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Procedia Engineering 59 (2013) 226 – 232

Procedia Engineering

www.elsevier.com/locate/procedia

3rd International Conference on Tissue Engineering, ICTE2013

Reconstruction of the injured spinal cord by implantation of a hydrogel based on chitosan and β-glycerol phosphate - motor behavior and ventilatory assessments

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Abstract

Over the last few years, increasing attention has been focused on the development of biomaterials that will be able to support axonal regeneration and will lead to successful recovery after spinal cord injuries. Especially hydrogels are being actively investigated due to their intrinsic properties that are favourable in spinal cord tissue regeneration. The main advantage of this type of biomaterials is the fact that their mechanical properties and structural architecture can be easily modulated. They exhibit excellent biological properties that allow on good cell-adhesion. Moreover, they provide a proper biocompatibility and biodegradability. The purpose of this study was to investigate the effect of hydrogel grafts based on chitosan and β-glycerol phosphate on spinal cord injury at the C2 level. Twenty six adult male albino Sprague-Dawley rats were divided into 3 experimental groups (Control, Lesion alone and Lesion+Hydrogel) and examined with the use of the following behavioural tests: ladder walking test, forelimb grip strength test and respiratory assessment by whole-body plethysmography measurement. Six weeks post-grafting, both the behavioral test results and respiratory data have indicated a better functional recovery of animals receiving hydrogel in the lesion cavity in comparison with animals with non-implanted. Chitosan-β-glycerol phosphate hydrogels could be excellent candidate for spinal cord regeneration, and should be used as adjuvant with other treatments in order to enhance functional recovery.

Selection and peer-review under responsibility of the Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria, Centro Empresarial da Marinha Grande.

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Selection and peer-review under responsibility of the Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria, Centro Empresarial da Marinha Grande
doi:10.1016/j.proeng.2013.05.115

Keywords: spinal cord injury, hydrogel, chitosan.

1. Introduction

The development of an ideal procedure to repair damaged axons by a spinal cord injury is up to date challenge for medical doctors as well as biomaterial engineers.

The most promising materials for central nervous system reconstruction are natural biopolymers [1, 2]. They are obtained from natural sources, exhibit similar properties to the tissues they are replacing and reveal good cell adhesion. Furthermore, they tend to accelerate regeneration processes due to specific chemical interactions within biological tissue (e.g. with extracellular matrix molecules).

One of the most influential factors determining the choice of biomaterial for spinal cord treatment is its biocompatibility [3, 4]. Such materials should evoke minimal chronic inflammation and immune responses. The host reaction to implanted biomaterial is influenced not only by its chemical properties but also may be dependent on its physical form of implantation: shape, size and porosity [5, 6]. Moreover, it is important to monitor the product of biomaterial degradation due to the fact that it can cause different inflammatory responses than those of parent material.

Novel approach to central nervous system reparation strategy is to use hydrogels basing on chitosan [3, 7-9]. Chitosan as the copolymer of D-glucosamine and N-acetyl-D-glucosamine is an aminopolysaccharide. It is derived from chitin, the most abundant polysaccharide found in nature after cellulose. Chitosan shows molecular similarity to the basal membrane and extracellular matrix glycosaminoglycans, which allows it to interact with molecules like collagen, laminin and fibronectin. Furthermore, chitosan reveals favorable biological properties including non-toxicity, biodegradability, and impressive adhesion to living tissues [10-12]. Recent research has proved that chitosan based scaffold is excellent support for neural stem and progenitor cells [13, 14].

The purpose of this study was to investigate the effect of hydrogel grafts based on chitosan and β-glycerol phosphate implanted in the spinal cord after a C2 hemisection interrupting sensitive and motor pathways that lead to the alteration of the ispsilateral forelimb properties, and to a deafferentation of the ipsilateral phrenic nucleus, which renders the hemidiaphragm paralysed [15]. Hydrogels obtained was temperature-dependent and formulate at physiological temperature of mammal body (37°C). Their structure was elastic, soft and highly porous. Six weeks post-grafting grafted rodents were examined with the use of the following behavioral tests: ladder walking test, forelimb grip strength test and respiratory assessment by whole-body plethysmography measurement.

2. Materials and methods

2.1. Animals

Twenty six adult male albino Sprague Dawley rats (Janvier®, Centre d'Elevage Roger JANVIER, Le Genest Saint Isle, France) weighing 360-460g were used in the experiment. The animals were kept individually in smooth-bottomed plastic cages at 22°C in a colony room with a 12h light/dark cycle. Nourishment (rat chow, SAFE®-Scientific Animal Food & Engineering) and water were available *ad libitum*.

Animals were divided into 3 experimental groups as illustrated in Table 1.

Table 1. Experimental groups.

Group	Characteristic
Group 1	Control, n=6 (no surgery)
Group 2	C2 left hemisection, n=7 (the lesion cavity was left empty)
Group 3	C2 left hemisection + chitosan, n=13 (the lesion cavity was immediately filled with a block of chitosan hydrogel)

One week handling and pre-training procedure were performed in order to accustom them to the laboratory environment.

All housing, surgical procedures, analgesia and assessments were performed according to the French law on Animal Care Guidelines, using protocols approved by the Animal Care Committee at Aix-Marseille Université (AMU) and the Centre National de la Recherche Scientifique (CNRS).

2.2. Hydrogel preparation

Thermogelling chitosan lactate hydrogel was prepared by dissolving 400 mg of chitosan (Sigma-Aldrich®, St. Louis, MO) in 18 ml of 0.75% lactic acid (Sigma-Aldrich®, St. Louis, MO). The obtained solution was stirred (under slow rotations) until complete dissolution. Then the sample was placed in the ice bath and chilled to 4 °C. Then 2 g of β-glycerophosphate disodium salt hydrate (Sigma-Aldrich®, St. Louis, MO) dissolved in 2.5 ml of distilled water was added dropwise under stirring to the chilled sample. The obtained solution was stirred (under slow rotations) for 20 min. Prepared sample was placed at 4°C. Then sample was kept at 37°C for 12h. In these conditions it undergoes phase transition from sol to gel.

Obtained chitosan lactate hydrogel was sterilized by autoclaving (Prestige médical®, France) at 120 °C, 1.05 bar and for 22 min.

2.3. Surgical protocol on the C2 vertebral level

The surgery on the C2 vertebral level was performed on animals from Group 2 and 3. Animals were anesthetized with an intra-peritoneal injection of chloral hydrate (500 mg/kg, Sigma-Aldrich®, St. Louis, MO). All surgical steps were performed aseptically with the help of dissecting microscope. The dorsal and lateral surfaces of cervical spinal cord were exposed after the left laminectomy at C2-C3. The dura was cut and then the spinal cord was hemisected on the left side. A 1 mm hemisection lesion cavity was made.

Animals from Group 3 were implanted with the hydrogel. The hydrogel implant was shaped *in situ* to the dimensions of hemisection lesion cavity. The sample was washed with sterile saline (0.9%, NaCl) before implantation.

Muscles and skin were sutured (Vicryl® 3-0, Ethicon, Issy Les Moulineaux, France) in anatomical layers and the wound was disinfected with Betadine (5%).

After surgery, the animals were kept in a heated room overnight. Animals were preventively treated during one week post-surgery. Analgesic (30mg/400mg, dextropropoxyphène paracétamol, Sandoz®, Basel-Landschaft, Switzerland) was placed in the drink water. State of hydration and gastrointestinal function were monitored twice a day. Saline (2 ml of 0.9%) was injected subcutaneously and bladders were manually expressed when necessary.

2.4. Locomotor behavioral tests

The ladder walking test. This test requires cortical control of fine sensorimotor coordination. The left forelimb of each animal was filmed during animal walking through a ladder (10 cm x 150 cm) placed under the angle of 45°. The rods had diameter of 0.4 cm and they were placed with the space of 2 cm. Every animal had to walk through the ladder 5 times (40 rods, 20 grips) during one test procedure. Depending on the manner of placing forelimb the animals were scored: 0, +1, +2, i.e. 0 when the paw goes between bars, +1 when paw is just placed over the bar without grabbing and/or pulling on it, then + 2 when the animal can fully grab the bar and pull then push to move upward the steep of the ladder. A ratio based on attempts, bars and score is then computed. This test was performed once a week.

The forelimb grip strength test. The grip strength test is a widely-used non-invasive method devoted to evaluate rat limb strength. It was designed to investigate the effects of neuromuscular disorders. The test is based on the natural tendency of the animal to grasp a bar or grid when it is kept by the tail. The forelimb grip strength (GS) test was performed on the grip strength measure apparatus (Bioseb®, Aix-en-provence, France). Animal was held with the tail and its two forepaws were brought into contact with the grid of the GS. The animals reliably grasped the grid bar, and then they were gently pulled away from the device. The GS measures the maximal force before the animal released the bar. Each session of animal testing was performed for 5 min. It was consisted of 5 trails/session. This measurement was performed once a week.

2.5. Ventilatory assessment

The measurement was performed on whole-body plethysmography apparatus (EMKA Technologies, Paris, France). An animal was placed in a closed chamber for 1.5 h. Plethysmograph measured pressure changes due to the animal's breathing. Tidal Volume (TV), Insipartory Time (Ti), Expiratory Time (Te), Peak Inspiratory Flow (PFI), Peak Expiratory Flow (PFE), Expelled Volume (EV), Time to expire 65% of the volume (RT), Minute Ventilation (MV) and breathing frequency (f) were recorded.

2.6. Euthanasia

At the end of the recordings, animals were euthanized. Euthanasia was performed according to the French law on Animal Care Guidelines, using protocols approved by the Animal Care Committee at Aix-Marseille University (AMU) and the Centre National de la Recherche Scientifique (CNRS).

2.7. Statistical Analysis

Data are given by mean +/- SEM. We used SigmaStat® 2.03 software (SigmaStat, Jandel Scientific Software), and we performed two way repeated measures analysis of variance (ANOVA) based on two factors (groups x times). Then all pairwise multiple comparisons were done with the Student-Newman-Keuls post-hoc method. Significant changes were considered when p value was below 0.05.

3. Results and Conclusion

3.1. Locomotor behavioral tests

The results of the ladder walking test for experimental groups are shown in figure 1.

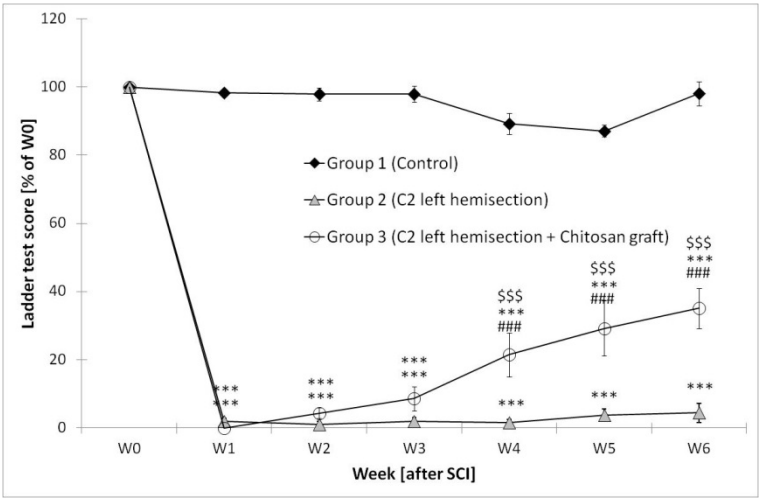

Figure 1. The results of the ladder walking test for experimental groups. The kinetics of recovery is given as a relative value, where reference value is W0 (W0=100%). *** indicate significant difference p<0.001 within group compared to W0. ### indicate significant difference p<0.001 within group compared to W1. $$$ indicate significant difference p<0.001 between groups 2 and 3.

Animals from Group 1 showed stable score from week 0 to week 6. Animals from Group 2 and Group 3 showed severe impairments one week after the injury. However, contrary to group 2, the ratio of correct foot placements to total steps of animals treated with chitosan graft (Group 3) showed some improvements over the period of testing. These improvements indicated a recovery of left forelimb movement capacity, mainly after the fourth week. From

week 0 to week 6, the score of group 3 was significantly higher than group 2, this latter remaining at the lowest score all along the protocol after injury. These results may indicate that neither the cortical control of fine sensorimotor coordination, nor the motor control and proprioceptive feedback in the spinal cord network level of rats in Group 2 were not restored or have recovered a functional activity after C2 left hemisection.

The results of the forelimb grip strength test for experimental groups are shown in figure 2.

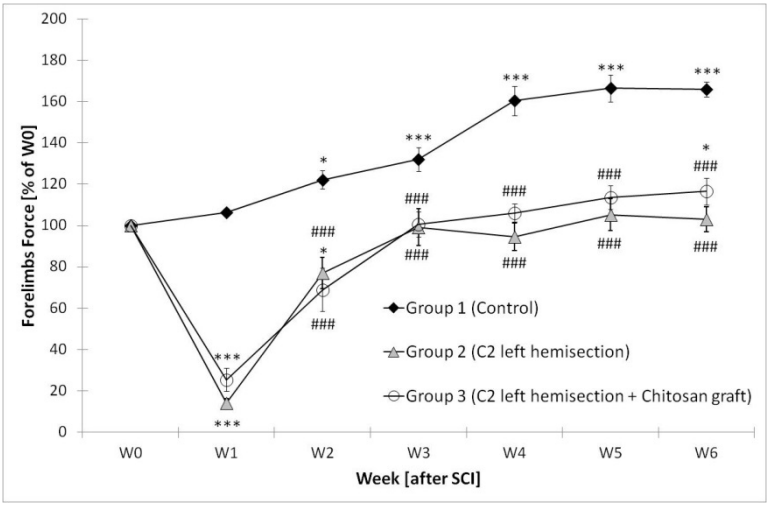

Figure 2. The results of the forelimb grip strength test for experimental groups. The kinetics of recovery is given as a relative value, where reference value is W0 (W0=100%). * and *** indicate significant difference $p<0.05$ and $p<0.001$ within group compared to W0. ### indicate significant difference $p<0.001$ within group compared to W1. $$$ indicate significant difference $p<0.001$ between groups 2 and 3.

Animal from Group 1 showed a strength increase during 10 weeks of the study. The increase in strength was due to animal growing, and consecutively its related neuromuscular efficiency. Animals from Group 2 and Group 3 showed severe decrease in grip strength on week after injury and high degree of improvement over the course of testing. However, their grip strength was lower than for control animals. Even if the strength recovery of animals on Group 2 and Group 3 looks similar over five weeks, the results obtained for week 6 indicate that their grip strength significantly exceeded the strength obtained at the beginning of the experiment in the group 3, whereas the data from group 2 just reached the level before injury.

3.2. Ventilatory assessment

The results of the respiratory flow analyzer parameters for experimental groups are summed up in table 2 (Appendix A). Briefly, all these ventilatory parameters remained stable or increased (TV, Te, EV) in group 1 due to animal aging. However, tidal volume, peak expiratory flow, peak inspiratory flow and expelled volume decreased in groups 2 and 3 after spinal cord injury. The values of group 2 remained significantly lower, whereas values of group 3 were close to the value of control group or values before injury, indicating partial recovery of ventilatory muscles functions altered after spinal cord lesion. Figure 3 shows results of the tidal volume (TV).

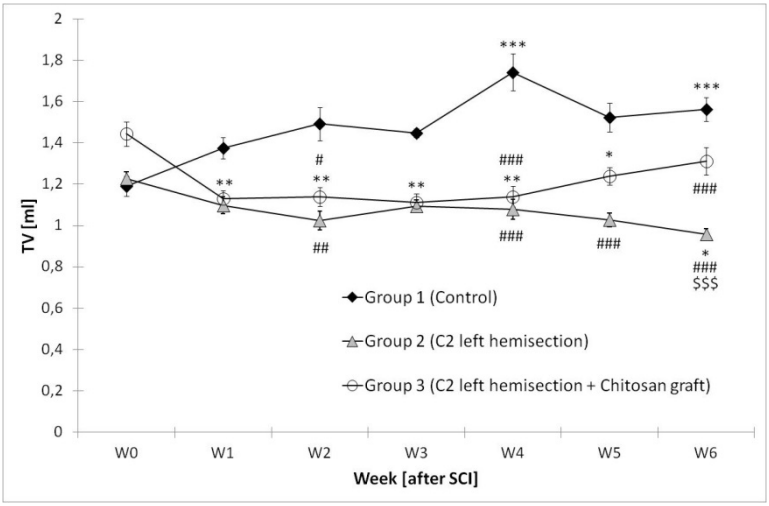

Figure 3. Measurements of the tidal volume (TV) for experimental groups. *, ** and *** indicate significant difference $p<0.05$, $p<0.01$ and $p<0.001$ within group compared to W0. #, ## and ### indicate significant difference $p<0.015$, $p<0.01$ and $p<0.001$ as compared to Group 1. $$$ indicate significant difference $p<0.001$ as compared to group 3 respective data.

The main purpose of this study was to test the hydrogel based on chitosan and β-glycerol phosphate as scaffold for spinal cord tissue regeneration. The graft was prepared in the form of hydrogel matrix in order to provide appropriate and stimulating environment for nerve regeneration.

Hydrogel obtained posses inherent flexibility and well developed three-dimensional structure, that mimics the *in vivo* extracellular matrix environment. It undergoes phase transition from sol to gel at physiological temperature of mammal body (37˚C). This property enables its direct delivery to a target area via injection. Moreover, hydrogel obtained can be easily shaped into desired forms *at situ* what makes them more ideal for implantation into complex spinal cord injuries. It exhibit good biocompatibility and biodegradability. In addition it has been proved that chitosan support adhesion of the nerve cells and neurite outgrowth.

The behavioral test results as well as respiratory data indicate a better functional recovery of animals receiving hydrogel in the lesion cavity in comparison with animals with non implanted. Based on the results obtained it seems that chitosan-β-glycerol phosphate hydrogels are excellent candidate for spinal cord regeneration, and could be used as adjuvant with other treatments in order to enhance functional recovery.

Acknowledgements

This work was supported by grants from National Science Center of Poland-Grant N UMO-2011/01/B/ST8/06686, Aix-Marseille University (AMU), Centre National de la Recherche Scientifique (CNRS) and “Combattre la Paralysie” Association.

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Appendix A : The respiratory data

		Groups			ANOVA	
		G1 Control	G2 Lesion	G3 Lesion + Chitosan	groups effect ($F_{2,76}$) p value	Interaction ($F_{4,76}$) groups x times (p)
TV (ml)	Before	1.19±0.05	1.2±0.03	1.44±0.06	F=10.45	F=12.80
	1 w after SCI	1.37±0.05 *	1.09±0.04 #	1.13±0.04 *** ##	p<0.001	p<0.001
	6 w after SCI	1.56±0.06 ***	0.95±0.03 ** ### $$$	1.31±0.07 * ##	G2 ≠ G1 and G3	
Ti (ms)	Before	239±9	237±19	240±8		F=4.79
	1 w after SCI	212±14	209±11	229±9	NA	p<0.01
	6 w after SCI	260±14	217±7 ###	212±7 *** ###		
Te (ms)	Before	355±15	339±28	366±13	F=8.97	F=13,47
	1 w after SCI	342±25	276±4 **	283±13 ***	p=0.001	p<0.001
	6 w after SCI	442±29 ***	271±11 ** ###	275±11 *** ###	G1 ≠ G2 and G3	
PIF (ml/s)	Before	8.02±0.44	8.88±1.46	9.94±0.66		F=4.40
	1 w after SCI	11.24±0.77	8.37±0.72	7.72±0.37 #	NA	p<0.01
	6 w after SCI	10.29±0.48	7.34±0.33 ## $$	11.25±1.14 *		
PEF (ml/s)	Before	8.49±0.29	9.44±1.22	9.29±0.45	F=3.72	F=4.25
	1 w after SCI	10.38±0.35	7.75±0.75 #	7.18±0.17 ** #	p<0.05	p<0.01
	6 w after SCI	10.29±0.39	7.20±0.40 * ## $	8.87±0.71	G1 ≠ G2 and G3	
EV (ml)	Before	1.20±0.05	1.24±0.03	1.39±0.06	F=11.01	F=13.90
	1 w after SCI	1.39±0.05 *	1.10±0.04 ## $	1.14±0.04 *** #	p<0.001	p<0.001
	6 w after SCI	1.58±0.06 ***	0.96±0.03 ** ### $$$	1.32±0.07 ** ##	G2 ≠ G1 and G3	
RT (ms)	Before	193±8	185±13	224±7	F = 4.00	F=8.35
	1 w after SCI	193±17	167±6	167±8 ***	p<0.05	p<0.001
	6 w after SCI	223±14	157±6 ###	168±6 *** ##	G1 ≠ G2	
MV (ml)	Before	128±5	149±26	152±9		F=3.12
	1 w after SCI	174±13	147±9	143±5	NA	p<0.05
	6 w after SCI	146±7	125±5 $	191±19 * ##		
f (bpm)	Before	112±4	125±23	111±6		F=2.73
	1 w after SCI	136±15	138±7	134±5	NA	p<0.05
	6 w after SCI	100±7	135±6 ##	155±10 ##		

Table 2. *The results of the respiratory flow analyzer parameters for experimental groups.*

TV = Tidal Volume, Ti = Insipartory Time, Te = Expiratory Time, PIF = Peak Inspiratory Flow, PEF = Peak Expiratory Flow, EV = Expelled Volume, RT = Time to expire 65% of the volume, MV = Minute Ventilation, f = breathing frequency

*, ** and *** indicate significant difference p<0.05, p<0.01 and p<0.001 compared to before value within respective group
#, ## and ### indicate significant difference p<0.05, p<0.01 and p<0.001 compared to similar value in Control group (G1)

$, $$ and $$$ indicate significant difference p<0.05, p<0.01 and p<0.001 compared to similar value in Chitosan group (G3)
NA , not applicable