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The Role of Chitosan Membranes on scarring process following lumbar surgery

Post-Laminectomy Experimental Model

Master Degree's Thesis on Experimental Pathology

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Abstract

Introduction: Post-operative scarring process on lumbar surgery is object of several studies mainly because of the epidural fibrosis formation. Chitosan have shown promising effect fibrosis prevention. The aim of this study is to determine the influence chitosan-silane membrane on the lumbar surgery scarring process. These membranes have improved mechanical strength which makes it suitable to mantain a predefined shape.

Materials/methods: Fourteen New Zealand male rabbits underwent two level lumbar laminectomy. Laminectomy sites were randomly selected for biomaterial or control. Chitosan membranes were prepared and care was taken in order to make it adapted to the bone defect dimensions in a way that it covered the totality of the defect and also the bone margins. Histological analysis was performed by haematoxylin/eosin and by Masson's trichrome staining four weeks after laminectomy.

Results: Microscope observations revealed the presence of a well-organized regenerating tissue, integrated in the surrounding vertebral bone tissue with a regular and all-site interface on the chitosan sites, in clear contrast with the presence of a disorganized regenerating tissue with aspects consistent with the persistence of a chronic inflammatory condition, on control sites.

Conclusions: The results of this study clearly demonstrated that chitosan had an organizing effect on post-operative scarring process. The presence of the chitosan membrane resulted on a well-organized tissue integrated in the surrounding vertebral bone tissue with signs of regenerative bone tissue in continuity with native bone. This can be a major feature on the dynamics of epidural fibrosis formation.

Introduction

Materials and Methods

Results

Discussion

Conclusion

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Lumbar disk herniation

The sciatic pain, which is pain radiating from the lumbar region to the inferior limb, is a condition previously described in ancient Rome and Greece manuscripts, although not associated with a spine disorder. In fact, the intervertebral disc was described much later, by Vesalius, in 1555. In 1764, Cotugno established an association between sciatic pain and the sciatic nerve and 15 years later, Pott established a relationship between this type of pain and a spine disorder. It's only in 1858 that Luschka, based on autopsy studies, observed and registered degenerative disc processes. In the early years of the XX century, several authors have proposed an association between sciatic pain and lumbar disc herniation (Sachs and Fraenkel, 1900; Bailey and Casamajor, 1911; Goldthwait, 1911) but without major acceptance. This perspective have changed in 1929 when the first case was presented by Dandy and, in 1934, Mixter and Barr cleared its pathophysiology on a 34 cases review. [1,2,3,4,5]

In these pioneer times, the surgical procedure proposed by Mixter and Barr involved a wide removal of the vertebral laminae and a discectomy throughout an intradural approach. It was in 1977 that, not only Yasargil, in Switzerland, as Caspar, in Germany, described microdiscectomy surgical techniques, by an extradural approach, and using the surgical microscope. [4,6]

Meanwhile, the acquired clinical experience led to the identification of additional factors enrolled on the sciatic pain pathogeny. Therefore, it's currently assumed that the pulpous nucleus

has a local sensitizing effect on the nerve and the pain is triggered by the local deformity induced by the herniation. [5]

Currently, as the disc herniation sciatic pain not associated with neurological deficits is a benign condition, that could resolve spontaneously, the therapeutic approach is usually initiated with conservative treatment, reserving surgery for selected cases. [7]

Lumbar discectomy

Actually, the lumbar discectomy is the most common neurosurgical procedure performed in the US, with about 300.000 lumbar discectomies being performed each year. This is a direct result of the near 15 million medical consultations for lumbar pain each year, together representing a direct and indirect cost of over US$ 50 billion, per year. [4]

The surgical technique for lumbar discectomy is performed under general anaesthetics, with the patient on a ventral decubitus position, with care taken to avoid pressure over the abdomen in order to minimise the epidural haemorrhage (which would be profuse if there is an elevated venous pressure). The patient hips and knees should be flexed in order to provide posterior distraction of the interdisc space. The skin incision is performed longitudinally, on the midline and to the level of the dorsolumbar fascia. [1,2,4,8]

A 10-12 cm long skin incision is suggested by Benzel that argues that a wider approach as the advantage of, among others, inducing less trauma to the paravertebral muscles. [2]

However, Javedan and Sonntag suggested shorter skin incisions that should variate arround 3 to 6 cm long and Koebbe et al. suggested even shorter skin incisions from 1,5 to 2,5 cm long. [4,8]

After the division of the subcutaneous fat and identification of the dorsolumbar fascia, the latter is divided laterally to the spinous process in order to preserve the interspinous ligaments. The paravertebral muscles are then separated from the bone through subperiosteal dissection and division of the ligaments inserted on the inferior edge of the cranial vertebra. From this stage on, most of the authors recommend the use of the surgical microscope; however, Benzel does not use it in his description of the standard discectomy technique.

Then begins the stage of bone removal which starts by the inferior edge of the superior hemilamina in its most medial portion, which is where the canal has it's maximum diameter, therefore minimizing the risk of lesion of the intracanalar structures. The bone removal extends laterally on the inferior edge of the cranial hemilamina with exposure of the ligamentum flavum. This ligament has insertions on the inferior edge and intracanalar surface of the cranial lamina therefore separating the lumbar dura from the plane of bone manipulation, minimising the risks of dural lesion. In this stage the bone removal have provided a comfortable space for opening of the ligamentum flavum. After removal of the exposed ligmantum flavum, its rostral insertions should be removed together with a bone rim. [1,2]

Performed this way, the opening of the vertebral canal should be about 20-25 mm long on the longitudinal axis and 10-15mm on the transverse axis. Such a window would be wide enough to allow a clear visualization of the tecal sac and the nerve root and would permit mobilization of these structures to the approach of the interdiscal space. During the bone and ligament removal, the handling of the epidural fat should be performed with great caution. The preservation of this tissue is considered of great importance because it could be protective against the perineural fibrosis formation. [1,2]

Before the nerve root medial mobilization to allow the approach to the intervertebral space, an ipsilateral foraminotomy should be performed in order to add space to the mobilization of the nerve root with minimal stretching. [8]

After the medial retraction of the nerve root it could be visualized herniated fragments which should be removed in order to expose the intervertebral space. The posterior longitudinal ligament and the annulus should be divided in order to proceed to the removal of the nucleus pulposus. After this removal, any epidural bleeding should be controled with bipolar coagulation or haemostatic agents. [1,2,8]

The closure will include suture of the dorsolumbar fascia, subcutaneous layers and skin. [1,2,4,6,8,9]

The lumbar disk surgery estimated success rates are between 49 and 90% with a reintervention rate of 4 to 15%.[10,11] On the retrospective study of 654 patients who underwent surgery on the Barrow Neurological Institute, Pappas et al. reported good/excellent surgical outcomes in most of the patients with a reintervention rate of 11% and with 3% of local relapse of lumbar disk herniation. The surgical technique described by the authors included the disk removal aside from the extruded fragment. The technique proposed by Williams, with exclusive removal of the herniated fragment had about 9% of relapse rate, against the 4% rate described by Yasargil and Caspar with radical disk removal and curettage of the vertebral plates. In the study performed by Pappas et al. there was no significant difference on the outcome of patients who underwent placement of fat graft after discectomy against those who underwent simple discectomy.[12]

On a 1996 paper, Fritsch et al. retrospectively evaluate 182 cases of FBSS lumbar surgical reinterventions and, reporting a post-discectomy reintervention rate of 10,8%, conclude that instability and epidural fibrosis are the most contributive factors to poor results and reinterventions related to lumbar disk surgery.[13]

Ambrossi et al., on a retrospective study of 156 consecutive patients who underwent surgery for lumbar disk herniation, at the Johns Hopkins Hospital, reported that 88% of patients had no symptoms or minimal symptoms which do not prevented their daily activities, at 1-year follow-up. They also reported 12% of patients with local relapse

of lumbar disk herniation. Of these 17 patients, 11 needed a second surgical intervention with an estimated cost of $39.836 USD vs. $2.315 USD for the 6 patients who underwent conservative treatment.[14]

Abramovitz and Neff, on the first prospective study designed to evaluate the outcome of lumbar disk surgery, reported 78,7% of good results at 3 months follow-up (with 19,2% of medium and 2,1% of poor results) and 79,3% of good results at 1 year follow-up (with 16% of medium and 4,7% of poor results). The authors argued that those results are related directly to a thorough patient selection. To illustrate this statement they point out the positive association between purely radicular pain and good surgical results and its negative association with predominantly axial pain. These authors relevance the patient's psychological evaluation, pointing it as a factor that will influence the surgical results.[15]

The patient psychological evaluation, albeit thorough, has no capability of surgical outcome prediction. However patients with the best surgical outcomes are mostly stable, cautious, defensive, confidant, with realistic concerns about their condition, could be mildly depressive but optimistic to the surgical outcome and with abilities to deal with setbacks. The patients with poorer surgical outcomes are less sable, unpredictable, depressive and with less ability to deal with stress and setbacks.[16]

These results, raised the debate over the role of surgery in the treatment of this condition. The SPORT trial (Spine Patient Outcomes Research Trial) is a prospective, multicentric, randomised study, in which the 2008 update gave advantage to surgical over conservative treatment (with 4 year follow-up) not only on pain relive but also on functional recovery. In this trial, the patients enrolled to the surgical

group were patients with pain lasting from two or more months and/or neurological deficit. The authors registered a post-discectomy recurrent disk herniation rate as high as 7% and a re-operation rate of 11%.[17]

Failed Back Surgery Syndrome

The so called Failed Back Surgery Syndrome (FBSS) it's a nonspecific condition that translate a surgical result that do not meet the expectations established before the surgery, nor for the patient, neither for the surgeon.[18] Therefore, its etiology could be related to poor criteria of patient selection, misdiagnosis, and a suboptimal selection of the surgical approach, poor surgical technique, and impossibility of achievement of the surgical goals and/or recurrent pathology.[19,20]

The structural causes of this syndrome could be identified in about 90% of the patients by complementary means. The most common causes of FBSS are the foraminal stenosis (25-29%), discogenic pain (20-22%), pseudarthrosis (14%), neuropathic pain (10%), recorrent lumbar disk herniation (7-12%), iatrogenic instability (5%), facet pain (3%) and sacro-iliac joint pain.[18,20,21] Waguespack et al. report similar causes for FBSS but they point out psychological problems as a cause of 3% of FBSS.[21]

The presence of epidural fibrosis is described in about one third of the patients following lumbar disk surgery.[10] However, the relationship between epidural fibrosis present on imaging post-operative studies and patients complaints is not entirely clear. Cinotti et al., on a lumbar disk surgery reoperation study had not found a clear relation between the amount of epidural fibrosis and the outcome of reoperations.[22] Nevertheless, the presence of epidural fibrosis is considered to be related with a poor surgical outcome for FBSS repeated surgeries. North et al. report a good outcome for those patients who do not present with post-operative epidural fibrosis in an amount that would indicate a reoperation.[10,20]

The epidural fibrosis after lumbar discectomies consists on a replacement of the normal epidural fat layers by post-operative fibrotic tissue. This fibrotic tissue enhances dura-mater and nerve root adhesions to the surrounding tissues, being the nerve changes resulting from those adherences pointed out, theoretically, as the main mechanism for recurrence of radicular pain following lumbar discectomy.[23,24]

Post-operative healing

The FBSS etiology is not clearly understand and local relapse of disk herniation and post-operative epidural fibrosis are pointed as the leading causes of this syndrome. The epidural fibrosis development after lumbar laminectomy is an unavoidable event and it develops 6 weeks to 6 months after surgery. The epidural fibrosis pathogenics is not fully cleared however, it's commonly accepted that the remaining debris on the surgical site, act as fibrotic stimuli.[25]

According LaRocca and Macnab, the membrane formed after laminectomy would be the result of the destruction of the epidural fat, surgical site hematoma and of the penetration of paravertebral muscles on the vertebral canal. The hematoma, present on the surgical site would be gradually absorbed and replaced by granulation tissue. The granulation tissue maturation would result on the fibrotic epidural tissue.[26]

Therefore, the epidural fibrosis is the result of post-operative hematoma invasion by thick fibrotic tissue, starting on the periosteum fibrous layer and fibroblast migration from the deep layer of the paravertebral muscles. This process can progress to the vertebral canal and create adherences to the dura-mater and nerve roots.[27,28] The fibrosis formation creates adherences that fix the root in the lateral recess and make it more sensitive to compressive or tension forces or to isquemia which would be, otherwise, assymptomatic. The factors included in the formation of perineural fibrosis include the amount of

trauma inflicted to the soft tissues during surgery and the amount of blood collected on the surgical site.

The epidural fibrosis could be associated to tissue damage processes mediated by inflammation and cellular adhesion molecules. The cellular adhesion molecules have been involved on the adhesion or scar tissue formation on the pathogenesis of some diseases, such as pulmonary fibrosis. In this context, there is some cellular adhesion molecules upregulation on the sites of active disease which promotes leucocyte recruitment and activation. The interaction between leucocytes and pulmonary parenchymal cells (and not the amount of inflammatory cells) will determine the pulmonary fibrosis formation. [29]

To test this hypothesis on the context of post-laminectomy epidural fibrosis, Sabuncuoglu et al. used monoclonal antibodies against intercellular adhesion molecule 1 and it's ligand, CD-18, on an experimental laminectomy model. They reported, with this strategy, an epidural fibrosis reduction on the treated animals.[30]

As appealing as this hypothetical model could be, some studies suggested that epidural fibrosis would be considered as a radiological entity without relationship with patient's complaints.[25,31]

In 1996, Ross et al. presented a prospective, randomized, controlled, blind and multicentric trial of 197 patients with a significative association between epidural fibrosis and recurrent radicular pain. However, this study presents some curious data. It is important to realise that only a fraction of patients who had severe fibrosis (19,4%) had recurrent radicular pain and, on the other hand, 6% of patients with less extensive epidural fibrosis, had recurrent radicular pain.[23]

The prevention of post-operative perineural fibrosis has been object of increasing interest as this condition has been pointed out has one of the main causes of lumbar surgery failure, commonly known as Failed Back Surgery Syndrome.

Therefore, it would be expected that a more minimalist and 'clean' surgery (concerning a thorough removal of the ligamentum flavum debris during fenestration and an effective haemostasis during closure), would have better outcomes.

Surgical options

For the therapeutic approach to lumbar degenerative disease, especially lumbar disk herniation, many treatment modalities were described in the early years of the 20th century. The first surgical technique description for lumbar disk herniation belongs to Mixter and Barr, in 1934.

On the attempt to minimize the exposure of soft tissues for the lumbar discectomy, Yasargil, in 1977 and Caspar and Loew in 1978, described microsurgical techniques with the objective of minimize the skin incision, the surgical trauma to paravertebral muscles and nervous structures and allow a better visualization of the intervertebral space with the surgical microscope.[32]

Javedan and Sonntag suggested the preservation of the medial portion of the ligamentum flavum in order to protect the intracanalar structures against post-operative scarring. On the technique described

by those authors it's not recommended the preservation of the epidural fat layer.[8]

There are descriptions of more minimalist approaches with minimal bone, ligamentum flavum and/or epidural fat removal, as the technique described by Williams.[6]

Facing the concern with FBSS, Williams developed the technique of microsurgical exclusive removal of the disk fragment responsible for the nerve root compression. He assumed that a more conservative approach would prevent most of the problems associated to the FBSS (epidural fibrosis, discogenic pain, spondilosis, arthrosis and instability). Williams recommend a minimal removal of bone, ligamentum flavum and epidural fat and he called this approach a sequestrectomy. It's postulated that the fibrous annulus opening from where the nucleous pulposus herniated, would close spontaneously. The literature about this technique reports 86-97% success rates.[6]

On a recent meta-analysis of the published series comparing sequestrectomy and conventional discectomy, it's reported that, on long-term, conventional discectomy presents with a higher incidence rate of lumbar pain and recurrent sciatic pain and, on the other hand, sequestrectomy presents with a higher incidence of recurrent lumbar disk herniation.[33]

Other authors have proposed small changes to the surgical technique with the goal of diminishing post-operative epidural fibrosis formation. Several authors described ligamentum flavum preserving techniques believing that, preserving this anatomical plane, it would act as a barrier to fibrosis formation and epidural adherences. [9,34,35,36]

The ligamentum flavum is the ligamentous structure which contains the highest percentage of elastic fibers of all human tissues. In

addition to its biomechanical properties, it physically covers the dura-mater, the nerve root, the epidural fat, acting as a protective barrier for those structures. The results for this type of surgery are similar to those of standard discectomy with less post-operative epidural fibrosis and with more easiness in a second surgery when needed.[9,34]

Oktenohlu et al. describe a technique of preservation of the ligamentum flavum with evidence of reduced local post-operative fibrosis at 6-months follow-up. On this technique, the authors propose a circunferencial incision in the ligamentum flavum forming a small flap that would be sutured into its position after the diskectomy was performed.[9]

The ligamentum flavum could be responsible for the development of perineural fibrosis when it's not fully removed during the surgical procedure and remnants persist under the lamina. Therefore, Benzel resumes the approach to the ligamentum flavum in two distinct options: minimal fenestration with near total preservation of the ligament or, complete removal, insisting on the thorough removal of the ligament under the rostral lamina, on the lateral recess and on it's inferior insertion, which is on the superior edge of the inferior lamina.[2]

The epidural fat is histologically different from the subcutaneous fat. The histological studies performed by Wolfram-Gabel et al., describe the epidural fat as a tissue composed by a homogenous adipocyte population (concerning shape and size) with a minimal amount of connective tissue. These characteristics are in contrast with those of the subcutaneous fat in which the adipocytes have different sizes and shapes and there are bundles of connective tissue that subdivide this layer in lobules. Despite all these differences the

distinctive feature of the epidural fat is the presence of empty spaces with a special arrangement.

These empty spaces would divide the epidural fat in several layers allowing each layer to slide over the adjacent ones and this characteristic earned the denomination of 'sliding spaces' from the authors. Therefore, the histological features of the epidural fat explain its semi-fluid characteristic and confirm its specificity. Its localization, at the level of the lumbar spine mobile segment (on adults, since on fetuses these tissue is uniformly distributed, surrounding the entire dural sac), suggests that this structure has an important role on the interface between the posterior surface of the dural sac and the bony-ligamentous structures forming the posterior wall of the vertebral canal.[37]

Therefore, the absence of the fat epidural tissue after lumbar surgery and the formation of epidural fibrosis could lead to compression and/or adhesion of the nervous structures. This is the main reason for, as said before, the recommendations of great care handling the epidural fat tissue during lumbar surgery.

Minimally Invasive Spine Surgery

On the field of minimally invasive discectomy, the first steps were given by Lyman Smith who, in 1963, injected a chemical agent, percutaneously, on the disk space with the objective of nucleous pulposus destruction. This was a papain-derived chemical agent which

gain the designation of 'chymopapain' and it's role on the dissolution of the nucleous pulposus had been previously observed by the author on experimental studies using a rabbit model. This process, known as chemical nucleolisis, was abandoned, mainly, because of its high rate of serious complications (anaphylactic reactions) and because it's efficacy was inferior to the standard discectomy. [38,41]

About a decade later Hijikata, in Japan, described a technique of manual percutaneous nucleotomy which consisted on the removal of the nucleous pulposus through a working tube placed on the disk space percutaneously. This process had the objective of reducing the intra-disk pressure with the following reduction of the irritative effect over the nerve root and fibrous annulus and peridisk area pain receptors. After successive changes and improvements of the technique and despite the high success rates observed (similar to the standard discectomy), these techniques are not used commonly because, mainly, the impossibility to approach the lateral recess and perform foraminotomy and the impossibility, on a high percentage of cases, to access the L5-S1 disk space percutaneously. [39,41]

In the mid-eighty's Gary Onik proposed an automated percutaneous lumbar diskectomy using a probe with simultaneous suction and cutting function. This technique also was of inferior efficacy to the standard diskectomy and it was contraindicated on patients with extruded hernia fragments (about 70% of the technique related failures was related with hernia fragments not visualised on the pre-operative imaging). The laser diskectomy technique was also used on the eighty's with very satisfying results but not superior to the results achieved with the standard diskectomy.[40,41]

The endoscopic diskectomy is a promising technique with some advantages over the other minimally invasive diskectomy techniques because it allows the approach and the direct visualisation of the disk and the nerve root compression. However, there are limited advantages over the standard diskectomy and it has a flat learning curve by which it's a low popularity approach.[40,42,43]

Endoscopic techniques are also under development for the treatment of FBSS, specially the endoscopic adhesiolisis, which allows either the local drug administration.[44]

Other strategies

Several materials have been studied with the goal of prevention of epidural fibrosis and it's complications, such as autografts (free and pedicled fat tissue grafts and repositioning of the ligamentum flavum), biomaterials used as mechanical barrier (polytetrafluoroethylene membranes, Gelfoam, Silastic membranes, Surgicel, Avitene, polymethyl methacrylate, carbon hydrates based synthetic polymers and Goretex), topical application of drugs for fibroblast function and infiltration reduction (urokinase, tissue plasminogen activator, mitomycin C, hyaluronic acid and glucocorticoids), intra-operative application of CO2-laser therapy and localized external-beam radiotherapy application peri-operativelly. [45,46,47,48,49,50,51,52]

A thorough hemostasis is suggested to avoid epidural fibrosis formation. Several approaches have been proposed for this purpose.

An absorbable gelatine sponge was tested and it was reported that it acted as a stimulus to fibrosis production and therefore it's use is unadvised.[2,45]

The platelet-derived growth factor (PDGF) is a mitogenic agent responsible for the migration and proliferation of miofibroblasts and which could have an important role on the epidural fibrosis formation within the local post-discectomy hematoma. Therefore, the prevention of hematoma formation on the surgical site is of extreme importance. The tissue reparation requires the fibrin removal either by leucocytic phagocytosis, as by fibrinolysis mediated by local release of plasminogen activator. Those hypothesizes are supported by the report of decreased fibrotic tissue formation with local application of recombinant tissue plasminogen activator (rt-PA), on animal models.[46]

Ceviz et al., on an experimental animal model, tested uroquinase on the prevention of peridural fibrosis assuming that this enzyme, acting on the surgical site, would accelerate the degradation of the hematic debris. Therefore, the authors verified an uroquinase epidural fibrosis preventive effect confirming the important role of the surgical site hematic debris for the fibrosis formation.[47]

The use of free fat grafts it's commonly utilized by spine surgeons. The rationale for the use of free fat grafts on the laminectomy site over the dura-mater is that it could reduce the epidural fibrosis and allow a better interpretation of post-operative imaging.[31] The effect of this technique is, however, questionable. The fat grafts besides producing a mass effect, they shrink over time, there are descriptions of fibrotic adherences between the graft and the dura-mater (probably with origin on a graft surounding hematoma) and a foreign body reaction itself could promote fibrosis and adherences. In addition to

those factors, it is almost impossible, technically, to apply the fat graft in the entire nerve root circumference.It has been reported some cases of graft displacement associated with neurological deficit. On the other hand, it was described that there is a reduction of the graft volume over time, presenting, over a year, with about 30% of its initial volume. Other complications associated to fat grafts are: fat graft inflammation and aseptic necrosis.[45,48,49]

One of the most popular gestures used with the objective of peridural fibrosis prevention is the topical application of steroids even though without clinical evidence based on scientific studies. The benefit of the topical steroid application was achieved on animal experimental models. However, some controversial results are published and there are reports on scaring delays and of frequent abscess formation.[50]

The Aloe Cepea extract, or 5-ureo-hidantoin, is a natural molecule derived from the onion with bactericidal and anti-inflammatory effects. The mix used by Temiz et al., on animal models, was effective on the reduction of epidural fibrosis with no inhibition of bone repair. Besides, it's a cheap material, liable to sterilization procedures and of easy applicability.[51]

Radiotherapy has been used on clinical practice to prevent post-operative keloid scar recurrence since the fifties. Radiation reduces fibroblasts proliferation which prevents keloid scaring formation. Radiotherapy as also been tested on heterotopic bone growth prevention after total hip replacement.

The ionizing radiation radiobiological target seems to be pluripotent mesenquimal and radiosensitive cells which have a stimulus to proliferation and differentiation due to a specific trauma, such as

surgery. Gerszten et al. verified an epidural fibrosis reduction, on experimental models, using a 700 cGy single dose administered through an 6 MeV electron beam performed before, during or after a laminectomy procedure.[52]

Also on experimental models, it has been verified that the radiotherapy inibitory effect over epidural fibrosis formation would be similar to the application of a Goretex membrane.[45]

However, when using radiotherapy one should be aware of its adverse effects, specially it's direct effect on the surgical wound repair (local erythema, suture dehiscence, hematoma or infections) and the risk of neoplastic lesions on the long run.[45,52]

The ideal agent for epidural fibrosis and adhesions prevention should have, therefore, the following characteristics: 1) Scar tissue to dural structures adhesions preventive effect; 2) leptomeningeal arachnoiditis preventive effect; 3) No effect on dural repair when it is lacerated with CSF leakage; 4) No inflammatory induction effect on perineural tissues.[53]

The Adcon-L, a gelatine hydrocarbonated polymer has proven to be effective on the reduction of perineural fibrosis, not only on histological animal studies as on human radiological studies.

In the late nineties it was tested the Adcon-L (Gliatech, Cleveland, OH, USA), an animal origin substance, absorbable, with a biological life of 6 weeks and which had demonstrated a significative reduction of scar tissue formation on animal models.[54]

This effect was equally demonstrated on human trials, 6 and 12 months post-surgery. In addition to this, on clinical trials, the patients treated with Adcon-L (Gliatech, Cleveland, OH, USA) showed a 50% improvement on activity related pain scores, compared with 37% improvement on control group.[55,56]

Adcon-L (Gliatech, Cleveland, OH, USA) become, therefore, the most promising material for epidural fibrosis prevention however, it was withdrawn from the market due to multiple CSF fistulas human reports, due mainly to a diminished capacity of dural repair when ADCON-L is used. It was also suggested that this compound would interfere with bone regeneration which could impair fusion.[53,57]

This biomaterial is applied all-around the nerve root.[54]

On animal models it was also tested the hialuronic acid which is used currently in other surgical fields with the objective of fibrosis reduction. The local use of high molecular weight hialuronic acid has shown to reduce the post-laminectomy epidural fibrosis formation, on an experimental study with an animal model. The authors suggested this use based on the fact that the fetal lesion repair doesn't have any

scar tissue involved, during the first two quarters of pregnancy.[58,59] The fetal lesions repair occurs through an extracellular matrix which creates an unique environment in which the key condition is the presence of high molecular weight hialuronic acid. The fetal tissue repair matrix also has low concentrations of hialuronidase and beta tumoral growth factor. These authors verified that the use of barrier materials was more effective on the post-surgery epidural fibrosis formation than the use of high molecular weight hialuronic acid or the gel form ADCON-L, on the surgical site.[58,59,60] However, the use of bone wax, synthetic membranes (as, for example, silastic) or fascia grafts were also tested, but it's use is not recommended because it was not verified, on clinical trials, a preventive effect for post-operative residual nerve root pain.[2]

Some authors, based on the use of heparin embedded collagen membranes for the prevention of post-laparoscopy intra-peritoneal adherences, have used this material on laminectomy experimental models. Temiz et al used a mixture of heparin, alantoin and Aloe cepea extract. The alantoin is known by its supportive effects on primary and secondary scaring processes.[51]

The polilactid resorbable film is used on clinical practice with the objective of preventing soft tissue adherences to visceral structures. Studies conducted by Klopp et al. and by Welch et al. showed that this material reduce epidural fibrosis and does not interfere with dural repair.[53]

It was verified that polyehtylene oxide and carboxymethylcellulose membranes prevented the formation of peritoneal adherences on animal models. Rodgers et al. tested that compound (Oxiplex; FzioMed, Inc., San Luis Obispo, CA), under a gel and membrane forms,

on animal laminectomy models verifying the same preventive effect on the epidural fibrosis and adherences formation, in the gel and membrane forms.[57]

Kim et al. tested the Oxiplex/SP gel composed of polyethylene oxide and carboxymethylcellulose. Pre-clinical studies showed that the carboxymethylcellulose reduced adhesion and epidural fibrosis formation. The polyethylene oxide, on is turn, interacted with proteins that, when organised, contribute to fibrosis formation. Based on favourable experimental results, these researchers conducted a blind, randomized, multicentric pilot clinical study with evidence of clinical benefit from the use of this biomaterial on 6 and 12 months follow-up. [61]

The use of a vicryl mesh was tested and got good results. Superior results were achieved when using a Goretex membrane. This is a very thin membrane (0,1mm thick), malleable, synthetic, biocompatible and non resorbable. This membrane presents two functionally distinct surfaces: one surface with texture due to 22 micron pores and a flat surface with small 3 micron pores which minimizes tissue adherences. Alkalay et al. have tested bioelastic, biosynthetic polymers successfully demonstrating its safety and efficacy on epidural fibrosis reduction, on animal models. Those authors also underline the importance of an accurate application of the material, overlapping the membranous material over the laminectomy bony limits in order to achieve a total filling of the bone defect which would prevent the penetration of fibrotic tissue on the vertebral canal.[27]

Akeson et al., based on post-laminectomy epidural fibrosis animal models, suggested that the application of a high molecular weight hyaluronic acid gel or the interposition of a Goretex membrane over

the bony defect would have similar effects on the post-operative fibrosis and adherences formation. The authors discourage the application of membranes inside the vertebral canal, over the dura-mater, and suggest it's application over the laminectomy borders. The membrane itself would lead to the formation of fibrotic tissue in order to form an envelope involving it and not in the vertebral canal.[60]

However, several studies stress that the most effective strategy would be the interposition of a barrier, based on collagen quantification studies, on experimental models.[58,59]

Kim et al. using the high molecular weight hyaluronic acid (Healon; Pfizer, Inc., New York, NY) epidural fibrosis preventive features have studied the behaviour of mice after laminectomy with Healon application. They've concluded that the animals who developed less epidural fibrosis was those with Healon application laminectomy and these mice presented with a reduction of post-laminectomy pain-related behaviour.[62]

Chitin and chitosan

Chitin is a co-polymer of N-acetyl-glucosamine and of N-glucosamine randomly distributed either in blocks or along the biopolymer chain, depending on the process method used to obtain it. When the number of N-acetyl-glucosamine units is superior to 50% it is called chitin and

when the number of N-glucosamine units is superior that biopolymer is then called chitosan. Of these two biopolymers, chitosan has been more used on research due to its prompt solubility on diluted acids which males this polymer more accessible for chemical reactions use. Chitin and chitosan are obtained from shellfish such as crabs and shrimps. It could be possible, on a near future, chitin or chitosan production through biotechnology techniques, especially if it's for medical applications.(63)

Since the mid-sixties that a considerable amount of chitin and chitosan related research comes from Asia, mainly from Japan.

Nowadays it is globally accepted that any material, to be considered implantable should be of natural origin. This happens because these natural materials have already showed a better capacity for promoting cure on a higher rate and, therefore, it believes that it would show a better compatibility with humans. In addition, new concepts over medical use implantable devices, mainly on tissue engineering, points to a combination of a biomaterial in which cells are sown and which would suffer a biodegradation process into non-toxic products.(64)

Chitosan hybrids

While chitosan matrices have low mechanical strength under physiological conditions and are unable to maintain a predefined shape after transplantation, their mechanical properties can be improved by modification with a silane agent, namely γ-glycidoxypropyltrimethoxysilane (GPTMS), one of the silane-coupling

agents which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups. Finally, the silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan, GPTMS and siloxane network. By adding GPTMS and employing a freeze-drying technique, an hybrid chitosan membrane with pores of about 110 μm diameter and about 90% of porosity is obtained.[65]

A synergistic effect of a more favorable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan hybrids, and the presence of silica ions may be responsible for the good results in promoting post-traumatic nerve regeneration. In fact, chitosan hybrids were successful in improving sciatic nerve regeneration after axonotmesis, both isolated or using non-differentiated human mesenchymal stem cells.[66,67]

Significant differences in water uptake between commonly used chitosan and hybrid chitosan membranes were reported and it has been shown that they retain about two times as much biological fluid. The addition of GPTMS improves the wettability of chitosan surfaces. The improvement of the wettability of chitosan surfaces is achieved by the addition of GPTMS. Wettability of material surfaces is one of the key factors for protein adsorption, cell attachment and migration, which can explain, at least partially, the good results on nerve regeneration studies.[66,67]

Despite this factors, the significant improvement of axonal regeneration obtained in crushed sciatic nerves surrounded by hybrid chitosan membranes suggests that this material may not just work as a

simple mechanical scaffold but instead may work as an inducer of nerve regeneration. The neuroregenerative property of hybrid chitosan membranes might be explained by the action on Schwann cell proliferation, axon elongation and myelination. Yet, the expression of established myelin genes may be influenced by the presence of silica ions which exert an effect on several glycoprotein expression.[66,67]

Other studies reveals chitosan ability to promote cell membranes fusion after damage as chitosan is able to form large phospholipid aggregates by inducing the fusion of small dipalmitoyl phosphatidylcholine bilayers — which are major endogenous components of the plasma membrane. Moreover, chitosan preferentially targets damaged tissues.[68,69]

On the other hand, on biocompatibility studies, hybrid chitosan membranes elicits a mild inflammatory response (consisting of a mixed inflammatory infiltrate and mild peripheral fibrosis) that decreases gradually until the third month. This findings are in clear contrast with the exuberant pyogranulomatous inflammatory reaction involving numerous neutrophils (PMN) and macrophages surrounded by a thick, immature and highly vascularized fibrous capsule (granulation tissue) developed by non-hybrid chitosan membranes.[70]

An amalgam of a mineral polymer originates a composite material with the polymer resistance and flexibility and the mineral strength and hardness. Over the last few years there were generated some polymer and calcium composites in which the potential advantage

could be not only an increase of the osteogenic potential through the calcium component but also the inhibition of the calcium component migration through the polymer matrix. In example, it has been demonstrated that a combination of a polymer with hydroxyapatite maximises the hydroxyapatite osteo-conductive behaviour which allows bone growth inside the device as the matrix is progressively reabsorbed.[71]

In a study conducted by Wan et al., chitin was combined with hydroxyapatite producing a composite material with a strength reduction but maintaining the polymer plastic properties. In the same way, those authors worked on chitin-mediated in situ calcification induction using chemically modified chitin with anionic carboxymethyl groups, which would act as calcium attraction sites.[72]

Ma et al. created cement with calcium phosphate and phosphorylated chitosan and, on rabbit models, didn't find adverse effects and presented histological evidence suggesting biocompatibility, bio-absorption and osteo-inductivity.[73,74,75]

Many other studies were conducted using chitosan as a main component on calcium based cements. Most of these studies have as goal the development of bone replacement materials using various techniques for achieving a material with the desired characteristics (in terms of resistance, plasticity, porosity, etc.) that are thought to have advantage in vivo.[76]

Therefore, it appear to be natural the development of more work using chitin and chitosan on the production of similar compounds and some of which with practical application, on the short-term.

Most of tissue engineering research is based on cell sowing/culture on biodegradable porous polymer matrixes. The limiting element for those studies is the availability of good biomaterials that work as temporary matrixes. Therefore, these biomaterials should have the ability of acquiring a porous form when prepared in a way to provide a channel between the matrix and receptor cells that would allow the tissue to growth into the biomaterial while this biomaterial is progressively degrading on non-toxic compounds.

Khor et al. produced a series of chitin porous matrixes with 100 to 500 um pores in wich they have verified growth and proliferation of human and mice fibroblasts.[77]

Many studies have been developed using chitosan combinations with several materials and it have been verified very appealing potentialities of the resulting biomaterials such as membranes without significative endothelial cells interaction (imunoisolating potential). These membranes in particular were used on tissue engineering research for the development of a trachea and in which chitosan was shown to exert a strong influence on nervous cells adherence and proliferation.[78]

Cho et al. prepared biomaterials combining chitosan and alginate and demonstrated hepatocyte adherence to those materials.[79]

Implantable chitin based biomaterials are a field of research in large expansion, especially on Asia. The interest is based on the fact that the chitin monomeric unit, N-acetyl-glucosamine, also present on hyaluronic acid, is of major importance on tissue repair. Therefore, it has been postulated that chitin should have certain characteristics favourable to the promotion of fast dermal regeneration. So, efforts have been developed to create biomaterials that would enhance tissue regeneration, from simple wound covering materials to complex matrixes of artificial skin.

Ueno et al. demonstrated that chitosan (on a cotton form) acted as an accelerator on wound repair since it has an important role on polimorphonuclear cells infiltration in the lesion site.[80]

On an attempt to increase keratin's tension properties, Tanabe et al. developed a composite keratin film with 10-30% chitosan. This material showed as main features fibroblasts adherence and proliferation and bacterial resistance.[81]

Park et al. studied the effect of modified chitin with anionic carboxymethyl groups on normal human fibroblast proliferation and on in vitro cultures of keloid fibroblasts. They have verified that this biomaterial inhibited the growth of keloid fibroblasts but exert no influence whatsoever on normal fibroblasts.[82]

Dung et al. presented good results on a three years clinical trial with the use of 'Vinachitin', a biomaterial with a membrane shape, for the treatment of near 300 patients with severe burn lesions,

orthopaedic trauma and ulcers. Results were presented on the 8th International Conference on Chitin and Chitosan.[83]

Chitin and chitosan - Applications on drug release formulations

Implantable chitin and chitosan applications either in the form of hydrogel or membrane, has been an important research field for these biomaterials. Several authors have been proposing chitosan as a component for interpenetrating networks.[84,85,86]

The creation of mucoadhesive membranes using an acrylic acid polymerisation with chitosan technique was used successfully on a drug transmucosal release study (in this case, triamcinolone acetonide) for the inflammation reduction on oral ulcers treatment.[87]

Citosan microsferes and nanosferes was also created for drug release and chitosan based microgranules were developed for dissodium diclofenac release.[88] In vivo studies with chitin microsferes showed 60% clearance 3 hours after administration, on an animal model, with most microsferes to be retained in the liver suggesting therefore, that these microsferes could be used for selective liver directed drug release.

Chitin and chitosan - Other biomedical applications

Sato et al. studied the efficiency of chitosan transfection as gene vehicle.[89] On other works chitosan was used to increase the adenovirus infectious rates to human cells on gene therapy research. Chitosan fibres could be used on the production of blood vessels because of the blood compatibility featured both by chitin and chitosan. This blood compatibility has been chitin and chitosan main characteristic since the early studies by Hirano, in 1985.[90] Recent publications from this author move forward to this direction. Another potential chitosan application is the bioadhesive materials production. Ishihara et al. prepared a chitosan and lactosis based material which they have verified to have an adhesive strength similar to fibrin glue.

Chitin and chitosan - Biocompatibility

The in vivo degradation rate has high values to chitin but it gradually decreases as deacethylation increases. However, it was described a mild reaction to chitonsan subcutaneous implants on an animal model.[91]

On another animal model water soluble chitosan biodegradation study on animal model, the authors verified that about 50% of deacethylated material was promptly degraded and eliminated and, therefore, it did not raised any bioaccumulation issues.[92] Other works

researched chitin and chitosan effect on biological systems. Thus, when chitin or chitosan are orally or intra-peritoneal administrated on animal models, chitosan triggers more reaction than chitin.[93]

Some studies point to a chitin mediated fibroblast proliferation accelerating effect while chitosan would have an inhibitory effect, probably due to bounding to growth factors that, otherwise, would bind to target sites exerting their functions. Thus, this point of view is polemic due to many different studies with contradictory results.[94,95]

Chitin and chitosan - Sterilisation

On the evaluation process of any biomaterial potentialities, its sterilisation features are an important topic to evaluate and it assumes a sine qua non condition when concerning implantable biomaterials. Thus, medical and pharmaceutical products common sterilisation methods, as dry heat, saturated steam, ethylene oxide and gama radiation, were tested on chitosan products.

Lim et al. studied the effects of dry heat, saturated steam and gama radiation on chitosan membranes and verified that dry heat exposure resulted on a decrease of chitosan water solubility and, on extreme cases, insolubility in acid aqueous environments. It was registered a 60% reduction on the tensional strength and 53% reduction on fracture point tension.[96,97] It was also verified that saturated steam significatively enhances the rate and extension of chitosan termal events. Chitosan became water insoluble and it loses 80% of its

tensional strength maintaining only 28% of its original fracture point tension.

Gama radiation has produced events of chitosan main chains breakdown. Chitosan membranes irradiated with open air 2,5 Mrad presented with a 58% elevation on tensional strength and a decrease of 22-33% on volumetric expansion capability. Performing radiation under anoxic conditions these membrane properties changes are considerably reduced.[98] It can be concluded that the best mean of sterilisation for chitosan products is 2,5 Mrad gamma radiation under anoxic conditions.

Economics of chitosan production

On the late seventies, the chitin/chitosan production costs were subject of interest. On those days, in the US, taking a plant capable of producing approximately 5×10^5 kg chitosan/year a spread of possible production costs were calculated ranging from $1.20/kg to $2.20/kg (chitin) and from $1.82/kg to $5.53/kg (chitosan). This gives an average cost of $3.50/kg, which at 2008 prices, based on the Consumer Price Index of the Federal Reserve Bank of Minneapolis, converts to $11.50/kg. However, these calculations made in 1977 were based on the assumption that the raw material, that is the waste from the shellfish processing plants, would be available at very low or even zero cost. This is one factor that has changed considerably and competition for the shellfish waste is now coming in particular from

animal feed stuff manufacturers, including those supplying prawn and shrimp farms. This conflict may become a major obstacle to increasing chitosan production if a large scale chitosan application was to emerge in the future. The use of other raw materials as waste mycelium, and the use of new production processes such as the electrochemical process, could both contribute to the overcoming of this potential shortage should it ever arise. Currently, the increasing chitosan medical applications interest inflated the chitosan production prices with ‘medical grade' chitosan being offered by several companies at a cost of approximately $25,000/kg.[99]

Introduction

Materials and Methods

Results

Discussion

Conclusion

References

Animals

For this study 14 New Zealand male rabbits were used. The animals average weight was 3kg. Each animal undergone two level lumbar laminectomy (L1 and L3). On each animal, one laminectomy site was randomly selected for biomaterial application and the other laminectomy site acted as control. The animals were kept on Veterinary Medical Teaching Hospital of Trás-os-Montes e Alto Douro, and were fed a standard rabbit regimen. All rabbits underwent a complete pre-operative neurological evaluation to ensure complete neurological integrity. After surgery, all animals were kept under close surveillance for infection or neurological deterioration screening. All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Chitosan membranes preparation

Chitosan (high molecular weight, Sigma, USA) was dissolved in a 0.25M acetic acid solution to attain a concentration of 2% (w/v). The hybrid membranes were obtained by adding GPTMS (Sigma, USA) to the

chitosan solution. After stirring at room temperature for 1 hour, the resultant chitosan and chitosan-silicate solutions were poured into polypropylene containers with cover, and aged at 60°C for 2 days. After aging, the membranes were dried at 60°C for 2 days. The chitosan and chitosan-gama-glycidoxypropyltrimethoxysilane membranes were soaked in a 0.25M sodium hydroxide solution to neutralize the remaining acetic acid, and then washed with distilled water, and dried at 37°C for 24 hours. Before the implantation procedure the membranes were sterilized with ethylene oxide gas and kept at room temperature for 1 week (Figure 1).

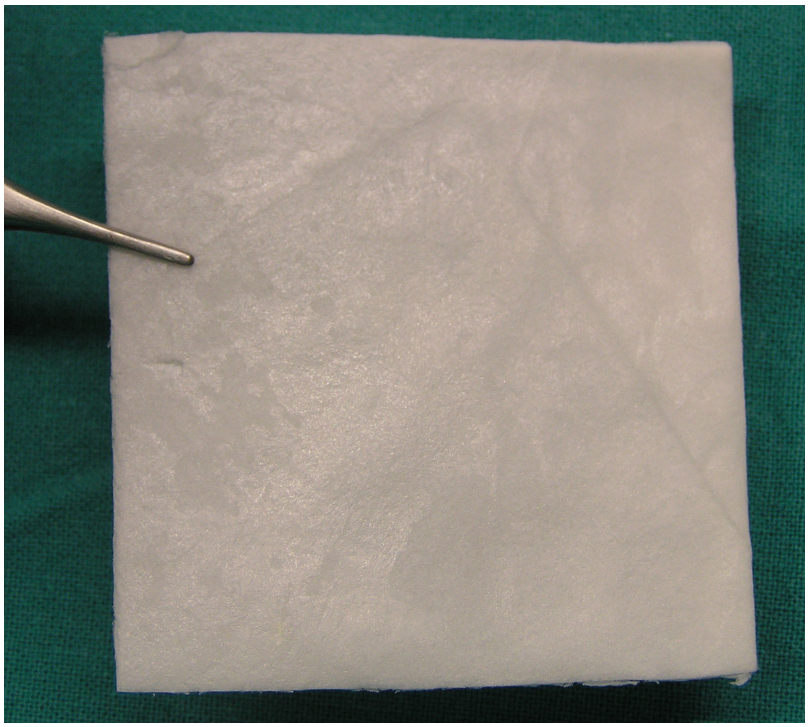


Figure 1: Implantable chitosan membrane.

Surgical procedure

Animals were anesthetized intravenously with ketamine (30 mg/kg) and medetomidine (0.1 mg/kg). Then, lateral spine radiographs were performed in order to identify the number of lumbar vertebrae and, thus, localize the segments to operate.

A midline incision exposed the spinal column at the L1–L3 level, and the paravertebral muscles were dissected bilaterally to visualize the transverse apophyses.

The segments to approach were identified intra-operatively by direct palpation. On each segment, the dorso-lumbar fascia was divided and a bilateral paravertebral muscles subperiosteal dissection was performed. On this stage, the paravertebral muscles were retracted using a retractor (Figures 2 and 3). It was then performed the bilateral laminectomy at L1 and L3 with a drill until dura-mater exposure (Figure 4).

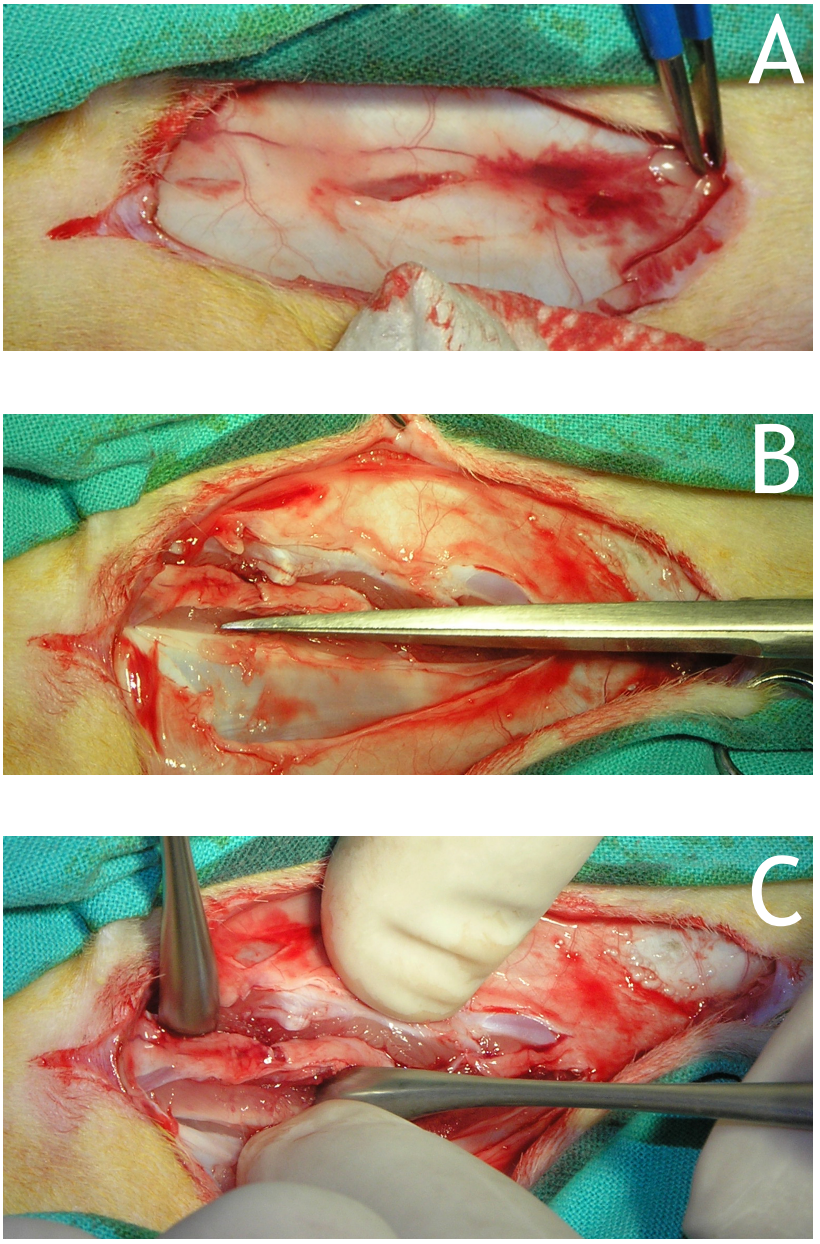


Figure 2: Surgical procedure images: (A) Midline skin incision at L1-L3 level, after spine localization plain radiograph and tricotomy; (B) Division of the dorso-lumbar fascia on each side of the spinous processes; (C) Bilateral subperiosteal dissection of the paravertebral muscles.

The laminectomy defects were measured to be approximately 10x5mm.

In order to diminish the influence of any level-specific variations the biomaterial implantation was placed on L1 laminectomy site (7 animals) and on L3 laminectomy site (7 animals), the defect was left empty in the other sites.

Care was taken in order to make a chitosan membrane adapted to the bone defect dimensions in a way that it covered the totality of the defect and also the bone of the margins (Figure 5). A thorough hemostasis was performed in order to reduce the potential post-operative haematoma. Dorso-lumbar fascia was closed with simple stitches of resorbable suture and the cutaneous layer was closed with intra-dermic continuous resorbable suture. The operative wound was cleaned with an iodopovidone solution.

During the procedure the animal's temperature, blood pressure and electrocardiogram were continuing screened. An ophthalmic gel (Lacryvisc, Alcon, Lisbon, Portugal) was applied to prevent drying of the eyes.

Postoperatively the animals were housed in individual cages and allowed normal activity. They were weighed daily for the first 7 days postsurgery and then weighed weekly. Postoperative care included injections of sulfadiazine and trimethoprim twice a day for up to 1 week.

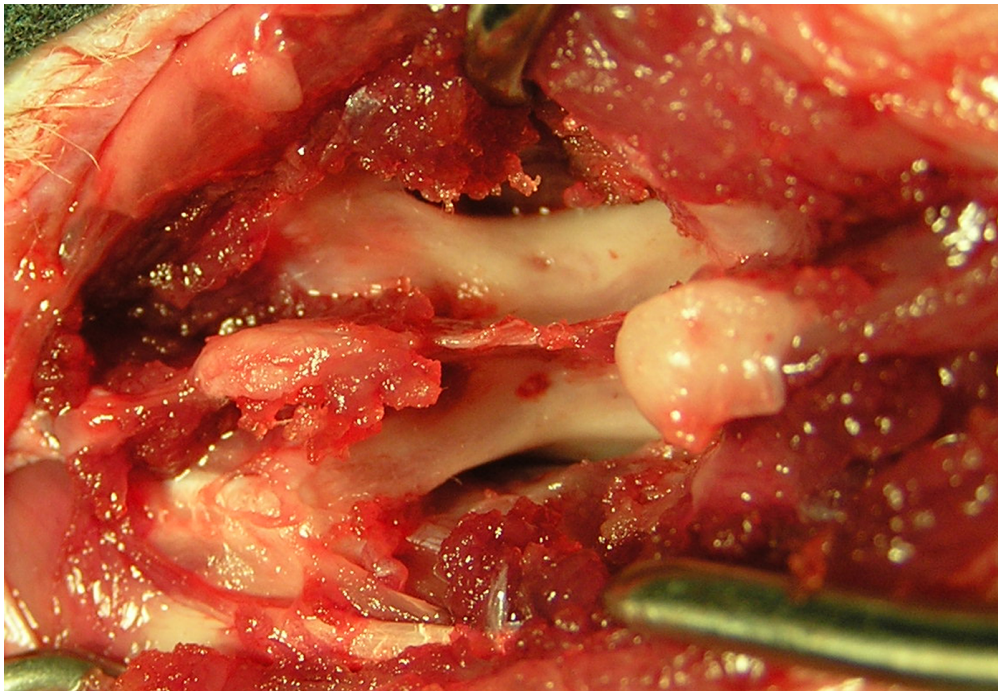


Figure 3: Posterior aspect of the lumbar vertebra after finishing paravertebral muscles dissection.

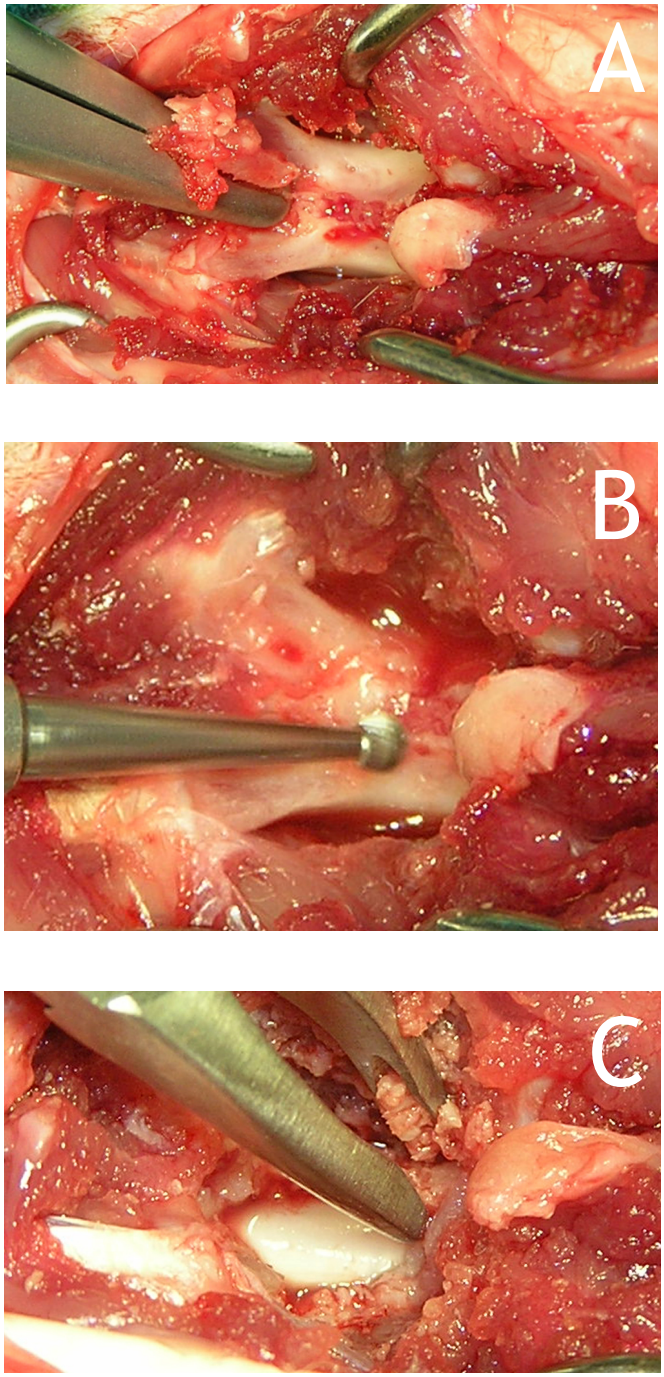


Figure 4: Surgical procedure images: (A) Spinous process removal with bone roungeur; (B) Laminectomy performed with high-speed drill; (C) Bone removal from the laminectomy borders with bone rongeur.

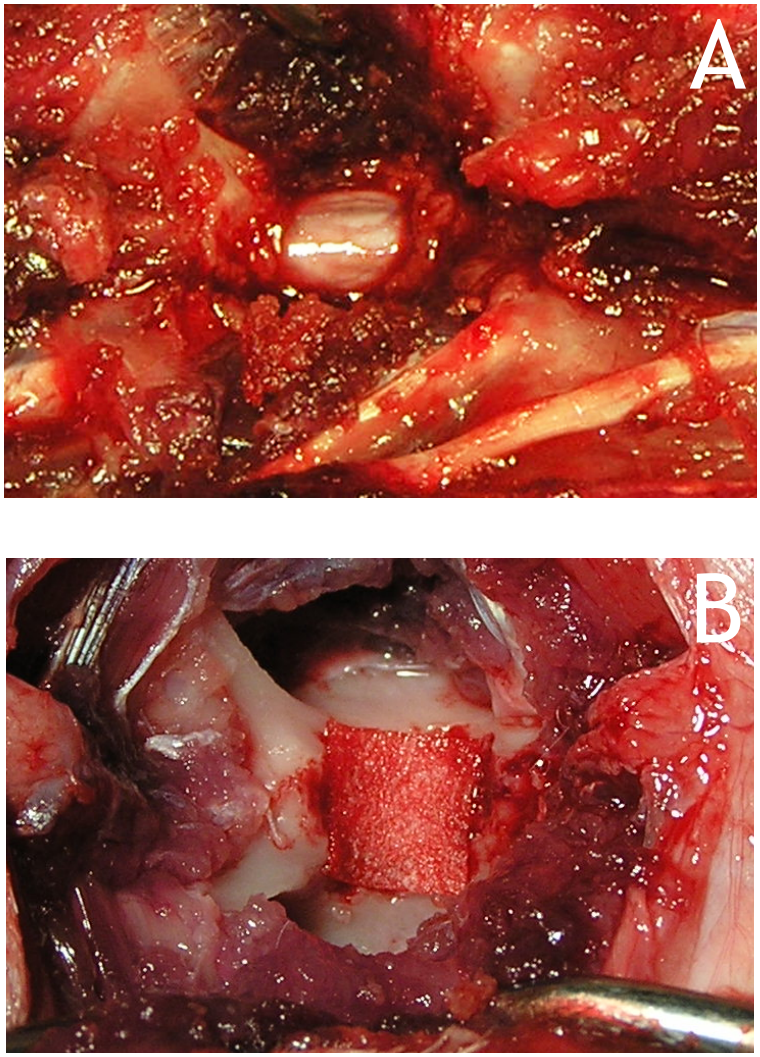


Figure 5: Surgical procedure images: (A) Laminectomy final aspect with visualization of blood vessel throught the thin lumbar dura; (B) Chitosan membrane over the laminectomy site covering all the laminectomy area and its bone borders.

The animals were euthanised 4 weeks after laminectomy.

The vertebral L1-S1 segment was removed en bloc during the animals necropsy and was fixed on 10% formaldehyde. Decalcification of the entire vertebral segment was achieved using the Morse solution. In brief, decalcification was obtained using sodium citrate/formic acid until chemical testing for the presence of calcium runs negative; the samples were placed in ten times their volume of the decalcifying solution and the solution was replaced daily. Chemical testing of the presence of calcium was carried out by adding 1cc of ammonium hydroxide to 5 cc of the decalcifying solution plus 0,1cc of a saturated ammonium oxalate solution. When calcium is still present in the solution, it precipitates, otherwise decalcification is complete (usually it takes around three weeks).

The columns were then cut in 1-cm-long segments transversely to their axis. Each column was then rehydrated with PBS and cryo-protected with three passages in increasing solutions of sucrose (7.5% for 1 hr, 15% for 1hr, 30% over-night) in 0.1M PBS. Thereafter, specimens are maintained in a 1:1 solution of sucrose 30% and optimal cutting temperature medium (OCT) for 30 min and then embedded in 100% OCT. Specimens must then be store at –80°C.

Sections were then cut by means of a Leica CM1850 Cryostat in a thickness range of 20-30 μm, placed on silane-coated microscope slides to improve slice adhesion, and stored at –20°C. Before staining, sections were taken out of freezer to room temperature and as soon as

they were acclimatized, they were further processed either by haematoxylin and eosin (the most commonly used stain for light microscopy observation in histology and histopathology) or by Masson's trichrome staining (that, in comparison to haematoxylin and eosin staining, it highlights also connective tissue).

For haematoxylin and eosin staining, the slides were immersed in 0.1% haematoxylin (we use the product from Ciba, Basle, Switzerland) for 10 min, washed in tap water for 15 min, then immersed in 0.1% eosin (we use the product from Ciba, Basle, Switzerland) for 5 min and washed in distilled water. The sections were finally dehydrated in ethanol and mounted in DPX (Fluka, Buchs, Switzerland).

For Masson's trichrome staining, we used a *Masson trichrome with aniline blue* kit (Bio-Optica, Milano, Italy): six drops of Weigert's iron haematoxylin (solution A) and six drops of Weigert's iron haematoxylin (solution B) were combined together and used to stain slides for 10 min. Without washing, the slides were then drained and incubated with ten drops of alcoholic picric acid solution for 4 min. After washing in distilled water, sections were stained with ten drops of Ponceau acid fuchsin for 4 min and washed again in distilled water. Further on, ten drops of phosphomolybdic acid solution were added to the section for 10 min. Without washing, the slides were drained and 10 drops of aniline blue are added to the section for 5 min. Finally, after washing in distilled water, dehydrating rapidly in ethanol and clearing in xylol/ Bioclear (Bio-Optica, Milano, Italy), the slides were mounted in DPX (Fluka, Buchs, Switzerland).

Sections were then analyised and photographed suing a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany).

Results of the histological analysis of rabbit columns at the site of laminectomy are illustrated in figures 6-9.

Extensive microscope observations, at different magnifications, showed, in the animals that did not receive chitosan application, the presence of a disorganized regenerating tissue (fig. 6A, asterisk) mingled with islets of bone tissue (fig. 6A, arrows). At higher magnification (fig. 6B), the regenerating tissue appeared to be rich in various-sized and round-shaped cells (including many lymphocytes) that indicate the persistence of a chronic inflammatory condition. With respect to the bone tissue organization at the border of the lesion site, lamellae did not show osteonic organization and were oriented without clear orientation (fig. 7A). Higher magnification observation (fig. 7B) showed that the interface between the regenerating tissue and the bone tissue (fig. 7B, arrow) has a limited extension without a clear orientation. Often, bone and regenerating tissue were completely detached and separated by large empty lacunae (fig. 7A, asterisk).

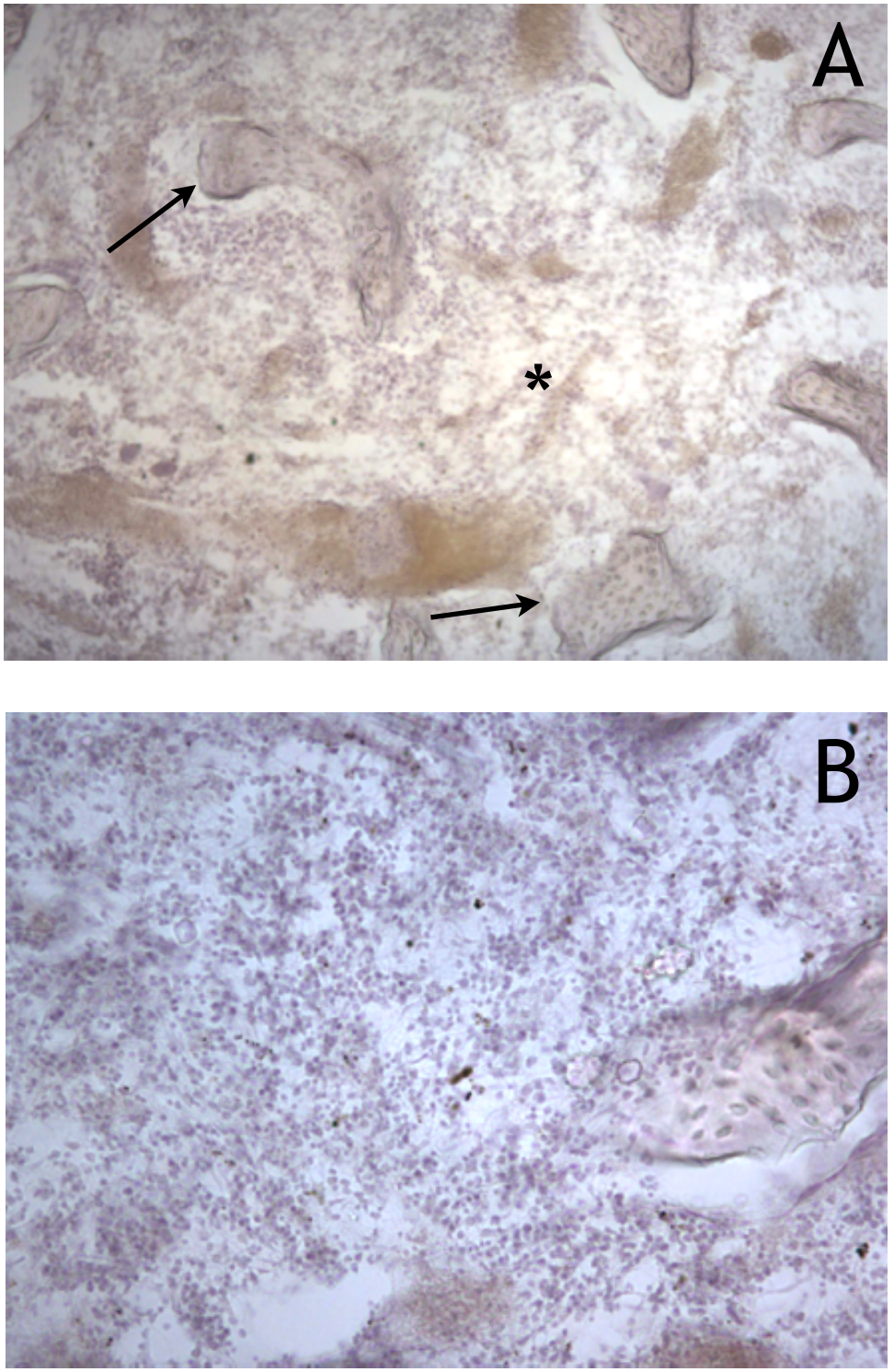
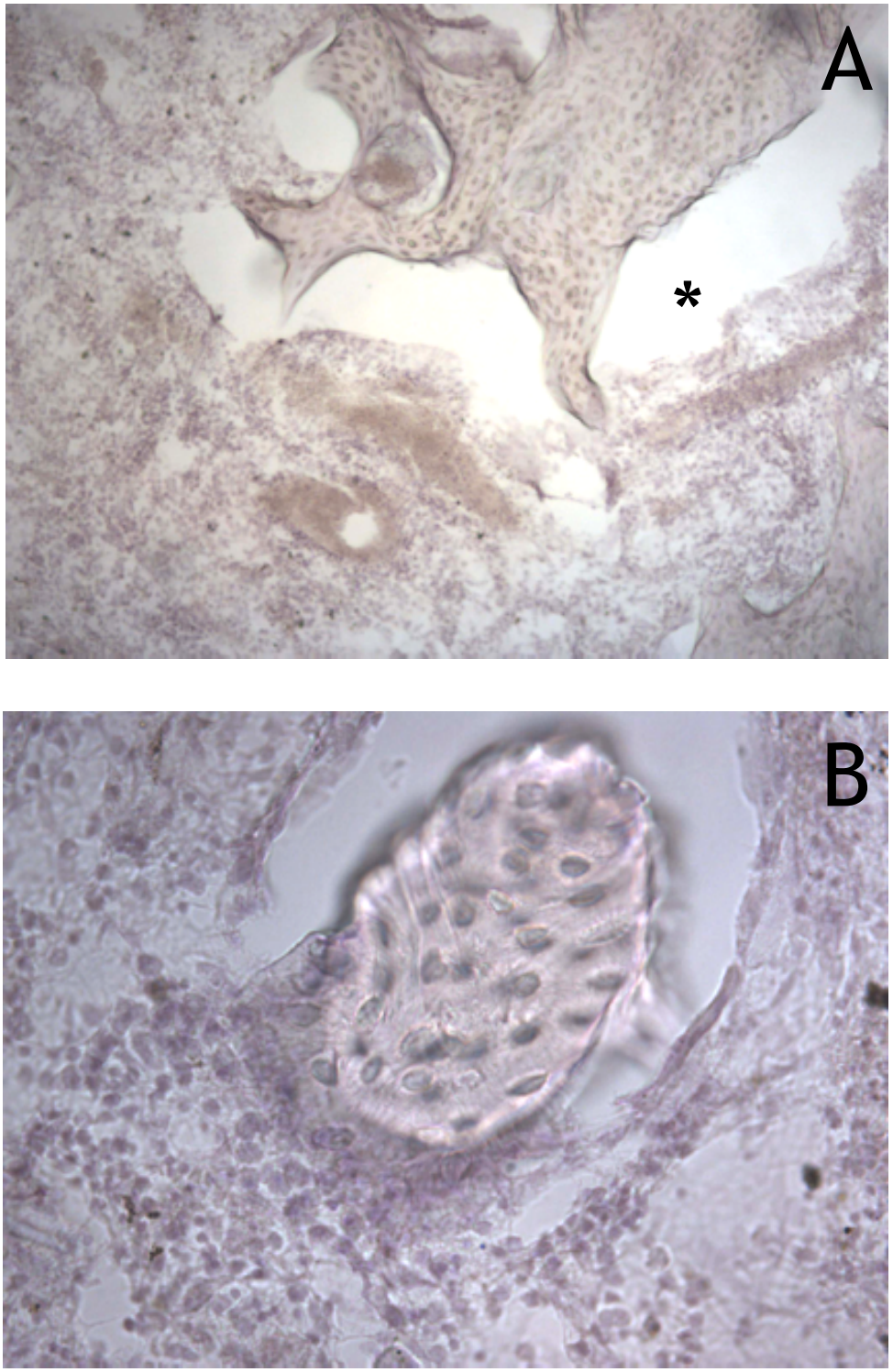


Figure 6: (see text for details)



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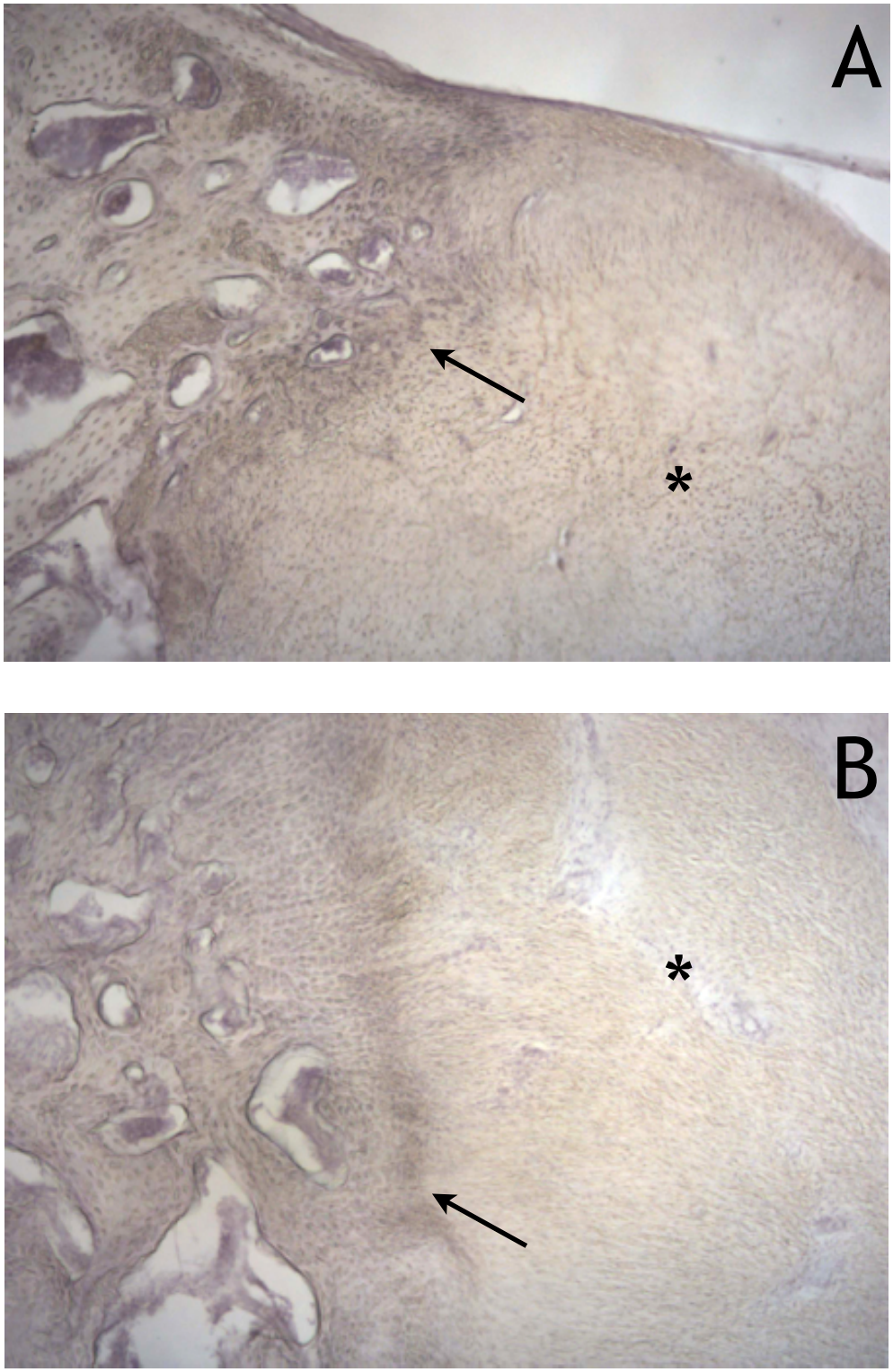

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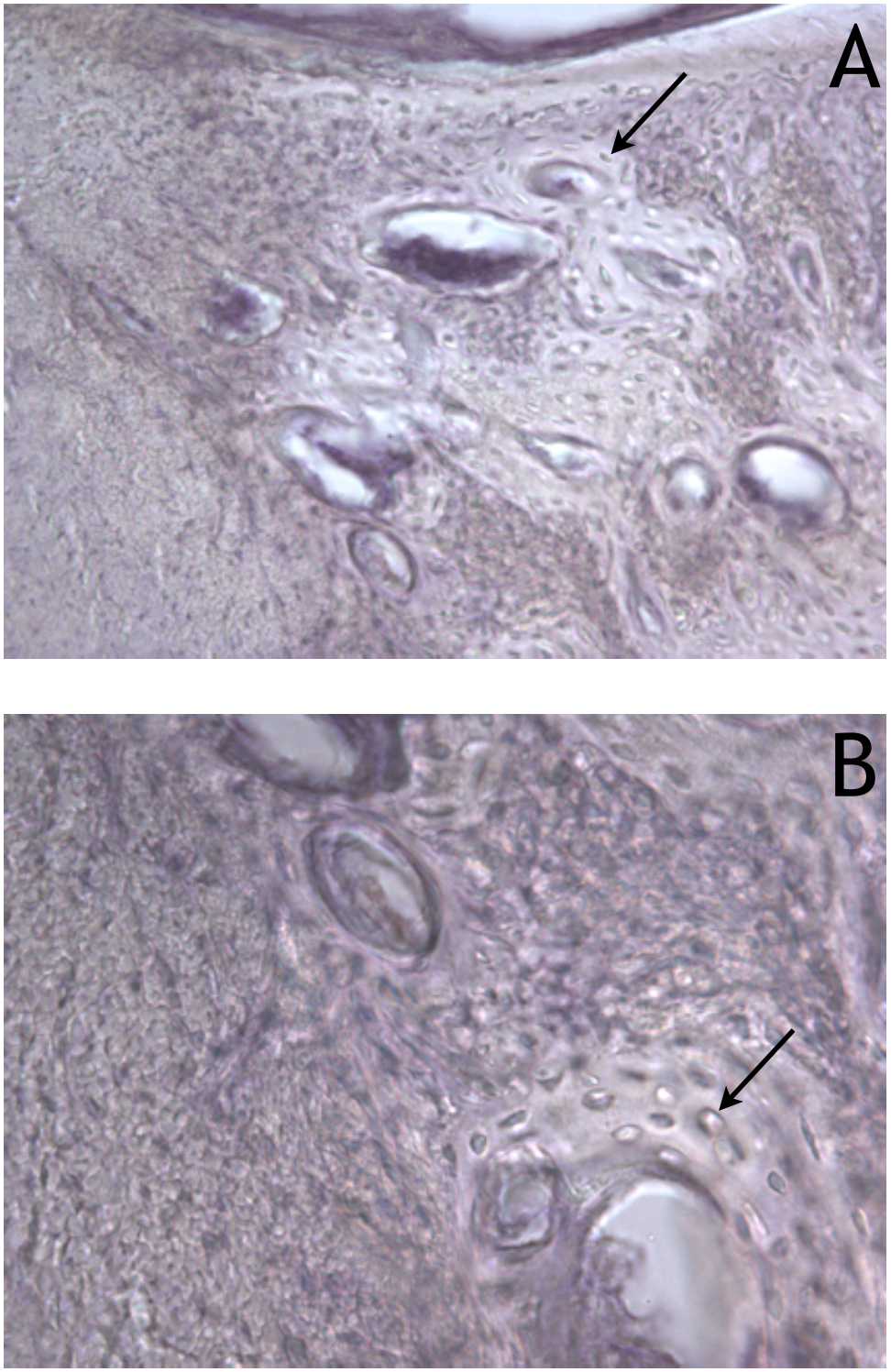

Figure 9: (see text for details)

Histological analysis of rabbit columns at the site of laminectomy in the animals that received chitosan application is illustrated in figures 8 and 9.

At lower magnification (fig.8), the presence of a well-organized regenerating tissue, integrated in the surrounding vertebral bone tissue though an interface (fig. 8A-B, arrows) that, contrary to what observed in the control group, was regular and extended from on side to the other side of the lamina. Although the location of islets of bone tissue inside regenerating tissue (fig. 8C-D) and the small extension of regenerative tissue in comparison to controls suggest that bone regeneration has progressed significantly filling the lamina defect. Yet the presence signs of osteonic reorganization (fig. 9A-B, asterisk) can be detected inside the regenerating tissue. However, since regenerated bone tissue can hardly be distinguished from native bone tissue, the amount of regenerated bone cannot be quantified.

Since the clarification of the sciatic pain pathophysiology, nearly a century ago, and its relationship to lumbar disk herniation, several surgical approaches were proposed. The surgical approach currently and wide-world used has its origin in the works of Yasargil and Caspar on the mid-seventies.[4,6] Currently it's the most common neurosurgical procedure performed in the US and there is evidence that the surgical treatment has advantage over conservative treatment.[4,17] The surgical technique involves a posterior approach through a small skin incision and an extradural disk removal. Several authors have descriptions of their technique and a large majority describe gestures for protection against post-operative scarring such as removal of the ligamentum flavum bone insertions after its excision, preservation of the medial portion of the ligamentum flavum or complete preservation and performing only a division of the ligament to access the vertebral canal, preservation of the epidural fat layer or application of fat grafts or steroids over the bone defect.[1,2,6,8,9] The prevention of post-operative perineural fibrosis has been object of increasing interest as this condition has been pointed out as one of the main causes of Failed Back Surgery Syndrome. Although the aetiology of this syndrome is not fully understand, post-operative perineural fibrosis is commonly pointed as an important etiopathogenic factor.[18,20,21] The 1996 prospective, randomized, controlled, blind and multicentric trial, presented by Ross et al. revealed a significative association between post-operative epidural fibrosis and recurrent radicular pain.[23]

The epidural fibrosis formation is the result of post-operative hematoma invasion by thick fibrotic tissue, starting on the periosteum fibrous layer and fibroblast migration from the deep layer of the

paravertebral muscles. This process can progress to the vertebral canal and create adherences to the dura-mater and nerve roots.[27,28] Wound healing consists of three phases: (1) the inflammatory phase, which consists of inflammatory cell migration to the healing wound; (2) the proliferative phase, which consists of new tissue formation such as angiogenesis, fibroplasia, epithelialization, and extra-cellular matrix accumulation; and (3) the tissue remodeling, which is the maturational phase.[100]

Fibroblasts, which invade the wound in the first few days of healing, have multiple functions important to wound repair, such as collagen synthesis, extracellular matrix reorganization, and wound contraction resulting in mature scar formation. It has been shown that chitosan can inhibit fibroblast growth.[101,102]

Chang et al. in a study for reduction of peritoneal adhesions using a Electrospun anti-adhesion barrier made of chitosan concluded that this later component can inhibit tissue adhesion by inhibiting proliferation and triggering apoptosis.[102] Apoptosis as a programmed, physiological mode of cell death that plays a key role in tissue homeostasis may be inhibited by deleterious stimuli such as hypoxia, contorting the balance of cellular proliferation, differentiation, and death, hence impairing the normal peritoneal wound repair process.[103] The results of the study by Chang et al. suggested that in the presence of chitosan, apoptosis would occur in a significant level. Previous studies reported that chitosan may interact with the cell membrane to trigger apoptosis.[104,106] N,O-carboxymethyl chitosan (NOCC) is a non-toxic absorbable agent that is also used to prevent postoperative adhesion formation. This agent is used as a peritoneal instillate and is suggested that, in addition to its mechanical action in separation of raw

peritoneal surfaces, it dilutes fibrin and fibrinous exudate released from traumatized tissue.[105]

On recent study evaluating the effectiveness of biophysical barriers on peridural fibrosis, Kasimcan et al. compared ADCON-L and Seprafilm® Adhesion Barrier on an animal model. ADCON-L is a bioresorbable carbohydrate polymer gel which is composed of a polyglycan ester and porcine derived gelatin in phosphate-buffered saline. It is suggested to prevent scar formation when it is applied on nervous structures. It is an antiadhesive and physical barrier gel that prevents fibroblastic migration in the laminectomy area. Seprafilm® Adhesion Barrier is a bioresorbable adhesion membrane composed of two anionic polysaccharides, sodium hyaluronate and carboxymethylcellulose. It is indicated in patients undergoing abdominal or pelvic laparotomy as an adjunct intended to reduce the incidence, extent and severity of postoperative adhesions between the abdominal wall and the underlying viscera. The authors observed low level density of peridural adhesion on both experimental groups however, the fibroblast count results were not found statistically different between experimental groups and controls. The authors postulate that although these barriers are almost physiologically inert materials, they are also physiologic bioresorbable and they are removed by macrophages which can also transform and behave as fibroblast. These materials may act as foreign bodies, and force the macrophages and histiocytes to migrate into the laminectomy area in long term period. So it's suggested that these materials could not block the inflammatory cell migration into the laminectomy area of the rat. [106]

For this study it was used an animal laminectomy model using New Zealand rabbits. The bone anatomy of these animals is favourable for a lumbar laminectomy model as the dorsal laminae are long which makes the surgical procedure brief and straightforward, therefore reducing post-operative complications. One of the major complications of this procedure in rabbits is post-operative neurological deterioration which is more common with laminectomies on lower levels. This fact determined the choice of the laminectomy level for this work. Thus, two level lumbar laminectomy were performed, on L1 and L3. At these levels, on the vertebral canal, there is the lower portion of the spinal cord. Therefore, on this particular aspect, this model does not mimic the human lumbar laminectomy conditions. For these concerns, some authors have suggested lumbar laminectomy models in the region of the cauda equina (suggesting different animals, such as the ovine model) which would allow biomechanical testing on nerve roots.[53,57] However, on the present work, the focus of the study was the histological characterization of the differences of post-operative fibrosis on a manipulated site (in the presence of the biomaterial) and on a control site. So, the results are still valid and applicable to the biomaterial effects on post-operative epidural fibrosis.

Another pitfall of the present study is that a simple laminectomy was performed to the rabbits and a discectomy was not done. So, the effect that the experimental material might have on scar formation at discectomy site and on anular ligament healing could not be assessed. This was not done because of the extreme difficulty to expose the nerve root and annulus fibrous without neurologic injury in a rabbit model.

Chitosan matrices have been shown to have low mechanical strength under physiological conditions and to be unable to maintain a predefined shape for transplantation. The improvement of their mechanical properties can be achieved by modifying chitosan with a silane agent. The gama-glycidoxypropyltrimethoxysilane (GPTMS) is one of the silane-coupling agents, which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules while the methoxysilane groups are hydrolyzed and form silanol groups. The silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the crosslinking between chitosan, GPTMS and siloxane network.[66,67] This chitosan membrane was used on the present work and was found to be very convenient to manipulate in order to cover the entire bone defect.

One important aspect of this study results was the finding that the chitosan membrane were consistently and totally degraded by the fourth week after the surgical procedure. This is an important feature as the barrier-effect for this biomaterial is important only in the early stages of the scarring process. Zhou et al. stressed this aspect on their work on reduction of post-surgical adhesion formation after cardiac surgery by application of N,O-carboxymethyl chitosan. Those authors described that the chitosan film used was effective on reducing post-surgical adhesion formation maintaining its structural integrity for five days and degrading by the seventh post-operative day.[107]

The results of this study clearly demonstrated that chitosan had an organizing effect on post-operative scarring process. The presence of the chitosan membrane resulted on a well-organized tissue integrated in the surrounding vertebral bone tissue. It was also observed signs of

regenerative bone tissue in continuity with native bone which, by the present technique cannot be distinguished from each other. In fact, the four-week duration of this study allows only the important conclusion of improved vertebral regeneration on a post-laminectomy model. This can be a major feature on the dynamics of epidural fibrosis formation.

Sandoval-Sánchez et al. achieved similar results using a bilayer chitosan scaffolding as a dural substitute on experimental models. On their work it was observed the biomaterial adapted to the defect and attached to the host tissue borders with organized collagen fiber deposits and neovascularization and with no evidence of fibrosis or abnormal healing or epidural or subdural adhesions.[108]

Chuang et al., using a combination of poly-lactic acid gel and autologous micromorselized bone observed significant lamina bone regeneration.[109] However it is still unknown if the regenerated vertebral lamina can be expected to confer stability to the spinal column and prevent peridural adhesion as a long-term outcome. Further research on ligament structure will confirm whether a newly generated vertebral lamina can provide an attached bed for soft tissue growth and enable ligament regeneration.

Nonetheless, these studies are aiming tissue regeneration after surgical aggression which is significantly different from other current studies that purely aim inhibition of fibrosis such as the use of mitomicin C.[100,110] Those studies, chasing the same goal, use an entirely different approach and their results cannot be compared with results from studies using regenerative approaches.

Therefore, these very interesting results open a wide gamut of possible future studies. It would be important to evaluate the

evolution over time of post-operative scarring using a chitosan membrane in order to access the maintenance of a well-organized regeneration tissue throughout the scarring process without the formation of perineural adhesions or formation of aberrant structures mainly inside de vertebral canal. These studies should use models that allow laminectomy procedures on the cauda equina lumbar levels. There should be used models to quantify the regenerated tissue formation and inflammatory markers survey would be of interest.

This studies would be an important contribution for giving substrate to human clinical trials. The transition from these studies to human trials poses a complex challenge. Despite the promising results obtained, the ideal chitosan-based material is not entirely defined as it's clear for the numerous studies using different chitosan-based formulations. The U.S. National Institutes of Health service ClinicalTrials.gov is a registry and results database of federally and privately supported clinical trials conducted in the United States and around the world. A search for 'chitosan' on this database (on January, 2012) retrieve 20 studies, from which only one involved an implantable chitosan-based biomaterial. This study, focusing joint cartilage regeneration and entitled 'Trial Comparing BST-CarGel and Microfracture in Repair of Articular Cartilage Lesions in the Knee' is designed for clinical testing of a chitosan-based biomaterial called BST-CarGel which was developed to stabilize the blood clot in the cartilage lesion by dispersing a soluble and adhesive polymer scaffold containing chitosan throughout uncoagulated whole blood. The efficacy and underlying mechanisms of action of BST-CarGel have been examined in several studies using animal models.[111,112] After promising results on animal studies, thirty-three human subjects were

treated with BST-CarGel from August 2003 to December 2004 under Health Canada's Special Access Program for medical devices that is designed to enable compassionate use. Notably, treatment occurred on a case-by-case basis and, by law, was not considered a clinical study. In particular, the absence of a control group as well as the wide ranging characteristics of the treated patients and lesions impeded a rigorous interpretation of outcomes. The safety of treatment with BST-CarGel was demonstrated because no uncharacteristic observations were made during physical examinations or blood analyses for all patients. BST-CarGel has not been approved at this time for sale in any country. Another implantable chitosan biomaterial has been tested in humans on cancer therapy research. Transarterial and percutaneous Holmium-166/chitosan (Milican, Dong Wha Pharmaceutical Co., Seoul, Korea) complex was evaluated for long-term therapeutic efficacy and safety on clinical trials in a group of patients with hepatocellular carcinoma. The chitosan-based biomaterial used by the authors possessed the useful property of transforming from a liquid to a gel state depending on the pH of the surrounding environment. It was readily dissolved in water to yield a clear solution under acidic conditions but was converted to the gel state under neutral or basic conditions above pH 6, such as those found in injected tumor tissues, thus effectively holding the bound holmium in place.[113,114]

A commercially available chitosan-based based implantable biomaterial is the OPTOMESH(TM) (Tricomed S.A., Poland) which is a non-resorbable surgical mesh covered by a microporous resorbable chitosan coating. This mesh was designed to be used for surgical abdominal wall repair after hernia surgery.[114] Recent studies on animal models suggest that these meshes are equal, in terms of quality

and anti-adhesion properties, to the globally recognized Dynamesh®-IPOM mesh.

Whereas a quantitative analysis of the type or tissue repair was not possible due to its polymorph complexity, extensive microscopy observation carried out in the present study allowed to show that the morphology of tissue repair was rather consistent, in each of the two experimental groups, and rather differentiated between them.

It can thus be concluded that chitosan application after laminectomy improves vertebral regeneration by stimulating the formation of a new and well organised cover tissue at the site of bone removal.

Moreover, it promotes its quick differentiation into a newly formed and well organised bone tissue that integrates rapidly with the native bone, filling the defect and recreating a vertebral structure which is close to the normal pre-lesion one.

Unlike previous studies focused on inhibition of the scarring process in order to prevent epidural adhesions, the present work using this chitosan membrane, has showed that the scarring process can be directed to improving tissue regeneration suggesting a reduction on epidural fibrosis.

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