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ORIGINAL ARTICLE

Investigation of the Effects of Calcium Channel Blockers with Cigarette Smoke Exposure to Nerve Healing at Peripheral Nerve Injuries

Periferik Sinir Yaralanmalarında Kalsiyum Kanal Blokerleri İle Birlikte Sigara Dumanı Maruziyetinin Sinir İyileşmesine Olan Etkisinin Araştırılması

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ABSTRACT

Objective: A study was planned to investigate the effect of exposure to cigarette smoke and the effects of calcium channel blockers in experimentally induced sciatic nerve injuries.
Meteryal-Metod: The sciatic nerve was cut on unilaterally in all the groups, and repair was performed at hour 0. After repair, single dose of 1 ml of 0.9% saline was administered to group 1, cigarette smoke to group 2, 1mg/kg calcium channel blocker to group 3, cigarette smoke and 1mg/kg Calcium channel blocker was given to group 4.
Results: At the end of the 12th week, gait analysis at and sciatic function index (SFI), in 30 minutes for 90 days the number of myelinated axons, axon areas and myelin diameters were assessed. There was a significant difference between the measured values of SFI in group 1 and group 3 and 4, and there was a significant difference between group 2 and group 3 and 4 ($p < 0.05$). In addition there was a significant difference between the number of axons in group 2 and group 3 ($p < 0.05$).
Conclusion: As a result, after peripheral nerve laceration repair calcium channel blockers have a positive effect on the sciatic function index while cigarette, smoke has a negative effect. In Moreover, according to the sciatic function index the negative effect of cigarette smoke can be resolved with calcium channel blockers. However, these data are not supported by the number of axon, axon area, and the myelin diameter

Key words: peripheral nerve, cigarette smoke, calcium channel blocker

ÖZ

Amaç: Bu çalışma deneysel olarak oluşturulan siyatik sinir kesisin de sigara dumanı maruziyeti ile birlikte kalsiyum kanal blokerlerinin etkisini araştırmak için planlandı.
Gereç-Yöntem: Tüm gruplarda siyatik sinir tek taraflı kesildi ve 0. saatte onarım yapıldı. Onarım sonrası grup 1 e sadece günde tek doz 1 ml %0.9 serum fizyolojik, grup 2 ye 90 gün 30 dakika sigara dumanı, grup 3 e 1mg/kg Kalsiyum Kanal Blokeri, grup 4 e 90 gün 30 dakika sigara dumanı ve 1mg/kg Kalsiyum Kanal Blokeri verildi. 12. hafta sonunda Yürüme Analizi ve Siyatik Fonksiyon İndeksi (SFİ), miyelinli akson sayıları, akason alanları ve miyelin çapları değerlendirildi.
Bulgular: Ölçülen SFİ değerlerinden grup 1 ile grup 3 ve 4 arasında anlamlı fark olduğu, grup 2 ile grup 3 ve 4 arasında anlamlı fark olduğu tespit edildi ($p<0.05$). Ayrıca grup 2 deki akson sayısı ile grup 3 teki akson sayısı arasında da anlamlı fark bulundu($p<0.05$).
Sonuç: Siyatik fonksiyon indeksi açısından periferik sinir kesilerinde onarım sonrası kalsiyum kanala blokerleri olumlu sigara dumanı da olumsuz yönde etki yapmaktadır. Ayrıca siyatik fonksiyon indeksine göre sigara dumanın yaptığı bu olumsuz yönde etki kalsiyım kanal blokerleri ile giderilebilir. Ancak bu veriler akson sayısı akson alanı ve miyelin çapı ile desteklenmemektedir

Anahtar Kelime: periferik sinir, sigara dumanı, kalsiyum kanal blokeri

Introduction

Peripheral nerve injury as a result of trauma is a serious condition that causes physical problems as well as psychosocial and economic ones. Regardless of the cause of the injury, incomplete healing of the nerve tissue or abnormal regeneration of the nerve often results in functional loss and pain (1). In addition, it is known that cigarette smoke and the toxic substances in it have a negative effect on the central nervous system and also on the peripheral nervous system (2). Ischemia, which occurs as a result of injury or after repair, causes the accumulation of many toxic agents, especially free oxygen radicals, in the injury site (3,4). Thus, membrane permeability is disturbed and influx of calcium into the cell begins, which causes the destruction of cell structures (5,6). The usage of calcium channel blockers early after injury may reduce the damage by disrupting this mechanism (7).

The smoking rate is high in patients with peripheral nerve injury and is increasing day by day. In these patients, smoking continues even after nerve repair. This situation seriously affects our clinical results negatively. The aim of this study is to investigate how the negative effect of cigarette smoke on nerve healing is affected by the use of calcium channel blockers.

Materials and Methods

This study was performed after the approval of Animal Ethics Committee of Necmettin Erbakan University Experimental Medicine Research and Application Center (KONUDAM) dated 23/08/2011 and numbered 2011-097. "Ethical Guidelines for the Use of Animals in Research" was followed in the study. A total of 28 adult female Wistar Albino rats weighing between 250 and 275 grams were used.

Groups:

Rats were randomly selected and divided into 4 groups of 7 rats each (Table 1).

Table 1: Groups and given agents

	Serum Physiological	Calcium Channel Blocker (Flunarizine)	Cigaratte smoke
Group 1	+	-	-
Group 2	-	-	+
Group 3	-	+	-
Group 4	-	+	+

Group 1 (7 rats): After the sciatic nerve repair, 1 ml of 0.9% serum saline was administered orally once a day for 90 days postoperatively.

Group 2 (7 rats): After the sciatic nerve repair, the animals were exposed to cigarette smoke with the cigarette smoke delivery system, approximately 30 minutes once a day for 90 days postoperatively.

Group 3 (7 rats): After the sciatic nerve repair, 1 mg/kg calcium channel blocker, flunarizine (Sibelium® 5 mg, 50 capsules, Jansen-Cilag, Colombia) was administered orally once a day for 90 days postoperatively.

Group 4 (7 rats): After the sciatic nerve repair, 1 mg/kg calcium channel blocker, flunarizine (Sibelium® 5 mg, 50 capsules, Jansen-Cilag, Colombia) was administered orally once a day for 90 days postoperatively. In addition, the animals were exposed to cigarette smoke with the cigarette smoke delivery system, approximately 30 minutes once a day for 90 days postoperatively.

The Cigarette Smoke Delivery System:

In this study, the cigarette smoke delivery system was used which was designed by us for experimental research. In this system, an enclosed environment was prepared and the cigarette smoke was transmitted to this enclosed environment by a closed transportation system, and the inside smoke was transmitted to the outside via negative pressure. The system could introduce 50 ml of cigarette smoke with each puff to the closed environment (figure 1).

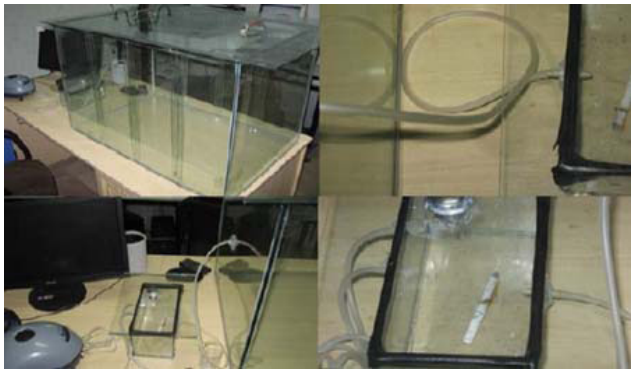

Figure 1: Cigarette smoke delivery system

In our study, the rat groups (7 rats together) were exposed to cigarette smoke for 30 minutes by using this system. Our machine was adjusted to give 1 puff every minute and each cigarette was consumed in approximately 10 minutes in the smoke machine by providing 200 ml/min air flow. In the study, 3 cigarettes were consumed in 30 minutes, and a total of 6 cigarettes were consumed per day (2 groups). In addition, cigarettes made of Turkish tobacco were used in our study and which contains similar toxic substances of the cigarette designed for experimental research by Tobacco and Health Research Institute (Lexington, KY). With this system, a similar effect to human exposure to cigarette smoke was created (8-9).

Preparation of Calcium Channel Blocker:

In our study, flunarizine (Sibelium® 5 mg, 50 capsules, Jansen-Cilag, Colombia) was used as a calcium channel blocker, which has a specific effect on peripheral arteries and its beneficial effect on nerve healing has been reported in the literature when administered daily at a dose of 0.33 mg/kg i.p. (10). In the study, 1 capsule of the agent was diluted with 5 ml of serum saline and 0.5 ml of this solution (0.5 mg) was administered orally to the rats every day.

Surgical Technique:

Surgical procedures were performed under appropriate anesthesia (Ketamine-HCl), with the appropriate surgical site cleaning and sterilization. While the rat was in the prone position, the skin and subcutaneous tissue were dissected through an oblique gluteal incision in the right lower extremity. The gluteus maximus and biceps femoris muscles were separated by sharp and blunt dissection, and the sciatic nerve was exposed (figüre 2A). The sciatic nerve was released from the surrounding tissues from the sciatic notch to the trifurcation region with starting the help of microsurgical instruments and was cut 1 cm proximal to this level with the help of a scalpel by a single stroke (figure 2B). Nerves repaired epineurally with 10/0 prolene via were microsurgical techniques under the microscope. (figure 2C). After the surgical procedure was applied to the subjects, the muscle

was sutured one by one with 4/0 vicryl and the skin was sutured with 4/0 prolene kontune and the rats were taken back to their cages. (figure 2D).

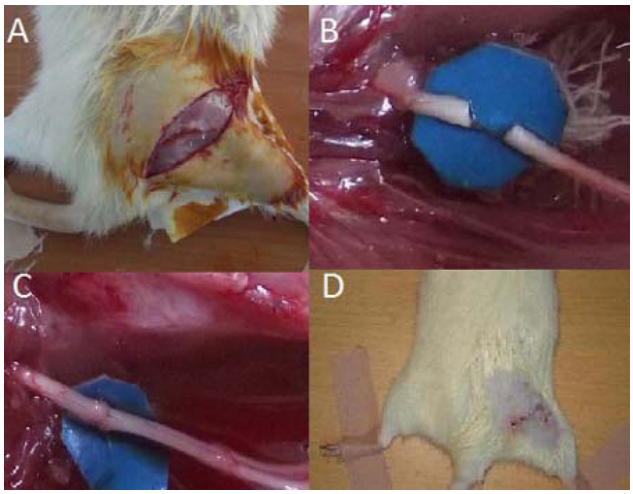

Figure 2 A: Rat preparation for operation and sciatic nerve dissection **B:** Cutting the sciatic nerve 1 cm proximal to the trification in one stroke. **C:** Nerve repair with 10/0 Prolene with epineural technique (at 10x and 16x magnification). **D:** Muscle and skin suturing.

Evaluation Methods

Gait Analysis and Sciatic Function Index

A walking path arrangement was prepared in accordance with the literature to ensure that the rats walk in the same direction (11). Lane white file papers were placed inside the walking lane. Both hind legs of the rats were pressed to the black ink-impregnated stamp and the footprints were taken by walking in the prepared lane (12) (Figure 3). The sciatic function index (SFI) was calculated with the obtained measurements by the formula developed by De Medinacelli and later modified by Bain et al. (13). The values scaled between zero and -100, in which zero indicates normal function and -100 indicates complete loss of function. It was statistically compared whether there was a difference between the groups in terms of SFI values.

Figure 3: Walking lane with walking path analysis

Histological (Histomorphometric) Evaluation:

At the end of the 12-week follow-up period, rats in all groups were sacrificed by cervical dislocation under anesthesia after walking tests were performed. The part of the sciatic nerve between the region where it exits from the gluteal notch and the bifurcation region was totally excised. The nerve was placed in 2.5% glutaraldehyde solution prepared in 0.12 M buffer (pH 7.4). After 6 hours of fixation in this solution, 1-millimeter samples distal to the anastomosis were taken.

The samples were evaluated according to the previously determined principles (14). The analysis of the sections obtained from sciatic nerves was performed by the Stereological Image Analysis System equipped with a digital camera (mbf/Bioscience, Qimaging), an image capture card (Flash Point 3D), a desktop computer containing a stage control unit (Prior), a computer-controlled microscope stage motor (Prior), a microcator (Heidenhein) and a research light microscope (Leica, DM400B). Stereological measurements by this system were carried out by means of a software (mbf/Bioscience, Stereo investigator, version 9) which controls the specified units and includes new stereological measurement tools. Sciatic nerve sections were placed in a 900 mm2 unbiased counting frame. After the frame was positioned, meander sampling was performed by placing 70µmx70µm steps on the tissue section according to the systematic uniform random sampling scheme and the number of myelinated axons according to the rules of the unbiased counting frame were determined at each step. The same system was also used to determine the axon area and myelin diameter.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the differences in axon numbers and SFI tests between the groups. Individual analyzes of data were performed by using ANOVA and Student-Newman-Keuls test. The level of statistical significance was accepted as $p<0.05$.

Results

General Evaluation Findings:

At the end of the 3-month follow-up period, no animals were excluded due to death and 28 animals were included in the evaluation. Paralysis was observed in the right lower extremities of all rats after the surgical procedure. At the end of the third month, partial improvement in paralysis and muscle atrophy were observed in all groups.

Walking Analysis and Sciatic Function Index Findings:

The sciatic function indexes of the groups according to the walking test were shown in Table 2 and Figure 4A. Statistically significant differences were found in SFI comparisons of the groups with the ANOVA test

(p<0.05). In comparison of SFI values obtained after 12 weeks of surgery with Student-Newman-Keuls test among the four groups, there was a significant difference between group 1 (mean -59.12) and groups 3 and 4 (mean -40.38 and -47.50, respectively), between group 2 (-67.15) and groups 3 and 4 (mean -40.38 and -47.50, respectively), but there was no significant difference between group 3 and group 4, and between group 1 and group 2 (figure 4A).

Table 2: Sciatic function indices (SFI) obtained from the groups (SFİ)

	Group 1	Group 2	Group 3	Group 4
1	-75.45	-68.93	-34.19	-47.17
2	-68.45	-61.55	-48.06	-43.39
3	-39.83	-68.68	-41.23	-52.49
4	-35.32	-59.13	-35.13	-42.80
5	-56.51	-70.20	-39.35	-47.25
6	-64.02	-72.64	-43.50	-46.93
7	-73.79	-67.90	-41.23	-52.49
Average	**-59.12**	**-67.15**	**-40.38**	**-47.50**

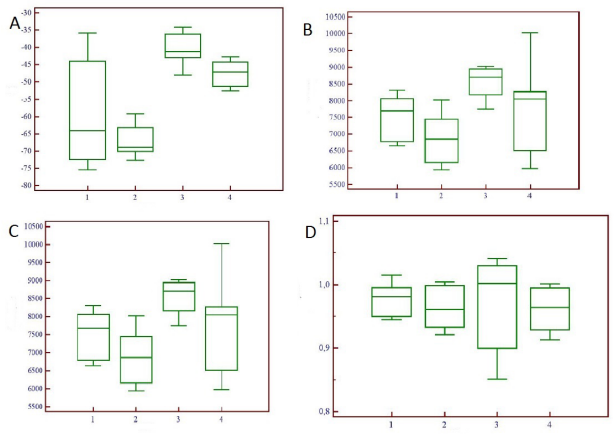

Figure 4: A: Sciatic function indices (SFI) obtained from the groups **B:** Number of axons obtained from groups **C:** Areas of axons obtained from groups µm2 **D:** Myelin thicknesses obtained from groups µm

Histological (Histomorphometric) Evaluation Findings:

At the end of the 12-week follow-up period, three parameters (the number of axons, axon area and myelin thickness) were evaluated from the materials taken for histological examination. The axon numbers of the groups are shown in Table 3 and Figure 4B. A significant difference was found when the axon numbers of the groups were compared with the ANOVA test (p<0.05). When the Student-Newman-Keuls test was performed for individual group comparison, there was a significant difference between the number of axons in group 2 (mean 6918) and group 3 (mean 8517). However, there was no significant difference amog the other groups (Figure 4B).

Table 3: Number of axons obtained from groups

	Group 1	Group 2	Group 3	Group 4
1	6821	6859	7742	8258
2	8307	5934	8738	5980
3	8182	6647	9021	8057
4	7714	8251	8155	10025
5	6647	6545	8221	8272
6	7695	7695	8712	5995
7	6770	8201	9031	8042
Average	**7448**	**6918**	**8517**	**7804**

The axon areas of the groups were shown in Table 4 and Figure 4C. When the groups were compared in terms of axon areas by the ANOVA test, there was no significant difference among the groups (p>0.05) (Figure 4C).

Table 4: Areas of axons obtained from groups

	Group 1	Group 2	Group 3	Group 4
1	13.415	13.963	14.709	13.558
2	13.950	14.171	14.307	11.928
3	15.729	17.686	10.423	12.215
4	12.763	13.850	14.586	14.143
5	17.686	17.587	14.601	13.573
6	12.752	12.752	14.261	11.943
7	13.115	15.415	10.445	12.201
Average	**14.201**	**13.863**	**13.330**	**12.794**

The measured myelin thicknesses of the groups were shown in Table 5 and Figure 4D. When the groups were compared in terms of myelin thickness by the ANOVA test, no significant difference was found among the groups (p>0.05) (Figure 4D).

Table 5: Myelin thicknesses obtained from groups

	Group 1	Group 2	Group 3	Group 4
1	0.946	0.925	1.022	0.961
2	0.963	1.004	0.997	0.991
3	1.015	0.957	0.851	0.918
4	0.998	1.001	1.032	1.001
5	0.998	0.921	1.041	0.964
6	0.981	0.961	1.002	0.996
7	0.945	0.992	0.867	0.913
Average	**0.976**	**0.965**	**0.972**	**0.963**

Discussion

It is known that many factors can affect healing and axonal regeneration after repair in peripheral nerve injuries. One of the most important factor is the vascular structure of the peripheral nerve. The vascular structure of the peripheral nerve is usually in better condition in clean wounds caused by a sharp object. However, despite this type of injury and early repair, intraneural microcirculation is impaired until collateral circulation develop. As a result, tissue ischemia develops in

the repair region. During ischemia, the increase of calcium ions in the cytoplasm, cell swelling and the formation of toxic oxygen radicals lead to neural tissue damage (15). In addition, calcium ions are important in maintaining neuronal homeostasis after trauma. The extracellular calcium activity is approximately 1000 times higher than the intracellular one. Therefore, calcium enters into the cell easily after the injury which increases the proteolytic enzyme activity and causes the destruction of cell structures. Furthermore, increased intracellular calcium disrupts the electron transport chain in the mitochondria, which results in free radical and vasoactive inflammatory substance release. This situation increases ischemia of the tissue and impairs healing by decreasing blood flow (16).

The exact mechanism of the positive effect of calcium channel blockers on nerve healing is unknown. However, this positive effect was mainly explained by two mechanisms in the literature. First, calcium channel blockers increase the resistance of the tissue against ischemia by the vasodilation effect which increase the nutrition of the axons in the injured area (17). The second mechanism is increases axonal budding and myelination in the growth cone (18). Apart from these two mechanisms, there is also literature stating that calcium channel blockers exert their protective and curative effect on the nerve by increasing the rate of muscle reinnervation and by giving rise to neovascularization for the nutrition of axons (19). Consistent with this literature, in our study, we found that there was a statistically significant difference in group 3 (which we gave only Flunarizine) when compared with other groups in terms of both the sciatic function index and the number of axons. This may show us that Flunarizine has a positive effect on nerve healing both functionally and histologically.

The effect of cigarette smoke on peripheral nerve and brain injuries has been studied in myocardial infarction, necrotizing enterocolitis and experimental animal ischemia-reperfusion models (20). Brett et al. attributed this negative effect of chronic nicotine exposure on neuronal tissue to hypersensitivity reaction and increased inflammatory response (2). In addition, Rinker et al. stated in an ischemia-reperfusion study in rats that cigarette smoke slows down the healing due to the non-functioning of the healing mechanisms after peripheral nerve injury (20). In our study, there were findings supporting this data. We found that there was a significant difference in the number of axons between group 2 in which we gave only cigarette smoke and group 3 in which we gave only flunarizine. In addition, when examined in terms of sciatic function index, we found that there was a significant difference between group 2 and group 4. These data suggest us that cigarette smoke has a negative effect on nerve healing.

There are studies investigating how the negative effect of cigarette smoke is affected by using calcium channel blockers (21). Rinker et al. reported that calcium channel blockers and cigarette smoke were administered after ischemia reperfusion injury to the rat sciatic nerve. The authors stated that there is no evidence that cigarette have smoke impairs healing, nor that calcium channel blockers affect this healing in a good way, histologically (22). However, when the authors compared the sciatic function indexes, they stated that there was a significant difference between the group in which only cigarette smoke was given and the group in which cigarette smoke and calcium channel blockers were given. In our study, we found a significant difference in terms of sciatic function index between group 2 in which we gave only cigarette smoke and group 3 in which we gave only flunarizine. This data suggest us that flunarizine has a positive effect on peripheral nerve injuries in terms of sciatic function index. Although we found a similar result in terms of axon numbers between group 2 and group 3, other histomorphological data did not support this finding. In addition, we did not find any significant difference in any of the parameters we specified between group 1 in which we gave only saline and group 2 in which we gave only cigarette smoke. This may show us that cigarette smoke did not impair nerve healing, similar to the study by Rinker et al. However, we found a significant difference in terms of sciatic function index between group 2 administrated only cigarette smoke and group 4 administrated cigarette smoke and flunarizine together, which may indicate that smoking has a negative effect on nerve healing and this effect can be reduced with flunarizine, but none of the histomorphological data supported this parameter. Moreover, the lack of an electrophysiological data in our study is a limitation in this respect. It should not be forgotten that functional recovery is faster than histomorphologic recovery in nerve healing.

Conclusion

In our study, the negative effect of cigarette smoke exposure and the positive effect of calcium channel blockers on nerve healing were shown according to some parameters. However, there is not enough data to show that calcium channel blockers reduce the negative effect of cigarette smoke. Although there was a significant difference between group 2 and 4 in terms of sciatic function index, there is a need for further studies using different evaluation parameters with longer follow-up time.

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