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DOTTORATO DI RICERCA IN CHIRURGIA SPERIMENTALE E MICROCHIRURGIA - XXX CICLO
Coordinatore Chiar.mo Prof. Paolo Dionigi

Nanotechnology and New Horizons in Postoperative Pain Management

Dott. ssa Claudia Filisetti
Matricola N° 432773

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Summary

PREFACE

As pediatric surgeon, every day I am in contact with children who experience postoperative pain. It is not always easy to understand the mechanisms that regulate pain, and not all the strategies available to alleviate or prevent it, are always known and applied. My research focused initially on the analysis of pain management in children and then moved on a comprehensive assessment of technological resources, in particular nanotechnologies, applicable for intralesional analgesia. An accurate analysis of the experimental models in the big animal followed in order to define the translational pathway research to enforce this investigation. These were the premises to get to design a study on the use of the local anesthetic chloroprocaine in continuous infusion through intralesional catheter. Based on the results obtained, we designed an original and brand-new nano device loaded with chloroprocaine and we studied its effects in vivo.

1. POST-OPERATIVE ANALGESIA

1.1 Pain management in children

Pain control after surgery is a popular matter when children are involved. Since the mid-1980s, the need to improve postoperative pain control started to be a cherished problem. In the last 20 years, pediatric hospitals have implemented pain management protocols improving postoperative pain relief of patients and families' satisfaction, although pain management continued to be a problem after pediatric surgery. Today, the importance of postoperative pain control is well recognized; pain is considered as the fifth vital sign, and needs clinical innovations in improving its care. Indeed, the enhancement in pain management results in a reduction of recovery time and complications. Pain is a complex and unsolved phenomenon, characterized by different pathophysiological changes potentially leading to relevant postoperative morbidity and can strongly affect the clinical outcome.

Pain in children requires special consideration due to physical and psychological immaturity. We have few data regarding clinical features and the pathophysiology of postsurgical pain in children. In particular, few studies have focused on postoperative pain and analgesia requirements in children following surgical procedures [1-11]. We reviewed this field using the following search criteria using PRISMA flow chart considering the last 5 years: "pediatric pain management"; "pediatric pain management guidelines"; "pediatric pain management review"; "post operative pain management in children; "pain management in pediatric surgery". We summarized in Table 1 the most

relevant papers published in the last 5 years (source PubMed/MedLine). None of these studies directly assessed functional limitation or provided detailed information about the type and duration of analgesia use. We were unable to identify any studies that systematically followed patients after specific surgical procedures through to full recovery. Stewart and coworkers described the clinical features (duration and severity) of postoperative pain and analgesia requirements in children after tonsillectomy, orchidopexy, or inguinal hernia repair [12]. The investigation on the different clinical and pathophisiolological features of the pain related to different surgical procedure is crucial to develop new strategies in the management of pain in children. From the data available the incidence of perioperative pain in children is high [13].

Until now, we cannot predict which patients will develop severe postoperative acute and/or persistent pain, and the standard analgesia is chosen by considering patient personal data (weight and age) and clinical conditions. We have to consider that little data are available about how painful the pediatric surgery is, due to the difficulty of pain assessment [13]. Nevertheless, in order to stem the problem of pain evaluation in neonates, infants, and children under 4 years, physiological parameters such as increases in heart rate, respiratory rate and blood pressure with the presence of sweating, high cortisol and cortisone levels, and endorphin concentrations are measured, together with the evaluation of the facial expressions. Considering that different receptors and neurochemicals are involved in postoperative pain pathways, pain management should be multimodal [14]. By using minor, moderately active or major analgesics, it is possible to act on the transduction of the stimulus into the

electrical signal, on the transmission of the signal along the pain pathways, and on its modulation and perception, which is also modulated by cognitive, emotional and cultural factors. Currently, the systemic multimodal approach includes different drugs, ranging from paracetamol (acetaminophen), to nonsteoroidal anti-inflammatory drugs (NSAIDs), opioids, adjuvants as ketamine and clonidine and regional anesthetics [15]. Another analgesic technique involves the On-Q pain pump, which allows a continuous infusion of continuous local analgesia by a catheter placed deeply in the fascial planes, without the risks associated to the epidural catheterization but with the advantage to provide similar analgesia [16]. Great advantages are associated with regional anesthesia, too. A substantial body of literature supports the safety and efficacy of performing regional anesthetic techniques in children; evidence-based literature shows that combined regional and general anesthesia in paediatric patients can decrease hospital stay and improve outcomes [17].

First author	Title	Year of publication	Journal
Chou R. et al.	Management of Postoperative Pain: A Clinical Practice Guideline From the American Pain Society, the American Society of Regional Anesthesia and Pain Medicine, and the American Society of Anesthesiologists' Committee on Regional Anesthesia, Executive Committee, and Administrative Council.	2016	J Pain
Van der Heijden MJ et al.	The Effects of Perioperative Music Interventions in Pediatric Surgery: A Systematic Review and Meta-Analysis of Randomized Controlled Trials	2015	Plos one
Baley K et al.	Intravenous acetaminophen and intravenous ketorolac for management of pediatric surgical pain: a literature review.	2014	AANA J
Shah RD et al.	Applications of regional anaesthesia in paediatrics.	2013	Br. J. Anaesth.
Association of Paediatric Anaesthetists of Great Britain and Ireland.	Good practice in postoperative and procedural pain management, 2nd edition.	2012	Pediatr Anaesth
Morton NS	The pain-free ward: myth or reality	2012	Pediatr. Anaesth.

Table 1. Relevant review published in the last 5 years.

Advances in ultrasonography and precise dosing regimens have facilitated widespread applications of regional anaesthetic techniques in infants, children, and adolescents. Prospective studies indicate that these techniques can be safely performed and positively impact on the outcomes of paediatric patients undergoing painful procedures and those who suffer from chronic pain [17]. In order to obtain maximum pain relief reducing the side effects as much as possible, new strategies are needed. New research about the inter-individual variability of pediatric patients in terms of genetics and epigenetics and new tools (based on nanotechnology), would permit a major use of local drugs and the personalized dosage of systemic drugs. We are confident that in near future molecular analysis and nanotechnology will help the traditional medicine, towards the personalization of postoperative pain treatment and an improvement of patient's quality of life.

1.2 The ideal pain control

One of the most important current objectives of the research in pain therapy consists in personalizing the treatments, by a multidisciplinary approach including new analgesic deliveries (based on nanotechnology) and a deeper knowledge about pharmacogenetics and epigenetics (in terms of histone changes, mRNA expression, and DNA methylation). Sprints and collaborators, already in 2012, discussed the key role of Nano medicine in the development of innovative pain management [18]. The importance of developing implantable devices for tailored local drug release, as nano-structured

devices, are due to the drug release only into the targeted tissue affected by the surgery, dissolving any side effect related to the systemic release of the traditional treatments. Several literature data demonstrated the importance of the genetic background in determining the inter-individual variability of pain sensitivity (such as COMT), drug pharmacodynamics (such as OPRM1) and pharmacokinetics (such as CYP2D6) in the postoperative setting [19-22]. Recent data show that modification of epigenetic processes are involved in the development of chronic pain [23,24]. Orlova and coworkers showed the modulation of microRNA in complex regional pain syndrome, and recent data demonstrated the dynamicity of microRNA due to opioids and opioid tolerance [25]. On the other hand, Dohering and coworkers observed an increase of DNA methylation in patients under chronic opioid use [26]. The same effect of opioids has been also described in the postoperative period, while a demethylating effect has been shown for the local anesthetics [27]. Shaw and Martin determined the potential of epigenetics on modulating surgery wound healing in a murin model. Their discovery also demonstrates, once again, the importance of animal studies in pain therapy, as recent discussed by Gigliuto and coworkers for big animal models [28]. We are confident that new tools and multidisciplinary approaches, in postoperative medicine, might improve pediatric patient outcome (Figure 1).

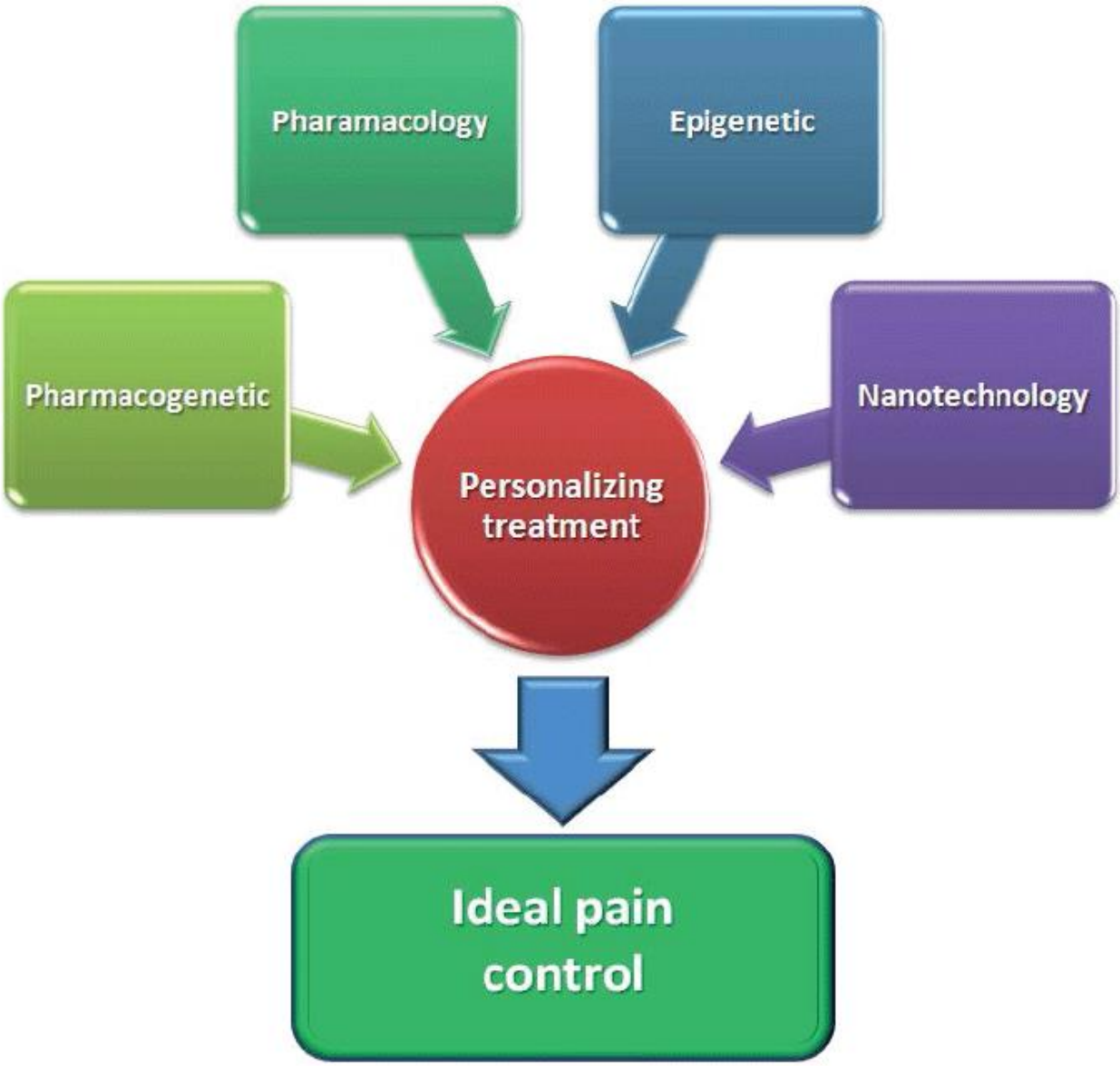


Figure 1. Multidisciplinary approach for an ideal postoperative pain management.

2. FROM MICRO- TO NANO-STRUCTURES

Local anesthetics block the transmission of painful stimuli to the brain by acting on ion channels of nociceptor fibers, and find application in the management of acute and chronic pain. Despite the key role they play in modern medicine, their cardio and neurotoxicity (together with their short half-life) stress the need for developing implantable devices for tailored local drug release, with the aim of counterbalancing their side effects and prolonging their pharmacological activity. This review discusses the evolution of the physical forms of local anesthetic delivery systems during the past decades. Depending on the use of different biocompatible materials (degradable polyesters, thermosensitive hydrogels, and liposomes and hydrogels from natural polymers) and manufacturing processes, these systems can be classified as films or micro- or nanostructured devices. We analyze and summarize the production techniques according to this classification, focusing on their relative advantages and disadvantages. The most relevant trend reported in this work highlights the effort of moving from microstructured to nanostructured systems, with the aim of reaching a scale comparable to the biological environment. Improved intracellular penetration compared to microstructured systems, indeed, provides specific drug absorption into the targeted tissue and can lead to an enhancement of its bioavailability and retention time. Nanostructured systems are realized by the modification of existing manufacturing processes (interfacial deposition and nanoprecipitation for degradable polyester particles and high- or low-temperature homogenization for liposomes) or

development of novel strategies (electrospun matrices and nanogels). The high surface-to-volume ratio that characterizes nanostructured devices often leads to a burst drug release. This drawback needs to be addressed to fully exploit the advantage of the interaction between the target tissues and the drug: possible strategies could involve specific binding between the drug and the material chosen for the device, and a multiscale approach to reach a tailored, prolonged drug release.

2.1 Local anesthetics: clinical relevance for their use in implantable delivery systems

Local anesthetics (LAs) temporarily and reversibly prevent nociceptive nervous fibers from sending stimuli to the brain. These drugs act by blocking sodium and other ion channels present in the nerve cell membrane, thereby stopping the electrical signal before it can cause feelings of pain [29,30].

LAs have been used for acute or chronic pain management, and their principal use is after surgery (eg, thoracotomy, laparotomy, cesarean section, breast cancer surgery, cosmetic breast surgery, limb amputation, and others) [31,32]. A recent study has highlighted the importance of LA administration after major operations to ward off chronic pain onset after thoracotomies and cesarian sections.

As nociceptive inputs from the wound play a major role, it is logical to “go back to the periphery”, acting locally, to prevent nociceptive inputs from reaching the central

nervous system and triggering endogenous pain processes [33]. Peripheral LA administration is a rational approach to control pain [34]. Continuous wound infusion (CWI) prolongs the action of LAs, thus improving their efficacy. Experimental models of incisional pain have demonstrated that the CWI of LAs modulates peripheral inflammation and sensitization in acute post-operative pain (PP) [35]. CWI is effective in controlling pain and reducing postoperative morphine administration after a major surgery [36]. CWI through an intralesional catheter presents several limitations such as risk of infection (external device), reduction in patient activity (compliance patient problems), difficulty in administration (need to fill the device many times), and systemic toxicity related to inaccurate administration of LAs or steroids, such as convulsions, arrhythmias, cardiovascular (hypo- or hypertension) and metabolic (eg, hyperglycemia) problems. Clinically used LAs possess common chemical structural characteristics, in which three chemical moieties can be identified: a hydrophilic amino group, a lipophilic benzene ring, and an intermediate chain (Figure 2). The intermediate chain can be an amide or an ester group, which allows classifying the drug as an ester-type or amide-type LA [37]. Some of the most used amide types are lidocaine, bupivacaine, dibucaine, mepivacaine, and ropivacaine, whereas the most common ester types are benzocaine, tetracaine, proparacaine, procaine, and chloroprocaine. Some molecules present isomerism: the most diffuse chiral molecules are bupivacaine and prilocaine, with the (S)-enantiomer being more active and less toxic than the (R)-form [38].

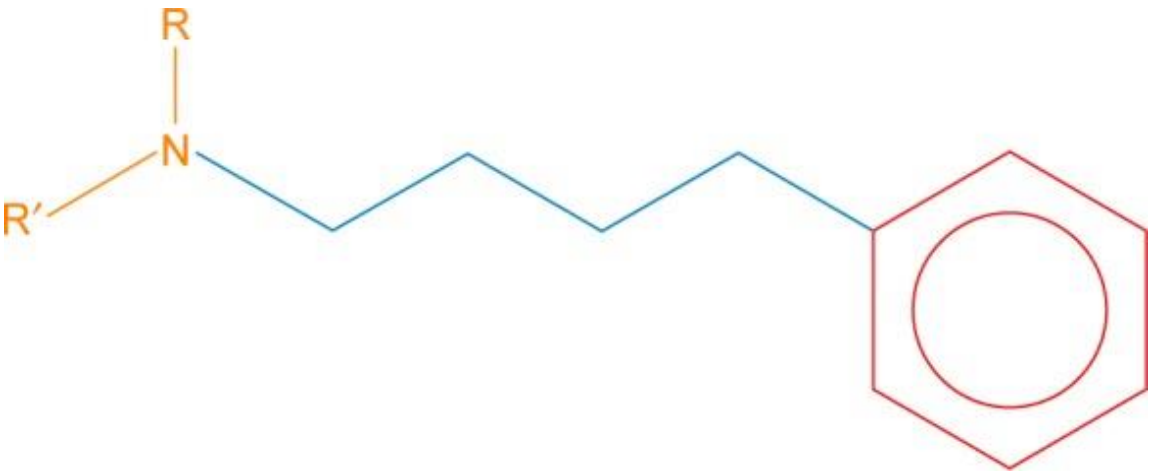


Figure 2. Typical structure of a local anesthetic: amino group (orange), benzene ring (red), intermediate carbon chain (blue).
Notes: The presence of an amide or ester group in the intermediate chain leads to the classification of the drug as ester-type or amide-type local anesthetic. R and R′ represent two different chemical groups.

The duration of a drug's effect is related to its protein-binding ability. Agents that penetrate the nerve membrane and attach more firmly to the membrane proteins show prolonged activity, eg, tetracaine, bupivacaine, and etidocaine. On the contrary, procaine binds poorly to proteins and possesses a short half-life [39]. LAs bind also to plasma proteins, albumin (low affinity), and α-1-acid glycoprotein (high affinity), leading to the same effects as tissue proteins [40]. The duration of drug's action is also related to its structure, length, and the groups present.

Amide type is generally more stable and possesses a longer half-life than ester type, which undergoes rapid hydrolysis by nonspecific esterases [40].

Most of the clinically useful LAs are available in two forms: as free base or as a salt. The salt form has greater solubility in aqueous media and thus is preferred for a drug

delivery system. The salt, being a weak base, can exist in solution as an un-ionized free base, which is more lipophilic, or as a positively charged cation; both species are involved in the process of conduction blocking [40,43]. The base crosses the neuronal bilayer more easily, and thus is preferred for optimal penetration [40]. The percentage of drug in the two forms depends on its pKa and the environmental pH. Each drug has a characteristic pKa, with ester type LAs typically having a higher value than the amide type [41]. The closer the pKa is to the pH of the tissues, the faster is the onset of action of the drug [43,44], since the percentage of free base formed increases. For this reason, in infected tissues, the LA effect is reduced or even delayed, as the environment tends to be more acidic than usual and the un-ionized form decreases [40].

Even though anesthesiologists often prefer them to general anesthetics, LAs present side effects like allergies and stroke [45,46] related to their chemical structure (eg, ester types are more problematic than amide types). In particular, lidocaine and bupivacaine have caused strokes leading to death of the patient [45].

Chloroprocaine, an ester type LA, has been demonstrated to be less cardiotoxic than most other LAs,15 but it has an extremely short half-life: <10 minutes in vivo [47].

Recently, drug delivery systems, according to different user needs obtained by a variety of materials and physical forms, have been developed to minimize severe side effects and to prolong the anesthetic action [29].

2.2 Local anaesthetic delivery: application of micro/nano-devices in the clinical practice

Injectable and implantable systems for LA delivery aim to reduce LA toxicity and prolong the anesthetic effect of drugs with short half-lives, shielding the drug from the environment of the implantation site [29].

New delivery platforms are necessary to obtain effective, lasting postoperative peripheral analgesic treatment for several days to block the nociceptive message and to reduce postoperative inflammatory response. This platform has to overcome the limitations of the external inaccurate delivery devices. The development of intraoperative nanogel delivery systems, able to provide anesthetics with planned dosages and timing, is a key step toward attaining the safest and most effective postoperative treatment. Nanotechnology plays a critical role in the development of patient-specific therapies and individualized medicine and offers great opportunities for combating the limitations of postoperative pain management.

One of the simplest systems for LA delivery is represented by the oil depot formulation: lipophilic drugs (eg, lidocaine [46,47], bupivacaine[49], or ropivacaine [49,50]) are dissolved in different biocompatible oils [46,47] and injected intramuscularly, subcutaneously, or intra-articularly.

In aesthetic medicine, lidocaine loaded-commercial fillers are commonly employed in patients. For these applications, the release profile is not studied because its characterization is not an intended scope, as they are not designed as drug delivery systems, with LAs added to reduce pain during the filler injection.

Calcium phosphates were loaded with lidocaine for bone defect reconstruction to provide relief from pain after implant [51,52]. Scaffolds were formed by sintering ceramic powers and infiltrating them with lidocaine solutions (60 mg/mL and 20 mg/mL). To treat pain after tooth extraction, other implantable devices were developed to fill the bone gap, mixing commercial bone putty (Xybrex; Orthocon, Inc., Irvington, NY, USA) and lidocaine [53] or developing sponge-like polycationic and polyanionic hydrogel fillers: lidocaine solution (up to 4 mg/mL) was incorporated into polymer solutions before cross-linking [54].

However, none of these systems for LA delivery was developed for a prolonged, tailored release.

Current achievements and future perspectives in pain therapy raise the question whether hospitalization without pain is still wishful thinking or can became a reality. Micro and then nanodevices are on the horizon of new technologies for pain management, which can overcome the limitations of traditional anesthetic delivery tools, such as drug delivery security and safety of the delivery system, simplicity of setting up, as well as maintenance and management. These systems consist in micro- and nanostructured films, particles, capsules (suspended in a solution or embedded in a gel matrix), and hydrogels, which can be implanted in the site of surgery and release LAs in a tailored manner. Irrespective of the origin of pain, management is necessary in clinical practice leading to essential benefits including shorter hospital stay and improved patient satisfaction. Otherwise, totally implantable devices (according to the US Food and Drug Administration [FDA], ISO 10993-1 micro/nanodevices must be

categorized as implant devices with prolonged exposure to the body) with their full integration with the biological milieu of the human body is still a crucial multidisciplinary task to solve. From a clinical perspective, the ideal pain management should cover the entire period necessary with as little discomfort as possible to the patient. The production of devices as biocompatible and small as possible can prevent discomfort. This concept drives this clinically translational research to manufacturing a device characterized by features from micro to nano if considering physical size as the major overriding issue (even if not the only one). LAs, as the main actors of drug delivery through implanted nanodevices, play a pivotal role for the entire system due to their peculiar characteristics: completely reversible action and the relative absence of collateral or side effects compared to other drugs generally used in the clinical scenario. Their use is nowadays limited to intravenous patient-controlled analgesia or patient-controlled epidural systems. Although these analgesic methods are commonly used, they are not free from important disadvantages including their invasiveness and the need for pumping equipment, tubes, or power lines, which could potentially limit patient mobility during, for example, the postoperative period. They also require exhaustive staff-time preparation and resources with the risk of catheter dislodgement or epidural hematoma (increased in case of coadministration of anticoagulant therapy). These limitations show why the development of LA drug delivery system administering therapy from inside the patient's own body is so crucial and, secondly, why nanotechnology has received a never-seen-before enthusiasm in this field.

Drug delivery systems described so far are the widest and simplest methods for LA administration. Among them, it is possible to find also commercial products (eg, EMLA cream). More advanced ones have been developed to treat the urgent issues represented by chronic and postoperative pains. These can be classified as films, or micro- or nanostructured systems, depending on the final shape and size of the devices constituted by different materials and realized using different techniques (Figure 3).

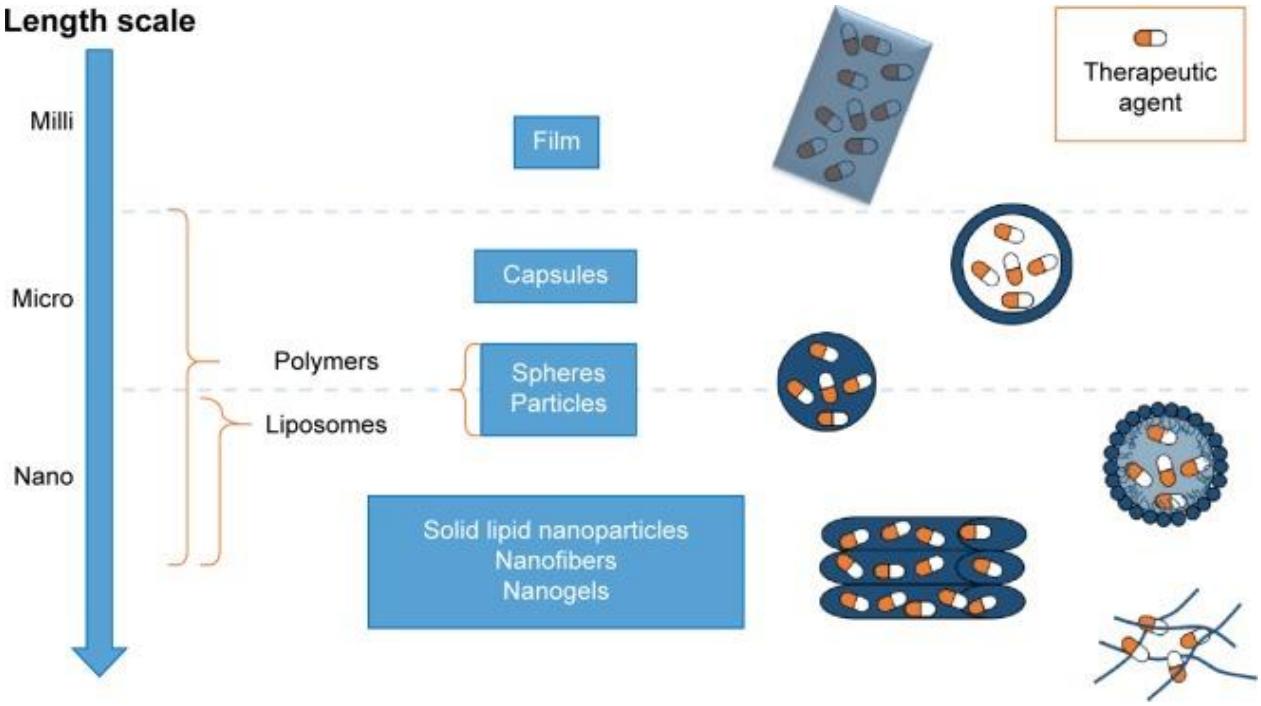


Figure 3 Local anaesthetic delivery systems can be produced at different length scales (milli, micro, or nano) using different physical forms: films, capsules, spheres and particles, liposomes, nanofibers, and nanogels.

2.2.1 *Films*

Drug delivery systems in form of films were developed in particular for wound treatment or as transdermal patch [56]. Implantable films were produced by UV polymerization [57,58] or solvent casting [59,60]. The UV curing technique consists of cross-linking polymer chains using UV radiation and loading the drug prior to the cross-linking. Depending on the type of materials, the loading can vary from 10 μg/g of film in the case of a semi-interpenetrated network obtained through UV-curing of poly(ethylene glycol) (PEG) diacrylate in presence of gelatin to ~25 mg of drug/g of film using N-isopropylacrylamide (NIPAAm) and 1-vinyl-2-pyrrolidinone [57]. Thermoresponsive polymer chains can lead to a more compact hydrogel network, slowing down the drug release.

Solvent casting technique can be applied by exploiting different strategies for drug loading, either during or after the solvent casting. Alternatively, drug loading was achieved by swelling a dried k-carrageenan film in dibucaine solution [59]. Simultaneous film casting and drug encapsulation were achieved by loading lidocaine, a lipophilic drug, in a poly(lactic acid) (PLA) solution in chloroform [60]. PLA was also employed to produce a composite film [61]. During in vivo test in rats, drug release in the plasma lasted up to 10 days.

Although different materials and types of drug were used, when the drug was loaded in the step after film formation [59], the release was faster than when the drug was loaded during the film preparation [60]. Drug incorporation in the polymer solution

can allow a homogeneous dispersion within the bulk of the material, but it is limited by the solubility of the drug in the solvent used for casting the film.

2.2.2 *Microstructured systems*

Microspheres, microparticles, and microcapsules are classified as microstructured systems, which can be implanted or injected once suspended in appropriate media, used as injectable matrices, or formed in situ (Figure 4).

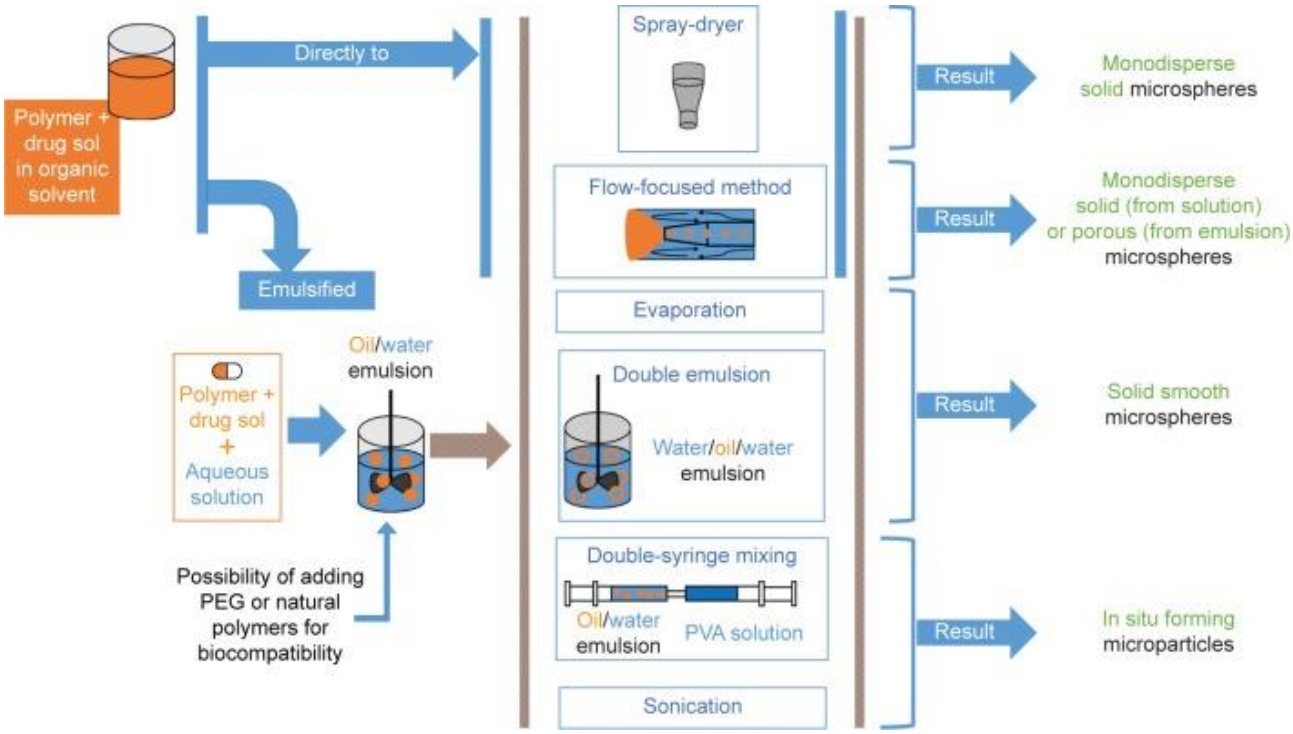


Figure 4. Scheme of production of microstructure systems.
Notes: Different techniques were developed depending on the materials used. Manufacturing processes starting from traditional synthetic polyesters begin from a solution of the polymer and drug or from an oil/water suspension of such a solution. Then they are processed through different methods (spray-drying, flow-focused method, evaporation, sonication, double-syringe mixing, and a second emulsion) to obtain microspheres.
Abbreviations: PEG, polyethylene glycol; sol, solution; PVA, polyvinyl alcohol.

Among the several proposed solutions, the most widely used materials for microparticle production are synthetic degradable polyesters such as polyglycolic acid (PLA) and polylacticglycolic acid (PLGA).

The use of these materials to produce microspheres is related to the relative simplicity of microsphere production. In most of the works, microparticles were produced by oil/water (O/W) [60,62–70] or water/oil/water (W/O/W) emulsions [70,71], and solvent evaporation techniques. Usually, particles produced using those methods are solid and smooth microspheres, with a prolonged drug release lasting from 12 hours to 3–4 days. In some cases, dexamethasone was used as the excipient, enhancing bupivacaine's in vivo effectiveness without modifying significantly the in vitro release profile [63,64].

Alternatively, microspheres were produced through a spray-drying technique, which consists of spray-drying an organic drug/polymer solution [73,74]. This technique requires higher and thus more expensive technology, but the cost of the spray-dryer is compensated by better results in terms of simplicity of the production process, narrower particle size distribution, and higher drug encapsulation efficiency [73]. Spray-drying can be exploited also to produce porous microparticles by using an O/W emulsion instead of a drug–polymer solution.

Porous microparticles were subsequently immersed in an aqueous solution of procaine (hydrophilic drug) and the particle pores were closed by adding an organic solvent in the procaine-loaded bath: in this way a hollow microcapsule was formed. This

unconventional method succeeded in reaching drug release up to 9 days and to produce microcapsules that were more easily degradable [76].

Another technique to produce monodisperse polymeric microspheres is flow-focusing [77-79]. This technique employs a microfluidic device consisting of coaxial capillaries. At the inlet of the capillary with the larger diameter, the drug (lidocaine [77,79] or bupivacaine [80]) and polymer organic solution follow in a certain direction, surrounded by polyvinyl alcohol (PVA) aqueous solution flowing in the same [77,79] or opposite direction [79]. By tuning the process parameters (eg, viscosity of lipophilic and hydrophilic solutions), it is possible to produce microspheres with final characteristics close to the desired ones and with very narrow particle size distribution [77]. Monodisperse microspheres showed slower drug release with reduced initial burst release compared to microspheres produced by traditional methods presenting similar average size but a broader size distribution. Moreover, if O/W or W/O/W emulsions were used instead of a drug-loaded polymer solution, it is possible to obtain porous microparticles. This approach was followed by Vladisavljević et al [79], who used numerical simulation to find the most suitable process parameters (eg, flow rate and solution viscosities) to tailor particle characteristics.

Porous microparticles could be produced also using a simpler W/O/W technique. In contrast to the previously described technique, the first emulsion consisted of a water-in-oil emulsion in which the oily phase was constituted by PLGA dissolved in chloromethane in presence of lidocaine, which was subsequently mixed with an aqueous solution of PVA [80].

In situ formation of microparticles was achieved by an emulsion of bupivacaine-loaded polymer (PLA with an emulsion stabilizer [81] or PLGA [82]) solution in 2-pyrrolidone with an oily phase: emulsions were formed both by sonication and by manually mixing the contents of two syringes (one containing the oily phase and the other the polymer phase) previously connected with a double-female luer lock connector.

In spite of prolonged LA release by synthetic polyesters (up to 15–20 days [75,77]), they could also cause significant inflammatory reaction.55 For this reason, alternative strategies were proposed.

In some studies, PLA or PLGA microsphere were produced in presence of PEG [37,42] which is well known for its nonfouling properties and can reduce inflammatory response. In one case, a mixture of the PLA and PEG was prepared, producing the microsphere by coacervation method, in which hexane (not able to dissolve PLA) was added to a stirred polymer solution (PLA + PEG + lidocaine) in methylene chloride. The coacervation droplets adhered to the suspended drug particles and coalesced around them to form polymer microparticulates [70].

In another study, PLA/PEG copolymer was used and particles were realized following the O/W emulsion technique. In this case, particles were coated with gelatin by solvent-casting of a gelatin solution to tailor the drug release, which reached 3-day release during in vitro tests in water at 37°C.37 Combination with natural polymers is a strategy also used in another study, where PLGA microparticles were produced by an

O/W emulsion in which alginate was dissolved in the aqueous solution. In vitro release of bupivacaine in PBS (37°C, pH 7.4) lasted for up to 24 hours[68].

Other studies focused their attention on the use of stimuli-responsive materials (Figure 5). Thermosensitive polymers such as polyN-isopropylacrylamide (PNIPAAm) [84] and copolymers [85,86] were used to produce microspheres by cross-linking preformed polymer chains: an aqueous polymer solution was acidified and glutaraldehyde was added as the cross-linking agent. The solution was then poured into light mineral oil containing lecithin as emulsifying agent. Drug loading was obtained by soaking the microspheres in a lidocaine solution. However, this method did not guarantee prolonged drug release in PBS even up to 100 minutes in vitro [85]. PNIPAAm microparticles were also embedded in an injectable chitosan thermogel matrix the embedding of microparticles was achieved through an innovative coinjection technique through a double-barreled syringe. In these cases, the chitosan matrix was useful to both prolonging bupivacaine release in PBS (>6 hours in vitro) and avoiding particle dispersion [87-89]. Chitosan injectable loaded matrices [90,91] were also studied with different results in terms of duration of anesthetic release in PBS: from 40 minutes [90] up to 48 hours [91]. Other polymeric materials used for loaded microsphere production were tetraethoxysilane[92-94] and poly(acrylic acid) [94].

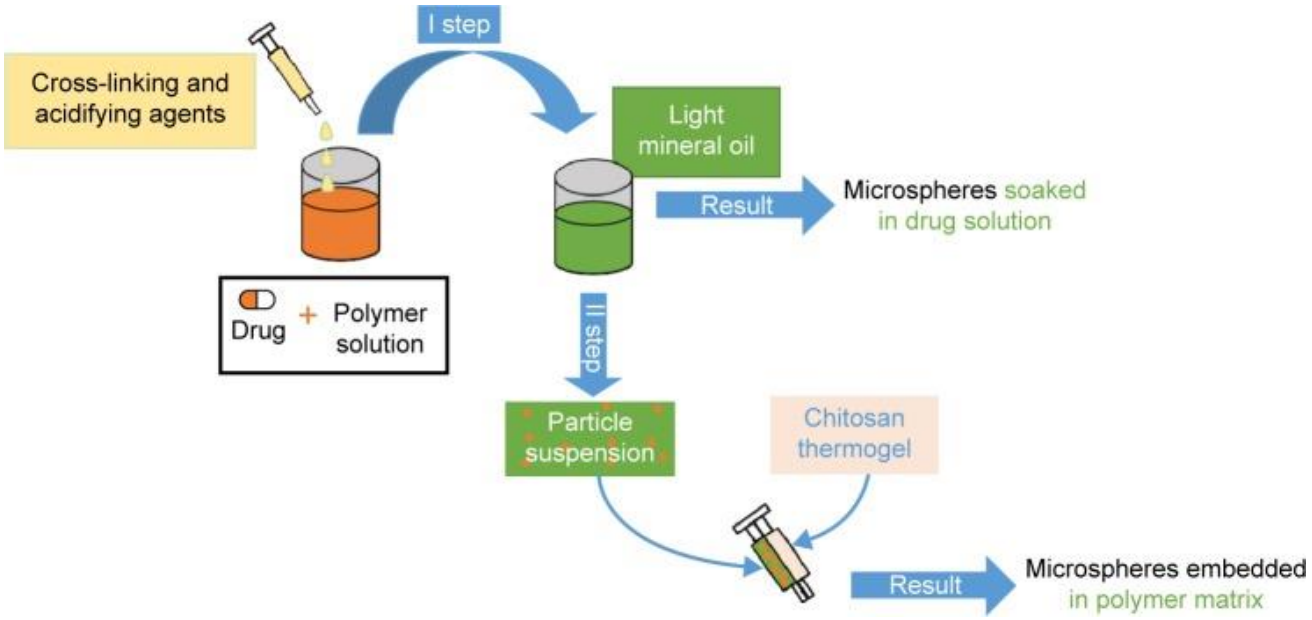


Figure 5. Fabrication process from thermoresponsive materials starts from a polymer–drug solution poured into light mineral oil to create a suspension of microspheres that can be mixed with another thermogel matrix to embed them in it.

Inorganic microparticulates can be loaded with LAs (Figure 6). Different methods of loading lidocaine on calcium phosphate powders were tested: wet granulation, isostatic compression, and a combination of both [95,96]. These techniques started with mechanically mixing calcium phosphate and lidocaine. In wet granulation, the powder was moistened with water and passed through a sieve. Isostatic granulation involves the application of high pressure to the mixed powder (calcium phosphate and lidocaine) [95]. Bupivacaine was loaded on calcium phosphate apatite: in this case, wet

impregnation of an ethanol solution of the drug resulted in a uniform loading than when mixing bupivacaine powder with calcium phosphate powder [96].

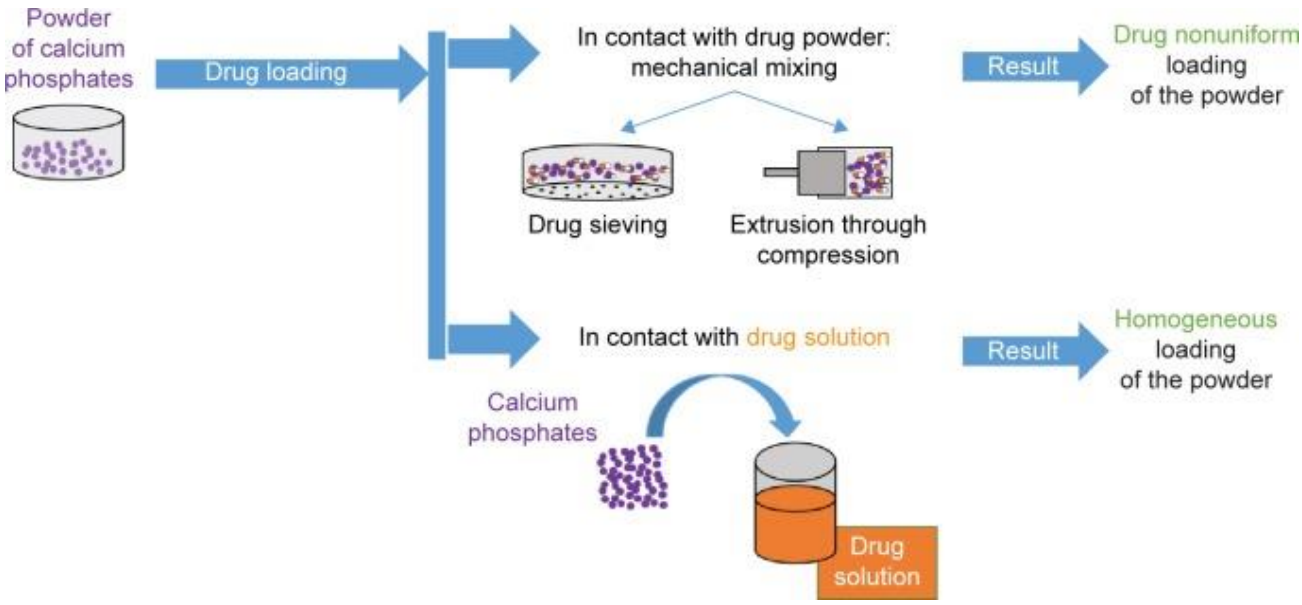


Figure 6. Processes exploiting the use of calcium phosphates involve mechanical mixing or contact between the powder and contact between the powder and drug solution, which leads to a homogeneous drug loading different from the mechanical mixing

2.2.3 *From micro to nano*

In the past decades, a large number of publications have focused on the advantages resulting from the adoption of nanosystems instead of microsystems and therefore adopting different strategies to obtain nanoscale devices (Figure 7).

The aim of the scaling down is to make the size of the drug delivery system more similar to the biological environment, which is nanostructured. In this way, the molecular interactions could be more specific, and the drug could be absorbed into the targeted tissue to attain an enhancement of its bioavailability and retention time. The nanosystems, indeed, were demonstrated to improve intracellular penetration.

In order to have a controlled and reliable LA release, the drug would be encapsulated in a biocompatible and biodegradable nanostructured system, to minimize tissue reaction and to control precisely the rate of drug delivery, which has to be comparable with the rate of generation of nontoxic degradation products. In fact, an initial uncontrolled large dose of drug release could be lethal for the organism and therefore must be avoided. Moreover, LA nanoencapsulation increases the drug's efficacy, specificity, tolerability, and therapeutic index [87].

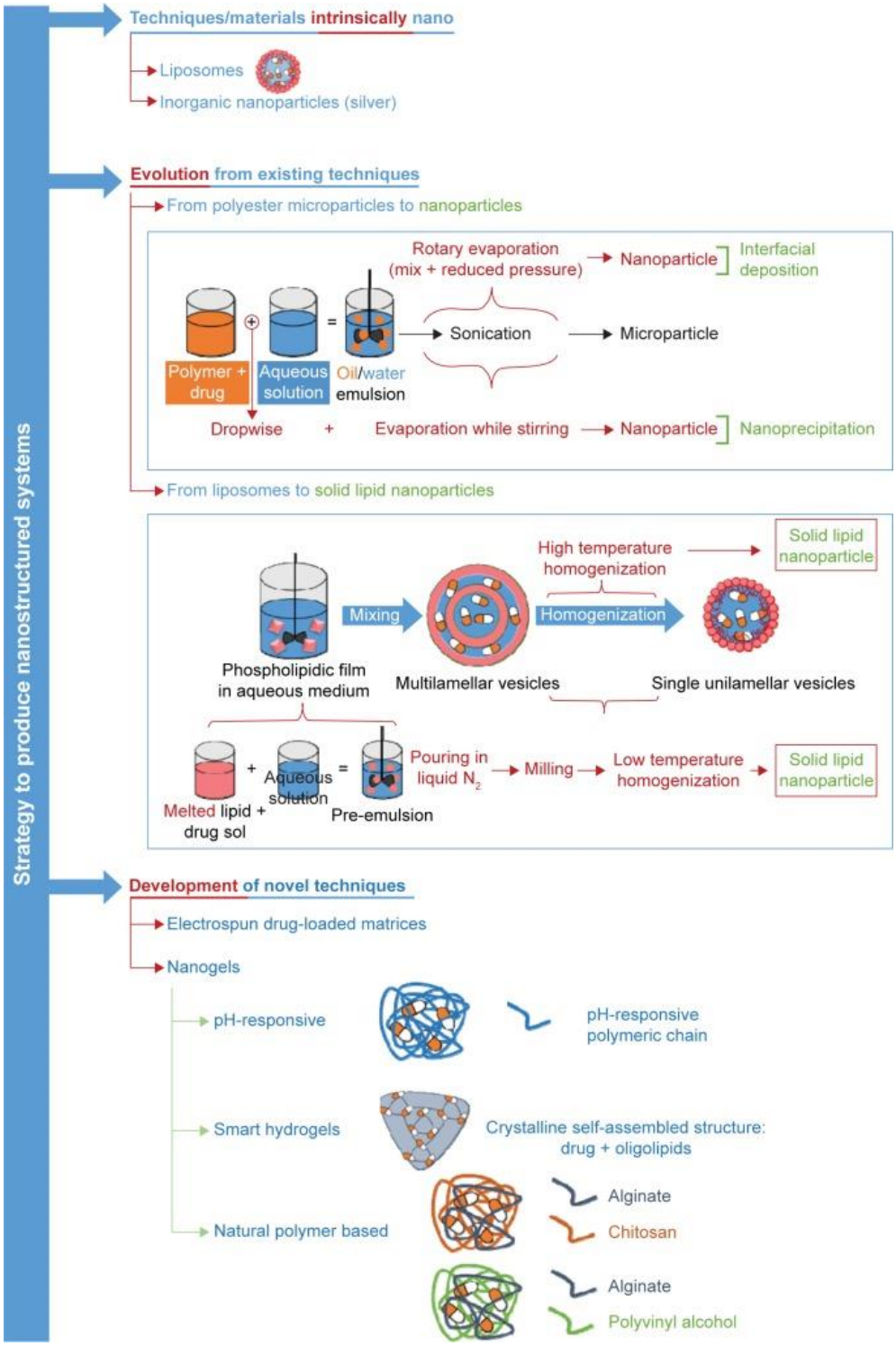


Figure 7. Scheme of nanostructured device production.

Notes: Nanostructured systems can be produced using techniques that intrinsically lead to nanoparticles (silver nanoparticles, liposomes) or evolve from other techniques. Interfacial deposition and nanoprecipitation have evolved from synthetic polyester manufacturing techniques, and solid

lipid nanoparticles are derived from liposome production technique. Eventually, new techniques are continuously developed: electrospinning of drug-loaded matrix and drug-loaded nanogels. The last class groups different production strategies and materials: pH-responsive hydrogels, self-assembling systems, and nanodevices based on natural polymers. Abbreviation: sol, solution.

The LA release kinetics and the drug loading efficiency are influenced by the size of the system. Larger particles could load a larger amount of drug, leading to a longer block duration and thus a longer effect. On the contrary, smaller particles have the advantage of reducing the release rate of the incorporated drug, thereby improving the drug binding, even though smaller systems show a rapid initial release of the drug, which could be toxic. This is due to the fact that on moving from micro to nanosystems the surface-to-volume ratio of the particles increases and therefore also the initial dissolution rate [92,93]. Therefore, the development of a nanostructured system has to take into account ad hoc strategies to exploit the possible advantages offered by those systems (in particular the higher encapsulation efficiency and specificity in targeting biological structures) and limit the drawbacks related to their size.

2.2.4 *Liposomes to solid lipid nanoparticles*

Liposomes are phospholipid-based carriers. Because of their multilayered structure, with alternating aqueous compartment and lipid bilayer, they are able to incorporate both lipophilic and hydrophilic drugs, which can pass the lipophilic barrier of cell membranes.

The potential benefits in the use of liposomes as injectable or implantable devices for LA delivery were highlighted when it was found that an injection of soybean phosphatidylcholine nanoemulsion reduced bupivacaine's cardiotoxicity [94].

Liposome synthesis consists in obtaining large multilamellar vesicles (LMVs) from dried phospholipid films by suspending the film in an aqueous solution and vortexing it. Though LMVs were initially used, in also small unilamellar vesicles (SUVs) were also used. From an LMV suspension in D2O, SUVs were obtained by freeze-drying the suspension [96,97]. Alternatively, SUVs could be obtained by homogenization [98] or extrusion [99,100] of an organic suspension of LMVs (Figure 8). Different phospholipids alone or mixed have been used in different works: egg phosphatidylcholine; soy phosphocholine and cholesterol; egg phosphatidylcholine, cholesterol, and α-tocopherol and hydrogenated soy phosphatidylcholine and cholesterol [100,101].

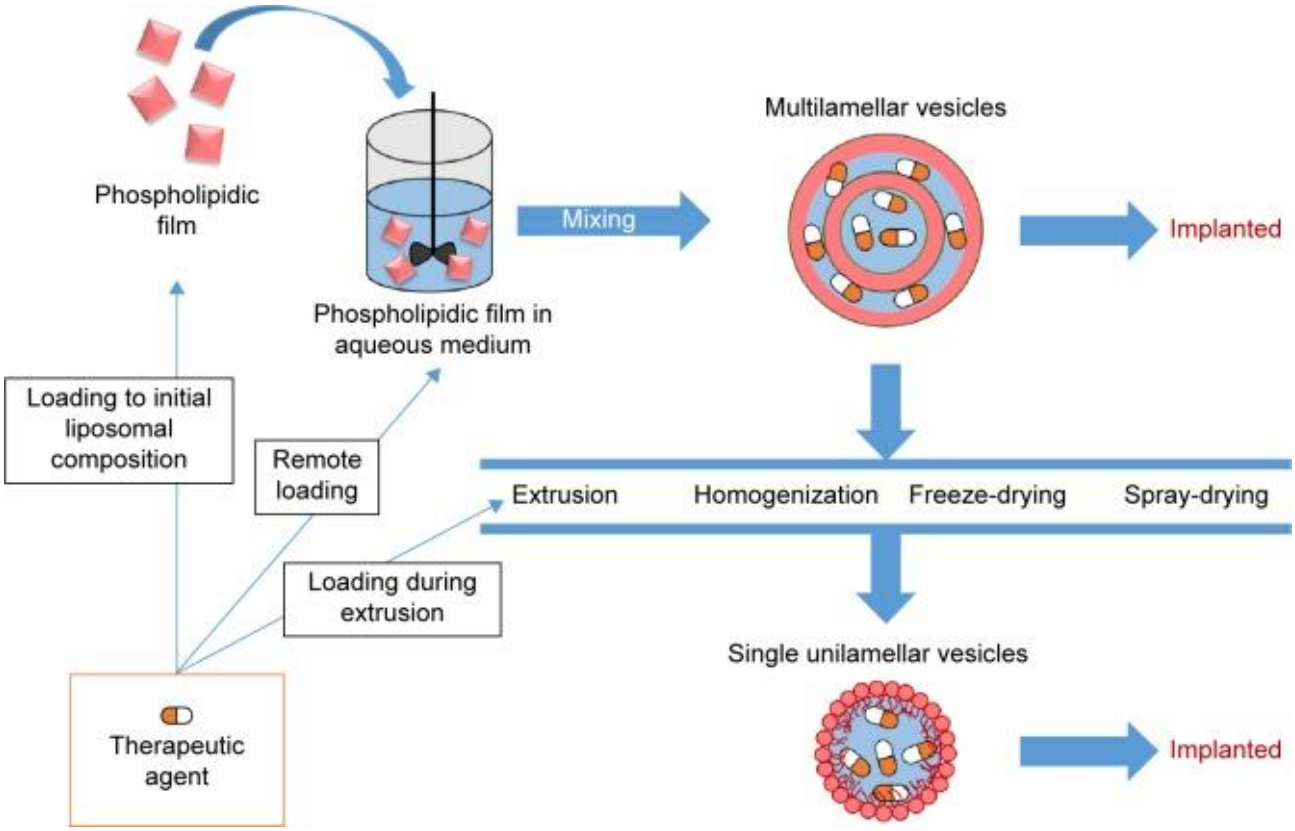


Figure 8. Production process of liposomes.

Notes: Multiple lamellar vesicles (MLVs) can be produced and ready for implant after a suspension of lipophilic film in aqueous solution. In an alternative method, starting from MLVs, single lamellar vesicles can be obtained through extrusion, homogenization, freeze-drying, and spray-drying. The therapeutic agent can be incorporated directly in the starting phospholipidic film, during the extrusion process, or when the suspension obtained (labeled as remote loading).

In general, drug loading can be done by simply adding the drug to a solution containing the phospholipids [101,102], or after an extrusion process through an ammonium sulfate gradient. The latter realizes remote loading of the drug in preformed LMVs produced in presence of (NH4)2SO4. (NH4)2SO4 present in the extraliposomal

medium is removed by dialysis against normal saline. In this way, an ammonium sulfate gradient is formed over the liposomal membrane. Bupivacaine was loaded by incubating the liposomal formulations with a concentrated bupivacaine solution at the pH range 5.0–5.5. Remote loading was found to be a more effective encapsulation method, since it led to 24–48 hours release in saline solution in vitro and 45 hours in vivo lasting of the anaesthetic effect, which is six to eight times more than the release duration obtained with other loading techniques.

Ultimate evolutions in liposome technology involve the addition of sugars in their composition, which leads to lipid–protein–sugar particles (LPSPs) [104,105], with the aim of accelerating particle degradation in the biological environment. In one study, also dexamethasone was added to the composition to prolong in vivo drug effectiveness. In this work, LPSPs were prepared through spray-drying: then di-palmitoylphosphatidylcholine and bupivacaine were dissolved in a given amount of ethanol; sugar (lactose) and albumin were dissolved in water and the solutions were mixed immediately prior to spray-drying. LPSPs provided significantly shorter tissue reaction to foreign body than conventional PLA and PLGA microparticles.

In one of the latest works, liposomes were coated with chitosan and alginate to overcome the major drawback associated with liposomes: their short shelf-life [105-114]. The coating process succeeded in prolonging liposome conservation, avoiding drug leakage when stored up to 2 years.

An alternative injectable nanocarrier to liposomes [115-117] is represented by solid lipid nanoparticles (SLNs), a nanosized colloidal system. SLNs have the advantage of

a production process that avoids the use of organic solvents, namely high-pressure homogenization. This process can be performed either at high or low temperatures. In the first case, the process is called hot homogenization [115-117]: the drugs (tetracaine or bupivacaine) are dissolved in the melted lipid, and the drug-loaded lipid is dispersed in a hot aqueous surfactant solution to form a pre-emulsion, which is subsequently homogenized. The whole process is conducted at 500–1,500 times the atmospheric pressure. In the second variant, called cold homogenization, after dissolving the drug in the lipid, solidification is done by pouring the drug-loaded lipid in liquid nitrogen. This leads to the formation of a solid solution (molecular dispersion) of the drug in the lipid matrix. After milling the solid solution, the particles are suspended in a poloxamer solution, and the resulting suspension is homogenized at room temperature and 1,500 times the atmospheric pressure. Even though this carrier can be considered safer than polymeric nanoparticles, through the avoidance of organic toxic solvents, it failed to provide prolonged drug release in phosphate–citrate buffer at 37°C (2 hours) [115].

2.2.5 *Nanostructured systems*

Nanostructured systems include nanospheres, nanocapsules, principally designed as injectable systems, and nanofibers. Similar to microstructured systems, synthetic degradable polyesters traditionally play an important role in the production of nanostructured systems.

Nanospheres have been produced by O/W emulsion [118-121] and nanoprecipitation [122-124]. The first technique consists of making a solution (oily phase) in which the polymer and the drug are dissolved in organic solvents (typically methylene chloride or chloroform). The organic solution is subsequently poured into an aqueous solution (which often contains PVA, which acts as the emulsifier) and the suspension is vortexed. The organic solvent can be eliminated at atmospheric pressure or by rotary evaporation followed by freeze-drying. On the other hand, nanoprecipitation consists in dissolving the drug in an organic polymer solution, which is added dropwise to an aqueous phase. The suspension is magnetically stirred until complete solvent evaporation is achieved.

Interfacial deposition (also known as solvent displacement) is an innovative method to produce polyester nanocapsules with hollow oily nuclei [125–127]. This method consists in creating an organic phase composed of the drug (benzocaine in all reported works), the polymer, acetone, oil, and the emulsifier. The organic phase is mixed under magnetic stirring with an aqueous solution containing an emulsifier in turn, and acetone is evaporated by rotary evaporation. This technique can be considered an evolution of

the O/W suspension, as interfacial deposition differs from it for solvent evaporation from the suspension while mixing it. In vivo duration of nerve blockage could reach 4–5 hours.

An advanced nanostructured device for LA delivery is represented by electrospun bupivacaine-loaded PLGA suture [131]. Sutures were produced by adding the drug to the polymeric solution used for the electrospinning process. They released their entire drug payload in PBS at 37°C in 12 days and maintained ~12% of their initial tensile strength after 14 days in vitro.

Silver nanoparticle suspension and LA solutions (procaine, dibucaine, tetracaine) were prepared to study the silver–anesthetic interaction: this study, however, is in a preliminary phase and far from any clinical application.

2.2.6 *Nanogels*

The most advanced research on nanostructured drug delivery devices focuses on nanogels.

Tan et al. [133,135] have produced pH-responsive nanogels by emulsion polymerization of methacrylic acid–ethyl acrylate cross-linked with di-allyl phthalate. In a more recent study, the same research group succeeded in coating the pH-responsive gels using a layer-by-layer technique. In this case, layers of poly(allylamine hydrochloride) and poly(sodium 4-styrenesulfonate) polyelectrolytes were alternated on the particle surface with the aim of tailoring drug release [135]. Interestingly, it was found that beyond a certain thickness of the coating, the drug release had a slow and continuous profile [135]. The emulsion polymerization technique is used also to produce functionalized thermoresponsive nanogels based on (PNIPAM). Thanks to the acrylic acid functionalization, the nanogel binds firmly to a cationic drug such as bupivacaine [136,137].

A smart production process of nanogels is the self-assembly technique. Self-assembled structures made of bupivacaine and liquid crystalline phases (monoglyceride and medium-chain triglycerides) have been studied [138,139]. Precisely, bupivacaine with appropriate concentrations was mixed with glycerol monooleate or binary glycerol monooleate/medium-chain triglycerides combination, and the mixture was heated at 40°C-50°C. The obtained isotropic solutions were hydrated by adding PBS and then homogenized by vigorous vortexing. The lipid composition led to different crystalline

nanostructures, which were shown to influence the drug release, lasting for up to 48 hours.

Also natural polymers such as alginate-based nanogels were investigated for nanogel production (Figure 9). Alginate/chitosan nanogels loaded with bupivacaine were produced starting from a dilute (0.0067% w/v) solution of sodium alginate (containing the drug) to which CaCl2 was added under mechanical stirring. To this solution, a dilute chitosan solution was added at a controlled flow rate. The second hydrogel consisted of alginate/anionic surfactant (AOT) nanogels, which were produced starting from an AOT solution in methylene chloride, which was added to an aqueous alginate solution containing bupivacaine, and homogenized. PVA solution was eventually added to the initial solution, creating a double emulsion, to which calcium chloride solution was added to ensure alginate cross-linking. Both compositions showed good stability and cytocompatibility. However, alginate/chitosan nanogels showed a slower drug release in PBS (pH 7.4) than alginate/AOT surfactant (13 hours vs 8 hours).

LAs are powerful drugs whose use can positively affect modern medical procedures. Together with their utility, they also suffer from safety issues, as most of them show different levels of neurotoxicity and cardiotoxicity. Among the strategies to overcome this drawback, it is possible to develop implantable devices capable of tailored drug release.

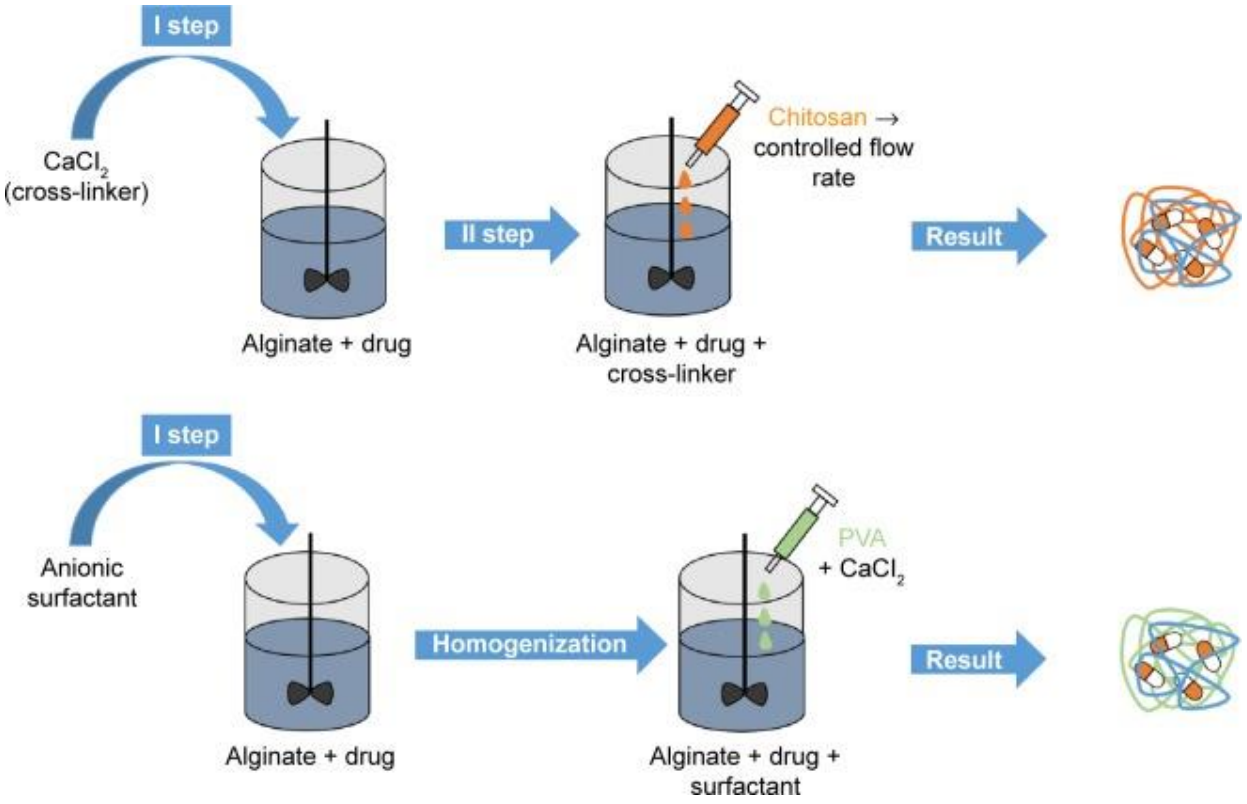


Figure 9. Production of hydrogel nanospheres, based on natural polymers.
Notes: To an aqueous solution of alginate and drug, a cross-linker or an anionic surfactant can be added. In the first case, chitosan is added at a controlled flow rate to the solution; in the second one it is added to it polyvinyl alcohol (PVA) and a cross-linker. In both cases, the result is an interpenetrated hydrogel structure embedding the local anaesthetic.

The most varied and studied delivery systems are microstructured PLA or PLGA microparticles. The many manufacturing processes available up to now provide full control of the particle size and dispersion, surface topology, and structure (particle or capsule). Most of the reviewed systems show great stability and prolonged drug release. Various strategies have also been developed to avoid initial burst release, which can worsen safety issues. Despite the maturity of the techniques and release systems, their application potential remains to be demonstrated in reality. Although

synthetic polyesters are often chosen for these aims, they show great stability in human body, which is not always required in this specific application and could lead to non-negligible foreign body response, in addition to acidic degradation products. Moreover, an acidic environment is not ideal for LA's effectiveness, as it could shorten the drug's half-life. Liposomes and SLNs do not release acids during degradation and can be produced without the use of organic solvents. These materials also have drawbacks, which are now being addressed, as the particles may be too stable in the biological environment but show poor stability on the shelf.

Scaling down to the nanoscale brings indeed several advantages with a device scale comparable to that of the nanostructured biological environment. The strategy to adapt techniques and materials developed for microstructures to produce nanostructures is not straightforward. Production of nanostructured systems could present some peculiar issues, mainly due to the high surface-to-volume ratio, which often leads to burst release of the drug. To this aim, strategies involving specific binding between the drug and the material and a multiscale approach are likely to be the key to reach a tailored, prolonged drug release. According to the progress in the field, the most promising approaches involve the presence of nanosystems in micro or macrodevices, to take full advantage of the different characteristics brought in a unique multiscale device.

2.2.7 *Loaded Drugs and their release kinetics*

The above mentioned unique properties of electrospun fibers such as high surface area, high loading, simultaneous delivery of diverse therapies, ease of operation, and cost-effectiveness expanded their use in drug delivery. Of the various applications, wound-dressing and local cancer treatments are two of the most investigated area.

Antibiotics and various antibacterial agents are the most preferred biocides to be loaded in nanofibers to afford antibacterial properties. Ignatova et al. summarized various antibiotics, such as tetracycline hydrochloride, ciprofloxacin, levofloxacin, and moxifloxacin, and anti- bacterial agents (eg, 8-hydroxyquinoline derivatives, itraconazole, benzalkonium chloride (BC), fusidic acid or silver nanoparticles) encapsulated in nanofibers for wound-dressing. In most cases, PLA, PLGA, and PCL were used as the carrier polymers, and other synthetic or natural polymers were added to regulate the biodegradability and hydrophilic nature of the fibers, thus regulating the release behavior. For example, in the early study on application of electrospun nanofibers for drug delivery by Kenawy et al, tetracycline hydrochloride was used as a model drug and poly (ethylene-co-vinyl acetate), poly(lactic acid) and their blends were used as polymeric carriers. The results demonstrated that drug release behavior was influenced by the nature of polymeric carrier and drug content. Smooth and regulated drug release over approximately 5 days was obtained by electrospinning 50/50 blend with relatively low drug content (5 wt.%). Higher drug content (25 wt.%)

induced much more rapid release than the 5 wt.% sample, due to the more surface-segregation of the drug in the former case.

In substance Electrospinning is an old technology for the fabrication of continuous nanofibers with simple setup. However, it attracted renewed focus of biomedical applications and other nano-techniques in recent years. Its inherent high surface to volume ratio, ease of operation, and cost- effectiveness are all appealing features for biomedical application. With the development of electrospinning techniques, such as co- and multi-nozzle electrospinning, co-axial electrospinning and emulsion electrospinning, a rich variety of materials, including natural polymers, synthetic polymers, and their composites have been electrospun into ultrathin fibers with controllable diameters and morphologies.. To date, the majority of the studies on the release of antibacterial agents or anticancer drugs from electrospun fibers were conducted in vitro. In depth and systemic in vivo studies are necessary before clinical translation or commercialization can be realized, especially those on in vivo drug release kinetics and dynamics, effects of drug dosage and release kinetics on therapy efficacy, bio-distribution of the released drugs and possible toxic effect on other organs, possible operation protocols, and metabolism of the carrier polymers.

3. LARGE ANIMAL STUDIES: THE CURRENT SITUATION

To date, the majority of publications on pain research have focused on humans. The greatest limitation of this human approach is the fact that these studies are primarily aimed at characterizing states of pain, and only a small percentage directly test the anatomical, biochemical, or pathophysiological mechanisms of pain [140]. If we are to make significant advances in our understanding and treatment of pain, animals provide an important resource for predicting analgesic efficacy, which can lead to the development of clinical drugs [141]. The last two decades have witnessed an evolution of animal models which has improved our understanding of the pathophysiology of inflammation, peripheral nerve disease, and bone infiltration by cancer cells. The animal models have also encouraged multidisciplinary analysis in the field of pain studies. Although the relative importance of genetic variability in human pain perception remains unclear, it is well-known that rodent populations display large and heritable differences in both nociceptive and analgesic sensitivity [142,143]. Improving our understanding of the genetic bases of pain-related traits may have important scientific and clinical implications, and could facilitate the development of novel analgesic strategies or improve treatment of pain using conventional therapies. The first evidence has recently been presented of epigenetic regulation in animals as a key factor in controlling states of pain [144]. Other "-omics" techniques are currently being developed, such as metabolomics, which has recently been associated with pain in the rat model [145].

Behavior resulting from pain can even be measured in decerebrate animals [146,147]. Withdrawal from algogenic stimulus (spinal reflex) [148], abdominal stretching and jumping (bulb-spinal reflex) [149], vocalization, biting, guarding, scratching, and licking (innate behaviors) are the most frequently used parameters in this type of evaluation. Unfortunately, some of these behaviors may be affected by a number of conditions (eg, reflexive withdrawal by surgical damage to motor neurons) which could affect the objectivity of the measurements [150], as well as their relevance to clinical pain [151].

Despite the many studies conducted on small animals, more efforts are necessary to develop tools for identifying pain and evaluating its intensity and form in large animals. Further research is needed on pain mechanisms and their phylogenetic bases across farm animal species [152].

In order to assess published data on pain in large animals, we conducted a literature search on PubMed by using the keywords: “animal model sheep and pain”, “animal model horse and pain”, “animal model cattle and pain”, “animal model cow and pain”, “animal model pony and pain”, and “animal model pig and pain”. We searched a full range of articles that explain how the authors assessed pain in these animal models.

For “pig” we conducted a further search for clinical trials only, with the words “pig and pain” and “pig and castration”. This additional search for “pig” reflected the importance of this animal model in the scientific community, despite the fact that other species are also used. We searched “pig and castration” because castration is one of the

most common operations to cause pain in this animal, so analyzing the evaluation of pain in this context could help us assess pain in pigs more effectively.

There are a number of reasons why rodent models have dominated the study of pain mechanisms: they cost less, are easier to manage ethically, and there is a large historical database of previous research with which authors can compare new findings. However, large animal models may predict human biology and pharmacology more accurately for a number of pain conditions. Validated pain models in these animals could facilitate the development of new and efficient analgesic drugs with few side effects, which could also be used in humans. In fact, the phylogenetic proximity of humans and large animals plays a key role for several reasons [153]: 1) species-specific variations in sequence patterns which result in different affinity or potency of the target; 2) they share a greater sequence homology than small animals and humans; 3) they experience different evolutionary pressure and therefore express pain in different ways; and 4) they have a different drug metabolism (large animals better predict human metabolism) [154]. Unfortunately, a universal method for identifying and recording pain objectively in large animal models has not yet been developed.

In studies involving cattle, locomotor function is used as the main indicator for pain. Bruijnis et al used a five-point scale, in which scores 1 (discomfort) and 2 (severe discomfort) represented a subclinical disorder visible on close inspection, where scores 3 (pain), 4 (severe pain), and 5 (very severe pain) represented a clinical disorder which causes lameness [155,156]. O'Driscoll et al used a locomotion score which considered four characteristics (spine curvature, tracking, head carriage, and

abduction/adduction), each of which was evaluated on a five-point scale, from less severe to very severe [157].

Rajkondawar et al compared a gait score (GS), a five-point score from “sound” to “severely lame”, and a lesions score (LS), in which lesion descriptions (such as “sole ulcer”, “interdigital dermatitis”, “puncture wound of the sole”, and “hemorrhage”) were associated with a score. This led to a better descriptor of lameness in cows [158]. The authors found that LS was a better descriptor of lameness than GS. Thoefner et al, in a study on heifers, considered lameness an indicator of pain, as well as other clinical signs, such as claw inflammation (warmth and increased pulsation), cardiovascular function, and gastrointestinal status [159].

Newby et al in a study for evaluating the effects of a label dose of ketoprofen after left displaced abomasums surgery in dairy cattle, used physiological (respiratory rate, heart rate, rumen motility, and rectal temperature) and behavioral (bright/quiet, alert, responsive, depressed, unresponsive) indicators of pain [160], whereas Saeed et al, after median sternotomy in calves, evaluated pain only by indirect signs such as heart rate, respiratory rate, and the animal’s ability to change posture [161].

Finally, Coetzee et al, in a study to evaluate plasma concentration of substance P and cortisol after castration or simulated castration in calves, also considered behavioral changes such as vocalization (scored on a scale of 0 (no vocalization) to 3 (continuous vocalization), and attitude or temperament, scored on a scale of 0 (unchanged from premanipulation behavior) to 3 (violent escape behavior).

Pippi et al analyzed three kinds of pain (superficial, deep, and visceral) to test analgesic drugs in ponies. The test for superficial pain used a heat source on a skin area, the test for deep pain used a current stimulus through a heating device on the surface of the radius, and the visceral test used a pressure stimulus through a rubber balloon into the cecum. In all the cases, the animal's reaction was movement away from the source in the superficial and deep tests, and a strong movement in the visceral test. This movement was recorded using an accelerometer [162,163]. Boatwright et al, in a study comparing two drugs for analgesia in a model of abdominal pain in ponies, used a cumulative pain score, a numerical ranking based only on physical criteria (kicking, pawing, head movement) with a score ranging from 0 (no pain) to 12 (maximum pain) [164]. Clinical signs of colic in ponies were used by Roelvink et al to evaluate pain in a comparative study of two analgesic and spasmolytic drugs. These signs (head shaking, kicking the abdomen, flehmen, stretching, pawing the ground and looking at the abdomen, yawning, restlessness, and leaning against the stocks) were evaluated before and after the administration of the drugs [165]. Fikes et al compared lidocaine and xylazina as epidural analgesics in ponies; to evaluate pain, they observed the reaction to a pin prick adjacent to the anus. Movements, such as attempts to kick or turn the head towards the stimulus site, were considered positive signs of pain. Epidural analgesia in ponies with carpal synovitis was reported by Freitas et al. Pain was assessed mainly through a lameness score, where 0 signified absence of visible lameness and 4 indicated severe lameness. Clinical signs such as heart rate, systolic

arterial pressure, respiratory rate, body temperature, and intestinal motility were also evaluated during the experiment.

In sheep, pain has largely been evaluated on the basis of escape-avoidance responses to different noxious stimuli. Dolan et al assessed withdrawal responses after formalin injection (into interdigital space) and mechanical stimulation with a pneumatic device [166-168]. Wilkes et al used a stiff von Frey filament tip connected to an anesthesiometer on the hind limb of the animal, exerting increasing pressure until it elicited a withdrawal response [169]. Stubsjøen et al inflated a tourniquet to a pressure of 300 mmHg (or until sheep showed signs of aversion) and Ong et al used four different electrical stimuli to produce a response [170,171]. Mather et al measured the pain threshold with a pneumo-mechanical pressure device on the animal's foreleg [172].

Some authors have used clinical signs and measurements (heart rate, blood pressure, eye temperature, rectal body temperature, electromyography), behavioral measures (appetite, vocalization, lip-licking, teeth-gnawing, ear posture, reaction to the environment, social isolation, feeding behavior), and motor function (abnormalities in gait, righting reflex, limping) as direct and indirect signs of pain [173-180]. Welsh et al compared a visual analog scale (VAS) and a numerical rating scale (NRS), developed by two veterinarians, to assess lameness in sheep. They found that, although the NRS and VAS are both repeatable and reproducible but not interchangeable, the VAS is intrinsically more sensitive [181]. An evaluation of electroencephalography (EEG) changes in young lambs before, during, and after seven treatments (tail-docking,

castration, sham-shearing, formalin injection, mulesing, ear-tagging, handling) was reported by Jongman et al [182], and showed a good correlation between EEG and painful procedure.

In studies involving horses as animal models, lameness is considered the main indicator of pain. Buchner et al used kinematic patterns of head and trunk, as well as the body centre of mass, to evaluate adaptations in movement during experimental lameness [183,184]. This indicator was applied to assess repair and the evolution of pain after removing calcified cartilage [185]. Other authors used a lameness score associated with other signs. Collier et al, for example, analyzed whether the surgical site was hot, painful, or swollen, and an increase in heart rate has also been used as a good indicator of the severity of lameness [186-191]. Cornelissen et al, using a five-point scale of lameness, considered indirect clinical and behavioral signs of pain such as heart and respiratory rate, rectal temperature, demeanor, appetite, and circumference and distension of the fetlocks; they also noted changes in local temperature by palpation [192]. Cayzer et al measured responses to pain through flexion tests and joint palpation, and noted the degree of swelling, scoring each indicator on a five-point scale [193]. Some authors have detected pain by monitoring mainly behavioral responses. Bussières et al, for example, used a composite pain scale (CPS) with multifactorial numerical rating, which was also used by Van Loon et al. [194,195]. The CPS considers physiological data (heart rate, respiratory rate, digestive sounds, rectal temperature), response to treatment (response to palpation of the painful area and interactive behavior), and behavior (sweating, appearance, posture, kicking at abdomen, pawing

on the floor, head movement, appetite). In the authors' opinion, these indicators are the most effective in identifying orthopedic pain. Scantlebury et al studied the incidence of recurrent colic in horses and the risk factors involved, using specific indicators of colic as signs of pain, including pawing, violent rolling, lying still, getting up and down, kicking belly, vocalization, rolling eyes, rapid breathing, and irritability. Other clinical signs, such as heart and respiratory rate, temperature, and borborygmi were considered. Clinical observations such as resting, respiratory rate, rectal temperature, carpal flexion angle, carpal circumference, carpal hyperthermia, and signs of carpal pain were scored on a scale from 0 (no warmth/signs of pain) to 2 (marked warmth/signs of pain). Miller et al considered indirect signs of pain, and evaluated the quality of analgesia during anesthesia in horses, using EEG, electrocardiography, heart rate, and blood pressure. The authors found that associating EEG and standard clinical parameters led to a better understanding of anesthetic management. Haussler et al assessed pain in horses with a pressure algometer, looking for avoidance reactions such as skin-twitching, local muscle fasciculation, lifting the thoracic limb, or stepping away from the pressure source [196]. Finally, Fureix et al hypothesized that chronic pain (for the presence of vertebral problems) in horses is associated with increased aggression towards humans. The authors found that chronic pain may behave similarly to acute pain, and therefore, it is important to include chronic pain as an influential factor in interpersonal relations and aggressive behaviour.

3.1 Focusing on pigs

In studies on pain, pigs seem to be the most suitable animal model. They share a number of anatomical and physiological characteristics with man. Moreover, their median body size facilitates the collection of samples such as biopsies, body fluids, and blood samples, which can be managed conveniently in standard facilities. Moreover, pigs are omnivorous, and anatomically, have a similar digestive apparatus to humans. The skin of pigs is similar to human skin in that it has little hair, and the pigmentation changes in different breeds [168]. The similarities between the skin of pigs and humans has been demonstrated in a number of studies, particularly when the animal is on average 12±4 weeks of age and the body weight is 20–27 kg [170]. Number, size, distribution, and communications of vessels in dermal porcine skin are similar to those in human skin, as are tissue turnover time and keratinous proteins in the epidermis. Transmission electron microscopy shows that the epidermal–dermal junctions in pigs and humans are similar, as well as immunoreactivity in the peptide nerve fibers, for example calcitonin gene related peptide, vasoactive intestinal polypeptide (VIP), and substance P. Changes in distribution and axonal excitability of nociceptive and non-nociceptive fiber classes are also similar in humans and pigs, which means that this animal model can be used to study the modulation of excitability in these C-fiber classes [171].

Unfortunately, pain in pigs can only be estimated by responses to nociceptive stimuli and none of these are ideal. Any reactions which are monitored are similar to other

animal models in that they are almost always motor responses ranging from spinal reflexes to complex behaviors. Motor responses, however, are not the only ones analyzed. In fact, Kluivers-Poodt et al, who studied the effect of pain relief in piglets, used vocalization as an indicator [172]. Using the common classification system described by Weary et al, they divided the calls produced by piglets into "high calls" (≥1,000 Hz) and "low calls" (<1,000 Hz), and these variations may reflect not only gross quantitative differences, but also the intensity and nature of the pain. The calls are registered to provide a record of their characteristics, using measures such as temporal parameters (eg, call rate and duration), waveform parameters (ie, peak-to-peak), and spectrum-based parameters (including peak amplitude and frequency, main frequency, and band width).

Other authors have used vocalization in association with other behaviors, but have evaluated quantitative characteristics, rather than qualitative ones as described above. Walker et al assessed the reaction of piglets after castration by monitoring the presence and degree of movements, and the presence or absence of vocalization.68 Reyes et al modified a method used by Firth and Morton, which considers behavioral parameters such as vocalization, lameness, aggression, restlessness, posture, isolation, appearance, sling time, agitation, and posture [173-179]. Possible scores ranged from 10 (no pain) to 35 (maximum pain). Other clinical parameters were heart and respiratory rate, blood pressure, and temperature. Lupu et al analyzed the response after a pinprick test – the usual response involved vocalization and/or withdrawal of the ipsilateral forelimb. On the other hand, Navarro et al, in addition to measuring withdrawal responses and

vocalization after a mechanical stimulus, analyzed motor function, muscle hypertonia and hyperreflexia, anal sensation, and the presence of allodynia to study a chronic spinal compression model in minipigs. Sutherland et al, in their study on reactions in pigs after castration with or without anesthesia, analyzed behavior such as lying down, nursing, sitting, standing, and walking. In terms of behavior indicating pain, the authors analyzed sitting, huddling, scooting, and stress vocalization, with an automatic system for monitoring stress calls [180]. Murison et al, in their study on pain behavior after laryngeal transplant, used a combination of locomotion scores to assess pain (the willingness of the pig to lie down, to stretch its neck and to approach its food, or how it moved), wound palpation (gently increasing pressure until the animal responded by turning away or grunting, for example), and other behavior (grunting, willingness to play with carers, appetite, or nesting behavior). Numerical scores were used, from 0 (no pain) to 10 (worst pain imaginable) [181]. In a study to evaluate the response to doses of ketoprofen, Mustonen et al examined clinical signs (locomotion, general temperature, respiratory rate, and general behavior) to form a total clinical score [182]. The same authors used a five-grade lameness scale to measure the efficacy of oral ketoprofen, recording lameness scores before and after the treatments as an index of pain relief [183]. Friton et al also used lameness as a primary parameter [184]. In a study to evaluate the safety and efficacy of meloxicam in non-infectious locomotor disorders in pigs, the authors used clinical examination classifications to describe and categorize “lameness at rest” and “lameness while walking”, as well as “feed intake” and “behavior”. Other authors have analyzed pain indicators in terms of behavior such

as inactivity, huddling up, trembling, tail-wagging, scratching, stiffness, sleep spasms, recumbency, coprophagy, aggression, depression, head-pressing, changes in activity, nursing, lying, body movement, muscle-twitching, and withdrawal [185-191]. These analyses allowed the authors to evaluate pain relief in pigs, distress after surgical procedures (such as castration), and the effects of some analgesics. Finally, some authors have used different methods, such as assessing postoperative analgesia after femoral fracture in pigs using a modified VAS for five different conditions: VAS1, observation; VAS2, contact; VAS3, ambulation; VAS4, vocalization; and VAS5, overall. The VAS chart consisted of a 100 mm horizontal line, where the left side represented no pain and the right side the highest level of pain [191]. Haga et al analyzed EEG, mean arterial blood pressure, and pulse rates as indicators of pain in post-castration piglets. The results showed significant differences between animals which had received intrafunicular or intratesticular lidocaine and the control group which had not received it [192]. Rukwied et al analyzed axon reflex vasodilatation by laser Doppler imaging as an indirect sign of C-fiber activation after irradiating pig skin to create erythema and applying mechanical (with von Frey needles) and thermal stimuli (with increased skin temperature to 45°C or 47°C) [193]. To measure the concentration of substance P and VIP involved in the transmission of pain, Cornefjord et al applied an ameroid constrictor at the root of the spinal nerve, and measured the gradual reduction in diameter of tissue samples from the nerve root (cranial to constrictor), and from the dorsal root (ganglion) [194]. The results showed that there was an increase of substance P but no significant differences in VIP concentration.

Reactions analyzed in the studies of all species include: 1) responses organized by centers which are “low” in the hierarchy of the central nervous system (termed “pseudo-affective reflexes”) – these include neurovegetative reactions (tachycardia, hyperpnea, arterial hypertension, etc), basic motor responses (contractures, withdrawal, etc), and vocalization [195,196]; and 2) more complex responses integrated by higher nervous centers, which include conditioned motor responses after a period of learning such as behavioral reaction (escape, avoidance, aggression, etc) or modifications of behavior (social, food, sleep, etc).

However, none of these evaluations are entirely satisfactory. They display a number of weaknesses, some of which are due to the types of stimuli or how they are applied, the plasticity of the animal or the way the data has been measured. Specific protocols will therefore need to be developed to improve estimates of pain in animal models, and in future, in patients who are unable to express their pain verbally, such as infants and comatose or cognitively impaired patients (Table 2).

Biological similarities between humans and large animals

1. Phylogenetic proximity:
   – Greater sequence homology
   – Better prediction of human metabolism
   – Less species-specific variations in sequence patterns
   – Different evolutionary pressure
2. Median body size
3. Humans and pigs are both omnivorous
4. Similar digestive apparatus with porcine species
5. Similarity with porcine skin:
   – Number, size, distribution, and communications of dermal vessels
   – Tissue turnover time

Table 2 Biological similarities between humans and large animals.

3.2 Future pain models in pigs

When we choose the animal species for a scientific study, it is important to consider the similarity of organs and tissues between the animal and humans. It is not possible, therefore, to make a general statement about animal models [197-200]. For example, some experts suggest that non-human primates should be used where there is no alternative, such as in studies involving neuroscience and brain function, or in diseases common to man and other primates (HIV/AIDS or tuberculosis). On the other hand, dogs are preferred in the development of anti-ulcer drugs because of the similarity

between the human and canine gastric mucosal membrane.

In terms of pain and its evaluation, it is important to emphasize that each species manifests pain in its own way, according to the behavioral repertoire of the species, and a particular reaction does not necessarily suggest that the animal is experiencing pain. Moreover, no single behavior is a definite sign of pain, and each reaction must be assessed in context (Committee on Regulatory Issues in Animal Care and Use, 2000) [201]. In fact, vocalization in piglets is not necessarily an indicator of pain. Piglets run a natural risk of being crushed by their mother, and they have developed a low threshold for screaming in order to alert her. Piglets will scream simply if they are picked up, but this does not mean they are feeling any pain. Equally, it is not true to say that piglets never feel pain when they scream. It only means that vocalization, as a single indicator, is not a sensitive parameter of pain.

Conversely, if it is difficult to find an indicator of pain, we can analyze signs of well-being that suggest the absence of pain, such as play (nudging and running, grabbing, biting chains, playful fighting), vocalization (calling the piglet or conversing in a group), strong growth, and good health [202,203].

Overall, pigs seem to be the most suitable animal model for studying pain. In fact, there are a number of reasons for choosing pigs. Firstly, their similarities with humans in terms of size, anatomy, and genetics make pigs suitable for studies where results can be reproduced in humans. Secondly, pigs are easily managed in the animal facility, which optimizes the conditions for study. Thirdly, the ethics committee is more likely to approve studies involving pigs as opposed to other animals such as dogs or primates,

and finally, pigs are most frequently used by the scientific community because they are likely the most practical for follow-up studies.

This evidence therefore indicates that pigs represent the best models for studying and assessing postoperative pain in humans.

It is necessary to identify new methods for evaluating pain (particularly postoperative pain) in large animals, and pigs represent the most suitable model because of their similarities to humans. The aim would be to transfer the results to human patients, to develop better pain evaluation and treatment. This would be especially useful in non-verbal patients to prevent complications in short- and long-term therapies due to incorrect diagnosis on the basis of pain.

Teamwork will be essential in this endeavour: biologists, anaesthetists, surgeons, pain therapists, and biomedical engineers will need to work together “from bench to bedside”, each contributing their specific skills to the task.

4. CONTINUOUS WOUND INFUSION WITH CHLOROPROCAINE IN A PIG MODEL: THE PATHWAY AND THE EXPERIMENTAL SCENARIO

Currently, drugs used for CWI are long-lasting LAs, such as ropivacaine, bupivacaine, and levobupivacaine. Such drugs are considered safe at clinical doses, and the kinetic profile and blood levels have already been investigated during epidural infusions. However, both from a clinical and a pharmacokinetic perspective, an ultra short-acting LA may be more appropriate for CWI, as it could be better modulated according to specific patients' needs with "on/off " profile. Chloroprocaine (CP) has fast onset (6–10 minutes) and short action (around 90 minutes), with a cholinesterase-based plasmatic metabolism. Early cases of toxicity after spinal anesthesia were documented, but were related to the sodium bisulfite antioxidant and to the solution's low pH. Nowadays, antioxidant-free, safer preparations are available, and interest has grown again because of CP's ideal profile for CWI. There is no study, to our knowledge, investigating CP during intrawound continuous infusion in an animal model; moreover, there are few data elucidating the effect on local and systemic inflammation of a continuous subfascial wound infusion of LAs and in particular for CP. We therefore evaluated the absorption associated with a continuous CP intrawound (subfascial) infusion in pig. We also investigated if CP at two different dosages may influence local and systemic inflammatory responses after surgical lesion both in vivo and ex vivo.

4.1 Materials and Methods: animals, surgical procedures and techniques

All animal procedures were performed according to the protocol approved by Institutional Animal Care and Use Committee (IACUC) – University of Pavia. All animal care and experimental procedures followed local, national and European Union guidelines for the animals' use (Directive 2010/63/EU – revising Directive 86/609/EEC on the protection of animals used for scientific purposes).

Piglets (20–30 kg) underwent pararectal laparotomy, extended for 10 cm. The parietal peritoneal membrane was then closed with a continuous absorbable synthetic suture. An intrawound, multiholed catheter (PAINFusor® – Plan- 1-Health, Udine, Italy) was placed between peritoneum and deep muscular fascia along the length of the wound and tunneled subcutaneously. Muscle layers and skin were finally closed with a continuous absorbable synthetic suture. Surgical procedure was performed under general balanced anesthesia, including mechanical ventilation via orotracheal intubation and mioresolution. A central venous catheter was placed in the right external jugular vein with open surgical technique and was used for drug infusion and for blood sampling. At the end of surgical procedures, piglets were set in the metabolic cage and were supplied with water and standard bran. Intravenous antibiotic (cefazolin 1 g) and omeprazole 40 mg were administered during follow-up to prevent wound infections and stress-induced gastric ulcerations. Analgesia was performed in all animals by intramuscular injection of tramadol 50 mg during the perioperative period, with no additional analgesics, according to our animal protocols for surgical trials. Animals

were randomly allocated to 3 groups with a 5 mL/h continuous infusion through elastomeric pump for 72 hours: saline (group A), CP 1.5% (group B) or CP 0.5% (group C). The randomization was based on a computer-generated list.

4.1.1. *Sample collection*

During the follow-up period, peripheral blood was collected for the determination of CP and its metabolite, as well as of the effects on systemic inflammation, at 0, 3, 6, 12, 24, 48, 72, 96, 102 and 108 hours from incision. Plasma was obtained by centrifugation at 3000 rpm for 15 minutes and stored at –20°C until analysis. Mononuclear cells (MNCs), for in vitro and ex vivo experiments, were obtained by gradient density on cell separation media (Cedarlane, Burlington, USA) at 2500 rpm for 20 minutes. The catheter was removed after 96 hours of infusion. Piglets were finally sacrificed, according to international guidelines.

4.1.2. *Determination of CP and its metabolite in plasma*

CP and its metabolite 4-amino-2 chlorobenzoic acid (CABA) were analyzed by liquid chromatography/tandem mass spectrometry using a high performance liquid

chromatography (HPLC) Agilent (model 1200) equipped with an autosampler (PAL HTS-xt; PAL System, CTC Analytics, Zwingen, Switzerland) directly interfaced with a triple-quadruple API 4000 (AB Sciex, Framingham, MA, USA), equipped with an electrospray ionization (ESI) source. CP was provided by Sintetica SrL, (Mendrisio, Switzerland) CABA by Sigma-Aldrich Co. (St Louis, MO, USA) and deuterated PABA (4-aminobenzoic acid), used as internal standard (IS) for chloroprocaine and CABA quantitation, by Santa Cruz Biotechnology Inc., Dallas, TX, USA. Methanol and formic acid were from Sigma-Aldrich, whereas HPLC grade water was obtained using a Milli Q water purification system. Chromatographic separation was carried out using a Synergi 4 micro-Hydro-RP 80 A, 150 × 2 mm × 4 μm column (Phenomenex, Torrance, CA, USA) at a flow rate of 200 μL/ min. A total of 10 μL of the sample was injected in a linear gradient system from 85% of solvent A (0.1% formic acid in water) to 90% of solvent B (0.1% formic acid in methanol) over 3 minutes. Final conditions were held for 3 minutes before equilibration back at starting conditions.

The triple-quadruple was operated in positive ions mode for multiple reaction monitoring (MRM). The following characteristic fragmentations were used for identification and quantitative analyses: CP from m/z 272 to 198.6 or 153.9, CABA from m/z 172.2 to 128 or 153.9, and d4-PABA from m/z 142.1 to 98.8 or 123.9. The most abundant transition was used for quantitative analysis, while the second transition was used as qualifier for additional identification purposes. A total of 100 μL of plasma samples were added with 20 μL (1 ng/μL) IS and 400 μL of methanol; samples were then put at –20°C overnight. After centrifugation at 13,000 rpm for 10 minutes, 450 μL

of the solution were recovered and evaporated using a rotary evaporator. Finally, the samples were resuspended in 100 μL of water/methanol (1:1) and 10 μL was used for liquid chromatography/tandem mass spectrometry analysis.

The calibration curves were prepared in drug-free pig plasma, from working solutions of CP and CABA of 1, 3, 10, 30, 100 and 300 ng/mL and processed as the biological samples. Calibration curves were obtained plotting the ratio of the chromatographic area of specific transitions for the compounds of interest over that of specific transition of the internal standard, and the correlation coefficient was always better than 0.99. The limit of quantification (LOQ) was 1 ng/ mL of plasma for both compounds. Data were acquired and processed using Analyst 1.6.1. Concentrations are presented as mean and standard error of the mean of n determinations.

4.1.3. *Histopathological analysis*

For histological examination, 3 samples of the full-thickness wound and 1 sample of the contralateral healthy abdominal wall were collected from each animal. Each sample was 0.5 cm thick. The samples were fixed in 4% neutral buffered formalin for 48 hours, dehydrated with gradient alcohol series, cleared in xylene and eventually embedded in paraffin. Serial sections (8 μm) were obtained using a Leitz (Wetzlar, Germany) microtome, stained with hematoxylin and eosin (H&E) and examined with a Zeiss

(Oberkochen, Germany) Axiophot microscope. We evaluated the presence of inflammatory cells: neutrophils, eosinophils, lymphocytes, macrophages and mast cells, in pericatheter blank tissues. A total of 15 sections were evaluated for statistical analysis (4 sections per group – corresponding to a different animal of each experimental group plus 1 contralateral healthy sample per group). For each section, at least 8 randomly determined areas of connective tissue were examined at 20× magnification and 100 cells per area were counted. The rate of inflammatory cells was assessed using a semiquantitative scale, with scores ranging from 0 to 4 by 3 independent observers (Table 3). Histological scores are reported as median and 25–75 percentile (interquartile range, IQR).

Score	Percentage of inflammatory infiltrate
0	0–10
1	10–25
2	25–50
3	50–75
4	>75

Table 3 Histological score for evaluation of inflammatory infiltrate.

4.1.4. *Systemic inflammation analysis*

Systemic inflammation was evaluated by quantifying pig interleukin-6 (pIL-6), pIL-10, pIL-8, pIL-1β and pig interferon (pIFN)-γ levels in plasma samples at each time point, after incision in all animals, by commercially available quantitative enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Inc., Minneapolis, MN, USA). Detection limits were as follows: 18.8 pg/mL for pIL-6, 31.3 pg/mL for pIL-10, 62.5 pg/mL for pIL-8, 39.1 pg/mL for pIL-1β and 39 pg/mL for pIFN-γ.

4.1.5. *In vitro and ex vivo cell proliferation suppression*

In order to evaluate in vitro cell proliferation suppression, CP and CABA were added at different concentrations (from 1.25 to 0.02 mg/mL) to resting and phytohemagglutinin (PHA)- activated MNCs obtained before treatment. In detail, 105 MNCs/well were plated in triplicate in flat-bottom microwells (Corning Costar, Euroclone, Milan, Italy), in RPMI 1640 medium (ThermoFisher Scientific, Waltham, MA, USA), 10% fetal calf serum (Euroclone, Celbio), without (resting) or with PHA (activated) (4 g/mL; Boehringer Ingelheim, Mannheim, Germany). After a 3-day incubation at 37°C in a humidified 5% CO2 atmosphere, 3 H-thymidine (3HTdR 0.5 mCi/well; Amersham, Buckinghamshire, UK) incorporation was measured during the

last 21 hours by standard procedure. Results were expressed as stimulation index (SI = cpm stimulated/ cpm unstimulated) and reported as residual proliferation. Moreover in order to define if CP in vivo treatment induced a change in cell response capability we evaluated ex vivo, with the same methodology, the effects of CP treatment on the response to PHA of MNCs obtained at different time points (basal, 96 and 108 hours) of pharmacological treatment in groups A (saline), B (CP 1.5%) and C (CP 0.5%).

4.1.6. *Cell viability*

To test if CP and CABA could affect MNCs viability, after in vitro culture, cells were evaluated by Trypan blue (Sigma, Milan, Italy) exclusion test. Percentage of cell viability is calculated as (number of viable cells/total number of cells [viable + nonviable] × 100).

4.1.7. *Statistical analysis*

Cytokine concentrations were compared between groups with analysis of variance for repeated measure. An ordinal logistic regression model was fitted using histological score as dependent variable and group as independent variable. To compare all groups,

post hoc estimation (between doses or between groups B and C and group A) and Wald tests were also performed. A p-value <0.05 was considered statistically significant. Quantitative variables are described as mean and SD. Comparisons between groups (A and B, C) and time were performed by means of analysis of variance for repeated measures. The same method was applied to analyze SI at different CP and CABA concentrations. Robust standard errors were considered to take into account the clustered nature of the data. A p-value <0.05 was considered significant. Stata v14.1 (StataCorp LP, College Station, TX, USA) was used for all the analyses. We did not perform a power analysis since previous literature does not provide data to support any power calculation.

4.2 Results

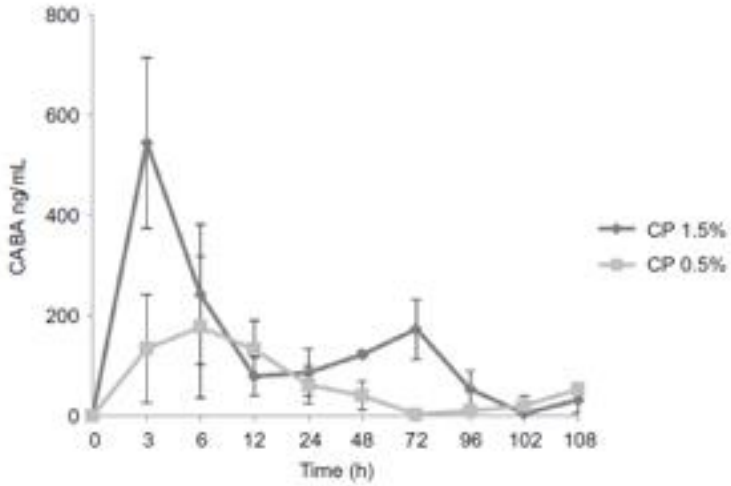
Four animals (25–30 kg, average weight 29.6 kg) were randomly allocated to each group; in group A 2 additional animals were studied as 2 animals were excluded as they developed fever and wound infections. All the other animals in groups B and C completed the follow-up period uneventfully and they were all treated without any deviation from the protocol.

We did not register any adverse effect or allergic reaction in all the animals used for the study.

4.2.1 *Concentrations of CP and its metabolites in plasma*

Concentrations of CP were mostly below the quantitation limit, with only 6 blood samples showing the presence of very low concentrations of the intact molecule (1–2.3 ng/ mL in animals perfused with CP 0.5%, and 1–20.8 ng/mL in animals perfused with CP 1.5%). CABA concentrations showed a sharp increase immediately after the infusion was started, peaking between 3 and 6 hours from the beginning of the infusion, and returning to basal level (zero) as soon as the continuous infusion was stopped (Figure 1). The peak in CABA concentrations appeared to be proportional to the

concentration of CP infused (Figure 10), averaging 177±141 and 543±169 ng/mL in animals perfused with CP 0.5% and 1.5%, respectively.



CABA plasma concentrations after continuous infusion with 0.5% (closed squares) and 1.5% (closed diamond) of chloroprocaine.

Note: CABA was measured by liquid chromatography/tandem mass spectrometry as described in the "Materials and methods" section. Data are expressed as mean and SEM (n = 4).

Abbreviations: CABA, 4-amino-2-chlorobenzoic acid; CP, chloroprocaine; SEM, standard error of the mean; h, hours.

Trend of the inflammatory infiltrate scores among the different treatments

Group	Catheter surrounding area (1 cm): median (IQR)	Contralateral area median (IQR)
A (Saline 0.9%)	4 (3–4)	0 (0–1)
B (CP 1.5%)	2 (1–2)	0 (0–1)
C (CP 0.5%)	3 (2–3)	0 (0–1)

Abbreviations: CP, chloroprocaine; IQR, interquartile range.

Figure 10. CABA plasma concentration after continuous infusion with 0.5% and 1.5 % of chloroprocaine.

4.2.2. *Histopathological analysis*

The presence of inflammatory cells was significantly higher in group A than in groups B and C ($p < 0.001$, in both cases). These results are well summarized by semiquantitative scores (Figure 10) that show a dose-dependent reduction of the inflammatory infiltrate within the wound, which is nearly halved with higher CP doses.

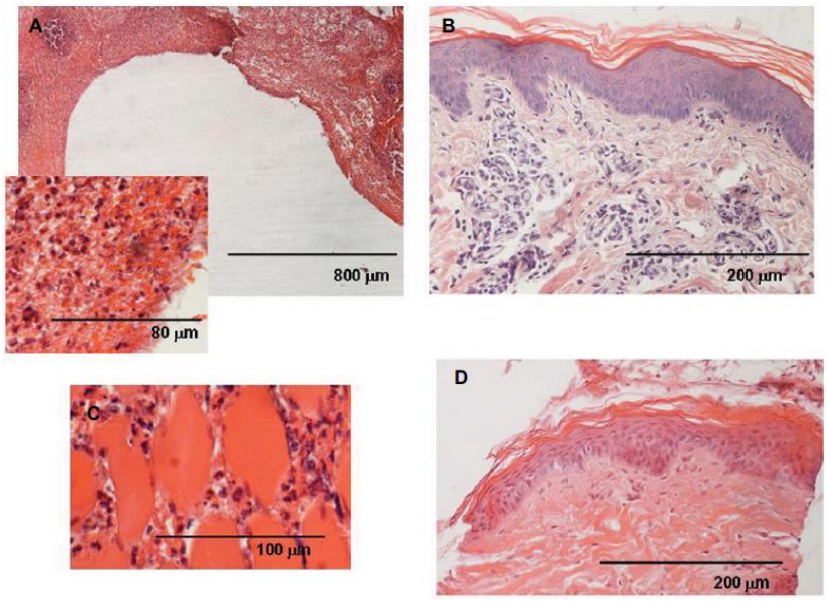


Figure 11. Group A (saline) animal

Notes: (A) Catheter blank (white in the picture) surrounded by connective tissue. A large amount of inflammatory infiltrate is evident, mostly round cells such as lymphocytes and some PMNs. (Inset) Right next to the blank, many cells appear pale as if suffering or necrotic; collagen fibers are contracted. Many red blood cells are present , probably due to catheter extraction. (B) Inflammation reaches the cutaneous connective tissue, while (C) a great number of leukocites surround muscular fibers (D) and contralateral healthy skin appears normal.

This reduction is even more evident in wound sections, where refined analyses on tissue damage and reaction are possible: pigs in group A (saline) have high amounts of

leukocytes and lymphocytes infiltrating wound layers up to connective tissue, and cells surrounding the catheter's blank display signs of necrosis (Figure 11). Signs of cell damage are less evident in CP-infused pigs, and inflammatory infiltrates are less pronounced and different in nature (less leukocytes); inflammation reaches the connective tissue to a lesser extent, and signs suggestive of tissue regeneration are evident (Figure 12). The reduction in inflammatory infiltrates and signs of tissue regeneration are more evident with higher doses of CP (Figure 13), however no statistically significant difference was observed between groups B and C (Figure 10).

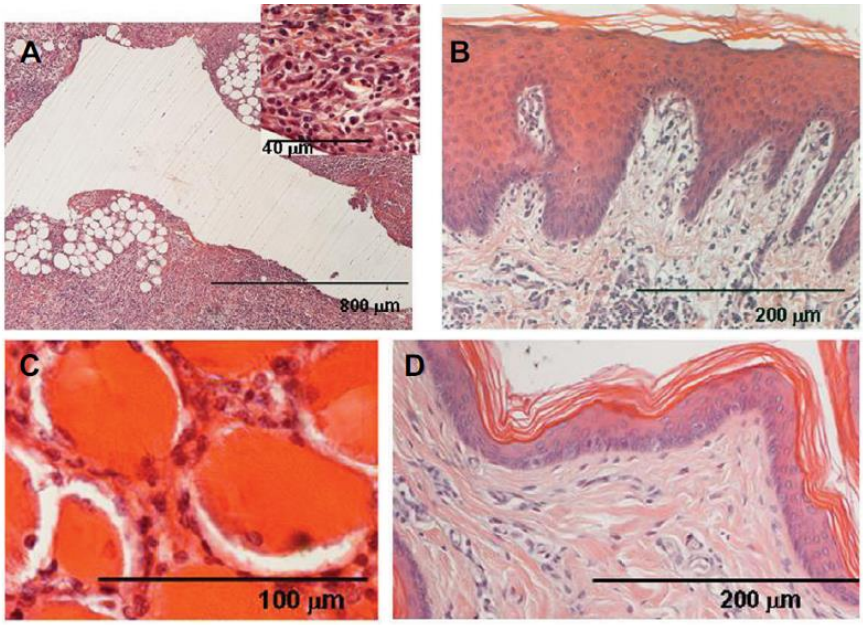


Figure 12. Group C (CP 0,5%) animal.

Notes: (A) Connective tissue of the blank wall contains active basophilic fibroblast, thin collagen bundles and a number of round white cells (inset); (B) mild inflammatory infiltrate affects dermal papillae and (C) intramuscular connective. (D) Contralateral healthy skin appears normal.

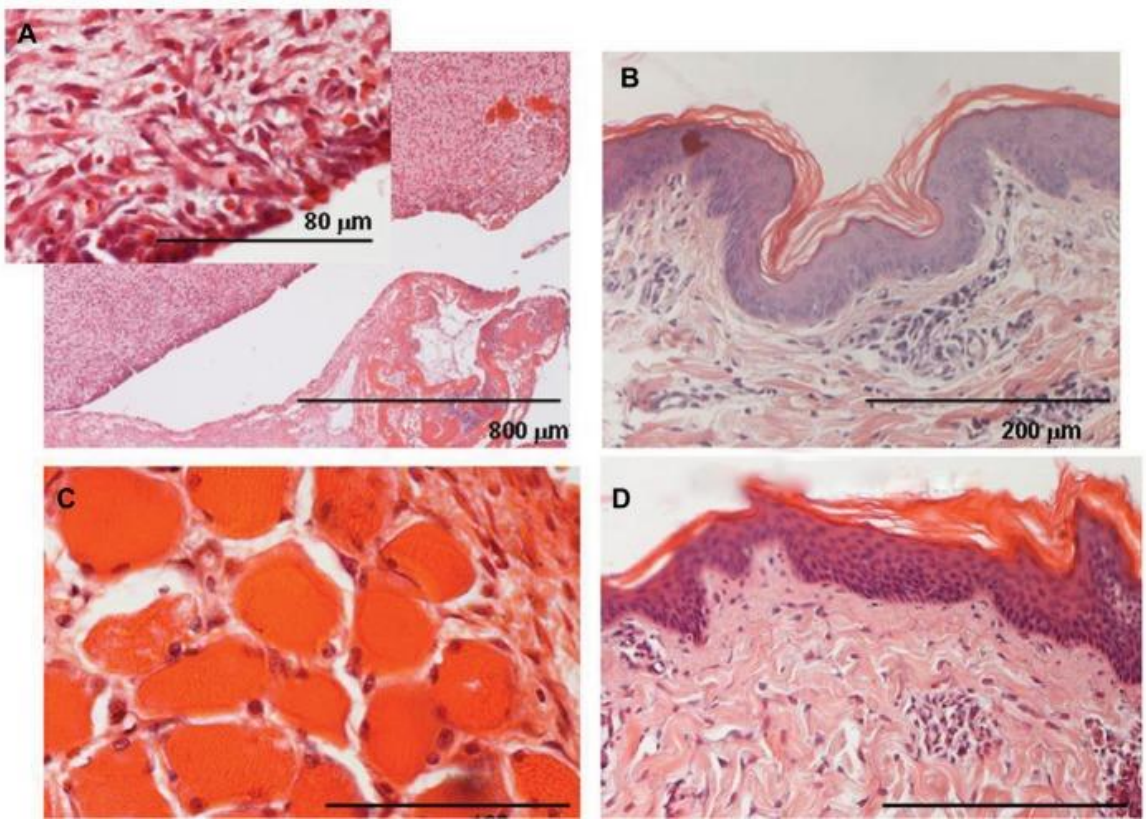


Figure 13. Group B (CP 1.5%) animal.

Notes: (A) Connective wall surrounding the blank shows no structural damage in comparison with controls; inflammatory infiltrate appears less evident, just few leukocytes are present, mostly lymphocytes; (inset) fibroblasts look plump and basophilic; collagen bundles are thin with an irregular arrangement and some tiny blood vessels are evident, suggesting tissue repair. (B) Cutaneous tissues contain a slightly higher amount of infiltrate than contralateral healthy skin (D). (C) Muscular fibers are surrounded by few leukocytes.

4.2.3 *Systemic inflammatory analysis*

Plasma cytokine quantification at different time points did not reveal any statistical differences between groups B and C versus group A, respectively, for the pro-inflammatory cytokines' levels (IL-1β, p = 0.8 and 0.4; IL-6, p = 0.7 and 0.9; IL-8, p = 0.6 and 0.6; IFN-γ, p = 0.06 and 0.2) and those of the anti-inflammatory ones (IL-10, p = 0.3 and 0.3) during the entire follow-up period (Figure 14).

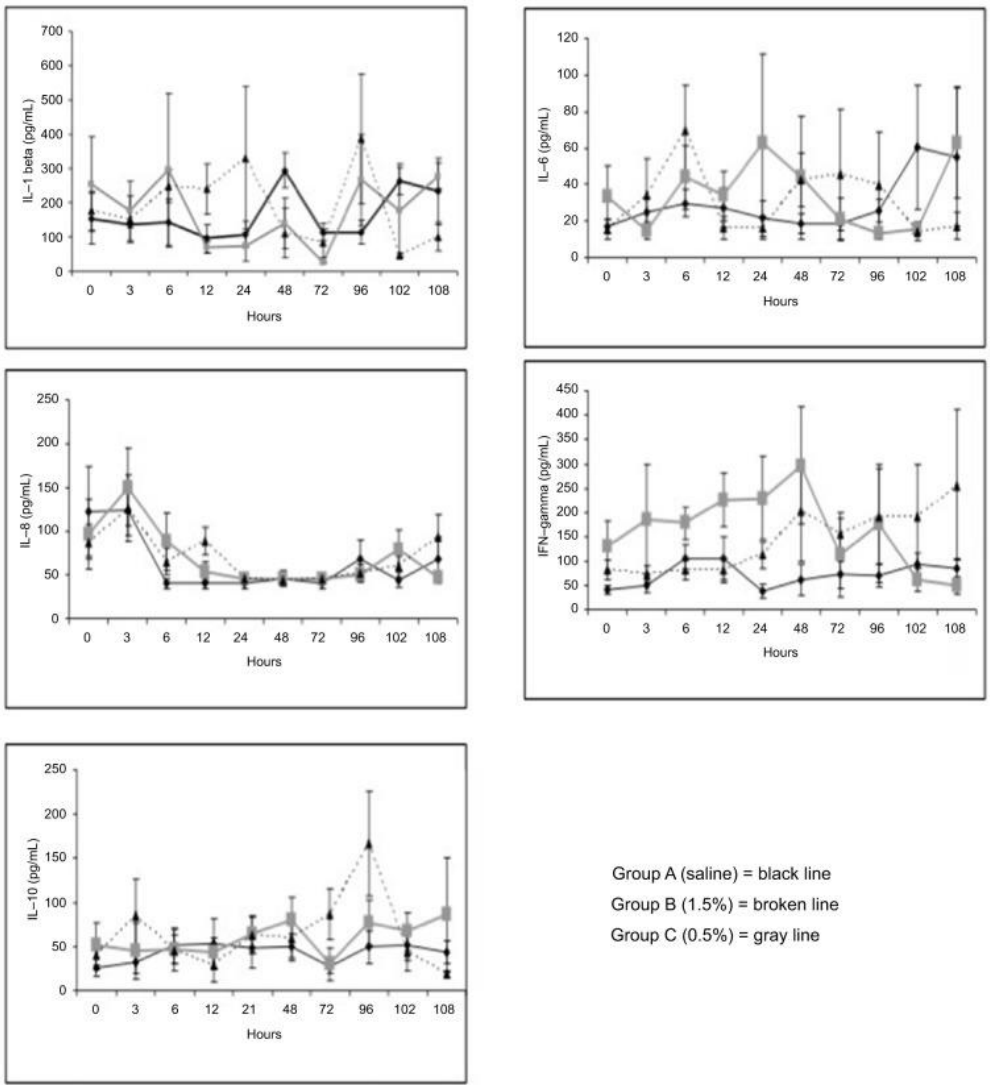


Figure 14. Trend of the blood cytokines levels in the three experimental groups. Notes: Group A, saline; Group B, CP 1.5%; Group C, CP 0.5%.

4.2.4 *In vitro and ex vivo cell proliferation analysis*

The effect of CP and its metabolite on the in vitro proliferation of pig MNCs stimulated with PHA was evaluated at different concentrations in 4 animals using MNCs obtained before any kind of surgical procedure and treatment. The results are shown in Figure 15.

PHA-stimulated proliferation of MNCs was significantly reduced by the addition of 1.25 and 0.6 mg/mL of CP, with a direct correlation between the concentrations and the degree of inhibition. A decrease in proliferation was also observed for CABA at 1.25 mg/mL, but it was not statistically significant ($p = 0.1$). Evaluation of cell viability, by Trypan blue exclusion test, showed that the addition of CP and CABA to in vitro resting and activated pig MNCs did not cause cell death, even at the highest concentration (percentage of viable cells 72% and 79%, respectively), suggesting the lack of cytotoxic activity of CP and its metabolite. Moreover, CP and CABA did not induce in vitro cell proliferation at any concentrations of resting MNCs, indicating no antigenic activity. In order to assess the effect of CP and CABA we evaluated ex vivo the response to PHA of MNCs isolated from blood collected at different time points (basal, 96 and 108 hours). Results did not show any significant differences between group A versus group B ($p = 0.22$) and versus group C ($p = 0.34$) at each time point.

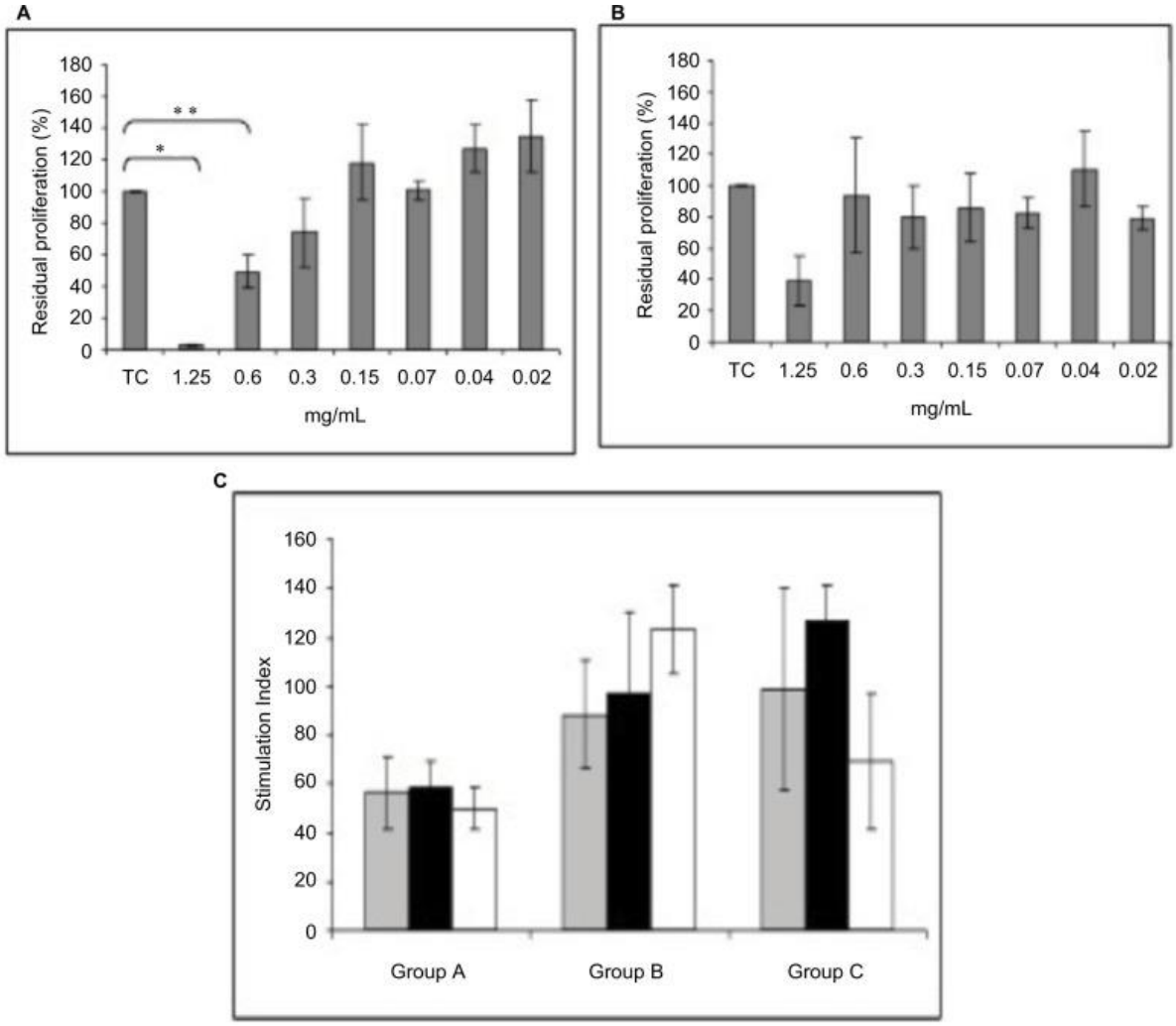


Figure 15. Treatment group in vitro proliferation. Notes: In vitro cell proliferation suppression by CP (A) and its metabolite (B) at different concentrations, on PHA-activated pig MNCs. Results are expressed as residual proliferation. CP induces significant reduction at concentration of 1.25 mg/mL (*p = 0.012) and of 0.6 mg/mL (**p = 0.052). (C) In vitro PHA-induced proliferation of MNCs obtained from group A, B, C animals at different time points (gray bars = T0, black bars = 96 hours and white bars = 108 hours). Results are expressed as stimulation index. No significant differences are observed between treated and control groups. Group A, saline; Group B, CP 1.5%; Group C, CP 0.5%.

4.3 Discussion

So far, long-acting LAs have mainly been used for CWI, despite their suboptimal pharmacokinetic profile and the lack of studies investigating their systemic exposure. New formulations of CP, an ultrashort acting LA with plasmatic pseudocholinesterase metabolism, theoretically display an ideal pharmacokinetic profile for CWI. In a translational perspective, and responding to the need of testing this molecule in an experimental setting, we investigated the use of CP in a large animal model (pig). The current investigation confirmed the hypothesis that CWI in our large animal model would result in relatively low amounts of CP being absorbed, and in light of the extremely short half-life of CP in plasma may definitely limit the possibility of systemic effects; indeed during the 96 hours of 5 mL/h continuous administration of both 0.5% and 1.5% CP, the overall amount of CP penetrating the systemic circulation (that is plasma concentrations of CP + its plasmatic metabolite CABA) reached an average Cmax at least 10-fold lower than that observed in obstetric patients receiving epidural CP during cesarian section. A dose-related increase in concentrations was observed after the beginning of the CWI and up to 6 hours, suggesting an initial blood vessel dilating response to CP, resulting in a peak of absorption that appeared to progressively decrease over time. We registered no side effects or allergic reaction in any of the enrolled animals. Our study confirms CP as an ideal local anesthetic for CWI, due to its fast catabolism, safety and on/off profile. The lack of a cholinesterase inhibitor at collection prevents the actual assessment of systemic exposure to intact CP

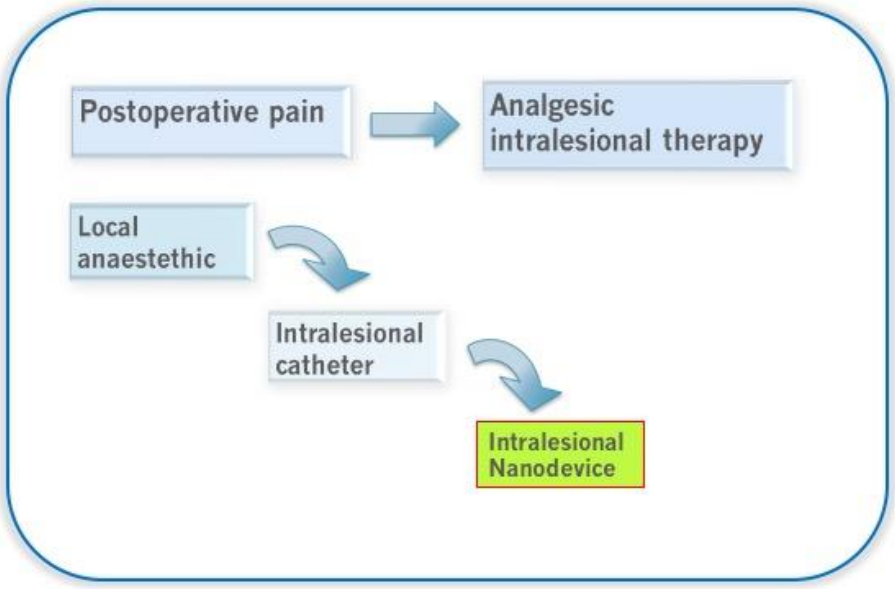
since CP degradation occurs very rapidly in plasma, but reporting the total amount of CP entering the bloodstream (that is CP + CABA) our study reliably describes the overall absorption of CP upon CWI, showing concentrations well below those observed upon epidural administration during either cesarean section or vaginal delivery, peaking between 3 and 6 hours and decreasing to undetectable levels after infusion was suspended. Therefore, also taking into account potential differences in esterase activities between the experimental animal used and humans, the systemic exposure to CP appeared very limited, as confirmed by the lack of effects on inflammatory parameters or blood cell activity. Indeed no correlation was detected between treatment and systemic inflammation, as assessed by determination of plasma concentrations of pro-inflammatory cytokines, suggesting that only local inflammation was modulated by the presence of CP. Additionally, local infusion of CP did not affect the ex vivo response of immune cells obtained from treated animals, even if PHA-induced proliferation of MNCs was suppressed by in vitro addition of CP, further supporting the lack of significant circulating concentrations of intact CP as suggested by the relatively low plasmatic concentrations of CABA. Our data also suggest that the continuous infusion with CP displays local anti-inflammatory effects. These effects were dose-dependent, as we observed a more pronounced reduction of inflammatory response in the group treated with higher concentrations of local anesthetic. Since we did not monitor pain, we cannot determine if the dose-dependent anti-inflammatory effect is associated with either better pain relief, or with a more pronounced reduction of neurogenic inflammation or with specific anti-inflammatory effect of CP.

Investigating the pain response would have provided additional support to our studies, but measuring pain in a large animal model can be very challenging, and a well-accepted model is still lacking. Nevertheless, apart from the assessment of the analgesic effect, our study was able to confirm in a large animal model results previously obtained only in small animals, the ability of nociceptive block to reduce local inflammation. In consideration of the promising results relative to the limited absorption and the anti-inflammatory effect of CP obtained with this study, future trials are warranted to address this topic. Despite the low sample size (typical of studies in big animal models), we confirmed the limited absorption and a local anti-inflammatory activity of CWI with CP. Once these results are confirmed in humans, they may show CP to be a good choice for CWI allowing immediate analgesic benefit and fast elimination; patients with liver or kidney diseases may take advantage of rapid plasmatic clearance of CP, preventing accumulation and toxicity. Clinical studies are needed to define the lowest effective dose of CP and investigate its anti-inflammatory effect in a human model.

5. NANOTECHNOLOGY APPLIED TO POSTOPERATIVE ANALGESIA

5.1 *Towards the application of a new Nanodevice (nanogel) for local drug delivery*

The term of our path is represented by the experimental application of Nanodevice (ND) in a large animal model. This investigation represents a consistent first translational step to apply nanotechnology to the intralesional pain treatment.



5.2 *The Nanodevice.*

Our new Nanodevice is a hydrogel made of alginate and chitosan, designed and generated using the electrospinning technique, as described before. All the technical procedures and the ND production have been done at the Laboratory of Biomaterial of

the Politecnico of Milano in collaboration with the Laboratory of the Department of Molecular Biology of the University of Pavia.

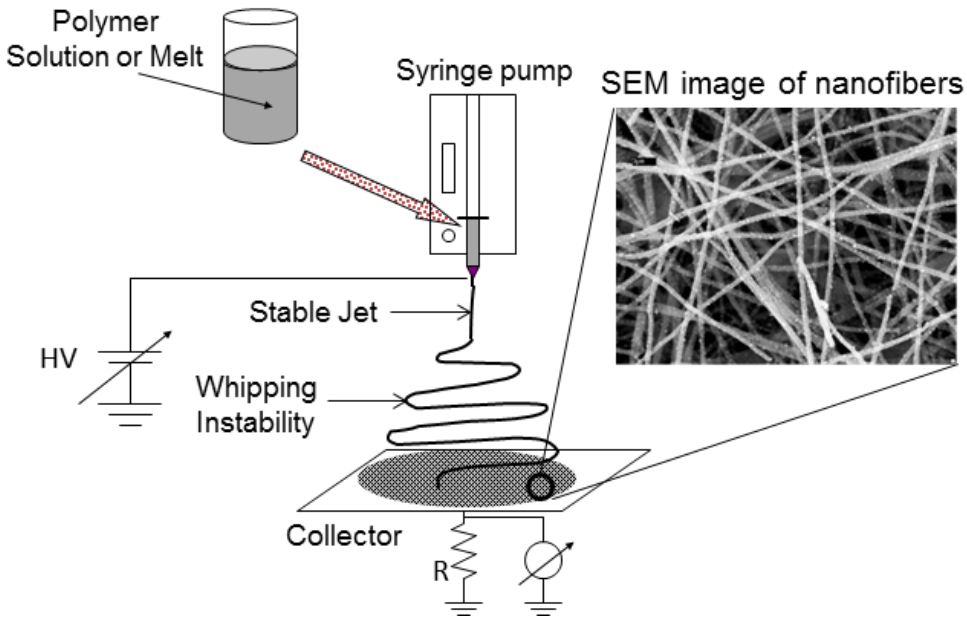


Figure 16. System for the production of the nanodevice.

The features of our device are: a high loading capacity; high encapsulation efficiency; ease of operation and cost effectiveness.

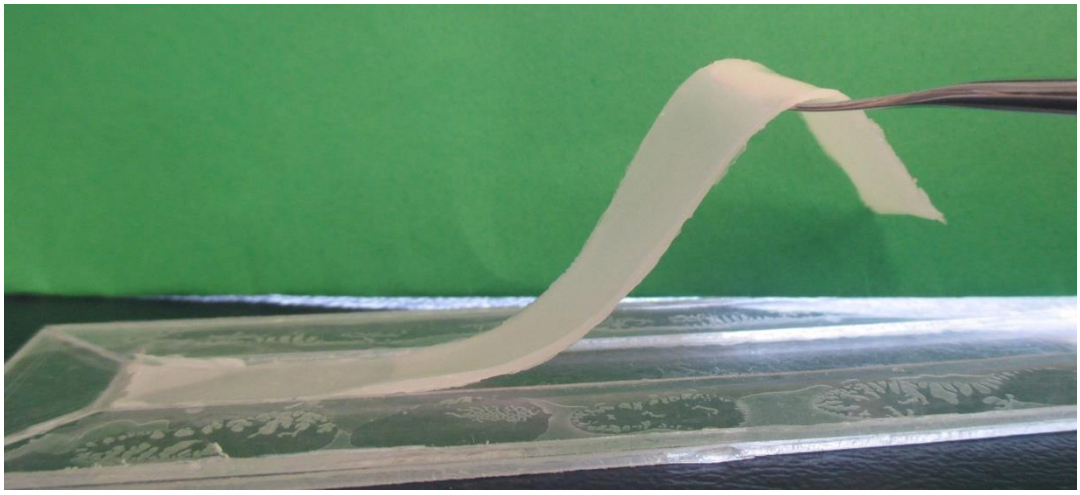


Figure 17. The nanogel ready for the intralesional application.

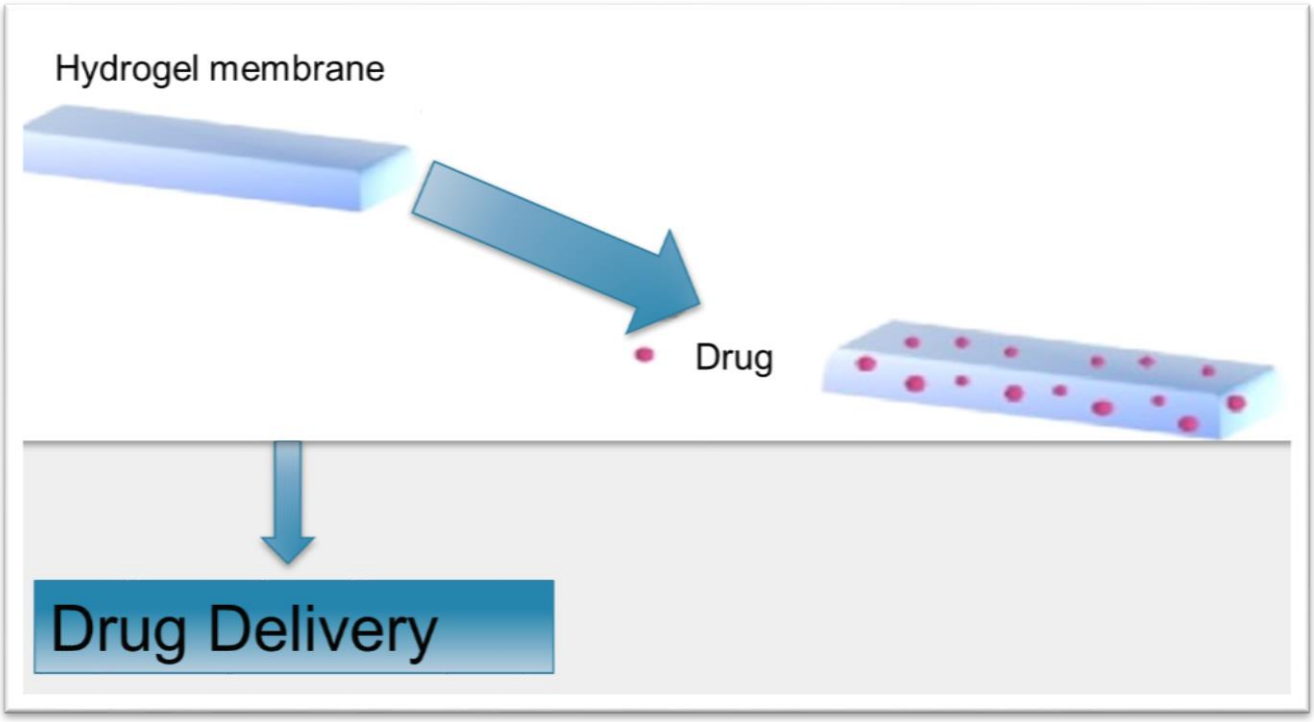


Figure 18. Scheme of the nanodevice and drug delivery

The ND has been loaded with chloroprocaine. The delivery system has been verified in vitro satisfying the two most relevant aspects: (1) efficient binding of the drug to the polymeric matrix and release of the drug in a “controlled” manner; (2) ability to release through a local or externally applied trigger by changing the binding affinity between the drug and the polymeric matrix. Therefore, a clear understanding on the interaction between the drug and the nanogel is critical for the development of an efficient drug delivery system, further in vitro study will be crucial for a better definition of a time controlled delivery of the drug.

5.3 *Materials and methods: in vivo assessment in a large animal model*

All animal procedures were performed according to the protocol approved by Institutional Animal Care and Use Committee (IACUC) – University of Pavia. All animal care and experimental procedures followed local, national and European Union guidelines for the animals' use (Directive 2010/63/EU – revising Directive 86/609/EEC on the protection of animals used for scientific purposes).

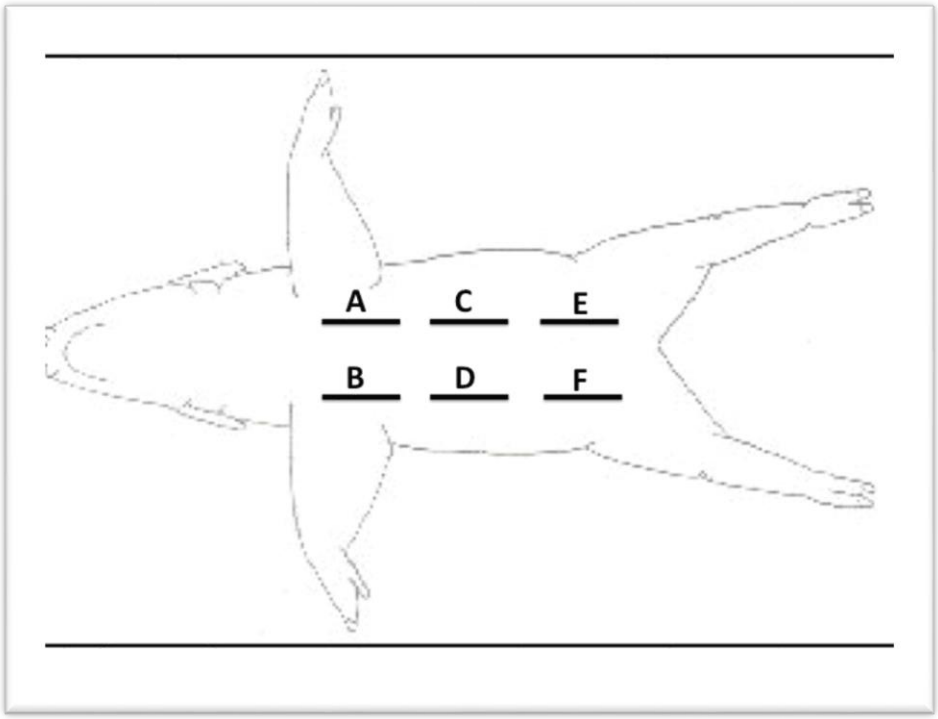


Figure 19.
In the animal model we performed six pararectal laparotomies extended for 7 cm
Nanodevice loaded with Chloroprocaine along the length of the wound.
Removal of nanodevice at different time point:

- A - control
- B - removed after 5 days
- C - removed after 10 days
- D - removed after 15 days
- E - removed after 20 days
- F - removed after 30 days

Under general balanced anaesthesia piglets (20-30 kg) underwent pararectal laparotomy, extended for 7 cm. Animals were divided in 4 groups: Control; ND alone; ND loaded with two different concentrations of CP (0,5% and 1,5%). We treated 3 animals for each group. The peritoneum was closed with a continuous suture and ND was implanted in the preperitoneal layer at the time of laparotomy closing. Animals were euthanized 30 days after surgery in order to investigate the long-term effects of nanomaterial. Tissue and blood samples were collected at 5, 10, 15, 20 and 30 days after the operation to describe the effect of the ND on the tissue and to perform histopathological, pharmacokinetic, local and systemic inflammation analysis.

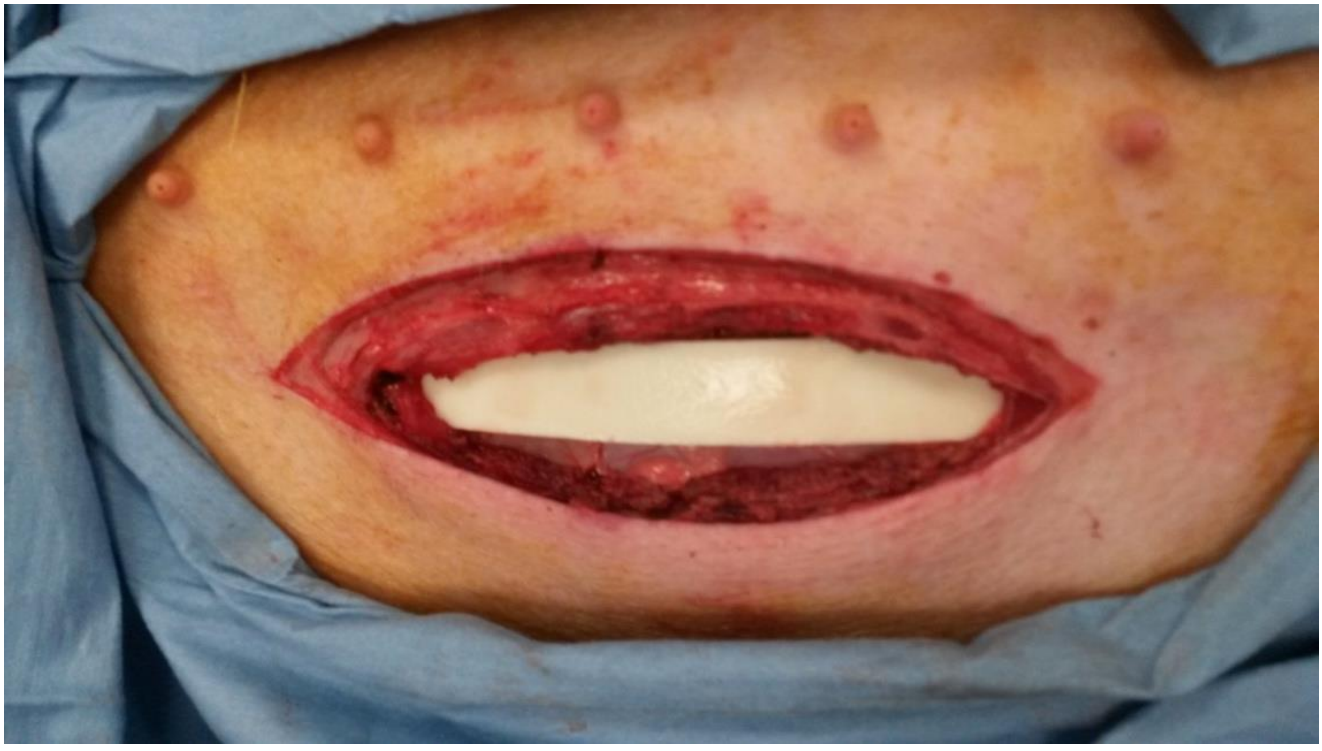


Figure 20. The Nanodevice applied in vivo.

Any adverse effects were registered. Histopatological analysis showed good biocompatibility with a moderate inflammatory response around the ND. The release of CP by the ND has been quantified, the inhibition of local inflammatory response at different concentration of CP loaded were dose dependent. No different systemic inflammatory response was observed in the different groups.

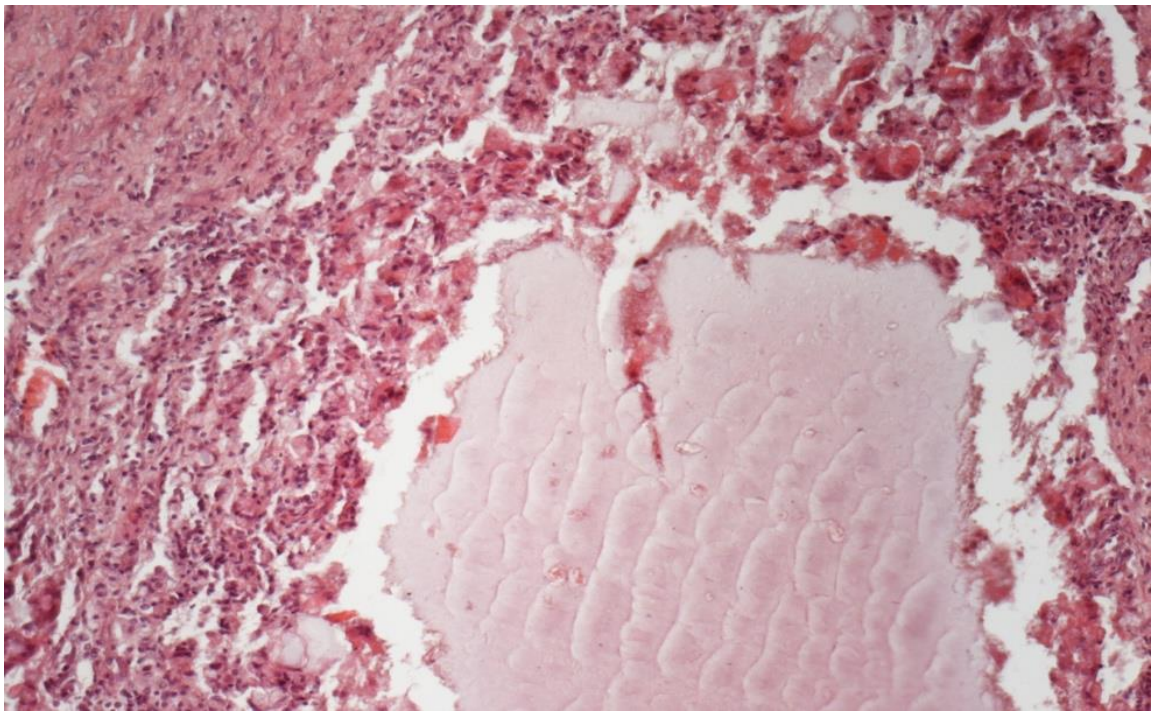


Figure 21. Sample of nanodevice and surrounding tissue in the control group.

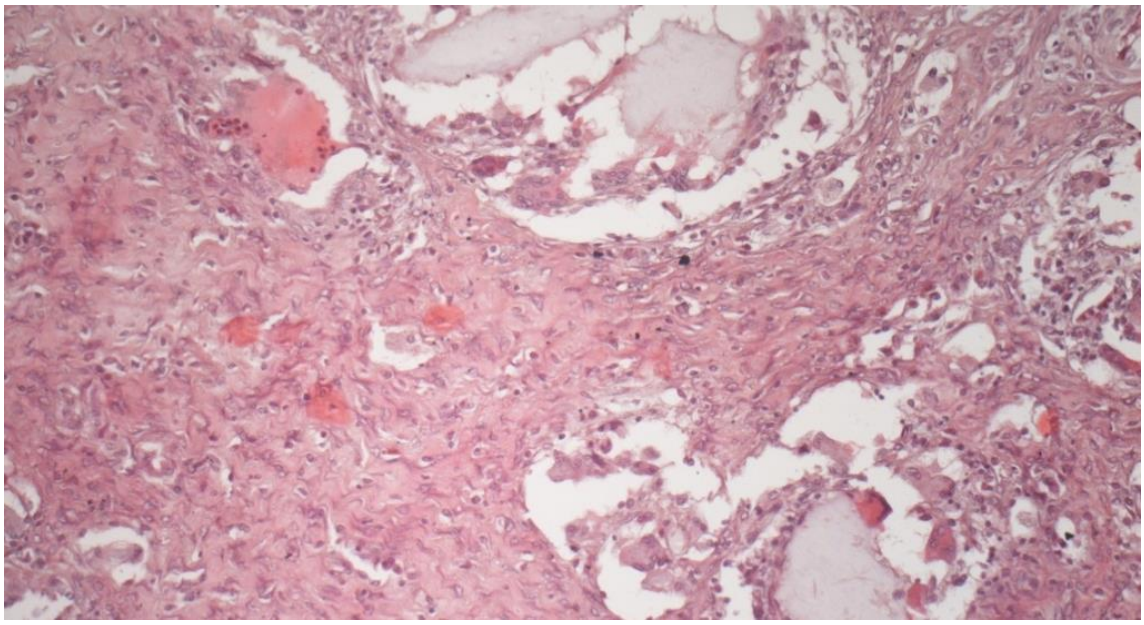


Figure 22. Sample of nanodevice and surrounding tissue in the group with chloroprocaine

Inflammatory response (Histology) in the nanodevice surrounding area	**Score (10 samples per group)**
Nanodevice alone	4 (3-4)
Nanodevice + Chloroprocaine 1,5%	2 (1-3)

Table 4. Inflammatory response around the nanodevice.

In the table 4 we summaries the inflammatory effect of the ND. We compared the groups in terms of biocompatibility, local inflammatory response with and without CP. The biocompatibility of the ND was optimal. The CP response was exactly similar to that described with the use of intralesional catheter.

The ND revealed a good biocompatibility, among the most desired features of nanogels, as with any nanotechnology used for therapeutic reasons, is that the materials be biocompatible. That means that they do not provoke any injurious biological responses at the molecular, cellular or organ levels when used. These include typical

foreign body responses to small particles that manifest as immunological, thrombogenic or mutagenic activation leading to undesired physiological or anatomical changes such as allergy, blood clot formation or induce disease state. The ND did not manifest any injurious biological responses. Further studies, in a translational setting, have to be done in order to investigate the long-term effect in vivo.

6. ACHIEVEMENTS AND CONCLUSIONS

We conceived an experimental path that clarifies the current scenario of intralesional postoperative pain therapy. We highlighted the requirements to satisfy this unmet medical need. We believe nano-technology can be applied successfully; however, experimental models in this field are scarce, in particular in large animal. We firstly consider what nanotechnology represents today by describing analytically and systematically the techniques applied to create nanotechnological devices. Afterward, we investigated which experimental models could be used, particularly in large-sized animals. Applying a new molecule (Chloroprocaine) and a new nanogel (Nanodevice) we began a brand new translational path that represents a model for future investigations.

In conclusion, continuous wound infusion using a new formulation of Chloroprocaine in a translational setting revealed:

- Low amounts of Chloroprocaine being absorbed;
- Very low systemic effects;
- No side effects or allergy reactions;
- No correlation between local infusion and systemic inflammation;
- Local infusion of Chloroprocaine has a local, dose dependent, anti-inflammatory effects.

About the new nanodevice designed and produced using electrospinning technique we can conclude that:

- Chloroprocaine is loadable to the nanodevice at selected concentrations;
- Nanodevice is manageable and technically implantable in the surgical site;
- Nanodevice do not generate side effects or allergy reactions after implant;
- Chloroprocaine loaded to the nanodevice has the same local effect compared to the local infusion using catheter.

7. GLOSSARY

AOT: alginate/anionic surfactant

CABA: 4-amino-2-chlorobenzoic acid

CP: chloroprocaine

CPS: composite pain scale

CWI: continuous wound infusion

D2O: deuterium oxide

GS: gait score

LAs: local anesthetics

LMVs: large multilamellar vescicles

LOQ: limit of quantification

LPSPs: lipid-protein-sugar particles

LS: lesion score

MNCs: mono nuclear cells

MRM: multiple reaction monitoring

ND: nanodevice

NRS: numerical rating scale

O/W: oil/water

PABA: 4-aminobenzoic acid

PBS: phosphate buffered saline

PCL: polycaprolactone

PEG: polyethylene glycole

PHA: phytohemagglutinin

PLA: poly lactic acid

PLGA: poly lactic glycolic acid

PNIPAM: Poly(N-isopropylacrylamide)

PVA: poly vinyl alcohol

RPMI: Roswell Park Memorial Institute medium

SLNs: solid lipid nanoparticles

SUVs: small unilamellar vescicles

VAS: visual analog scale

W/O/W: water/oil/water

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