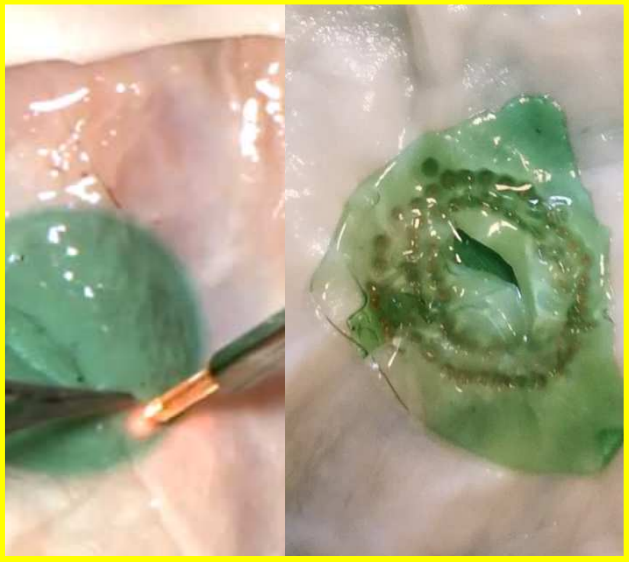

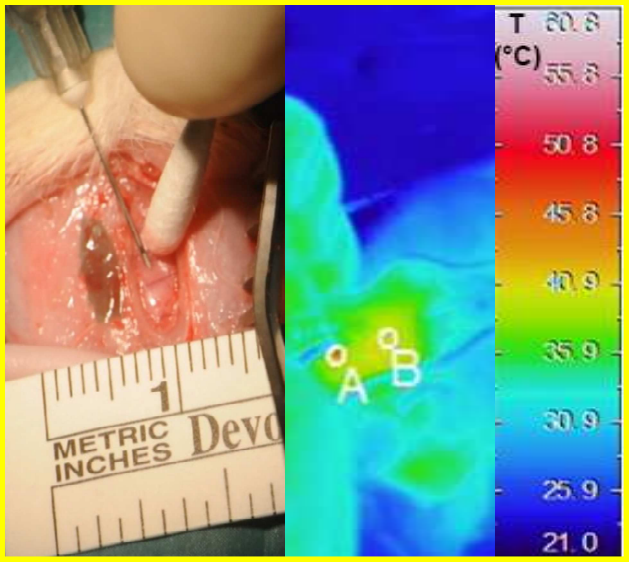

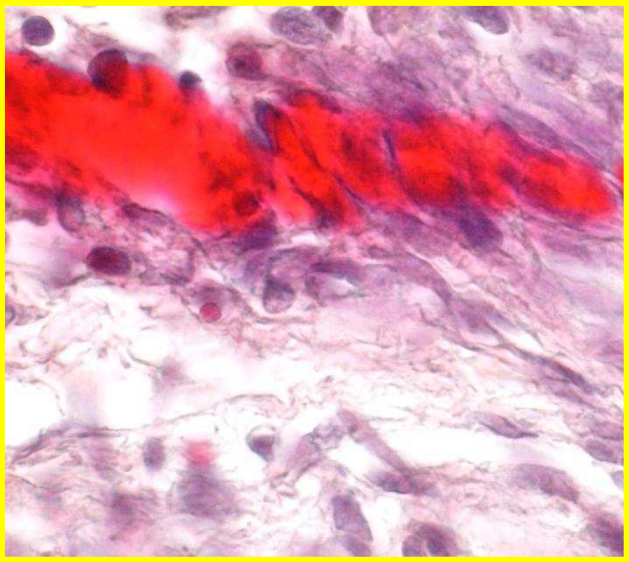

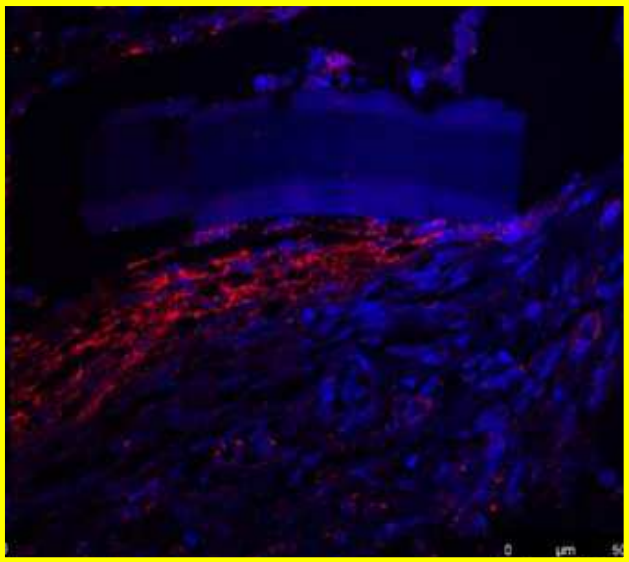

Università Politecnica delle Marche
Ph.D. in Human Health
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Laser welding of dura mater by means of indocyanine green-infused chitosan patches

Ph.D. Thesis

Dr. Roberto Colasanti

Tutor:
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July 2020

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Alla mia famiglia

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Preface

This Ph.D. thesis, titled “Laser welding of dura mater by means of indocyanine green-infused chitosan patches”, is submitted as a requirement for obtaining the Ph.D. in Human Health (Università Politecnica delle Marche).

The work presented in this Ph.D. thesis has been carried out during the three-years research activities conducted by the author, Roberto Colasanti, at the Department of Experimental and Clinical Medicine, Section of Neurosurgery, Università Politecnica delle Marche (Ancona), under the supervision of Prof. Massimo Scerrati and Prof. Maurizio Iacoangeli, and with the great help of many Doctors *(Dr. Alessandro Di Rienzo, Dr. Denis Aiudi, Dr. Erika Carrassi, Dr. Martina Della Costanza, Dr. Alessandra Marini).*

The work has been performed in collaboration, and with the great support of Researchers of the following institutes:

INRCA, National Institute Rest and Care for the Elderly, Ancona, Italy *(Dr. Fiorenza Orlando, Dr. Mauro Provinciali, Dr. Gianni Genga, Dr. Marta Balietti);*

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Roberto Colasanti

Ancona, July 2020

Abbreviations

α-SMA, Alpha-smooth muscle actin

BSA, Bovine serum albumin

DAPI, 4',6-diamidino-2-phenylindole

ICG, Indocyanine Green

I.N.R.C.A, Istituto Nazionale di Riposo e Cura per Anziani

PBS, Phosphate buffer saline

PBST, PBS solution containing 0.25% Triton X-100

List of original publications

This thesis is based on the following publications:

I. Colasanti R, Iacoangeli M, Marini A, Aiudi D, Carrassi E, Di Rienzo A, Scerrati M, Orlando F, Provinciali M, Giannoni L, Pieri L, Fagnani F, Dallari S, Magni G, Matteini P, Ratto F, Pini R, Rossi F, "Preliminary ex vivo and in vivo evaluation of laser bonding in dura mater". *Abstract from the SPIE BiOS 2020, San Francisco, California, United States, February 1-6, 2020, Proceedings Volume 11225, Clinical and Translational Neurophotonics 2020;* 112250G (2020) https://doi.org/10.1117/12.2543750.

II. Colasanti R et al., "Preliminary ex vivo evaluation of laser bonding of dura mater by means of indocyanine green-infused chitosan patches in a porcine model". *Manuscript under review.*

III. Colasanti R et al., "Preliminary evaluation of laser bonding of dura mater by means of indocyanine green-infused chitosan patches in an ex vivo human model". *Manuscript draft.*

IV. Colasanti R et al., "In vivo laser welding of dura mater by means of indocyanine green-infused chitosan patches in rats". *Manuscript draft.*

V. Colasanti R et al., "In vivo laser welding of dura mater by means of indocyanine green-infused chitosan patches: a comparison in adult and old rats". *Manuscript draft.*

VI. Colasanti R et al., "Laser bonding for dura mater and skull base reconstruction: literature review and our experience". *Manuscript draft.*

Abstract

Dura mater reconstruction represents a crucial step of a neurosurgical procedure. Indeed, an inadequate dural closure may determine dreadful consequences that significantly increase morbidity and mortality rates. Different dural substitutes/sealants have been used with suboptimal results. To overcome this issue, we previously proposed a laser-based approach to the bonding of porcine dura mater by means of Indocyanine Green (ICG)-infused chitosan patches, evidencing the feasibility of the laser assisted procedure. In this work, we present the optimization of the laser bonding approach for dura mater reconstruction, both ex vivo (in porcine and human tissues) and in vivo (in adult and old rats).

An 810 nm continuous-wave diode laser was used to weld the ICG stained chitosan patch to the dura. The ex vivo tests enabled to optimize the laser parameters, using histology and leak pressure evaluation to study the bonding effect. The in vivo tests were performed on 32 adult and 16 old Wistar rats: laser bonding was carried out in 16 adult and 8 old rats *(treatment groups),* while a non suturable collagen matrix was used for duroplasty in the remaining animals *(control groups)*. Then, at the planned follow-ups (respectively, the adult rats at 20, 60, 90, or 120 days after surgery, and the old rats on postoperative day 20 or 90), four rats in the treatment and four rats in the control groups were anesthetized for fluid leakage pressure test of the duroplasty, as well as for obtaining specimens for standard histology and immunohistochemistry.

The results of this study pointed out that the laser bonding procedure provides, both ex vivo and in vivo, an immediate dural closure without the need of any standard suturing, thus potentially reducing surgery times. In all the different models, the fluid leakage pressure values were always higher than the normal intracranial pressure. In addition, in the murine model, the laser bonding allowed to achieve fluid leakage pressure values statistically higher than those recorded with the collagen matrix as dural graft. No postoperative complication/neurological injury occurred in rats. The histological analysis evidenced a good adhesion between the chitosan patches and the dura mater, without a significant thermal effect. Chitosan patches effectively worked as biopolymeric scaffolds for dura mater regeneration, as evidenced by the histological analysis and the confocal microscopy. The laser bonding of dura mater was safely and effectively employed in old rats. Our data were in favor of the suggested age-related delay in the healing process. However, no significant functional differences were revealed in terms of fluid leakage pressure. Moreover, the maximum measured temperature after irradiation (<55°C) was, by far, well below the value reported in the previous studies on dural closure with laser tissue welding.

Hence, the technique can be proposed as a safe and valid alternative to traditional procedures for dural closure, in particular when the conventional suture technique is risky and/or extremely difficult, i.e. when working through narrow and deep surgical corridors (e.g. key-hole or endoscopic approaches), in case of reoperation, or following radiotherapy.

1. Introduction

Dura mater reconstruction represents one of the last but extremely important step of a neurosurgical procedure. Indeed, an inadequate dural closure may determine dreadful consequences (such as cerebrospinal fluid leaks, meningitis, neural tissue herniation, hypertensive pneumocephalus, pseudomeningocele, and adhesions) that significantly increase morbidity and mortality rates, while prolonging the hospital stay and raising healthcare costs.[1–12]

Different dural substitutes have been used when autologous dura mater is not available. However, no one of the so far developed dural substitutes is ideal. In fact, most of these substitutes have poor resistance to elongation, many cannot be sutured, they are very expensive and not always available in undeveloped countries. Moreover, these substitutes have been associated with complications such as aseptic meningitis, infections, adhesions, etc..[1–12]

Dural sealants have permitted to lower the overall incidence of the post-operative cerebrospinal fluid leak (the most frequent complication of an inadequate dural closure) from 4-32% to 0.9-10.7%. However, dural sealants are also high-priced and their application may determine complications, both acute (e.g. immune responses, rarely anaphylaxis) as well as chronic (e.g. adhesions, tissue fibrosis).[1,2,9–11]

Cerebrospinal fluid leaks remain a major concern after skull base surgery, especially following extended endoscopic endonasal approaches. In this regard, the introduction of vascularized pedicled flaps for cranial base reconstruction has reduced the rate of postoperative complications. However, vascularized pedicled flaps are used in combination with dural substitutes and/or sealants in most of the cases.[2–5,9–11,13–15]

Laser bonding could represent an alternative for the repair of dural defects. This is a technique that has been proposed in various surgical fields to overcome some problems that may arise with the standard suturing procedures. It has been widely demonstrated that in laser-assisted closuring of wounds several problems related to the use of stitches and needles can be overcome: there is no foreign body reaction, a better and faster healing phase, a reorganization of the treated tissue architecture, with no scar formation and an immediate watertight closure of the wound, thus reducing the risk of infections.[16–19] However, the results from experimental studies comparing the efficacy of laser bonding with traditional dural closure techniques have been inconsistent so far, and have blocked its introduction in clinical practice.[20]

In previous studies, we proposed a new laser-based approach to the bonding of biological tissues: biopolymeric materials were designed and prepared to be used as a patch covering a surgical wound.[21–23] The use of natural biopolymers for tissue repair has gained importance in the last few years. In particular, polysaccharides as hyaluronan and chitosan and their derivatives are suitable candidates for laser welding mainly because of their high affinity for the biological matrix, biodegradability and low cost. Moreover, chitosan is characterized by antimicrobial, haemostatic and wound healing-promoting activity. It naturally tends to form films with high mechanical strength and good elasticity.[24,25] Indocyanine green (ICG)

is commonly used in the laser welding procedures and it has been included in the biopolymeric matrices. The inclusion of chromophores into biopolymeric (chitosan) matrices has been exploited to construct tailor-made patches, in order to enable vascular healing process and wound closure. Moreover, the chosen biopolymer can play an active role in the post-surgical period by inducing a better reorganization of the tissue, decreasing the formation of scars and even preventing microbial infections. The matrix may also be loaded with additional drugs or factors which may optimize or speed up the wound healing process. In addition, the activation temperature range of chitosan (60-65°C) is 5°C lower than that of albumin solders (65-70°C), thus potentially reducing the risk of tissue thermal damage.[26] Furthermore, the inclusion of the chromophore in the biopolymeric matrices to be bonded may be helpful to further decrease the rise in the local temperature (typically in the range of 50 to 65°C).[27]

We published a preliminary ex vivo test of laser bonding of porcine dura mater and vocal fold by means of ICG-infused chitosan patches, evidencing the feasibility of the laser assisted procedure.[28] In this work, we present the optimization of the laser bonding approach for dura mater reconstruction, both ex vivo (in porcine and human tissues) and in vivo (in adult and old rats).

2. Review of literature

A literature search on Pubmed/Medline database was performed using the keywords “duraplasty”, “dural substitutes”, “dural graft”, “pericranium”, “galea”, “chitosan”, “cerebrospinal fluid leak”, “gelfoam”, “neurosurgery”, “tissue fusion”, “laser”, “dura mater”, “repair”, “soldering”, “welding”, “bonding”, “fibrin glue”, “dural defect repair”, “skull base”, “fascia”.

All records were carefully reviewed. Pertinent articles written in English were included. Cross check of references of the selected articles was performed in order to complete bibliographical research. Results were grouped on the basis of the welded tissue (dura mater and sinonasal mucosa for skull base reconstruction).

A total of 15 articles (11 on laser bonding for dura mater repair and 4 on laser welding of sinonasal mucosa for skull base reconstruction) were collected and analyzed.

2.1. Studies on laser bonding for dura mater reconstruction

In 1988, Hadley et al. evaluated in twenty-two greyhound dogs three different techniques of dural closure after transoral intradural operation: primary suture closure (running 8.0 nylon suture), laser fascia lata patch weld, and fibrin glue fascia lata patch closures.[12] All the animals survived up to the planned postoperative Day 14 follow-up, except one dog in the primary suture closure subgroup that developed a meningitis and was killed on Day 12. The laser and the glue closure subgroups showed similar good results 14 days postoperatively presenting an incomplete dural closure only in one out of seven animals of each subgroup. Conversely, 5 cerebrospinal fluid leaks were registered in the 8 animals of the primary suture closure subgroup, but the results were not statistically significantly different ($p=0.06$). However, a significant difference in closure competency at the time of operation was observed between the three subgroups ($p<0.01$). Indeed, every animal in the primary suture closure subgroup had a cerebrospinal fluid leakage, as well as all the seven animals who underwent the laser patch weld closure (even if for higher intrathoracic pressures in 3 cases), whereas the fibrin glue patch closure guaranteed an immediate and complete dural seal in all the cases supporting a pressure of more than 40 mmHg. Two animals (one in the primary suture closure subgroup, and one in the laser patch subgroup) had an abscess in the nasopharyngeal tissues close to the dural defect area. The authors concluded that fibrin glue is excellent for repairing complex dural defects and superior to both primary suture closure and laser patch weld closure.[12]

In 1996, Foyt et al. compared, in fresh human cadaveric dural fragments, the tensile strength of three different dural closure techniques: 4-0 silk suture (group 1, n=25), diode laser weld-reinforced 4-0 silk suture (group 2, n=20), and laser welding alone (group 3, n=25).[29] Moreover, leak pressure was measured on additional dural fragments. A 1-cm incision was carried out in the middle of the specimens, which were then divided into three further groups according to the closure technique: interrupted 4-0 silk suture (group 4, n=

25), diode laser welding alone (group 5, n=26), and interrupted suture reinforced with a laser-welded closure (group 6, n=25). The authors also performed an in vivo comparative study in fifteen Lewis rats. After a unilateral convexity craniectomy, the created 1-cm dural incision was closed by interrupted 9-0 Prolene sutures in 5 animals, laser weld closure alone in other 5 rats, and laser weld reinforcement of a suture closure in the last 5 rats. Two animals in each group were euthanized immediately, whereas the remaining three rats in each group were killed on postoperative day 14, thus allowing both early and late histological examinations of the tissues. Tensile strength of the welded closure alone (4.6 ± 1.4 Kgf/cm^2) was significantly weaker ($p<0.0001$) than that of the silk suture (13.3 ± 2.1 Kgf/cm^2). The laser solder reinforcement of the silk suture significantly increased the tensile strength (21.4 ± 2.4 Kgf/cm^2) over suture alone ($p = 0.0001$). Regarding hydrostatic burst pressures, suture closure alone (9.4 ± 1.7 mmHg) did not significantly increase the bursting pressure compared to the specimens in which no closure was attempted (4.5 ± 1.5 mmHg), while the bursting strength of the laser closure (26.2 ± 3.7 mmHg) was significantly higher than that of the interrupted 4-0 silk suture closure ($p<0.0001$). Weld-reinforced suture closure bursting strength (64 ± 6.7 mmHg) was significantly higher than all other types of closures ($p < 0.001$). Histological examination demonstrated no adverse effects in 8 out of 10 specimens analysed. Burn injury in the superficial layers of brain cortex was noted in one early (postoperative day 0) and one late (postoperative day 14) specimens. The authors concluded that laser welding might be a useful and safe dural closure technique, especially when an immediate leak-free closure is required. In addition, the tensile strength could be increased by combining laser welding and conventional suturing.[29]

In the same year, Menovsky et al. used scanning electron microscopy to investigate the structural changes of dura mater (n=10) and tibial nerve (n=10) taken from New Zealand white rabbits irradiated by CO_2 laser.[30] The specimens were divided into two groups: solder-assisted laser welding (using egg white as solder) and laser welding alone. They demonstrated that the bonding of the tissues was stronger in the solder group. The observation under electron microscopy showed that the collagen fibrils were swollen, densely packed, and fused together after laser irradiation, whereas (when a protein solder was used) the coagulated solder formed a solid bridge between the tissue edges. Hence, the authors concluded that laser welding mechanism depended on structural changes in collagen and that solder assisted laser welding caused coagulation of the protein solder that became the matrix in which the tissues were embedded.[30]

In 2005, Gil et al. performed fifty-seven experiments on specimens from fifteen adult pigs by using a CO_2 temperature-controlled laser system.[2] The fascia of the biceps muscle was used to patch the generated dural defect, and an aqueous 47% solution of bovine serum albumin (BSA) served as a solder. The weld line was approximately 2 cm long. The force required to detach the fused tissues and the leak pressure were measured. Further five experiments were carried out by suturing the fascia and dura with 6-0 continuous Prolene stitches. Reliable laser welding was obtained in 54 out of 57 experiments (95%), with an average peak adhesive strength of

82 ± 3 mN/cm^2 (as compared to a value greater than 10 N/cm^2 with the conventional suturing). The measured mean leak pressure of the fascia–dura soldered by CO_2 laser was 66 ± 5 mmHg, while conventional suturing resulted in an immediate cerebrospinal fluid leakage. The authors concluded that their CO_2 laser tissue-soldering technique for dural reconstruction was feasible, reliable and allowed to withstand cerebrospinal fluid pressure up to six times higher than normal intracranial pressure in a porcine model.[2]

In the same year, Forer et al. published their preliminary study regarding the feasibility of temperature controlled fiberoptic CO_2 laser welding for dural defects repair.[31] They investigated the reliability of laser soldering in in vivo (n=5) and in vitro swine models. In the in vitro experiments, a circular dural patch overlying the parietal lobe was excised and welded twice to a fascial patch by laser, using 47% BSA as solder. Then, the soldered area was subjected to burst strength measurements. The in vivo study was carried out in 5 female pigs in the same way. The animals were followed up for 10 days to monitor any clinical changes. At the end of this period, the welded area was microscopically observed to rule out any leakages and then taken, with the underlying brain, to examine the presence of necrosis, thermal damage or inflammation. The mean burst pressure of the soldered patches was significantly higher than intracranial pressure. No animal showed postoperative complications. Histopathological studies exhibited an accepted level of inflammatory reaction and showed no thermal damage to the underlying brain tissue.[31]

In 2006, they continued to investigate the matter by comparing the above mentioned laser soldering technique with and without microscope magnification.[32] The in vitro study was carried out by creating a circular dural defect over the parietal lobe, filled with fascial patch and lased. They divided 27 pig corpses into three groups: corpses operated without the use of the microscope (n=9), soldering done under microscope magnification with the outer "fatty" side of the fascial patch facing the dura (n=8) and laser welding realized under microscope control with the inner "muscular" side of the fascia facing the dura (n=10). The third group achieved the highest burst pressure (mean 258.5 ± 117.3 cmH_2O), which was significantly higher than the values registered in the other two groups, and more than ten times higher than normal intracranial pressure. The in vivo study was conducted in five pigs that underwent a 8 mm durectomy over the parietal lobe, repaired with fascial patch and lased. The animals were followed up for 10 days, and then re-operated to rule out any leakage. At the end, the animals were sacrificed and the reconstructed area was taken for histological analysis. The results showed no leakages and no neurological symptoms in all the pigs. Histological examination demonstrated the absence of necrosis or thermal damage. Therefore, the authors concluded that the CO_2 laser system created a watertight bond and did not cause thermal injury to the brain.[32]

In 2007, the same group compared the temperature-controlled fiberoptic CO_2 laser soldering with fibrin glue for in vitro dural repair in two equal groups of 10 pig corpses.[33] After a wide craniotomy, they created a circular 8-mm wide dural defect that was filled with a circular 15-mm wide autologous fascial patch in both groups. The patch was placed subdurally, then the

two above-mentioned reconstruction techniques were carried out. Finally, the reconstructed area was taken for burst pressure measurement. The difference in burst pressure between the two groups was highly significant in favour of the laser technique, with a mean burst pressure of 258.5 ± 117.3 cmH_2O versus 76.8 ± 47.2 cmH_2O in the fibrin glue group. One corpse in the fibrin glue group was excluded from the statistical analysis because the duroplasty was not watertight. In addition, the authors tested in vivo in five pigs the dural reconstruction with the laser system. At a 10 days follow-up, the soldered area was microscopically observed to rule out the presence of a cerebrospinal fluid leak, and then it was excised with its underlying brain tissue. Histological analysis revealed fibroblast infiltration of the longissimus et lumborum fascia, with no signs of necrosis. A complete sealing of the dura-fascia interface was evident. No architectural changes or signs of injury of the underlying brain tissue were visible, except for some mild recent bleeding at the level of the arachnoid and pia mater. The authors concluded that temperature-controlled laser soldering allows a safe and effective dural repair, creating a strong tissue bonding without any thermal damage to the tissue.[33]

In 2006, Ozisik et al. compared the effectiveness of four different dural closure techniques in eighty rats, after performing a 5 mm in diameter right parietal craniectomy, and a transverse dural opening of 3 mm.[34] The rats were randomly divided into two groups: group A (n=40) to test the tightness of dural closure achieved on postoperative day 1 with the different techniques and to perform an histopathologic analysis of the granulation tissue between the dura and dural graft after 14 days; group B (n=40) to assess the effects on the brain tissue underlying the duraplasty area 14 days after the operation. These 2 groups consisted of 5 subgroups: control, methyl metacrylate, n-butyl cyanoacrylate, fibrin glue, and CO_2 laser. The galea of the rats was used to harvest dural grafts (4 mm in diameter). In the control subgroup, the dural graft was positioned on the dural opening without any attachment technique. All animals were killed on the postoperative day 14. Regarding the competency of the dural closure on postoperative day 1, the n-butyl cyanoacrylate subgroup presented only one cerebrospinal fluid leak out of eight animals (p=0.0005). Good results were also noted in the methyl metacrylate and fibrin glue subgroups, with an incomplete dural closure in two out of eight rats in each subgroup (p=0.001), whereas 6 cerebrospinal fluid leaks were observed in the eight animals of the CO_2 laser subgroup (p=0.05). The histopathologic evaluation on postoperative day 14 showed moderate fibrous tissue development in the control subgroup. Methyl metacrylate had the thickest granulation tissue development. The CO_2 laser was the second subgroup for granulation tissue development, with collagen fibers forming bundles, and a capillary network. Conversely, in the fibrin glue and n-butyl cyanoacrylate subgroups, there was less evidence of inflammatory cell invasion. The analysis of group B revealed that there was not statistically significant difference of lipid peroxidation levels between the control and fibrin glue subgroups (p=0.44). The other subgroups had lipid peroxidation levels statistically significantly higher than the fibrin glue and control subgroups. The authors concluded that fibrin glue was the safest material for dural repair, with a

cerebrospinal fluid leakage risk higher than n-butyl cyanoacrylate, but significantly lower than control and CO_2 laser subgroups.[34]

In 2012, Zhong et al. investigated the CO_2 laser effectiveness in dural repair and the potential role of exogenous basic fibroblast growth factor (bFGF) or transforming growth factor-beta1 (TGFβ1) on wound healing in minipigs.[35] All animals underwent a craniotomy and a dural defect creation. An autologous temporalis fascia patch was inserted under the dural defect. The study was carried out into two phases. In the first part they used 10 minipigs, divided into 2 groups on the basis of the dural reconstruction technique: fibrin glue (group Ia), and CO_2 laser soldering (group Ib). One week after the repair, the specimens were subjected to burst pressure measurement and immunohistochemical analysis. In the second part they used 36 minipigs divided into 3 equal groups: CO_2 laser soldering alone (group IIa), laser soldering and fibrin glue enriched with recombinant bFGF (group IIb), and laser soldering and fibrin glue enriched with porcine TGFβ1 (group IIc). The specimens were subjected to burst pressure measurement after 1, 2, 3, and 4 weeks. All animals were followed-up for the scheduled time and then were subjected to a new craniotomy to expose the reconstructed area, which was observed under microscope to rule out any leaks, also in case of increased intracranial pressure provoked by injection of 0,1% methyl violet solution in the subdural space. After burst pressure measurement, the animals of part I were sacrificed and the specimens were taken for immunohistochemical examination. In Part I, the dura specimens showed the presence of only bFGF in group Ia, and of both bFGF and $TGF\beta_1$ in group Ib. Burst pressures were higher in Group Ib than in group Ia (98.00 ± 21.41 mmHg versus 70.80 ± 15.09 mmHg; $p<0.05$). No leaks were found. In Part II, group IIc had burst pressures significantly higher than group IIa ($p<0.01$). The burst pressures of group IIa tended toward stabilization after 3 weeks of growth, while that of groups IIb and IIc after 2 weeks of growth. The authors concluded that CO_2 laser soldering was a reliable technique for dural reconstruction, and that their superiority in the healing process could be related to higher expression of growth factors, whose secretion might be stimulated by CO_2 laser. In addition, exogenous bFGF or $TGF\beta_1$ could be involved in shortening the wound healing time.[35]

Later, in 2014, the same group analyzed the effect of exogenous bFGF or TGFβ1 on the healing process after dural reconstruction by CO_2 laser soldering in a minipig model.[36] The study was carried out with the same methods of the previous one. All animals recovered in 1-4 days without any neurological impairment and were killed 1 week after surgery in part I and after 1, 2, 3 and 4 weeks in part II. In part I, the CO_2 laser subgroup showed statistically significant higher fibroblast cells density than the fibrin glue subgroup. The CO_2 laser subgroup (Ib) showed a statistically significant higher optical density than fibrin glue subgroup (Ia) at immunohistochemical staining with antibodies against bFGF, and a positive staining with antibodies against TGFβ1, while fibrin glue subgroup was negative. In part II, the optical density of subgroup IIb was higher than that of subgroup IIa for specimen's immunohistochemical staining with anti-bFGF antibodies. Immunohistochemical staining with anti-TGFβ1 antibodies showed a higher optical density in both subgroups IIb and IIc than in the

control group (IIa). The authors concluded that CO_2 laser may trigger fibroblast proliferation, and that topic administration of exogenous bFGF and TGFβ1 could accelerate dural defect healing.[36]

In 2016, they carried out another study to test the feasibility and reliability of CO_2 laser for dural defects repair in the early postoperative and late follow-up periods in a minipig model.[37] For this purpose, they used 10 minipigs, divided into two groups: Group A (n=5) tested and sacrificed 5 minutes after dural reconstruction, and Group B (n=5) followed up for a month and then killed. All animals underwent a bilateral fronto-parietal craniotomy and a 2x1 cm dural defect creation, which was repaired by laser soldering. Also in this study they found that laser soldering was a reliable technique to repair dural defect without damaging the underlying brain. Indeed, the mean intracranial crest pressure was higher than the normal intracranial pressure, and histological analysis showed no brain injuries.[37]

2.2. Studies on laser bonding of sinonasal mucosa for skull base reconstruction

Bleier et al. developed a laser tissue welding technique for repair of sinonasal mucosal defects.[38–43]

In 2007, they published the first study regarding the feasibility of laser welding in endonasal tissues.[38] They conducted an ex-vivo study in sheep tissues, and in situ New Zealand white rabbit maxillary sinus mucosa in order to compare the burst strength of laser weld to traditional suture closure. Burst pressures in sheep tissues were tested in the nasal septal mucosa, hard palatal mucosa and periosteum under four conditions: intact tissue, 1 cm tissue incision, 1 cm tissue incision sutured with three interrupted 5-0 nylon sutures, and 1 cm tissue incision laser welded. In rabbits, the maxillary sinus mucosa was incised and laser welded in the left side, whereas it was left intact in the right side. Each condition was tested five times. In the sheep tissues, the laser welding was associated with burst pressures significantly higher than those registered with suture closure, and the septal mucosa had the highest burst pressures among the examined sheep tissues. In rabbits, the burst thresholds of the intact mucosa were significantly higher than those of the laser weld. The authors concluded that laser tissue welding could be successfully applied to endonasal tissues to produce instantaneous welds.[38]

A year later, they presented their in vivo experience in twenty New Zealand white rabbits.[39] After performing a 3 mm incision in maxillary sinus mucosa bilaterally, a strip of periosteum was placed over each mucosal defect. The periosteum on the left was soldered with the diode laser, whereas the one on the right was left in place without any further reconstruction and used as control group. Four rabbits were sacrificed on postoperative day 0, eight both on postoperative days 5 and 15. Burst pressures were significantly higher in the laser welds than in the control group on postoperative days 0 (120.85 ± 47.84 mmHg versus 7.85 ± 0.78 mmHg) and 5 (132.56 ± 24.02 mmHg versus 41.7 ± 7.2 mmHg), but no significant difference was detected on postoperative day 15 (169.64 ± 18.49 mmHg versus 160.84 ± 14.16 mmHg). The histological analyses did not find any thermal injury in the

specimens. No differences regarding overall inflammation or fibroplasia were noted between the laser and the control groups. The authors concluded that laser welding of sinonasal mucosa may safely and effectively determine tissue bonds that withstand even pathologically elevated intracranial pressures. In addition, the convergence of the burst pressures between the two groups on postoperative day 15 highlighted how the weld could act as a scaffold, preventing cerebrospinal fluid leak until native scar occurrence.[39]

In 2010, the same group published a prospective study performed on 10 patients who underwent endoscopic diode laser tissue welding using an albumin and hyaluronic acid-based solder for sinonasal mucosa injuries repair.[41] The surgical indication was a cerebrospinal fluid leak or skull base lesion in eight patients, and an inflammatory sinus disease in 2. The mean follow-up was 72 days, ranging from 12 to 138 days. No solder was evident in any patient beyond postoperative day 26. Regarding overall inflammation, thermal injuries or edema scores, no significant differences were found between the laser and the control group, and no complications occurred throughout the study. The authors concluded that endoscopic laser tissue welding represent a feasible, fast, and safe technique for sinonasal mucosa repair.[41]

Moreover, in 2013, they designed an in vivo study to evaluate the burst strength of a novel polysaccharide soldering gel composed of cyclodextrin, BSA and ICG dye, while defining its thermal and inflammatory profile in both rabbit respiratory mucosa and mouse dura mater, and its persistence time in tissues before complete resorption.[42] In the rabbits group (n=10), they performed bilateral maxillary sinus mucosa incisions, the left one welded with a diode laser irradiating the gel over the cut and the right one left unrepaired. Burst pressure was measured on postoperative days 0 and 5 in 4 rabbits before histological analysis, whereas the remaining 2 rabbits were used for histological examinations on day 45. In the mice group (n=4), they made a 3 mm craniotomy leaving the underlying dura intact. In 2 mice the gel was applied over the dura and lased, while in the remaining 2 ones the dura was lased for 60 seconds without any interposed solder. All mice were sacrificed on postoperative day 7 for histological examination. Within the rabbits, the burst pressures were significantly higher in the laser welded group than in the control one on postoperative days 0 and 5, without any significant differences regarding the thermal and the inflammatory profiles on postoperative days 0, 5 and 45. Moreover, no solder was detectable after 45 days from surgery. Also in the mice group, no significant differences in thermal and inflammatory patterns were found between the laser and the control groups. No neurological symptoms were developed in lased mice. The authors concluded that the gel solder taken into exam was safe and capable of creating mucosal repairs able to withstand over four times normal intracranial pressure without additional grafting materials.[42]

3. Aims of the study

I. To optimize a novel laser bonding technique for dura mater reconstruction by means of ICG-infused chitosan patches in an ex vivo porcine model, and to compare it with the conventional suture technique in terms of fluid leakage pressure.

II. To validate the feasibility and the effectiveness of our laser bonding technique for dura mater repair in an ex vivo human model, while making a comparison with the conventional suturing regarding the fluid leakage pressure.

III. To test and verify the feasibility and the validity of our laser assisted procedure for dural repair in vivo in adult rats at different follow-ups (in terms of safety, fluid leakage pressure, dura mater regeneration), while performing a comparison with a control group where a non suturable collagen matrix was used for duroplasty.

IV. To validate our laser bonding technique in vivo in old rats at different follow-ups, as well as to compare the dura mater healing process in adult and old rats.

4. Materials and methods

4.1. The ex vivo porcine model

The experiments were performed in the Photobiolab facilities (Florence) on fresh porcine tissues of 9-months old animals, which were sacrificed in a local slaughterhouse 3 hours before experiments. A wide craniotomy of the entire superior aspect of the skull was performed, thus exposing the dura mater and the underlying cerebral cortex. After the incision of the dura mater, the duroplasty was performed by a laser welding by means of ICG-infused chitosan patches in the *"treatment group"* (Figure 4.1), while a conventional suturing was performed in the control group.

Figure 4.1. *Laser bonding of the dura mater in the ex vivo porcine model. A characteristic green to beige transition of the patches evidenced the effective laser welding.*

4.2. The ex vivo human model

The experiments were performed in the animal research facility of the I.N.R.C.A (Istituto Nazionale di Riposo e Cura per Anziani, Ancona, Italy) on human dura that was harvested during routine post-mortem examination of the cranial cavity. Average time after death to harvesting of dura was 3 hours. The dura mater was immediately cryopreserved. Then, the tissue was gradually thawed to carry out the experiments at suitable times. After the dural incision, the opening was repaired by laser bonding in the "treatment group" (Figure 4.2), while a conventional suturing was carried out in the control group.

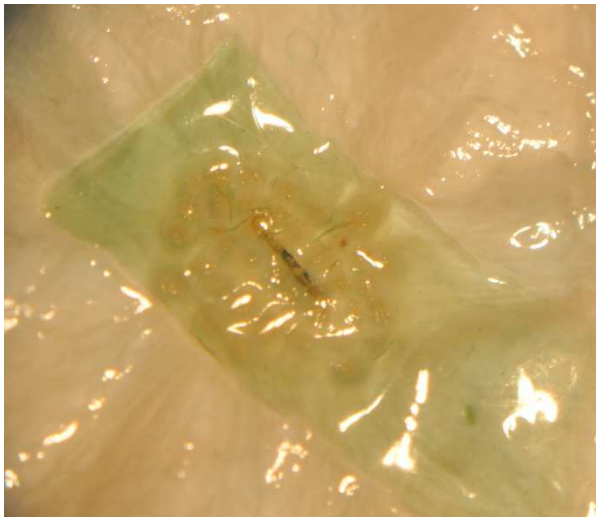

Figure 4.2. *Laser weld closure of the dura mater in the ex vivo human model. Note the typical green to beige transition of the patches in the bonding sites.*

4.3. The ex vivo and in vivo murine models

The ex vivo experiments were performed in the Photobiolab facilities (Florence) on fresh rats tissues within 5 hours from animal sacrifice. The ex vivo tests were used to optimize the laser parameters.

The in vivo tests were performed on 32 adult (7-12 months, weighting 500-700 g) and 16 old (20-24 months, weighting 800-1000 g) Wistar rats that were housed under pathogen-free conditions at the animal research facility of the I.N.R.C.A. Rats were anaesthetised by intra-muscular injection of ketamine/xylazine (50 mg/kg and 8 mg/kg, respectively) and the scalp was shaved and washed with povidon iodide propranolol solution. In addition, before the skin incision, the administration of a 18% mannitol bolus (on average 8 ml) was performed through the tail vein under aseptic surgical conditions. Under the operating microscope, a scalp incision was carried out in the midline and, using an electric drill, we accomplished a right parietal craniectomy. Then, the dura mater and the arachnoid were opened with a 25 G needle to allow full cerebrospinal fluid communication. At this point, laser bonding of the dura mater was carried out in 16 adult and 8 old rats *(treatment groups),* while a non suturable collagen matrix (Duragen; Integra LifeSciences Corporation, Plainfield, New Jersey, USA) was used for duroplasty in remaining 16 adult and 8 old rats *(control groups)*. Skin was closed with surgical clips (Figure 4.3).

The animals were left to recover after the procedure in individual cages under constant temperature (22°C) and humidity with a 12-hour light/dark cycle and with free access to food and water. Throughout the entire postoperative period, the rats were monitored for the presence of fever, cerebrospinal fluid leakage, and infection. Then, at the planned follow-ups (respectively, the adult rats at 20, 60, 90, or 120 days after surgery, and the old rats on postoperative day 20 or 90), four rats in the treatment and four rats in the control groups were anesthetized for fluid leakage pressure test of the duroplasty, as well as for obtaining specimens for standard histology. The experimental protocol was approved by the Institutional Animal Care Committee of the Ministry of Health (Italy, authorization number 808/2018-PR) and by the Animal Research Ethics Committee of I.N.R.C.A..

4.4. The laser system

The laser used in the research tests is an AlGaAs diode laser (produced by Quanta System Spa, Milano, Italy), emitting at 810 ± 10 nm. The device is enclosed in a compact cabinet and equipped with a fiber optic, with different available inner core diameter, ranging between 300 and 800 μm (Figure 4.4).

4.5. The ICG-loaded patches

The biopolymeric patches were prepared by pouring a dispersion of chitosan powder (low-molecular weight, Sigma-Aldrich, St. Louis, Missouri, USA) in an aqueous acidic solution of ICG (0.07% (w/v)) on circular moulds. After evaporation of the solvent, the samples were neutralized with alkali and finally washed.

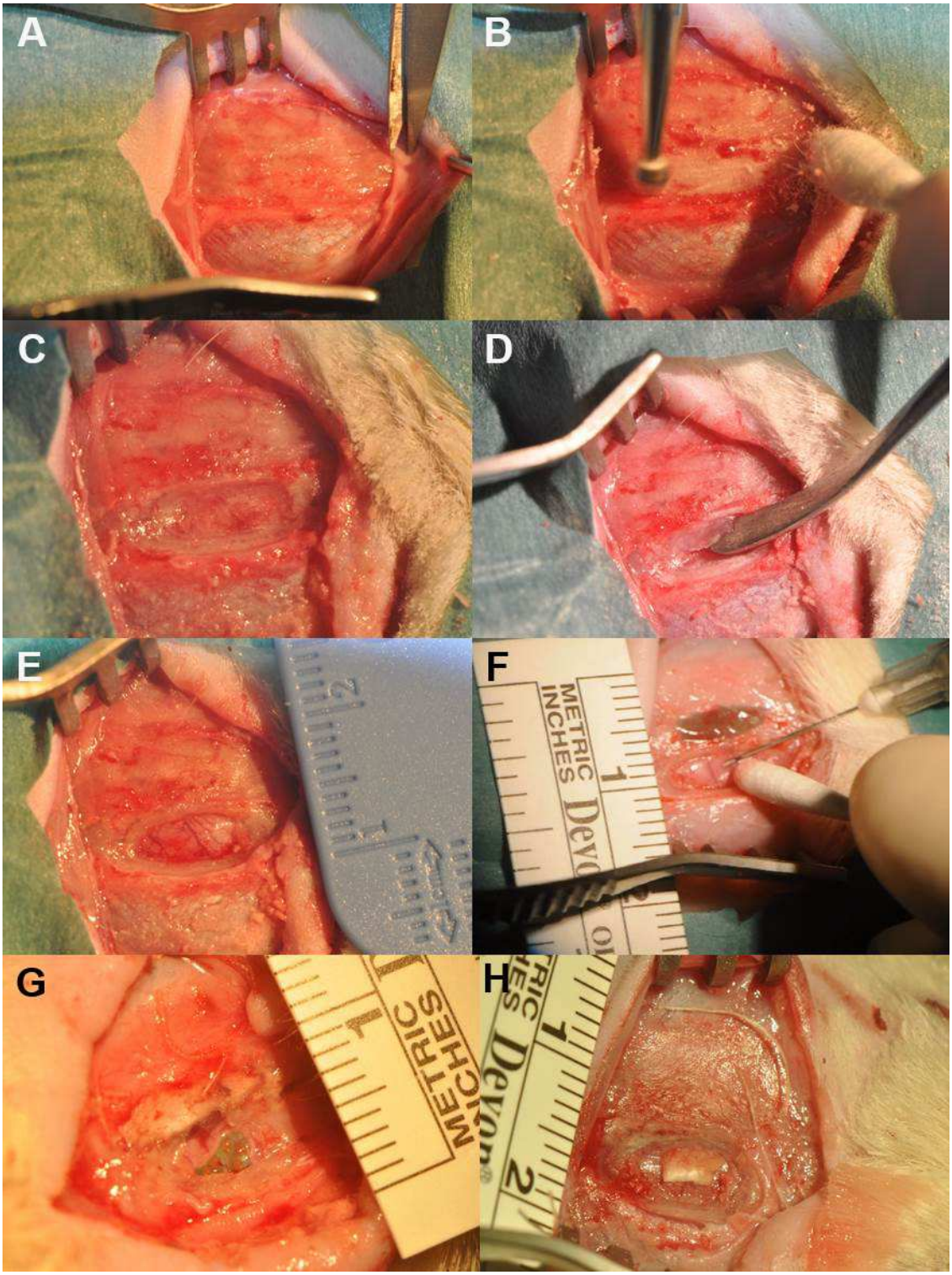

Figure 4.3. *The duroplasty in vivo in rats. After a midline scalp incision (A), under microscope magnification and with the aid of an electric drill, a right parietal craniectomy was gradually performed exposing the dura mater (B-E). At this point, the dura and the arachnoid were opened with a 25 G needle (F). Then, the duroplasty was performed by a laser welding of dura mater by means of ICG-infused chitosan patches (G, treatment group) or using a non suturable collagen matrix (H, control group).*

The so-obtained films (0.8 cm diameter, ~ 40 μm thickness) were resistant, pliable and stable in physiological environment. Moreover, the absorption properties resulted stable in time: the absorption peak was in the near infrared region, thus matching the diode laser wavelength (Figure 4.5).[28]

Figure 4.4. *The AlGaAs diode laser (Quanta System Spa, Milano, Italy), emitting at 810 ± 10 nm.*

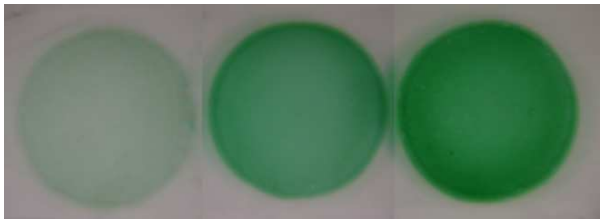

Figure 4.5. *The chitosan films with different ICG concentrations.*

4.6. Laser bonding

The laser bonding was performed with the aid of the surgical microscope. The dura mater was incised and then bonded. A diode laser emitting at 810 nm, equipped with a 500 μm diameter optical fiber was used to weld the patch onto the tissue, by delivering laser pulse burst to induce local patch/tissue adhesion. In Figure 4.6 the laser bonding procedure is depicted. The fiber tip was kept in contact with the chitosan patch, onto the tissue and the surface has been maintained hydrated during the treatment.

The first ex vivo tests were used to optimize the laser parameters. In the ex vivo porcine model, the fluence was about 25 J/cm^2 per pulse (30 pulses). A similar setting was used in the ex vivo human model (23 J/cm^2 per pulse, 30 pulses). The fluence was about 16 J/cm^2 per pulse (40 pulses) in rats.

Temperature control was performed during the laser bonding procedure: even if it is not possible to follow the temperature dynamics during treatment (as the fiber tip is in contact with the patch), it is possible to estimate the final temperature at the treatment end (Figure 4.7).

The weld line was approximately 1.5 cm long in the ex vivo porcine and human models, while about 0.7 cm in rats.

4.7. Control groups

In the ex vivo porcine and human models, a conventional suturing was performed in the control groups. After the incision of the dura mater, its margins were closed using 3-0 silk sutures evenly spaced 3 mm apart and 3 mm from the incision edge itself.

In the in vivo murine models (adult and old Wistar rats), as above written, a non suturable collagen matrix was used for duroplasty in the control groups.

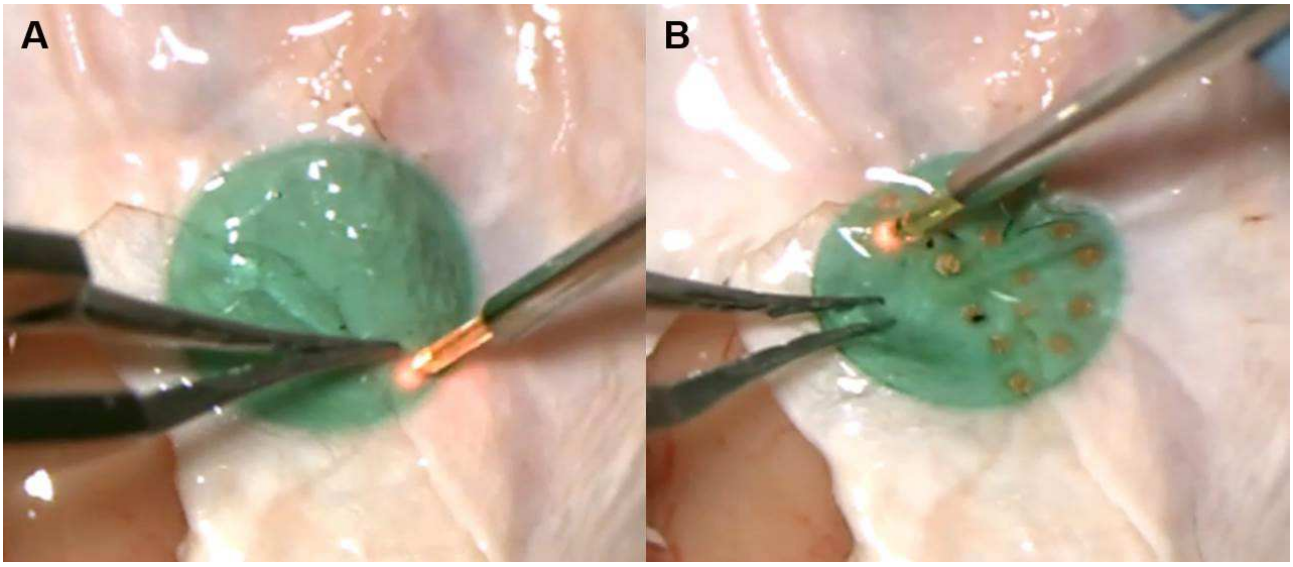

Figure 4.6. *The laser bonding procedure in the ex vivo porcine model. The green area is the ICG stained chitosan patch, the red spot is the pointer of the fiber optic used to the deliver the near-infrared laser light, the white tissue is the dura mater covering the underlying cerebral cortex. On the left, the beginning of the procedure is depicted. On the right, at a later stage of the welding procedure, the typical green to beige transition of the patches in the bonding sites is evident.*

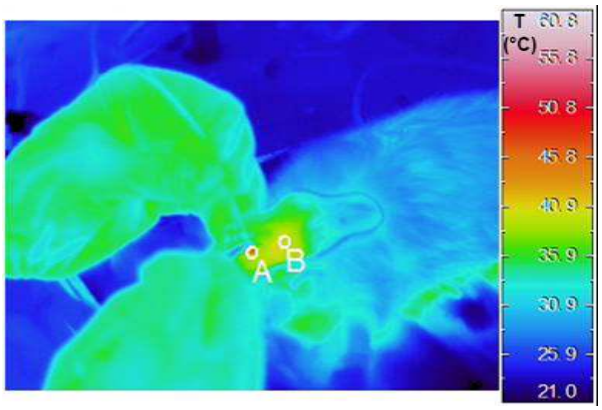

Figure 4.7. *Thermographic image at the end of the laser bonding procedure in rats in vivo. The point A is the welding point and the image is taken at the end of the welding procedure, as soon as the fiber tip is removed from the tissue.*

4.8. Fluid leakage pressure test

Tests were performed to evaluate the leak pressure of the duroplasty.

In the ex vivo porcine and human models, the specimens were tightly fixed to the rim of a 5-ml syringe by using a 2-0 silk suture. The 5-ml syringe was filled with saline solution and was placed in series with a pressure transducer (usually employed for intracranial pressure monitoring, Integra LifeSciences Corporation, Plainfield, New Jersey, USA) and a 10-ml syringe that was used to gradually raise the water pressure until fluid was noted to leak from the duroplasty (Figure 4.8).

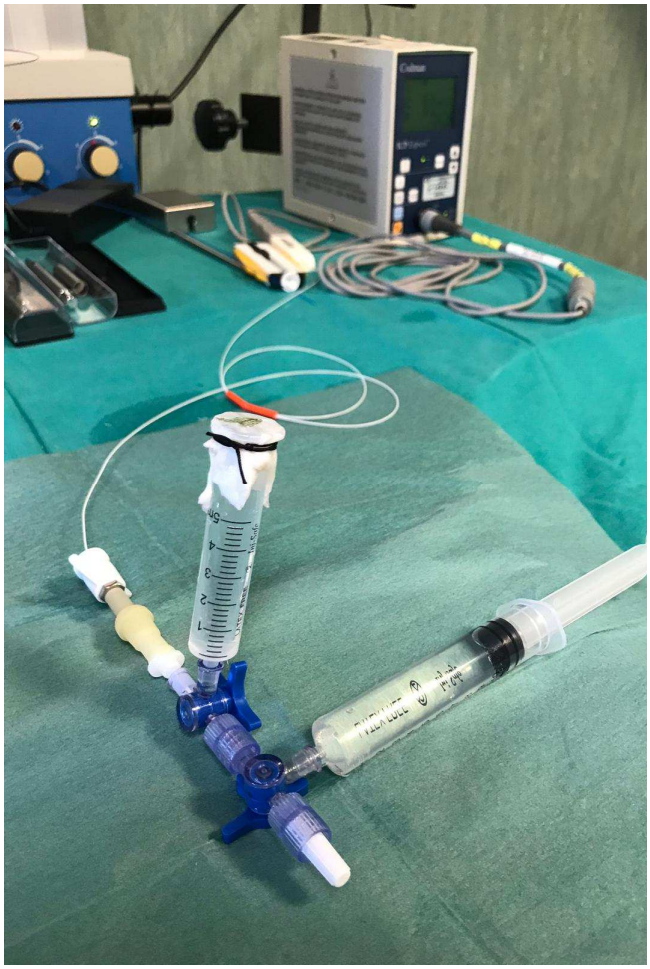

Figure 4.8. *The system used for measuring the fluid leakage pressure in the ex vivo porcine and human models.*

In the in vivo murine models, at the various follow-ups, we anesthetized the animals and exposed again the region of the duroplasty. Then, we carried out a left parietal craniectomy and, under microscopic guidance, we inserted into the subarachnoidal space a 25 G needle that was placed in series, as above, with a pressure transducer and a syringe that was employed to slowly infuse saline solution in the subarachnoidal space itself.

The fluid leakage pressure was read from the monitor connected with the pressure transducer.

4.9. Histologic examination

Samples of the tissues were harvested after laser treatment in the ex vivo models, as well as at the different follow-up times in the in vivo murine models, and prepared for standard histology analysis.

The specimens were immediately fixed in 10% neutral formalin, dehydrated through a gradated series of alcohol (or ethanol), cleared in Histo-clear (National Diagnostics, Atlanta, Georgia, USA), and embedded in paraffin. The 4-5 µm thick sections obtained were stained with hematoxylin & eosin for light microscopy evaluation.

4.10. Immunohistochemistry

In the in vivo murine models, immunohistochemistry was performed on 5 µm-thick brain sections collected on microscope slides after the application of antigen retrieval protocol as follow. The target antigens were retrieved by microwaving the sections submerged in sodium citrate buffer (10 mM sodium citrate, pH 6.0). The samples were heated in a microwave at 800 W for 3 min to initiate boiling of the solution and then for 3 min at 400 W to continue boiling, after that, the sections were cooled for 15 min at room temperature. The heating procedure was repeated twice with fresh buffer. After the last cooling, each sample was washed twice with phosphate buffer saline (PBS) and incubated in PBS solution containing 0.25% Triton X-100 (Sigma-Aldrich, USA) (PBST). After three washes in PBS, to block unspecific sites, the samples were incubated with 1% of BSA (Sigma-Aldrich, USA) in PBST

(PBST-BSA) for 30 min. After that, the samples were incubated overnight at 4°C in a solution of rabbit anti-alpha-smooth muscle actin (α-SMA) (AbCam, Cambridge, UK) diluted 1:250 in PBST-BSA. Cells were then washed three times with PBS and incubated 1 hour at room temperature with specific secondary antibody, AlexaFluor555 (AbCam, UK, Cambridge), diluted 1:500 in PBST-BSA. Cellular nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) contained in the mounting medium for cover slip (Sigma-Aldrich, USA) The images were obtained by a SP8 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany), using a 63X oil-immersion objective (NA 1.40) and the collected images were analyzed with an open source software (ImageJ, version 1.49v National Institutes of Health, Bethesda, MD, USA).

4.11. Statistical analysis

Statistical analysis to detect any differences among groups was carried out using Pearson's chi-squared test and Student's t-test. All analyses were performed using SPSS software (version 20; SPSS Inc., Chicago, IL, USA). The statistical significance was set at $p < 0.05$.

5. Results

5.1. The ex vivo porcine model

The experiments of laser bonding of porcine dura mater by using ICG-stained chitosan patches were performed on 30 specimens from six 9-months old animals. An immediate laser induced adhesion of the ICG-infused chitosan patches was observed. The effective laser bonding was evidenced by a slight modification of the appearance of the green patches in the bonding site (a characteristic green to beige transition).

The histology performed on treated samples evidenced a good adhesion between the patch and the dura mater tissue, with a spatially confined and limited thermal effect. A normal cortical cellular morphology and architectural pattern was observed under the laser bonding site (Figure 5.1).

The mean fluid leakage pressure was 216 ± 104.91 mmHg (range, 100 – 350 mmHg).

In the additional five experiments where the closure of the dura was performed with 3-0 silk sutures, the fluid leakage pressure test showed an immediate fluid leakage between the stitches and along the suture line in all the cases.

5.2. The ex vivo human model

The laser bonding was performed on 30 human dura mater specimens that were harvested from five post-mortem examinations. The prompt and effective laser induced adhesion of the ICG-infused chitosan patches with the underlying dura mater was always revealed by a typical green to beige transition of the patches in the bonding site.

The histologic examination confirmed a good adhesion between the chitosan patch and the dura mater (Figure 5.2).

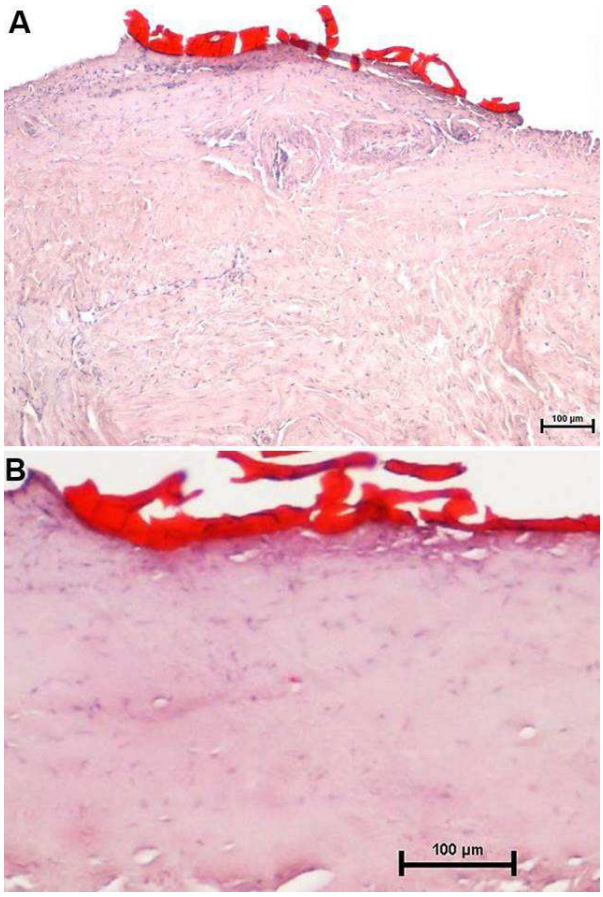

Figure 5.1. *Histology of laser bonded porcine dura mater; tissue samples were stained with H&E. The red tissue is the ICG chitosan patch, clearly welded to the dura. The thickness of the thermal damage in the tissue is around 40 µm and it is spatially confined in the area of the laser spot (A scale bar 10x; B scale bar 20x)*

The mean fluid leakage pressure was 218.33 ± 103.60 mmHg (range, 95 – 350 mmHg).

A conventional suturing of the dura using 3-0 silk sutures was carried out in further five

specimens. In these samples, used as control group, the fluid leakage pressure test evidenced an instantaneous fluid leakage between the stitches and along the suture line in all the cases.

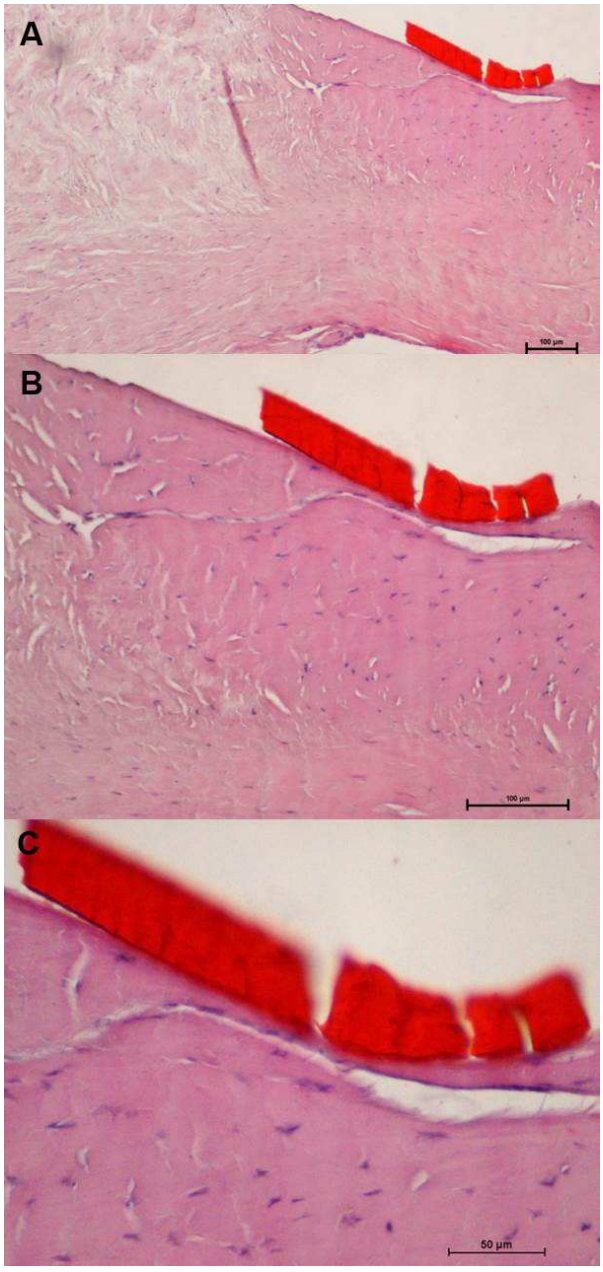

Figure 5.2. *Histology of laser bonded human dura mater; tissue samples were stained with H&E. The red tissue is the ICG chitosan patch, clearly welded to the dura (A scale bar 10x; B scale bar 20x; C scale bar 40x).*

5.3. The in vivo murine model: adult rats

Laser bonding of the dura mater was carried out in 16 adult rats *(treatment group),* while a non suturable collagen matrix was used for duroplasty in other 16 adult rats *(control group).*

All animals recovered uneventfully by 3 days from the surgical procedure. No postoperative complications (cerebrospinal fluid fistula, fever, infection, etc.) were observed.

Table 5.1 summarizes the fluid leakage pressures at the different follow-ups, both for the treatment and for the control groups. The registered fluid leakage pressures exceeded the normal range of the intraoperative cerebrospinal fluid pressure in both groups. The laser bonding of the dura mater by using ICG-stained chitosan patches allowed to obtain fluid leakage pressure values statistically higher than those recorded with the collagen matrix as dural graft.

The histological analysis showed a colonization by fibroblast and mononuclear cells at 20 days that was greater in the chitosan patches, but without statistical significance. At the longer follow-ups, fibroblasts were progressively replaced by fibrocytes and, then, by collagen fibers (Figure 5.3).

Qualitative analysis performed using confocal microscopy confirmed the presence of myofibroblasts populating the chitosan patches. Total cells were revealed by the fluorescence of DAPI that marked all the cellular nuclei, while the myofibroblast cells were identified by the fluorescence colocalization of DAPI and the α-SMA signals (Figure 5.4). Unfortunately, may due

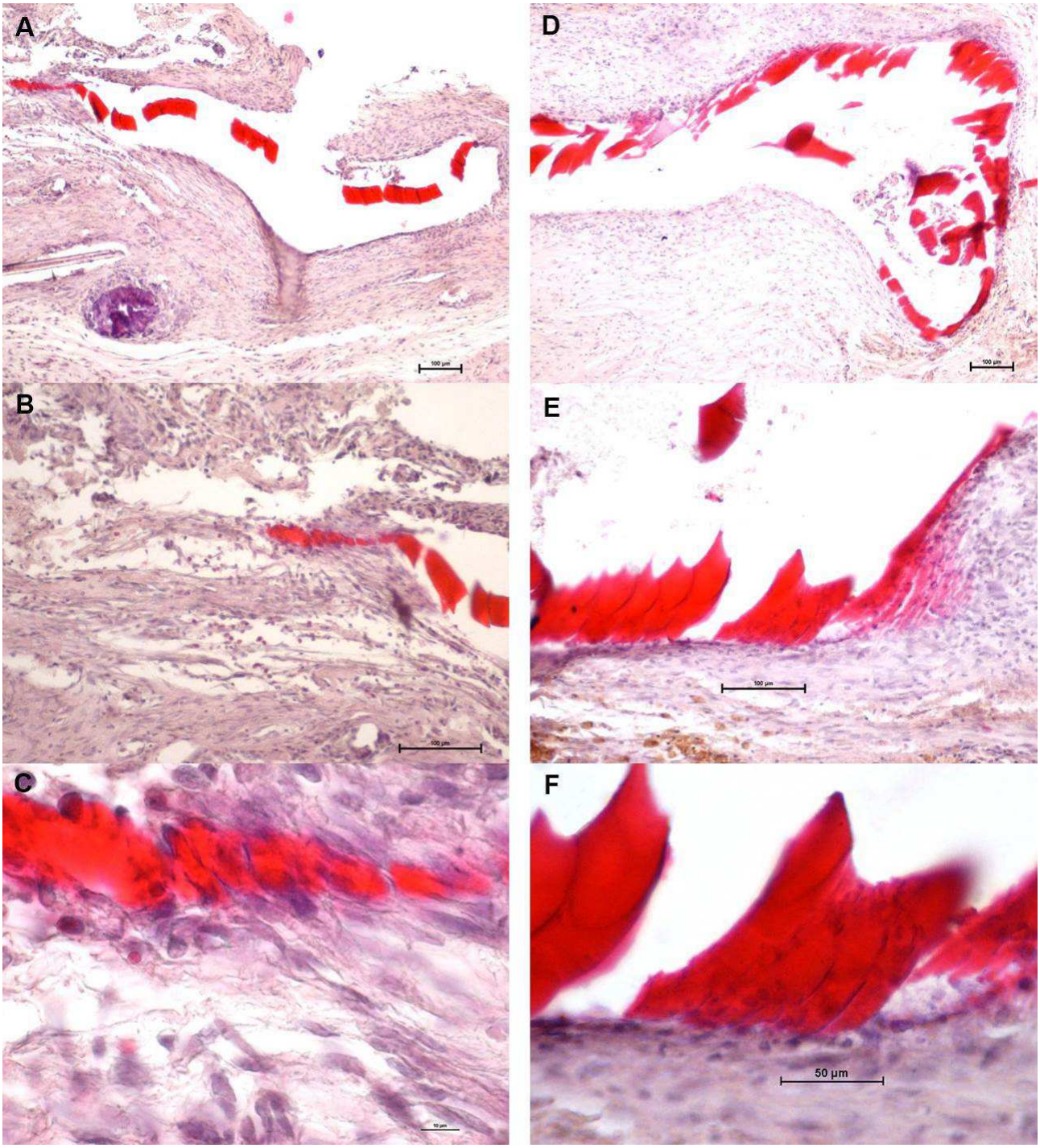

Figure 5.3. *Histology of laser bonded adult rat dura mater; tissue samples were stained with H&E. A-C, 20 days follow-up. The red tissue is the ICG-infused chitosan patch, clearly welded to the dura and colonized by fibroblast-like cells (A, scale bar 10x; B, scale bar 20x; C, scale bar 40x). D-F, 60 days follow-up. Fibroblasts and fibrocytes colonize the chitosan patch (D scale bar 10x; E scale bar 20x; F scale bar 40x).*

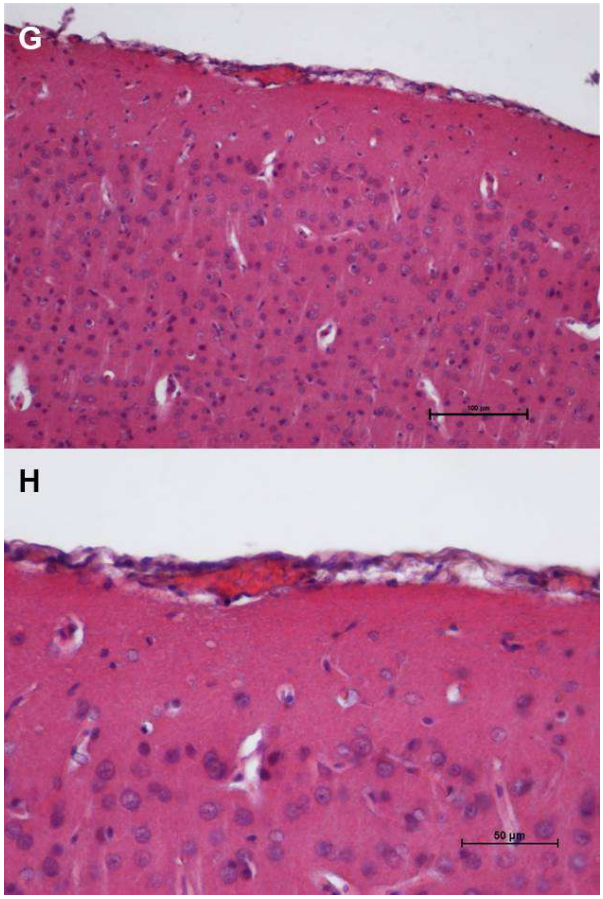

Figure 5.3. (Cont.) *Histology of laser bonded adult rat dura mater; tissue samples were stained with H&E. G-H, 90 days follow-up. Patch residues continue to be seen; the patch is now almost completely replaced by new dura mater tissue (G scale bar 20x; H scale bar 40x).*

to the sample processing and the antigen retrieval protocol applied to the samples, the chitosan patch appeared detached from the tissue. Nevertheless, the migration of the myofibroblasts in the tissue was clearly evidenced.

5.4. The in vivo murine model: adult *versus* old rats

Laser bonding of the dura mater was carried out in 8 adult and 8 old rats *(treatment groups),* while a non suturable collagen matrix was used for duroplasty in 8 adult and 8 old rats *(control groups).*

All animals recovered within 3 days, and no neurological impairment occurred. No postoperative complications (cerebrospinal fluid fistula, fever, infection, etc.) were experienced by the animals.

Table 5.2 summarizes the fluid leakage pressures for the two types of dural grafts in old rats at the different follow-ups. The values of the fluid leakage pressure were always superior to the normal range of the intraoperative cerebrospinal fluid pressure in both groups. The use of the laser bonding technique permitted to reach fluid leakage pressure values statistically higher than those recorded with the collagen matrix as dural graft.

Table 5.3 presents a comparison between adult and old rats of the fluid leakage pressure for the two types of dural grafts at the different follow-ups. The mean values were higher in adults than in old rats and achieved a statistical significance with the laser bonding technique at the 90 days follow-up.

A colonization by fibroblast and mononuclear cells of the two types of dural grafts was observed both in adults and in old rats at 20 days. The fibroblast and mononuclear cells density were higher in the treatment group than in the control

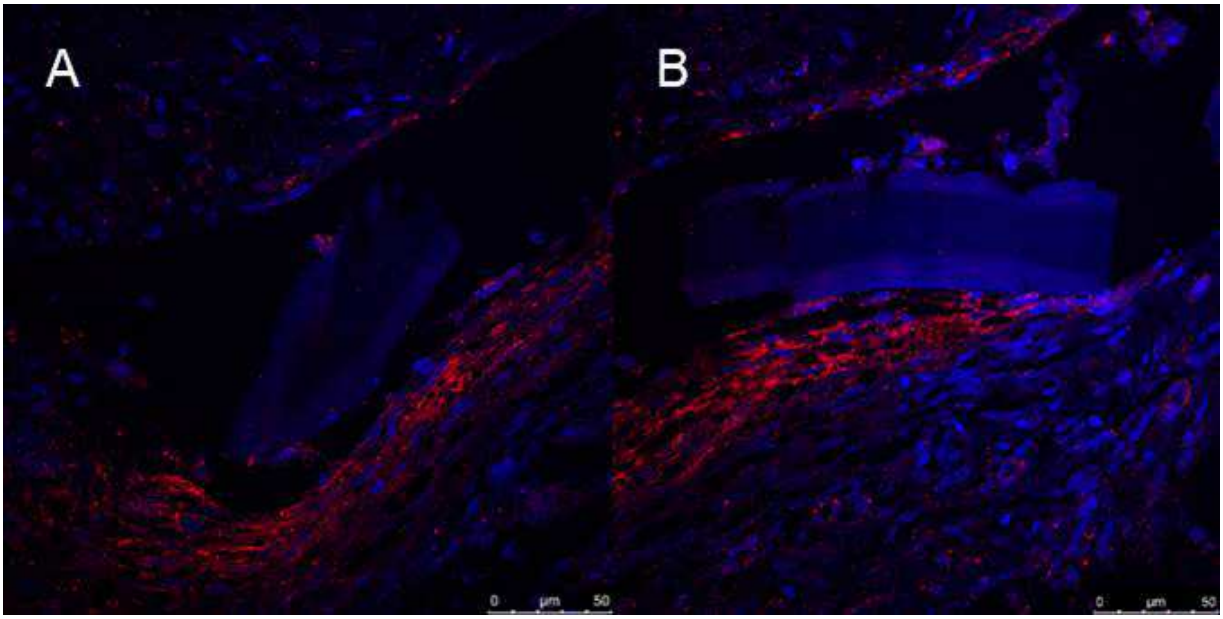

Figure 5.4. *A,B. Confocal microscopy of rat brain slice where cellular nuclei are stained in blue and α-SMA in red. The scale bar is 50 μm.*

Table 5.1. *Fluid leakage pressure for the two types of dural grafts in adult rats at the different follow-ups.*

Follow-up	**ICG-stained chitosan patch (mean ± SD, range) (mmHg)**	**Collagen matrix (mean, ± SD, range) (mmHg)**	**Univariate analysis, p-value**
20 days	301.25±37.05, 250-335	112.5±18.48, 95-135	<0.0005
60 days	321.25±16.52, 300-340	141.25±24.03, 119-168	<0.0005
90 days	346.75±13, 332-360	149.25±25.47, 125-180	<0.0005
120 days	368.75±13.15, 350-380	160.75±26.60, 134-192	<0.0005

ICG, indocyanine green; SD, standard deviation.

Table 5.2. *Fluid leakage pressure for the two types of dural grafts in old rats at the different follow-ups.*

Follow-up	**ICG-stained chitosan patch (mean ± SD, range) (mmHg)**	**Collagen matrix (mean ± SD, range) (mmHg)**	**Univariate analysis, p-value**
20 days	286.75±35.25, 238-321	103.25±15.41, 85-120	<0.0005
90 days	324.25±7.37, 316-333	136±26.14, 113-162	<0.0005

ICG, indocyanine green; SD, standard deviation.

Table 5.3. *Comparison between adult and old rats of the fluid leakage pressure for the two types of dural grafts at the different follow-ups.*

Follow-up	Adult rats - ICG-stained chitosan patch (mean ± SD, range) (mmHg)	Old rats - ICG-stained chitosan patch (mean ± SD, range) (mmHg)	Univariate analysis, p-value	Adult rats - Collagen matrix (mean ± SD, range) (mmHg)	Old rats - Collagen matrix (mean ± SD, range) (mmHg)	Univariate analysis, p-value
20 days	301.25±37.05, 250-335	286.75±35.25, 238-321	0.591	112.5±18.48, 95-135	103.25±15.41, 85-120	0.471
90 days	346.75±13, 332-360	324.25±7.37, 316-333	0.024	149.25±25.47, 125-180	136±26.14, 113-162	0.495

ICG, indocyanine green; SD, standard deviation.

group, both in adults and in old rats, but without statistical significance. In addition, the colonization by fibroblast and mononuclear cells at 20 days was slightly superior in adults than in old rats, but without statistically significant differences. At the 90 days follow-up, fibroblasts were replaced by fibrocytes and collagen fibers (Figure 5.5).

5.5. Temperature associated with the laser bonding procedure

The maximum measured temperature after irradiation was always below 55°C in all the different performed experiments, both ex vivo and in vivo.

As written in the previous paragraphs, even if it was not possible to follow the temperature dynamics during treatment (as the fiber tip was in contact with the patch), we controlled the temperature throughout the laser bonding procedure, and measured the final temperature of the welding area at the treatment end, as soon as the fiber tip was lifted from the patch. These observations were also validated by the analysis of the tissue through the histology, evidencing the eventual thermal damage and its degree.

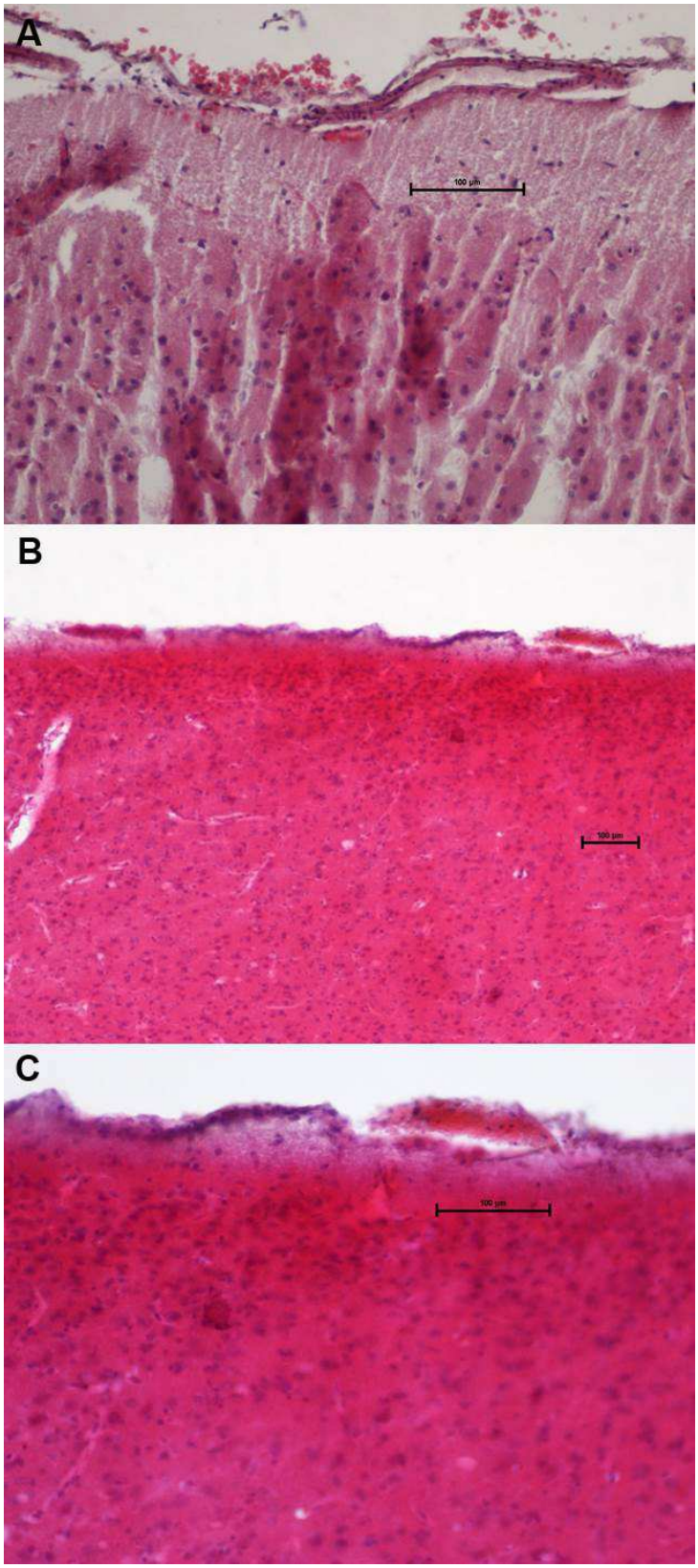

Figure 5.5. *Histology of laser bonded old rat dura mater; tissue samples were stained with H&E. A, 20 days follow-up. The red tissue is the ICG-infused chitosan patch, clearly welded to the dura and colonized by fibroblast-like cells (A, scale bar 20x). B-C, 90 days follow-up. The chitosan patch is almost completely replaced by new dura mater tissue (B scale bar 10x; C scale bar 20x)*

6. Discussion

An inadequate dural closure after a neurosurgical operation still remains a major concern, as it may determine detrimental clinical consequences, as well as an increase of healthcare costs. Dural substitutes and sealants have allowed to lower the overall incidence, but they are expensive and have been associated with various complications, both acute as well as chronic. Consequently, a standardization of dura mater reconstruction is still lacking.

6.1. Previous studies on laser bonding for dura mater reconstruction

Laser bonding represents an alternative for the repair of dural defects.

Foyt et al. performed one of the first study to repair dural lacerations, in fresh human cadaveric dura and in live Lewis rats, using a diode laser and a solder composed by albumin, hyaluronic acid and ICG dye. Dural closure with laser bonding alone allowed to achieve an immediate leak-free closure, but with poor tensile strength when compared with conventional interrupted sutures closure technique. In the human cadaveric dura, the laser bonding provided a greater mean leak pressure than conventional suturing (26.2 versus 9.4 mmHg), while a mean leak pressure of 64 mmHg was registered when a laser solder reinforced suture closure was utilized. In the murine model, histologic examination of welded dura and underlying brain tissue showed no evidence of thermal injury except for 2 of the 10 specimens examined, where a superficial cortical cell layers desiccation, i.e. a burn injury, was observed.[29]

In 1988, Hadley et al. evaluated in a canine model three different techniques of dural closure after transoral intradural operation: primary suture closure, laser fascia lata patch weld, and fibrin glue fascia lata patch closures. The laser and the glue closure subgroups showed similar good results 14 days postoperatively presenting an incomplete dural closure only in one out of seven animals of each subgroup (conversely, 5 cerebrospinal fluid leaks were registered in the 8 animals of the primary suture closure subgroup). However, all the seven animals who underwent the laser patch weld closure had an incomplete dural closure with cerebrospinal fluid leakage at the time of operation, whereas the fibrin glue patch closure guaranteed an immediate and complete dural seal in all the cases supporting a pressure of more than 40 mmHg.[12]

Other authors reported more encouraging results.

In a porcine model, Forer et al. employed a CO_2 laser system to heat a 20µm albumin surface, thus promoting the welding of a fascial patch to the dura mater. The laser bonding was associated with a significantly higher mean leak pressure than that of fibrin glue (258.5 ± 117.3 cmH_2O versus 76.8 ± 47.2 cmH_2O). An infrared computerized feedback system was used to maintain the surface temperature within a narrow range of 65 ± 3°C, thus avoiding inadvertent thermal damage to the bonded tissues and the underlying layers.[33] However, the CO_2 laser

wavelength is absorbed by the whole water content of tissues, thus it is not selective. The use of ICG or other chromophores in combination with a laser wavelength poorly absorbed by the tissue enables to avoid accidental thermal damage in healthy and not-stained tissue, while the laser energy is transferred only to the stained tissue.[16,17]

According to the postoperative histological analysis performed by Wang et al., dural repair by CO_2 laser soldering could determine a higher fibroblast cell density than conventional fibrin glue bonding, thus potentially expediting the healing process by stimulating more secretion of bFGF and TGFβ1.[35–37]

Bleier et al. developed an endoscopic endonasal laser tissue welding technique for repair of sinonasal mucosal injuries, using a hyaluronic acid-based solder with a diode laser, that was successfully and safely employed in 10 patients (eight with a cerebrospinal fluid leak or a skull base lesion, and the remaining two with an inflammatory sinus disease).[38–41] However, several solder-related drawbacks need to be addressed. As in many studies on laser bonding for dural repair, the active component of the solder was human albumin (the hyaluronic acid was used as a gelling agent), thus making the viral transmission a potential risk.[1,30,44] Moreover, as recognized by the authors, the viscosity of their solder was not enough to resist gravitational sedimentation, thus hindering and prolonging the lasing stage.[41] In addition, their solder was soluble in biological fluids (e.g. blood, cerebrospinal fluid, etc.) that could easily displace it from the region of application as a consequence.[1,41,44]

To summarize, after the first unsuccessful dural reconstruction attempts by laser bolding, various authors have obtained promising results.[2,12,29,33–37,41] However, different factors (such as the risk for thermal injuries, difficulties in the standardization of the procedure, solder-related concerns, etc.) have prevented the adoption of this technique in the clinical settings so far.[2,12,29,33–37,41]

6.2. Relevance of our laser bonding model

In this work, we present the optimization of a laser bonding technique we previously developed for dura mater reconstruction by means of ICG-infused chitosan patches.[28]

As written in the introduction, several fundamental properties such as excellent biocompatibility and biodegradability make chitosan a very suitable material for laser welding.[24,25] Chitosan naturally tends to form films with high mechanical strength, good elasticity and rather slow biodegradation, which could be helpful to promote a better reorganization of the tissue in the postoperative period. Moreover, its low cost, as well as its antimicrobial, haemostatic and wound healing-promoting activity could make this natural biopolymer an ideal dural substitute.[24,25] Furthermore, the activation temperature range of chitosan (60-65°C) is 5°C lower than that of albumin solders (65-70°C), thus potentially reducing the risk of tissue thermal damage.[26] In addition, the inclusion of the chromophore (ICG) in the biopolymeric matrices to be bonded may be useful to further decrease the rise in the local temperature (typically in the range of 50 to 65°C) and to improve the spatial selectivity of the procedure.[27]

However, there have been few reports so far of the use of chitosan as a substitute for dura mater.[1,45] Sandoval-Sánchez et al. compared in a New Zealand rabbit model three different techniques of dural reconstruction: primary suture closure of the autologous dura mater, duroplasty with a non suturable collagen matrix, and duroplasty with a bilayer chitosan scaffolding fixed with sutures. Chitosan allowed effective dural repair without cerebrospinal fluid leak. No significant differences in fluid leakage pressures between the three groups were reported. Moreover, chitosan promoted an organized regeneration with fibroblasts without evidence of fibrosis.[1]

Tissue repair with laser-activated chitosan films has been proposed,[46–48] but there have been no reports of their use for dural closure.[28]

6.3. The ex vivo porcine model

In a previous study, we demonstrated the feasibility of the laser bonding technique for dura mater reconstruction in an ex vivo porcine model by means of ICG-infused chitosan patches.[28]

In the present work, we optimized the procedure, resulting in an immediate watertight dural closure without the need of any standard suturing. The mean fluid leakage pressure was 216 ± 104.91 mmHg. Even if the range of the registered fluid leakage pressures was quite wide (100 – 350 mmHg), thus underlining the need of a further standardization of the laser bonding procedure, the lowest value was potentially sufficient to withstand a sudden rise in intracranial pressure, i.e. during coughing or Valsalva's maneuver. In other words, the laser-welded dural closure easily exceeded the normal range of the intraoperative cerebrospinal fluid pressure, whereas conventional suture closure did not.

The histological analysis evidenced a good adhesion between the chitosan patches and the dura mater, with a negligible and spatially confined thermal effect. The brain tissue underlying the laser bonding site had the regular pattern of neurons and glial cells that showed normal histological features.

The maximum measured temperature after irradiation (< 55°C) was, by far, lower than those reported in the previous studies on dural closure with laser tissue welding.

6.4. The ex vivo human model

We validated the feasibility and the effectiveness of our laser bonding technique for dura mater reconstruction in an ex vivo human model, on the basis of the good results we obtained in the ex vivo porcine model and in view of a future clinical experimental study. Indeed, it is widely well-known that could be important species-to-species variation in the structural characteristics of the dura mater, in part due to the supine versus upright nature of the species, as well as the following different cerebrospinal fluid pressure determined by the gravity.[49–51]

Our laser assisted technique for dural reconstruction by means of ICG-infused chitosan patches permitted to achieve an immediate watertight dural closure without the need of any standard suturing. The mean fluid leakage pressure was quite similar to that registered in the

porcine model (218.33 ± 103.60 and 216 ± 104.91 mmHg, respectively). Differently from the conventional suture closure, our laser bonding technique for dural repair was associated with fluid leakage pressure values that were superior to the normal range of the intracranial pressure. However, in a similar way to the porcine model, we observed a wide range of the fluid leakage pressure values (95 – 350 mmHg), which again emphasizes the fact that a standardization of our laser bonding technique is paramount to safely and effectively adopt the procedure in different clinical settings.

The histological analysis confirmed a good adhesion between the chitosan patches and the dura mater.

As in the ex vivo porcine model, the maximum measured temperature after irradiation was < 55°C. Only a limited reduction of the laser fluence, as compared to the porcine model, was required to successfully bond the cadaveric human dura mater (from 25 J/cm^2 to 23 J/cm^2 per pulse), due to quite similar structural characteristics.[49–51] It is reasonable to believe that minor adjustments of the laser setting could be required for the application of our proposed technique in vivo.

6.5. The in vivo murine model: adult rats

Our laser bonding technique for dura mater reconstruction was validated in vivo in rats. All animals recovered uneventfully within 3 days from the surgical operation. No postoperative complication was revealed, and no neurological injury occurred, as confirmed by repeated clinical evaluations. These findings represented indirect signs of the lack of any significant thermal effect to the underlying brain parenchyma following the dural laser bonding. The activation temperature range of chitosan, as well as the inclusion of the chromophore (ICG) in the patches, allowed to achieve an effective laser welding with a laser fluence of 16 J/cm^2 per pulse and a maximum measured temperature after irradiation always < 55°C.

The employed laser setting was obviously a direct consequence of the specific characteristics of the dura mater of rats, markedly thinner and more fragile than human and porcine dura. On the other hand, the subarachnoid space is also comparatively thinner in rats and, consequently, there is less room for an efficient heat sink between the dura and the brain parenchyma, thus potentially amplifying the thermal spread.[42] Hence, the absence of any thermal damage in vivo in the murine model, confirmed also by the histological analysis of the collected samples at the different follow-ups, represents a paramount finding and further implies that our laser bonding technique of dura mater could be safely used in vivo in larger animals, as well as in a clinical setting, without significant risk to the underlying neurovascular structures.

Moreover, the histological analysis and the confocal microscopy evidenced a colonization by fibroblast and mononuclear cells that was more pronounced in the chitosan patches than in the collagen matrix, as previously reported by Sandoval-Sánchez et al.,[1] but without statistical significance. At the longer follow-ups, fibroblasts were progressively replaced by fibrocytes and, then, by collagen fibers. Hence, chitosan patches effectively worked as biopolymeric scaffolds for dura mater regeneration.

The fluid leakage pressures at the different follow-ups were always considerably superior to the normal intracranial pressure, both for the treatment and for the control groups. However, the laser bonding technique allowed to achieve fluid leakage pressure values statistically higher than those recorded with the collagen matrix as dural graft.

6.6. The in vivo murine model: adult *versus* old rats

The incidence of postoperative cerebrospinal fluid leaks is determined by various factors such as the location of surgery, the size of dura opening, as well as by patient-related factors such as age and comorbidities.[10] Indeed, it is well recognized that ageing could be associated with a delay in the rate of the healing process.[52–54] In addition, the consequences of an inadequate dural closure could be worse in older than in younger patients in terms of outcomes, since older patients usually have multiple comorbidities, and are more prone to develop post-operative systemic complications such as thromboembolic events and pneumonia.[13,55,56] Hence, we reasoned that it could be worthwhile to validate our laser bonding technique in old rats, as well as to compare the dura mater healing process in adult and old rats.

All animals experienced an uneventful postoperative recovery within 3 days, without complications nor neurological impairment. The laser fluence (about 16 J/cm^2 per pulse) and the maximum measured temperature after irradiation (< 55°C) were identical in adults and old rats. The histological analysis confirmed the lack of any thermal injury to the brain parenchyma following the application of laser bonding for dural reconstruction. In addition, the histologic analysis confirmed the potential role of chitosan patches as biopolymeric scaffolds for dura mater regeneration.[1] Indeed, chitosan patches were largely colonized by fibroblast and mononuclear cells at the 20 days follow-up, more than the collagen matrix but without statistically significant differences. The fibroblast and mononuclear cells density at 20 days was slightly superior in adults than in old rats, but again without statistical significance.

Our laser bonding technique permitted to reach fluid leakage pressure values statistically higher than those recorded with the collagen matrix as dural graft, also in old rats. On the other hand, the mean values were higher in adults than in old rats and achieved a statistical significance with the laser bonding technique at the 90 days follow-up. This could reflect the different rate of the healing process in adult and old rats. However, it is worth to underline that the registered fluid leakage pressures were always higher, by far, of the normal intracranial pressure.

To summarize, our laser assisted technique for dural reconstruction was safely and effectively employed in old rats. Our data were in favor of the suggested age-related delay in the healing process. However, no significant functional differences were revealed in terms of fluid leakage pressure.

6.7. Limitations

Further experimental studies are needed in order to improve the proposed technique, as well as to standardize the results in different clinical scenarios. First and foremost, the technique needs

to be validated in vivo in humans. In this regard, the preliminary tests conducted in rats showed encouraging results.[57]

Moreover, some modifications of the laser applicator could allow to expedite and further simplify the laser bonding process. In addition, a computer program could detect the characteristics of the welding area (e.g. the local temperature, the exact thickness of the dura mater, etc.), and automatically adjust the laser output, if needed, while minimizing the potential thermal damage. Finally, the adhesiveness of chitosan patches could be potentially enhanced by the use of other compounds in the same patch.[58,59]

7. Conclusions

I. We optimized a novel laser bonding technique for dura mater reconstruction by means of ICG-infused chitosan patches in an ex vivo porcine model, resulting in an immediate watertight dural closure with fluid leakage pressure values higher than the normal intracranial pressure. The histological analysis evidenced a good adhesion between the chitosan patches and the dura mater, without a significant thermal effect.

II. Our laser bonding technique for dura mater repair was validated in an ex vivo human model, registering fluid leakage pressure values higher than the normal range of the intracranial pressure. The mean fluid leakage pressure was quite similar to that registered in the porcine model (218.33 ± 103.60 and 216 ± 104.91 mmHg, respectively). A good adhesion between the chitosan patches and the dura mater was confirmed by the histological analysis. Only a limited reduction of the laser fluence, as compared to the porcine model, was required.

III. The validity of our laser assisted procedure for dural repair was verified in vivo in adult rats at different follow-ups. No postoperative complication/neurological injury occurred. The lack of any significant thermal effect to the underlying brain parenchyma was confirmed by the histological analysis. Chitosan patches effectively worked as biopolymeric scaffolds for dura mater regeneration, as evidenced by the histological analysis and the confocal microscopy. Even if the fluid leakage pressures were considerably higher than the normal intracranial pressure with both techniques, the laser bonding technique allowed to achieve fluid leakage pressure values statistically higher than those recorded with the collagen matrix as dural graft.

IV. Our laser assisted technique for dural reconstruction was safely and effectively employed in old rats. No complication was recorded after the procedure. The histological analysis did not show any significant thermal injury, but revealed a colonization of chitosan patches by fibroblast cells, progressively replaced by fibrocytes and collagen fibers. The laser fluence was identical in adults and old rats. The laser bonding technique permitted to reach fluid leakage pressure values statistically higher than those recorded with the collagen matrix as dural graft, also in old rats. Our data were in favor of the suggested age-related delay in the healing process. However, no significant functional differences were revealed in terms of fluid leakage pressure.

In summary, the laser assisted technique for dura mater repair was optimized in combination with the use of ICG-stained chitosan patches, both ex vivo (in porcine and human tissues) and in vivo (in adult and old rats). The procedure enables to provide an immediate dural closure without the need of any standard suturing, thus potentially reducing surgery times. In addition, the maximum measured temperature after irradiation (< 55°C)

was, by far, lower than those reported in the previous studies on dural closure with laser tissue welding. The positive results of direct observations, fluid leakage pressure tests, and histological analysis evidenced that this technique can be proposed as a valid alternative to traditional procedures, in particular when the conventional suture technique could be risky and/or extremely difficult, i.e. when working through narrow and deep surgical corridors (e.g. key-hole or endoscopic approaches), in case of reoperation, or following radiotherapy.[1,2,10,11,40,41,43] Moreover, the use of biocompatible patches open up new strategies to provide local treatments, e.g. by the introduction of dural healing factors or drugs which can be loaded within the polymeric matrix.

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