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The effect of human placental suspension on rat sciatic nerve healing

Sıçan siyatik sinir iyileşmesinde insan plasenta süspansiyonunun etkisi

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Amaç: İnsan plasenta süspansiyonunun sıçan periferik sinir rejenerasyonundaki etkisi araştırıldı.

Çalışma planı: Çalışmaya ağırlıkları 250-300 gr arasında değişen, sekiz adet erişkin Sprague-Dawley türü dişi sıçan alındı. Sıçanlar kontrol (n=4) ve denek (n=4) olmak üzere iki gruba ayrıldı. Sıçanların her iki siyatik siniri anestezi altında eksplore edilip kesildi ve hemen tamir edildi. Tamir bölgesine işlem sonunda ve ikinci ve beşinci günlerde, test grubunda insan plasenta süspansiyonu, kontrol grubunda ise serum fizyolojik uygulandı. Cerrahi öncesinde ve sekizinci haftanın sonunda fonksiyonel değerlendirme için sıçanların her iki alt ekstremitesine elektromiyografi yapıldı. Sekizinci hafta sonunda histopatolojik değerlendirme için sıçanların yaşamı sonlandırıldı ve tamir bölgesi ve distalinden alınan kesitler incelenerek miyelinli akson sayımı yapıldı.

Sonuçlar: Elektromiyografik değerlendirmede her iki grupta da girişim sonrasında, girişim öncesine göre latans değerlerinde uzama, amlitüd değerlerinde azalma görüldü. Bu değişimler test grubunda biraz daha az olmasına rağmen kontrol grubuyla arasında anlamlı fark yoktu (p>0.05). Kontrol grubunda en belirgin bulgu endonöral kollajen artışı iken, test grubunda rejeneratif aksonal değişikliklerin varlığıydı. Her iki grupta da distaldeki akson sayıları tamir bölgesinden fazla bulundu. Tamir bölgesindeki ve distal bölgedeki miyelinli akson sayımı test grubunda daha fazlaydı; ancak, bu farklılık sadece tamir bölgesindeki akson sayısında anlamlı düzeydeydi (p=0.001).

Çıkarımlar: İnsan plasenta süspansiyonunun periferik sinir rejenerasyonunu olumlu etkileyebileceği sonucuna varıldı.

Anahtar sözcükler: Hastalık modeli, hayvan; sinir rejenerasyonu/fizyoloji; plasental ekstrakt; sıçan; siyatik sinir/yaralanma.

Objectives: We investigated the effect of human placental suspension (HPS) on rat sciatic nerve regeneration.

Methods: Eight adult female Sprague-Dawley rats weighing between 250 and 300 g were randomly divided into control and study groups equal in number. Both sciatic nerves were explored under anesthesia, transsected and then immediately repaired. At the end of the operation, and on the second and fifth days, HPS and saline were administered to the operation zone in the test and control groups, respectively. For functional examination, electromyographic activity was measured in posterior extremities of all rats preoperatively and at the end of eight weeks. The rats were then sacrificed to obtain transections from the repaired area and its distal region for histologic examination and the number of myelin-sheathed axons was estimated in both regions.

Results: Electromyographic study showed delayed latency and decreased amplitude following operation in both groups, being less severe in the study group without reaching a significant difference from the control group (p>0.05). The most notable histopathologic finding was increased endoneural collagen in the control group and regenerative axonal growth in the HPS group. The number of axons was greater in the distal region in both groups. In both regions, the number of myelin-sheathed axons was greater in the HPS group, but this difference was significant only for the number of axons in the repair zone (p=0.001).

Conclusion: Our data suggest that HPS may exert a favorable effect on peripheral nerve regeneration.

Key words: Disease models, animal; nerve regeneration/physiology; placental extracts; rats; sciatic nerve/injuries.

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Following total laceration of perpheral nerves axonal budding starts on the most distal ranvier's nodes proximal to the lecaretion site. These axonal buddings enter the area distal to the laceration site and try to contact target organ to regain function. Most important factors that accelerate axonal grow up towards target organ are, neurotrophic factors which are secreted from the target organ and proximal stump following Wallerian degeneration.[1,2]

Success of regenaration phase depends on multiple environmental and cellular factors such as patient's age, the site of laceration (e.g. proximal or distal), meticulaous repair with maximum possibilities and techniques.[3] This phase consists of a couple of steps which are, advancing of regenerated axons till repair site, their passing over the repair site towards target organ on distal stump and starting connection and lastly functional recovery.[4,5] A study made in human radial nerve showed that it takes 4-5 weeks for axons to cross over the lacerated site following adequate nerve repair.[4]

The mentioned duration time for axonal crossover for the repair site is in optimum conditions. When the laceration site is more proximal and factors such as perineural fibrosis following repair slows the regeneration process resulting with delay in crossover time. The prolongation of this time results with irreversible functional casualties in target organ. These factors direct the physicians towards new studies-investigations that promote less fibrosis in repair site and fasten regeneration. For this reason many growth factors, hormones and pharmacologic agents are studied on.[6-15]

There is not any studies that mention the acceleration effect of human placenta on regeneration of nerves following repair and reducing scar tissue in thorough search of the literature. However there are similar studies that report acceleration of nerve tissue regeneration and reducing of scar tissue on repair site with amniotic fluid.[7,8,16] This effect is suggested to be due to placental lactogen hormone (PLH), placental growth hormone(PGH) and hormones such as leptin,[17,18] which increase insulin like growth factor (IGF)-1 and 2 and their receptors, nerve growth factor (NGF), fibroblast growth factor (FGF) and hyaluronic acid (HA) release from repair site. Especially nerve growth factor (NGF) which is comprised by amniotic fluid is the first and most important neurotrophic factor known.[17,19]

Some studies are made to determine the content of hormone, various factors and protein in human placenta. According to these studies in human placenta and it's attachments(desidua, fetal membrane, amniotic membrane and chorionic villus) contain NGF 12.5-70 pg/g,[20] TGF alpha 6-100 ng/ml,[21] TGF beta 10 ng/ml,[22] NADPH 0.018±0.003 mg/ml,[23] fibronectin 0.3 mg,[24] leptin 259±118 pg/g/minute, hCG 31±13 mU/g/minute, hPL 1.16+/-0.19 μ/g/minute,[25] hyaluronane 1.1-2.9±0.2 mug/mg.[26] However Verhague et al.[18] reported that these factors could not pass the placental barrier and show their effects on the fetus by affceting the placenta itself. These findings makes us think that placenta is rich of hormones and growth factors at least as amniotic fluid.

In this study we aimed to show the effects of human placenta suspension(HPS) on rat sciatic nerve regeneration.

Materials and method

Following ethical comitee approval eight adult female Spraque Dawley female rats between 250-300 gr of weight were included in our study. Two groups were formed control and study group each consisting of four rats. In both of the groups both sciatic nerves of the rats were used. So in each group eight and total of 16 sciatic nerves were used(test =8 and control = 8).

For human placenta suspension(HPS), 1.5 ml injectable forms which contain human placenta in 10% NaCL solution that was free of cross reaction-allergic inflammation were used with generic name Placenta – Human (P-H)(Wiedemann – Pharma, Deutschland).[22,27,28]

To evaluate nerve regeneration in our study hystopathologic and electromyographic(EMG) methods were used.[29]

Normal sciatic nerve EMG values were measured before surgery in all rats with EMG device (Dantec Cantata, Copenhagen, Denmark).(Table 1) In electromyographic evaluation latency and amplitude in nerves and spontaneous activities in muscles were evaluated.

In normal instances the nerve signaling must be evaluated in relation with distance.[30] For this reason EMG measurements were made from the same distance (30mm) before and after the surgery.

Table 1. Comparison of latency and amplitude values.

	Latency(ms)			Amplitude(mV)		
	Test (HPS)	Control (S)	*p*	Test (HPS)	Control (S)	*p*
Before surgery	1.36±0.29	1.42±0.17	0.616	7.36±1.07	6.58±0.66	0.106
After surgery	1.80±0.35	2.18±0.74	0.204	5.30±1.15	4.17±2.31	0.240

ms: Milisecond; mV: Milivolt; HPS: Human Placenta Suspension, S: Saline

Surgical procedures were made in supine position with Standard dorsal gluteal approach [31] following anesthesia with 20 mg/kg Ketamine Hydrochloride and 10 mg/kg Xylazine Hydrochloride.[32] Sciatic nerve was reached between the posterior muscle group with blunt dissection. Then sciatic nerve was transsected with a sharp scalpel 1 cm proximal to it's peroneal and tibial branch division. Immediately after transsection epineural nerve repair was carried out under 16 x Zeiss ® brand biomicroscope magnification with 10/0 nylon sutures. In test group 0.3 ml of HPS,[7-9] whereas in control group same amount i.v. saline was injected to the repair site. The wounds were closed in anatomical layers. In both groups same dose of same medications were injected to the repair site percutaneously on the second and fifth postoperative days respectively. At the end of eighth postoperative week following repair EMG evaluation was made for both lower extremities. The rats were sacrificed following EMG with high dose Ketamine injection.

Latency and amplitude values were evaluated with SPSS 11.0 using independent samples test (Student t).

For histologic evaluation sciatic nerves were explored again. 1.5 cm of sciatic nerves were was harvested under biomicroscopic magnification 0.5 cm proximal and 1 cm distal to the repair site. From these samples cross sections were obtained from the repair site and 0.5 cm distal to it. All of the cross scetion specimens were immediately fixed with Karnosky fixative and postfixation was made with 1% osmium tetroxide there after. They were then copiously treated with ethanole and embedded into epon for dehydration. 1 μm thick sections were dyed with toluidine blue and evaluated with light microscope. Following decomposition of the tissues 500 – engström thick sections were obtained and placed into copper grids. Then these were evaluated with Zeiss EM 900 transmission electron microscope following dying process with uranyl acetate and lead citrate. After light microscopic evaluation, the slides were transferred to comuter media with BAB BS 2000 DOC module for myelinized axonal count. Here with 100x magnification all of the myelinated axons were counted with classical(manual) method in three seperate fields measuring 240.000 μ.[29] Myelinated mean axonal counts on the repair site and distal to the repair site were compared statistically with double t-test and independent samples (Student t) test.

Results

Electromyographic findings

When compared with the control group the latency and amplitude values in the test group showed closer results before and after the repair.

According to these data there was elongation in latency periods in both control and test groups. Despite that the elongation in latency periodin the test group was shorter then the control group there was not any statistically significant difference between two groups(p=0.204).

Similarly there was decreasing in the amplitude values in both groups. Altough the decreasing in the test group was smaller then control group, this also did not constitute a statistically significant difference between the two groups(p=0.240)(Table 1). Additionally there was no spontaneous activity following repair in both groups.

Hystopathologic results

Evaluation with light microscope revealed axonal and myelin degeneration, cellular remnants and macrophages additional to regenerated axons in different phases (Figure 1).

Electron microscopic evaluation showed axonal dystrophic changes and demyelinizations (Figure 2). In both groups there was foreign body reaction in the

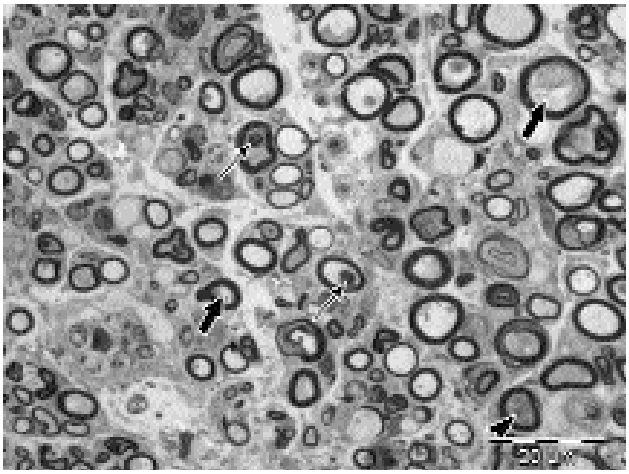

Figure 1. Transverse section of the distal site in control saline group (Thick arrow: axonal degeneration; Arrow tip: axonal regeneration and Thin arrow: axonal and myelin degradation(toluidine blue x 1000)

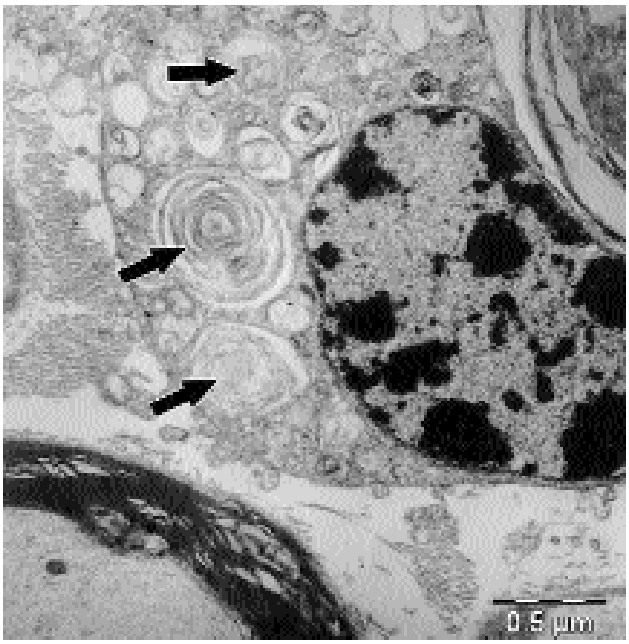

Figure 2. Transverse section of the repair zone in control saline group. Electron micrograph(Arrow: multiple membrane degradation products in Schwann cell cytoplasm(uranyl acetate-lead citrate x 14000)

light microscopic evaluation on the repair sire due to suture material(Figure 3). Also in both groups axonal degeneration, endoneural and perineural fibrosis was seen(Figures 4,5). Findings that have been observed with light microscope were confirmed with electron microscope. Typically the most evident finding in the control group was endoneural collagen increase, whereas in the test group regenerative axonal changes were more evident.

Axonal count showed that axonal counts were greater on the distal part when compared to the repair site. This numeral increase in axonal counts was statistically significant in each group(p=0.000) but not significant between two groups(p=0.308). In the samples prepared for test group the axonal count values were greater in both repair and distal to repair site when compared with the control group. Comparison of myelinated axons in the repair site mean axonal count in the test group was sginificantly greater(p=0.001) (Table 2). Axonal count distal to the repair site was also greater in the test group but this difference was not statistically significant (p=0.308) (Table 2).

Discussion

Despite the anatomy and pathologies of the peripheral nerves are understood better each day and despite new innovations and technologic developments for repair and screening procedures, functional results following repair are usually not sufficient.[6] Scar tissue that develops in the suturated site following surgical repair is an important factor that adversely affects nerve regeneration.[7] Another factor that adversely affects the result is the long healing time required neuromuscular restoration. In order to decrease the long healing time and reduce post repair scar tissue many factors such as pharmacologic agents and hormones were used. [7,10,12,13] Human placenta is one of these materials that is known to be rich of these factors. Toti et al. in their studies performed in 2006[33] have reported that

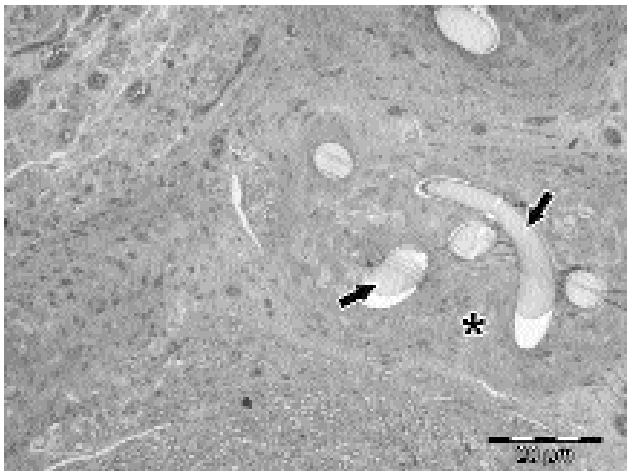

Figure 3. Transverse section of the repair site in control saline group. (Arrow: suture material, Star: Granulomas and fibrine(toluidine blue x 1000)

Table 2. Myelinated axonal counts on the repair site and distal site

	Repair site			Distal site		
	Test (HPS)	Control (S)	*p*	Test (HPS)	Control (S)	*p*
Myelinated axons	63.95±1.07	61.09±1.56	0.001	78.22±4.51	75.96±4.00	0.308

HPS: Human Placenta Suspension, S: Saline.

human placenta and with it's attachments is a strong neuroendocrine organ that secretes NGF and proteins. Again in the study aiming NGF derivation from human placental tissue made by Goldstein et al.[30] it is shown that greatest NGF amount is found in placental cothyledons. Similar studies have also reported that aminotic fluid fastens regeneration and decreases scar tissue on repair site.[7,16,34] Amniotic fluid by it's HA and HA stimulating activator (HASA) content increases the endogenous HA release in the repair site. Hyaluronic acid also is known to decrease the scar tissue in the repair site by decreasing lymphocyte migration, prolipheration, chemotaxis, granulocyte phagocytosis, degranulation and macrophage motility.[16]

Electron microscopic evaluation offers very valuable knowledge about morphological axonal count, axonal diameter and cellular - collagen orientation inside endoneurium. Recently investigation of the peripheral nerve lacerations by automatic methods are favoured. However standardized pathologic cross section derival and measurement, to determine axonal count, axonal diameter and myeline thickness is a very hard and delicate process.[29] Because of this reason we have counted the myelinated axons with classical manual method. This method also is technically difficult and time consuming but gives more accurate results then automatic method. [29] It is known that there is a straight relationship between regeneration and axonal size-myelin sheath thickness.[34] In our study because of technical inabilities and difficulties in standardization we have counted all of the myelinated axons during axonal count as Almquist et al.[35] has done.

Because of perineural fibrosis, suture granuloma and axonal disorganization, axonal counts on the repair site is calitative and does not yield so valuable information regarding regeneration. Mostly axonal counts distal and proximal to the repair site give more valuable information regarding regeneration.[29]

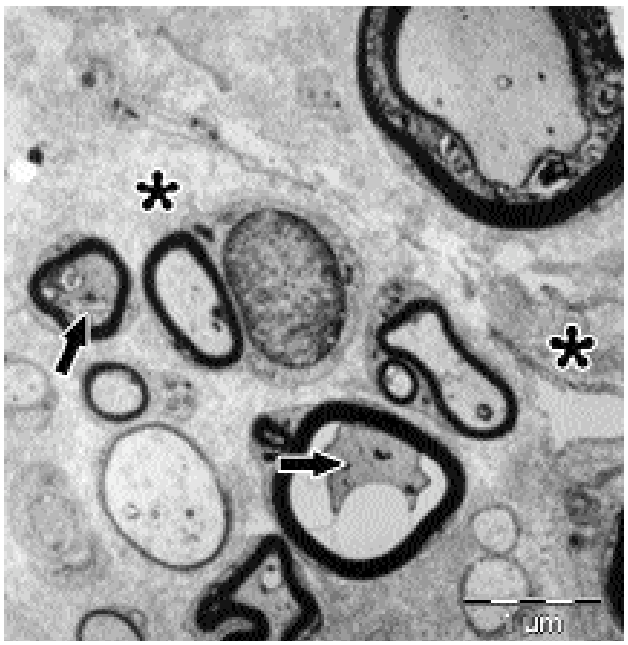

Figure 4. Transverse section of the control saline group (Arrow: Regenerated axons in different phases, Star: increased endoneural collagen (uranyl acetate and lead citrate x 8800)

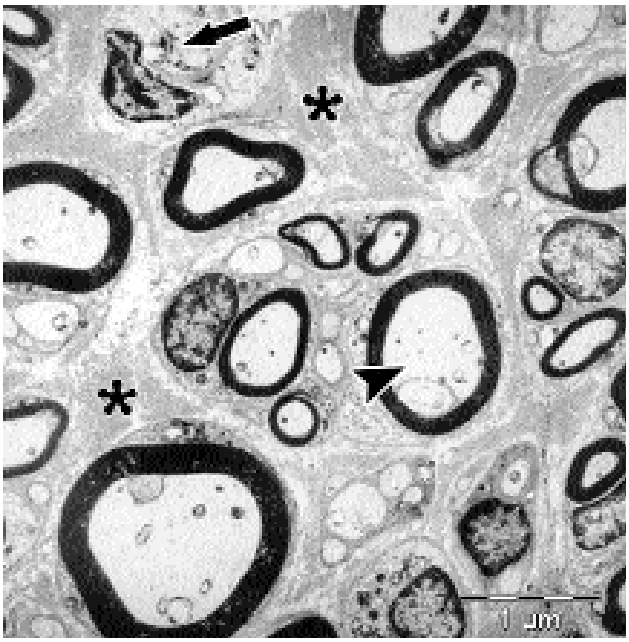

Figure 5. Transverse section of the repair site in test human placenta suspension group (Arrow tip: intact regenerated axons, Arrow: rare regenerated axons, Star: Fibrosis(uranyl acetate - lead citrate x 8800)

Althought this fact is known, to show the effects of HPS on scar tisue cross sections derived from the repair site were also examined and myelinated axonal count in repair site in the test group was found to be significantly greater than the control group(p=0.001; Table 2). On the cross sections derived from the distal segment in both group there was statistically significant(p=0.000) increase in myelinated axonal count compatible with the literature;[29] however despite that this increase was greater in the test group then control group results were statistically insignificant(p=0.308)(Table2).

Prolongation of latency values shows delay in transmission and axonal degeneration.[36] Decrease in amplitude values shows axonal loss and degeneration. [37] Normally spontaneous activity has to be negative, positivity of spontaneous activity shows target muscle mass degeneration and fibrosis.[30,34,36] We have found shorter latency values following repair in test group when compared to the control group. Similarly compatible with literature[7,8,34] amplitude values in the control group were found to be decreased when compared with the test group. However both of these findings were not statistically significant (p=0.204 and p=0.240 respectively) (Table 1).

Statistical insignificancy between the two groups regarding latency and amplitude values can be attributed to; fast regeneration process in rats(3.6±0.5–5.1±0.5 mm/day),[4,5,38] consequently 8 weeks of long period for evaluation following repair, inefficancy or low dose of HPS. Activation of NGF derived from human placenta suspension or the amniotic fluid is only posible if it is obtained in certain circumstances. [19] Also the pregnancy week that it has been obtained is important because of HA content; because HA content is 20 mg/l between 16-20 th gestation week and after 30th week until birth it continues as 1 mg/l.[16] In the HPS that we have used in our study it is reported by the manufacturing company that high molecular structural proteins, vitamins, enzyme and hormone content; are original and kept in constant values because they have been produced with specificaly directed derivation processes.

According to Frykman et al.[29] there is straight correlation between the electromyographic measurements and motor recovery. However the sensitivity of the technique decreases it's usefulness and repetition properties. That's because the amplitude changes according to the placement of the electrode in the muscle and electrode only gives information for the muscle fibers that it is inserted in.[6,29] Frykman et al.[29] have reported that for the amplitude evaluations to be useful, the measurements would be rather done before and after repair period as made in our study; or must be compared with the intact opposite side. As a result spontaneous activity and latency values obtained by electromyographic tests are reliable, whereas amplitude values are less reliable.[35]

Similar studies in the literature report that nerve regeneration evaluations made after repair are done between 4th and 12th weeks.[7,9,12,29,34] We also made the EMG evaluation 8 weeks following repair. However we suggest that this period is longer then the period needed for normal sciatic nerve regeneration in rats. Anyway lack of spontaneous activity in both groups means that nerve muscle regeneration is completed, and this supports the absence of differency regarding latency and amplitude values between control and test groups.

As a result, despite there is not any statistical significancy, in the electromyographic evaluation of the subjects that HPS had been injected factors such as; shorter latency period when compared to control group, relatively greater amplitude values, statistically significant and insignificant increase in the myelinated axonal count on the repair site and distal segment respectively can be evaluated as accelerated nerve regeneration effect and repair site scar reducing effect of HPS. Neverthless new studies with more subjects in number and detailed functional evaluation are needed.

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