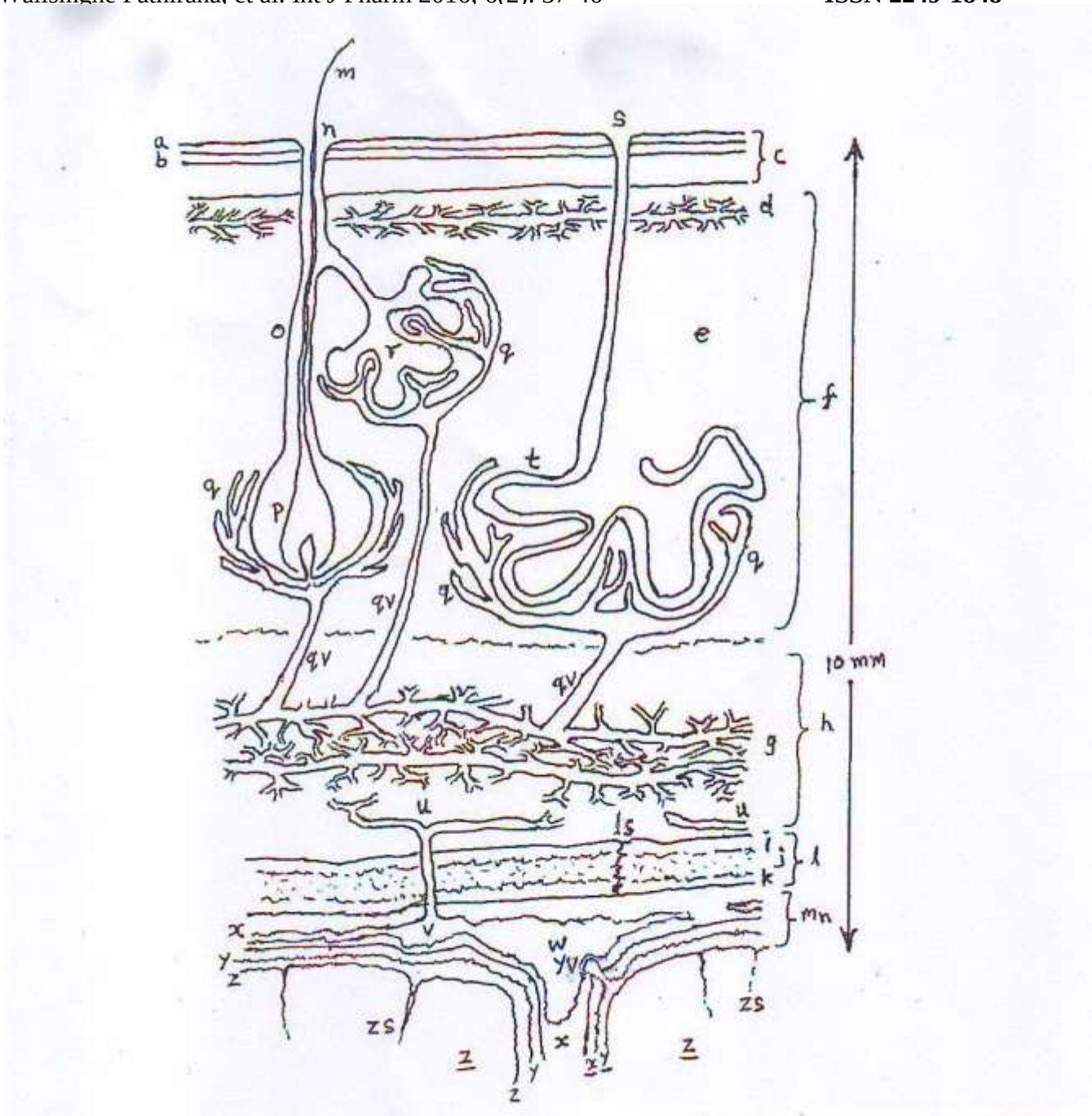

Figure 2: Line drawing of coronal section through calvaria with scalp depicting anatomy related to transcranial brain targeted drug diffusion pathways. Skin appendages drawn magnified.

a. Stratum corneum, b. Stratum lucidum, c. Epidermis, d. Superficial arteriovenous plexus, e. Stroma of dermis, f. Dermis, g. Deep arteriovenous plexus, h. Hypodermis, i. Cranial bone outer table, j.Diploe, k. Cranial bone inner table, l. Cranial bone, ls. Suture, mn. Meninges, m. Hair strand, n. Pore of hair follicle, o. Hair follicle, p. Hair bulb, q. Arterio-venous tufts, qv. Venule, r. Sebaceus gland, s. Pore of sweat gland, t. Sweat gland, u. Emissary vein, v. Lacuna, w. Superior saggital sinus, x. Dura mater, x. Subdural space, y. Arachnoid mater, y. Subarachnoid space, yv. Arachnoid villi, z. Pia mater, zs. Sulci, z. Brain.

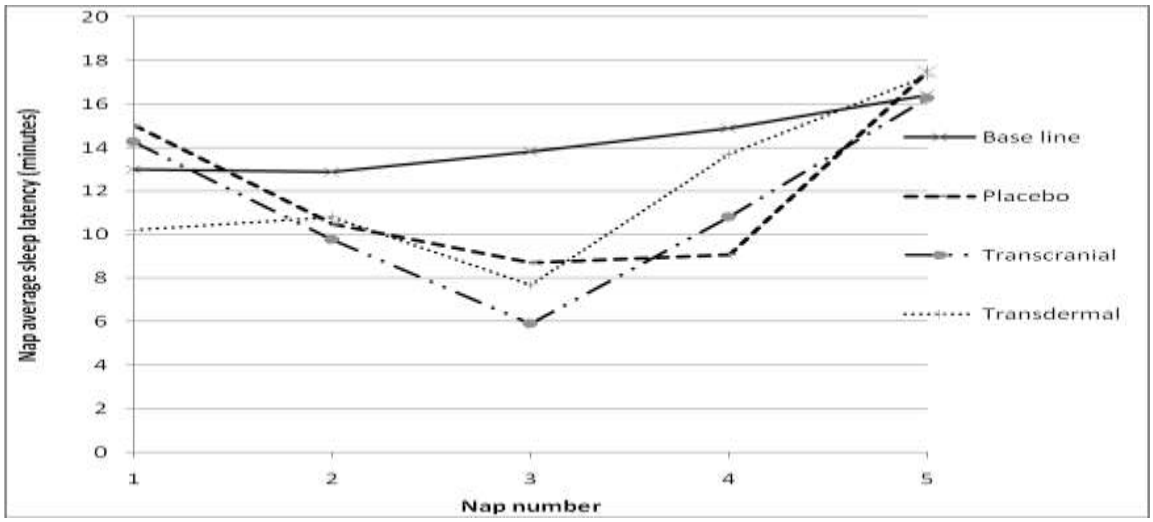

Figure 3: Graph indicating curves for nap vs average sleep latency for the given nap.

Table 1: Summery of sleep latency test results of six volunteers.

MSLT	Nap	M1 min	M3 min	M4 min	M5 min	F1 min	F2 min	Nap av. min
Day 1 Baseline	1	9.5	10	20	20	6.5	12	13
	2	4	20	20	5	8.5	20	12.9
	3	3	7.5	20	18	14.5	20	13.8
	4	15	12	20	20	12	10.5	14.9
	5	15.5	16.5	20	20	6.5	20	16.4
MSL	-	9.4	13.2	20	16.6	9.6	16.5	14.22
Day 2 Placebo	1	12	13	20	20	5.5	20	15
	2	8.5	7.5	12	8	7	20	10.5
	3	4.5	7.5	6	8.5	5.5	20	8.7
	4	4.5	8.5	7.5	3	11	20	9.1
	5	14.5	20	18.5	13.5	20	20	17.5
MSL	-	8.8	11.3	12.8	10.6	9.8	20	12.16
Day 3 Transcranial	1	17.5	12	20	5.5	10.5	20	14.25
	2	16.5	7	5	1	9	20	9.75
	3	3.5	6.5	1.5	5	5	14	5.9
	4	9.5	12	6.5	4.5	20	12.5	10.8
	5	8.5	20	20	9	20	20	16.25
MSL	-	11.1	11.5	10.6	5	12.9	17.3	11.4
Day 4 Transdermal	1	6.5	10	20	2.5	2	20	10.2
	2	7.5	10.5	20	4	3	20	10.8
	3	5.5	5.5	13.5	9.5	4	8	7.7
	4	20	9	20	6	7	20	13.7
	5	20	20	20	8.5	15.5	20	17.3
MSL	-	11.9	11	18.7	6.1	6.3	17.6	11.94

MSLT; Mean sleep latency test, MSL; Mean sleep latency, Nap denotes a time period of 30 minutes after asking the volunteer to sleep, M1-M5; male volunteers (M2 disqualified after Day 1 test), F1and F2; female volunteers, min; minutes, av; average.

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