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The pig model of chronic paraplegia: A challenge for experimental studies in spinal cord injury

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ABSTRACT

The regenerative medicine techniques that are beginning to be applied to the nervous system have led to increased hope in the treatment of diseases that have been considered incurable and that require experimental models on which to test new therapeutic strategies. We present our experience with adult pigs (minipigs) that have undergone a traumatic spinal cord injury (SCI) experimental model, and that have been followed for 1 year. We describe the surgical aspects of our SCI model by acute compression and also describe protocols for daily care and rehabilitation that are necessary to maintain the paraplegic pigs in good health during the months following the injury. Furthermore, we provide in detail the main complications that arise with this experimental model and the treatments used to address these complications. Suitable housing conditions, daily rehabilitation and prevention of complications (i.e., taking the same care applied to patients following SCI) are essential for achieving the absence of mortality and long-term maintenance of the animals. We consider the model that is described here to be feasible and useful for preliminary testing of novel therapeutic strategies aimed at regeneration of the injured spinal cord in paraplegic patients.

Contents

Abbreviations: BP, blood pressue; CNS, central nervous system; ECG, electrocardiogram; MEPs, motor evoked potentials; MRI, magnetic resonance imaging; ROM, range of motion; SCI, spinal cord injury; SSEPs, somatosensory evoked potentials.

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1. Introduction

Spinal cord injury (SCI) is a clinical entity that has been known since antiquity. SCI has great importance both medically and socially, as it represents a major cause of disability with serious personal and family consequences. Trauma causes 70% of SCI cases. Each year, there are 50 new SCI cases per million people. SCI mostly affects people under 40 years of age and is associated with mortality rates that vary between 5% and 20%, depending on the spinal cord level of the lesion (Rodríguez-Boto and Vaquero, 2009).

In recent decades there has been a breakthrough in the treatment of patients with SCI, mainly in regard to the prevention of complications. However, although there are many open lines of research in this field, there is still no effective treatment that leads to the full functional recovery of patients, whose deficits are generally permanent and irreversible (Ridet et al., 1997; Silver and Miller, 2004; Esclarin de Ruz, 2010).

It is therefore understandable that one of the most important challenges in the field of neurosurgery is the search for new strategies for the treatment of traumatic SCI. This requires the development of animal models in which to test possible treatments that will subsequently be applied to humans (Blesch and Tuszynski, 2009). In these animal studies, the initial aim is to demonstrate safety and then efficacy.

There is a large body of literature on SCI, from the studies of Ramón y Cajal (1914) to the present day. These studies of experimental animals, mainly rodents, have been mostly aimed at increasing the low capacity of the central nervous system (CNS) to regenerate after damage. In upper mammals, this low capacity for regeneration is due in part to the apparent inability of the CNS to induce spontaneous axonal regeneration in the area of injury (Ramón y Cajal, 1991; Goldberg and Barres, 2000). This is possibly due to an inhibitory microenvironment that is partly attributed to glial cells (Schwab et al., 1993; Ridet et al., 1997; Fournier and Strittmatter, 2001; Morgenstern et al., 2002; Zhao and Liu, 2004).

Since 1940, some publications have described transplants of various neural tissues, such as the peripheral nerve, nodosum ganglia or brain tissue, in attempts to restore anatomical continuity in the sectioned spinal cord (Brown and McCough, 1947; Barnard and Carpenter, 1950; Kao et al., 1977; David and Aguayo, 1981). In recent years, despite the technical difficulties of such models, there has been increasing interest in this field of research. This increased interest coincides with the hope that is offered by modern techniques of cell therapy (Chopp et al., 2000; Hofstetter et al., 2002; Fraidakis et al., 2004; Ohta et al., 2004; Parr et al., 2007; Zurita et al., 2008; Vaquero and Zurita, 2011).

2. Models of SCI

Most research on traumatic SCI has been performed on adult rats because these animals are easy to handle. With regard to the severity of the injury, numerous studies have been done on models of incomplete SCI. In studies of incomplete lesions there are fewer problems with achieving the survival of the animals. However, the effectiveness of different treatments is more difficult to determine because spontaneous recovery can occur in rats with incomplete lesions (Li et al., 1998; Takami et al., 2002; Verdú et al., 2003; Bravo et al., 2004; Gorska et al., 2007, 2009). Therefore, some researchers study complete SCI. These studies generally focus on the thoracic level because it is an area where experimental and clinical studies showed that the possibility of some degree of spontaneous functional recovery is poor (Ramsey et al., 2010; Harrop et al., 2011). However, complete paraplegia models are clearly much more complex, especially with regard to postoperative care and monitoring of animal evolution.

To advance our understanding and to uncover possible cures of SCI, it is important to develop an experimental model that is able to reproduce the effects of acute and chronic SCI in humans. The literature shows different experimental models of traumatic SCI that differ in the nature of the injury-causing agent. The contusion model, in which a weight generates a measurable and reproducible acute compressive force on the spinal cord, is one of the most widely used (Allen, 1911). This model is applicable to human clinical practice, because contusion is the underlying cause in 49% of paraplegic patients (Potter and Saifuddin, 2003).

The compression model of SCI is used as an alternative to the contusion model. In the compression model, a continuous force is applied to the spinal cord over a period of time. This model, originally described by Tarlov (1957), involves placing an inflatable balloon in the extradural space; the balloon is inflated acutely or at set intervals of time. One variation on this model is the application of an aneurysm clip to the spinal cord, which produces an acute compression injury of varying severity, depending on the time that the clip is applied and the force applied by the clip (Rivlin and Tator, 1978; Joshi and Fehlings, 2002a,b).

When a researcher aims to work with an experimental model of SCI, it is important to establish the time period from when the injury takes place until when the treatment in question is applied. Therefore, we must distinguish chronic phase studies (Zurita and Vaquero, 2004, 2006; Fraidakis et al., 2004; Lu et al., 2007; Muñoz-Quiles et al., 2009; Hu et al., 2010; Gelain et al., 2011; Dulin et al., 2011), in which the spinal cord lesion is chronically established, from acute phase studies (Carvalho et al., 2008; Lutton et al., 2012;

Zhao et al., 2011), in which the animal is subjected to treatment within hours or days after the injury.

The long-term survival of animals is a priority when trying to assess functional recovery, as a long follow-up period may allow more time for the development of effective axonal regeneration. With short periods of evolution, the absence of functional responses may simply be due to a lack of time (Lu and Ashwell, 2002; Lu et al., 2002). However, chronic models are rarely used, because the maintenance of paraplegic animals to reach a state of chronicity involves a great deal of special care. Studies in the literature rarely list in detail the basic care that must be performed daily (Santos-Benito et al., 2006; Ramsey et al., 2010). Furthermore, none of these studies makes a special mention of the rehabilitation of paraplegic animals, an aspect that we consider extremely important for progress in this field of research. In our opinion, it is necessary to establish protocols that reflect the specific rehabilitation exercises that are required for chronically paraplegic animals to prevent complications, especially of the skin, joints or muscles.

The limited information in the literature on this subject may be due to the fact that, in most studies, SCI animals are subjected to different therapies very soon after the lesion (Carvalho et al., 2008). In this situation, the degree of muscle and joint deterioration is much lower than in the case of animals with a long evolution time after injury.

It is possible that the lack of detailed protocols for long-term paraplegic animals, especially with regard to basic care and rehabilitation, has contributed to the lack of studies of chronically established paraplegia models. Moreover, models of traumatic SCI in adult upper mammals are scarce. Most of these studies have been done in primates in an acute phase after SCI; these studies usually have very short periods of evolution. In order to apply new therapies to humans, it is clearly desirable to know the potential effectiveness of these therapies on upper mammals (Deng et al., 2005; Santos-Benito et al., 2006; Rosenzweig et al., 2009; Brock et al., 2010). Theoretically, primates are excellent candidates due to their proximity to humans, but the ability of these animals to use their upper limbs makes housing and long-term care difficult. In primates it is difficult to maintain urinary catheters or incontinence pads and to perform skin care and healing of pressure ulcers. The alternative is to restrict their movements and keep them in cages, but this leads to a clear decline in their quality of life. Moreover, although some authors have applied active rehabilitation to primates suffering incomplete spinal cord lesions (Babu et al., 2007), the application of rehabilitation techniques to primates with complete paraplegia is often reduced to passive exercises; this therapy is far from the rehabilitation protocols that have been developed in humans with SCI. Although some patterns of care in upper mammals have been reported (Santos-Benito et al., 2006; Piedras et al., 2011) descriptions in the literature are scarce, possibly due to the difficulty of these experimental models. Furthermore, it should be noted that many research centers do not have regulatory approval for the housing of primates.

During the last 20 years in our laboratory, we have gained experience with cell therapy protocols for the treatment of adult rats suffering traumatic SCI. We have used a model of chronically established paraplegia along with long-term maintenance of animals (up to 18 months of evolution). With this model, we demonstrated that paraplegic rats could obtain functional recovery several months after intralesional transplantation of stromal cells from bone marrow (Zurita and Vaquero, 2006), a finding that was later confirmed by other authors (Alexanian et al., 2010; Kishk et al., 2010; Pal et al., 2010; Wu et al., 2011). However, before considering the application of these techniques to humans, it is necessary to demonstrate the effectiveness of such treatments in upper mammals. The functional organization of the rodent nervous system is less complex than that of the human nervous system, and therefore the success of a treatment in rodents raises questions about its potential efficacy in paraplegic patients. For this reason, in the last 10 years we have developed a model of traumatic SCI in the adult pig (minipig) to obtain long-term survival after the administration of cell therapy in these animals. The choice of a model of complete paraplegia is due to the fact that incomplete SCI in upper mammals can make it difficult to evaluate the results of any treatment. As in rodents, the spinal cord of upper mammals retains some plasticity when the lesion is incomplete, and this may result in spontaneous recovery (Tuszynski et al., 2002a,b; Fouad et al., 2004; Rosenzweig et al., 2010). Our model is feasible, allowing for adequate rehabilitation of paraplegic animals, and it is extremely useful for demonstrating long-term efficacy in chronically established paraplegia. Moreover, the adult pig is an animal that is commonly used for organ transplantation research. Some studies on pigs subjected to SCI have been reported, but these studies have focused on acute stages and with short follow-up times (Meylaerdts et al., 2000; Kuluz et al., 2010; Lim et al., 2010; Zahra et al., 2010).

3. The chronic paraplegic pig model

Our present work is the result of experience gained with the first model in the literature of chronically established paraplegia in the adult pig. The model was designed in our laboratory for testing cell therapy strategies prior to a possible use of these techniques in humans. The 20 consecutive animals that form the basis of this study underwent complete SCI and were followed for 1 year without mortality. Our goal has been to confirm the feasibility of the chronic paraplegic pig model and to study possible complications and their resolutions. The only criterion we have considered to exclude an animal to be a candidate for this model is the absence of complete paraplegia. In all cases we have followed a consistent protocol for care after the injury, which has allowed us to attain the conclusions and recommendations that we describe below.

3.1. Housing of animals before SCI

For this type of study it is appropriate to use female adult minipigs weighing 15–20 kg. This allows for better management in the daily care and rehabilitation processes without causing problems for overweight animals.

It is important that animals be housed in a housing area dedicated solely to them for at least 4 weeks before being subjected to SCI. This "adjustment period" before SCI minimizes stress and produces more reliable data (Obernier and Baldwin, 2006). During this time, the minipigs not only become accustomed to the facility where they will live, but they are also able to socialize with the people who will be responsible for their care. This greatly facilitates subsequent handling, as the animals show significantly reduced stress and exhibit greater collaboration in rehabilitation (Yelvington et al., 1985). During this period, the animals become familiar with the various rehabilitation exercises; we are also able to perform functional tests at this time, such as blood, urine, or evoked potentials studies, to be used as reference values (Table 1).

We believe it is advisable not to work with more than 3–4 animals at a time. Given the complexity of care, working with more animals would not allow us to adequately perform all rehabilitation protocols unless we were to have access to more human resources and larger installations. Our minipigs are housed in a room of 40 m^2 (Fig. 1) that has cycles of 12 h light/dark, controlled temperature (24 °C) and controlled humidity (55–65%), and automatic drinkers to supply water *ad libitum*.

Table 1
Main physiological parameters in minipgs. The values (mean) refer to adult female minipigs, weighing between 15 and 20 kg.

Hematologic values	
Red blood cell count	$6–7 \times 10^6$ cells/mL
Hematocrit	30–35%
Hemoglobin	9–11 g/dL
Reticulocytes	2–3%
White blood cell count	$16–17 \times 10^9$/L
Platelet count	$300–400 \times 10^9$/L
Thrombin time	25–30 s
Prothrombin time	11–12 s
Hemodynamic values	
Mean systolic pressure	140 ± 15 mmHg
Mean diastolic pressure	96 ± 14 mmHg
Heart rate	103 ± 14
Daily urine output	1.5–2 L
Rectal temperature (°C)	38.9 ± 2
Biochemical serum/plasma	
Glucose	101 ± 2 mg/dL
Total proteins	4.5 ± 3 g/dL
Albumin	2.6 ± 0.1 g/dL
Creatinine	0.6 ± 0.01 mg/dL
Calcium	11.8 ± 0.7 mg/dL
Alkaline phosphatase	492 ± 15 UL

We installed special cribs in this room for sleeping (Fig. 1b and c), with automatic water troughs and air mattresses to prevent pressure sores. The mattresses are protected with impermeable covers and fitted sheets to prevent staining or biting. We performed a change of sheets at least once a day for hygienic reasons. Air mattresses allow the preservation of the integrity of the skin after injury; once they are made paraplegic, minipigs move in their cribs with jerky movements and drag their hind legs. This can cause skin ulcerations due to pressure and friction. Before surgery and during the adaptation period, the animals slept in cribs in order to become accustomed to this new situation. During the day they were free and played throughout the room.

Animals ate an exclusively vegetarian diet alternated with dry food (SAFE, scientific and engineering animal food) containing fresh fruits and vegetables. Food was given three times a day controlling the amount provided to prevent weight problems so common in these animals (Swindle, 2007).

The room has two video cameras that allow us to monitor the animals for 24 h a day. We are able to record their movements continuously and systematically analyze their behavior.

The installation has music piped in and an area full of toys that the animals can access. This creates a welcoming and comfortable environment for them, as they will be housed for long periods of time. The maintenance of these housing conditions has been very effective in allowing us to gain the cooperation of the paraplegic animals with respect to their care and especially their rehabilitation.

All protocols described below are based on 20 years of experience studying a rat model of chronic traumatic paraplegia and 10 years of experience using this model in minipigs. Moreover, treatments that have been used for our animals have been adapted from procedures that have been performed in humans. All procedures were approved by the Welfare Animal Committee of our hospital, in accordance with European and Spanish guidelines.

3.2. SCI procedure

The day of SCI, an animal that had not had access to any type of intake the night before was pre-medicated with intramuscular ketamine (20 mg/kg) and diazepam (0.2 mg/kg). Anesthesia was induced with a face mask, through which 5% sevoflurane and oxygen were administered. The animal was then washed with hot water and chlorhexidine soap and was placed on the operating table in the prone position. Then, 20G and 22G teflon catheters were placed in the ventrolateral ear vein and the central ear artery, respectively. The arterial catheter was connected to a calibrated pressure transducer for direct arterial measurement of blood pressure (BP), and self-adhering patches were applied to the skin for electrocardiogram (ECG) and heart rate recording. The venous catheter was used for drug administration and for continuous infusion of lactate Ringer solution (10 mL/kg/h) to avoid hypovolemia in case of excessive bleeding. Once an adequate level of anesthesia was achieved, a 6-mm internal diameter endotracheal tube was introduced and secured, and its cuff was inflated. Urethral catheterization was performed with a 6F or 8F Foley catheter bladder. Then, the animal was placed in the lateral position on its right side. After cleaning the surgical area with povidone-iodine and waiting 3 min, a waterproof, transparent 15×28-cm^2 film dressing (Opsite post-op®, Smith & Nephew, Barcelona, Spain) was placed on the skin.

A laminectomy was performed at the Th12–L1 vertebral level, and a 4-cm length of spinal cord was exposed. SCI was caused by the epidural application of two surgical Heifetz's clips for 30 min (Fig. 2). In our experience, after this period of acute spinal cord compression, there is complete and irreversible paraplegia in 100% of animals (Zurita et al., 2008). If, during the course of the lesion (placing or removing the clip), the dura tears, it must be repaired using 6/0 sutures (Prolene 10/0; Ethicon EH111HE, Edinburgh) to avoid the risk of cerebrospinal fluid (CSF) fistula after surgery. A sheet of polytetrafluoroethylene (Gore®-Preclude® Dura Substitute, W.L. Gore & Associates Inc., Flagstaff, Arizona, USA) was

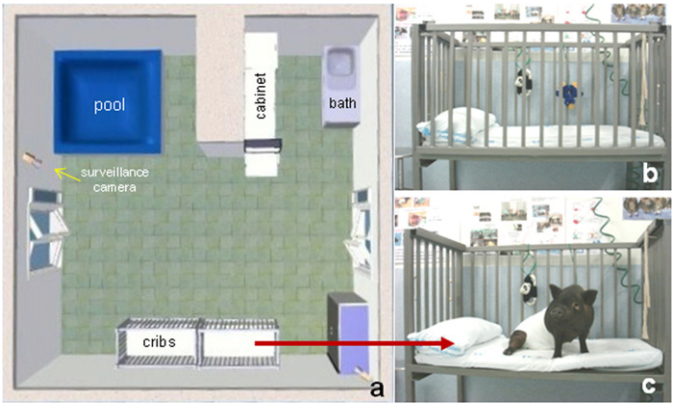

Fig. 1. (a) General view of the room for housing paraplegic minipigs. (b and c) Cribs for rest of the animals.

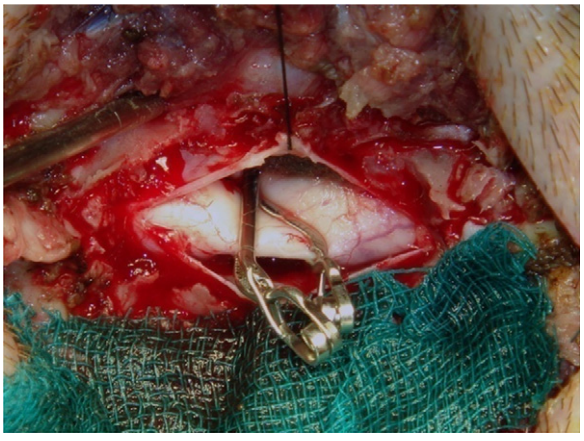

Fig. 2. Surgical field obtained in the course of SCI procedure showing spinal cord compression by two Heifetz clips.

placed over the lesion site to prevent scarring of connective tissue (Kurt et al., 2009). Although arachnoidal reaction with the use of epidural polytetrafluoroethylene in pigs has been reported (Haq et al., 2005), it is a non-absrorbable material that placed epidurally in our experience not cause significant epidural scarring and facilitates a new approach to the spinal cord (Vaquero et al., 1999).

The paraspinal musculature and subcutaneous tissues were subsequently closed with 3/0 absorbable sutures. Finally, chlorhexidine gluconate 1% was applied to the skin. The wound was covered with a hydrofiber dressing (Aquacel® Ag, ConvaTec. Inc. USA), which was secured with an adhesive bandage (Elastomull® haft, Smith & Nephew, Barcelona, Spain).

During the course of surgery, anesthesia with sevoflurane in oxygen (2 L/min) was maintained using a small animal anesthetic circle system connected to a volumetric ventilator. Neuromuscular block with intravenous vecuronium (0.2 mg/kg/h) was achieved, and intravenous morphine (1 mg/kg) was administered for intraoperative analgesia. In addition, to prevent infection, intravenous cefazolin (30 mg/kg) was administered before the start of surgery, and this administration was repeated every 12 h for a total of 4 doses. None of our animals has shown surgical infection.

The ventilator settings were adjusted to maintain an end-tidal CO_2 value of 35–45 mmHg. To achieve this value, the tidal volume was set at 10 mL/kg body weight and the peak airway pressure was set at 8–10 cmH_2O, with a respiratory rate of 15 breaths per minute. Temperature, hemoglobin oxygen saturation, and end-tidal sevoflurane and CO_2 concentrations were continuously monitored during the procedure. Pulse oximetry (SpO_2) was recorded continuously by placing a pulse oximeter in the tail, and rectal temperature was monitored and maintained at normothermia (between 37 and 38 °C) by means of a total temperature management system.

3.3. Immediate postoperative period: Phase of spinal shock or acute phase

Immediately after surgery, the pigs were placed in separate cribs to facilitate the intravenous administration of fluids and medication. Analgesia was obtained with morphine (1 mg/kg, intravenous) and meloxicam (1 mg/kg, intramuscular) and was maintained for three postoperative days. Hydrofiber dressings should be maintained until the skin sutures are removed 1 week after surgery.

Animals should be monitored continuously for at least 2 days after SCI in order to control parameters such as temperature, weight, behavior, activity, pain, aspect of the surgical wound, heart rate, BP and diuresis. These parameters serve as indicators of the animal's status and allow for the detection of any serious complications that may occur; the 48 h following the lesion are critical for the care of animals with severe SCI. This period corresponds to the start of what is known as "spinal shock" and can sometimes extend for up to several weeks (Guttmann, 1981; Roldan, 2002). This period is characterized, in general terms, by underactivity, flaccidity and areflexia of the voluntary motor system are seen below the injury site. This situation is maintained in 100% of our animals throughout the acute phase. We should note that, during the first week, there is a rapid and progressive atrophy of the muscles of the lower extremities (Dupont-Versteegden et al., 1998) that contributes to weight loss. Generally, there is a 10% loss of weight in the first week, but this loss is expected throughout the entire acute phase after injury and should not exceed 20%. Any weight loss in the chronic phase, or weight loss of more than 20% in this acute phase, should be considered a sign of disease. The second criterion is total loss of infralesional sensation (100% of cases). The third criterion is loss of the autonomic system, leading to paralysis of the bladder with urinary retention (100% of cases) and urinary tract infections (35%), possible ileus (10%), abdominal distension and constipation (50%) and vasomotor complications with lack of venous and arterial response. These can cause hypotension (100%) and even hypothermia (10%). Hypotension is usually due to the loss of vasomotor tone, causing decreased venous return and arterial contractility, which lead to vasodilation and decreased systemic vascular resistance.

3.4. Care during spinal shock and treatment of possible complications

We describe here the care that must be given to the animals at this phase and the most important complications that may be encountered.

3.4.1. Fluid intake and hypotension

After surgery, animals are kept in their cribs with oxygen for several hours if necessary. The venous catheter placed in the ear vein should continue to supply fluids (Ringer Lactate) for at least the first 72 h (Fig. 3). At this phase hypotension is a frequent finding, but given that this is generally not caused by hypovolemia, but rather by a lack of vascular tone, it is important to avoid indiscriminate fluid volume overload (Roldan, 2002). In our experience, under normal conditions, all animals overcome this situation without the use of alpha-adrenergic drugs to restore vasoconstriction and systemic vascular resistance. In some cases, if necessary, animals with severe hypotension can be treated with vasopressors such as dopamine. This increases heart rate and can be administered by continuous infusion at a dose of 2–4 mg/kg/min.

3.4.2. Orthostatic hypotension

Orthostatic hypotension due to lack of response to changes in position is very common (90% of our cases, with mean systolic BP between 110 and 120 mmHg). Consequently, during the shock phase, animal movements are performed slowly and with care to avoid hypotension. This is especially important when incorporating a toilet in the cribs and during postural changes. Control of BP must be maintained throughout the acute phase; if the systolic BP becomes less than 70 mmHg the animals are less active and vomit. If this happens (in our experience, this occurred in 2 cases) and it cannot be resolved with intravenous administration of lactate Ringer, the animal should be treated with plasma expanders (dextran), controlling BP and urine output at least every hour. If the animals vomit, the administration of Metoclopramide can be effective.

We determine the systolic BP of the animals daily from the median artery, using a noninvasive method ("doppler method"). The first step is to place the cuff around the forelimb. The cuff is connected to a manometer and a pump for inflation. After applying gel to the transducer and identifying the artery with a beep, we inflate the cuff until suprasystolic BP is reached (200–250 mmHg) and the sound has ceased. We then begin to remove air from the cuff until the appearance of an audible signal, which indicates systolic BP. The sleeve should be approximately 30–40% of the circumference of the limb on which it is placed; a very broad sleeve underestimates BP, while a very narrow sleeve overestimates BP. Neonatal and pediatric inflatable cuffs used in humans, with a diameter ranging between 1 and 8 cm can be used.

3.4.3. Control of body temperature

Malignant hyperthermia (a hereditary disease that arises as a reaction to certain anesthetics) is one of the most serious problems that can occur in experimental surgery in pigs (Muir et al., 2003). Although it is rare to find cases of this disease, it can be fatal. Hyperthermia appears in the immediate postoperative period (a few hours after surgery) and consists of a temperature increase

Fig. 3. Care of the animals immediately after surgery. (a) Intravenous catheter into the ear vein. (b) Oxygenotherapy. (c) Animals in their cribs after SCI. (d) Removal of blood from the saphenous vein.

that can even reach 46 °C. It is manifested not only by increased temperature (>42 °C) but also by tachycardia, tachypnea, sweating and increased muscle stiffness with spasms. The best treatment involves the administration of Drantolene® (2–6 mg/kg intravenous or 20 mg/kg, oral), 100% oxygen, and physical cooling. Anti-inflammatory drugs may be used as a supportive treatment. The animal should be lying prone (sternal) in a calm, cool and well-ventilated environment. In our series we had no case, but we must be aware of this complication, which cannot be prevented, because animals are not usually studied from the point of view of genetic analysis.

On the other hand, the pig has a low body surface area in relation to body mass, with few sweat glands and poor thermoregulatory mechanisms, and hyperthermia, which can be up to 40 °C, represents a frequent complication after surgery. It occurred in 50% of our animals and has been successfully treated by administration of Metamizol (1 g intramuscular/8 h).

3.4.4. Prevention of venous thrombosis

To eliminate the risk of venous thrombosis during this phase, all our animals received low molecular weight sodium heparin 1% as a prophylaxis (0.04 mL, subcutaneously) every 24 h, similar to that which is used in humans. If there is thrombophlebitis, with swelling or edema in a limb, the dose of heparin can be doubled and buprenorphine can be added (0.1 mg/kg im). Diuretics (furosemide) can also be used, maintaining bladder catheterization.

In our series, we have only a case of thrombophlebitis, possibly due to poor management of prophylaxis, and was detected by redness and swelling of the limb and hyperthermia. It was resolved in 4 days with the administration of low molecular weight sodium heparin 1% (0.08 mL, subcutaneously, every 24 h) and buprenorphine (0.1 mg/kg im).

3.4.5. Gastrointestinal complications

The occurrence of gastrointestinal complications in the acute phase is relatively common in this experimental model. The presence of bowel sounds must be verified by abdominal auscultation to confirm peristalsis; when there is abdominal distension, this denotes decreased peristalsis (Chen and Nussbaum, 2002). If we suspect ileus, animals are subjected to fasting. If defecation is absent over the course of 48–72 h, we begin treatment with enemas or oral laxatives. In our studies, we observed 2 cases (10%) of severe intestinal paralysis with impaired intake and vomiting. In both pigs this complication was resolved by the administration of metoclopramide (0.4 mg/kg, three times daily, orally) and intravenous administration of Ringer lactate. We observed the reappearance of bowel sounds and feces 48 h after starting this treatment. In extreme situations, methylsulfate neostigmine can be used at a dose of 0.25 mg, subcutaneous or intramuscular, every 6 h for 2 days to encourage the return of peristaltic activity. In most cases, transient constipation appears, and it is resolved with rectal administration of glycerin suppositories. Sometimes fecal impaction appears as a result of the sluggish colon and the involvement of the reflexes that control defecation. Finally, due to decreasing anal sphincter tone, fecal incontinence can occur, but it is not important in the bowel habits of these animals. To prevent these digestive problems, animals must be maintained during the first 2 days after surgery with a minimum intake of food, preferably with administration of saline only during the first 24 h. Later, the administration of a very soft diet (based on fruit purees) can be started. The food should be administered in small quantities and its administration should be suspended if vomiting occurs. Thereafter, if the food is well tolerated, a soft diet that is rich in fiber should be maintained for at least 1 week after injury.

3.4.6. Urinary complications

Urinary complications at this stage are also very common. Voluntary and reflex pathways are abolished, causing paralysis of the bladder detrusor muscle. Moreover, the internal and external sphincters are closed, which can lead to bladder distension in first few hours. Therefore, during spinal shock, the animals should have a urinary catheter. The catheter should be permanent at first, and then animals can be subjected to intermittent bladder catheterization in order to compensate for the lack of automatic bladder function. This helps to maintain bladder muscle tone and prevents the accumulation of urine that causes deformation of the detrusor muscle and hampers further bladder rehabilitation (Zermann et al., 2000). We use a Foley bladder catheter (No. 6 or No. 8), which is kept under strict aseptic conditions. When a good catheterization technique is used, there is no increase in the incidence of urinary tract infections, and in our series we did not observe complications in the form of urethral injury, as has been described by some authors working on models of SCI in primates (Lisenmeyer, 2002; Piedras et al., 2011).

In our model, suprapubic stimulation and the Crede maneuver have not been sufficient to resolve urinary retention; bladder catheterization has always been necessary, at least until bladder reflex activity is re-established and animals are able to urinate by overflow. The absence of bladder catheterization during this phase is related to mortality in 100% of cases.

The catheter should be kept attached to a urine collecting bag (preferably with a vacuum device that also facilitates the collection of samples for analysis). In addition, this bag should be protected by an incontinence diaper, providing additional hygiene and preventing the loss of the urinary catheter due to movements of the animals in their cribs. Moreover, a thorough survey of urine output should be made by observing not only the amount (diuresis in pigs is 1–1.5 L a day) but also the appearance and smell of the urine. The observation of these parameters may suggest a presence of a urinary infection. In the case of a urinary catheter malfunction, it must be replaced, as it is common to find a sediment blockage.

If possible, intermittent catheterizations of the bladder are preferable during this period. Hydrophilic catheters should be used to minimize the risk of bladder trauma and urinary tract infections (Chartier-Kastler and Denys, 2011).

Possible causes of urinary infections in animal models of paraplegia are incomplete bladder emptying, resulting in urine retention, or improper handling of the catheter. In our animals, urinary infection was detected during the spinal shock phase in 70% of cases and is often manifested by increased spasticity, fatigue, dark and foul-smelling urine, and sometimes fever. It is adequately controlled by changing the catheter and oral administration of antibiotics for at least a week, according to the results from urine culture.

On the other hand, occurrence of benign urinary infections is very frequent (70% of cases) and can be detected by alterations in the color and odor of the urine. Usually these are not associated with clear symptoms, and urinary culture is often negative. Given this situation, the urinary catheter must be changed, and antibiotics must be administered (ciprofloxacin 20 mg/kg every 12 h for 1 week).

The presence of blood in the urine (hematuria) is relatively common during the first few weeks after injury (70%) and may generally be due to urinary tract infection or urinary bladder distention. In the first case, it disappears rapidly after treatment with antibiotics; in the second case, it is resolved by placing a urinary catheter or by replacing an existing catheter that is usually blocked.

For the pig model of traumatic SCI, female animals should be used; they offer greater ease in the placement of urinary catheters because the urethra is short, straight and shows a clearly identifiable meatus. By contrast, in the male, the urethra is longer and has a spiral path that makes urinary catheterization virtually impossible (König, 2004).

3.4.7. Care and complications of the skin

Impaired skin integrity is among the most frequent complications seen in paraplegic animals, as in humans (McKinley et al., 1999). The risk of skin lesions (pressure ulcers) is primarily related to a prolonged decubitus body pressure due to immobility. However, lesions can also be caused by friction or rubbing, especially in cribs (Fig. 4). Another possible reason is irritation of the skin by urine or stool (incontinence ulcers). The best treatment is prevention; this can be accomplished by daily monitoring of the skin, mainly in areas of frequent occurrence of pressure ulcers, such as the sacral region, groin, knee and ankle (O'Connor, 2002; Kirshblum et al., 2004).

The main guidelines to avoid the appearance of skin ulcers are as follows: (a) Keep the skin very clean and moisturized by application of hyper-oxygenated fatty acids (Mepentol®, Bama-Geve SL, Barcelona, Spain); (b) Inspect the most vulnerable zones on a daily basis; (c) Avoid prolonged immobilization of the animals (postural changes must be made every 3–4 h) and exercise should be maintained as long as possible; (d) Alleviate pressure on the most critical areas of support. If a pressure ulcer appears, its severity must be determined, and appropriate treatment must be applied.

In animals, as in humans, we can differentiate the following four grades of pressure sores (Sopena et al., 2009):

Grade I. It is an ulcer that affects the most superficial layer of skin (epidermis) and characterized by a defined erythematous macula, with bright red aspect. Erythema is reversible and after removal of pressure the skin regains its normal appearance. This complication is common in paraplegic pigs (100% of animals). When it is detected, the skin must be kept clean and adequately moisturized. Avoid massage, rubbing or friction as much as possible.

Grade II. It is an ulcer with partial loss of skin (epidermis and dermis or both). This appears as an abrasion surface and presents a high risk of infection. The skin does not recover its original appearance after removal of the pressure. This complication was observed in 20% of our animals. It was treated by cleaning the wound with a saline solution. In addition, the skin should be protected to prevent bacterial contamination.

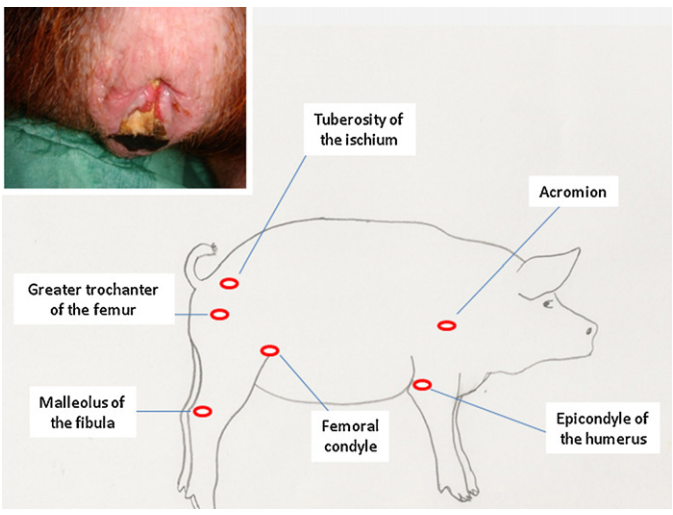

Fig. 4. Main zones of decubitus ulcers, in paraplegic minipigs. At the top left, the image shows a grade III ulcer in the perianal area.

Grade III. In this case, the lesion involves the subcutaneous tissue but does not affect the adjacent fascia. A blackish, necrotic area indicates that the tissue is dead. In the presence of these lesions, one should wash the wound, debride necrotic tissue and protect the skin with semipermeable dressings (hydrogels, hydrocolloids, alginates or polyurethane film, according to the exudate) to prevent infection. In our series these lesions appeared in one case (5%).

Grade IV. These lesions are characterized by extensive destruction, with tissue necrosis to muscle, bone or supporting structures, and can lead to death if they are complicated by serious infections (osteomyelitis or septic arthritis) which can lead to generalized sepsis by germs that enter the bloodstream. The wound need to be cleaned, and it is also necessary to debride necrotic tissue and protect the skin with semipermeable dressings (hydrogels, hydrocolloids, alginates or polyurethane films, according to the exudate) to prevent infection. We did not observe grade IV ulcers in our present series. On the other hand, we found no self-harm of the skin or self-mutilation of the lower limbs, as has been described by some authors in rodents and monkeys (Levitt and Levitt, 1981; Piedras et al., 2011).

Given the absence of previous experience in the literature on chronic models of paraplegia in adult pigs, there are no descriptions of self-aggressive behavior of these animals. However, these animals may bite each other's incontinence pads during periods of play and may cause injury to the anal area from a lack of sensitivity (1 case, 5%). We resolved this situation by using cotton underwears to cover incontinence pads.

3.5. Housing conditions in the immediate post-operative period

In the immediate postoperative period, special care is taken with paraplegic animals to ensure their welfare, representing an adaptation of that which is used in human clinical practice (Table 2).

After surgery (spinal shock phase), the animals remain in cribs all day and undergo postural changes every 3–4 h. After 2 weeks, if there are no major complications, pigs gradually begin to undergo the rehabilitation protocols described below. Rehabilitation starts gradually; when rehabilitation is begun, animals are only in their cribs to sleep for 12 h during the night. For hygienic reasons, animals are maintained at all times with incontinence pads (to which they readily adapt) and are changed every 4 h. At the time of each change we cleaned the perianal area with soapy water (following extensive washing) or with baby wipes to keep the skin clean. Hyperoxygenated fatty acids are then administered for protection and hydration of the skin. During the day, when animals are not in rehabilitation and when their physical condition permits, the animals are kept free in wheelchairs specially adapted for them (Fig. 5). These chairs allow animals to be positioned on all limbs, at least for certain time intervals, and permit independent movement for playing and relaxing during leisure time. Adaptation to wheelchairs must be slow and gradual to allow the animals to acclimate to their new situation and so that they can learn to move, at first with assistance from caregivers. In our experience, pigs adapt to the chair in around 4–5 days.

3.6. Care and possible complications in the chronic phase

During the acute phase after SCI there is a complete loss of sensory and autonomic function, as in other species, but this is not a permanent state. Autonomic reflexes begin to appear after several weeks. Paralysis, while flaccid at first, becomes spastic in some cases. This is a result of an increase in tone below the level of the injury and can even reach a state of hyperreflexia. The appearance of reflexes coupled with an increase in bladder tone often serves as an indicator of the onset of the chronic phase. From this time the management of the paraplegic pigs must be in accordance with the following guidelines:

Table 2
Protocol for daily care and rehabilitation of paraplegic minipigs. Some of these activities are not performed on the stage of spinal shock (see text).

8:00 am	
Toilet: bathroom and skin care. Placing incontinence diaper	
Mepentol application to moisturize the skin	
Change linens	
Measuring body temperature	
Measuring blood pressure	
Heparin (only during the first month)	
Review of the bladder catheter and measure of the diuresis (phase of spinal shock)	
Test urine dipstick pH, leukocytes, glucose, protein (once per week)	
Abdominal and cardio-pulmonary auscultation	
Stool examination: appearance and weight (phase of spinal shock)	
Blood drawn (once a week and then once a month)	
Control of animal weight (weekly)	
Placement in a wheelchair (since the first 15 days)	
Administration of food: breakfast (200 g dry food)	
Free play in wheelchairs	
10:00 am	
Neurological examination (weekly)	
Measurement of muscle mass (weekly)	
Passive rehabilitation (daily)	
Rehabilitation in pool (daily)	Fruit juices: kiwi, orange, pear
Free play in wheelchairs	
Treadmill rehabilitation (daily)	
Walker rehabilitation (daily)	
14:30 h	
Toilet: placing diaper. Mepentol application	
Administration of food: 200 g dry food	
Rest in crib	
16:30 h	
Toilet: placing diaper. Mepentol application	
Passive rehabilitation	
Rehabilitation in treadmill or pool, alternating days	
Free play in wheelchairs	
20:00 h	
Toilet: placing diaper. Mepentol application	
Measuring body temperature	
Administration of food: 200 g of dry food	
Sleeping in crib at night	

3.6.1. Control of food and management of digestive complications

In this phase animals receive special dry vegetarian food (Scientific Animal Food and Engineering, Panlab, Barcelona, Spain) to prevent constipation and excessive weight gain. The food is administered three times a day (breakfast, lunch and dinner) and supplemented with fruits and vegetables that are usually offered during rehabilitation. During the chronic phase there should be no major gastrointestinal complications if the animals take a proper diet and if water intake is controlled (water intake should be abundant), but the frequency and type of defecation must still be monitored.

3.6.2. Control of diuresis and urinary tract infections

Daily urine output should be monitored (color, turbidity and volume). At least one strip test a week (Urine strip 10C, Dialab Diagnostics, Hondastrasse, Austria) should be taken to determine some general biochemical values such as pH, leukocytes, glucose, proteins, etc. However, a complete biochemical study should be performed at least once per month. In the first weeks of the chronic

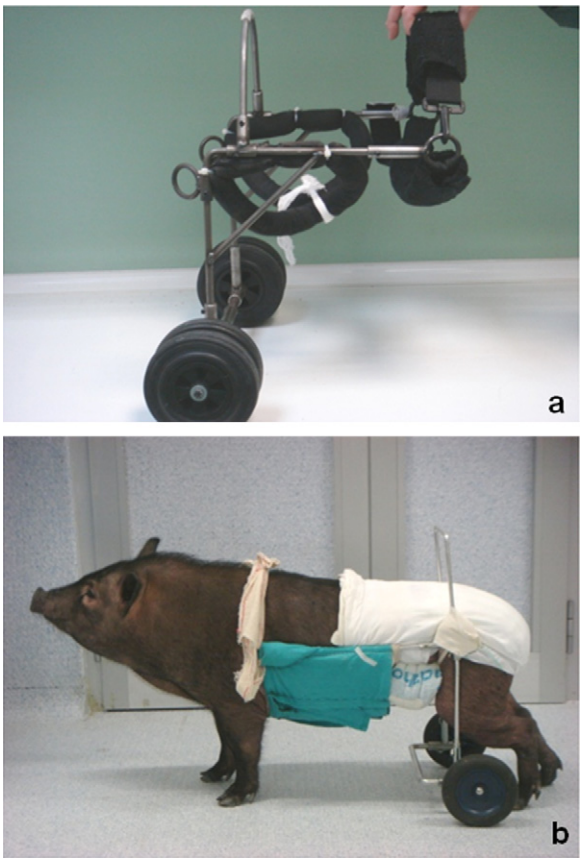

Fig. 5. Wheelchairs adapted to paraplegic minipigs (a and b).

phase (4 or 5 weeks after injury), during which some of the animals remain with a urinary catheter, the functioning of the catheter must be controlled. When in doubt, it should be washed with 0.9% saline to avoid infections or other urinary complications. However, it is advisable to maintain intermittent catheterization for at least the first 2 months to allow for the safe recovery of bladder automatism. During this time, bladder retraining techniques should be applied. Suprapubic massage can be used and, if needed, bladder emptying can be achieved by the Valsalva or Credé maneuvers.

One of the most common complications in the chronic phase is urinary infection, so we subjected our animals to prolonged treatment with a prophylaxis (1 month of treatment, another break and then a new treatment for 1 month) using fosfomycine, 1 g orally every 48 h. Despite this prophylaxis, we continued to monitor the existence of signs and symptoms that may indicate the presence of a urinary infection. Asymptomatic bacteriuria is often detected but should not be treated, to prevent development of resistance (Lisenmeyer, 2002). When an obvious urinary infection is detected (20% of cases), it is almost always solved by the oral administration of ciprofloxacin at a dose of 20 mg/kg/12 h for 7 days.

3.6.3. Care of the skin

The risk of skin lesions is primarily related to the immobility of the minipigs, especially in the crib. Together with urinary tract problems, skin lesions are some of the most frequent complications during the chronic phase. Although the animals at this stage remain in cribs for a short time, daily monitoring of the skin must be continued. The skin must be cleaned and moisturized. In addition, at this stage care must be taken with wheelchairs and other rehabilitation equipment, pressure points must be avoided on certain body areas. At this stage we found that 10 animals developed a grade I ulcer (50%) on the tuberosity of the ischium, and 2 animals developed a grade II ulcer (10%) that was caused by pressure from the wheelchair on the femoral condyle.

3.6.4. Spasticity

Spasticity occurs in the chronic phase of the injury as an increase in muscle tone and an exaggerated increase in reflex activity that is associated with involuntary movements (spasms) in the muscles below the injury. The appearance of mild spasms was observed in virtually all of our animals in the chronic phase, but only one animal (5%) experienced disabling spasticity. The intense rehabilitation to which our animals were subjected was likely able to prevent a higher incidence. Only one animal developed severe spasticity, which was associated with significant deformities and contractures of the lower limbs. In this case, spasticity was not followed by pain; it was treated with baclofen at a dose of 1 mg/kg (daily, oral) and improved partially.

To assess the degree of spasticity in animals, we used two assessment scales that are used in humans: the PENN scale (Penn et al., 1989) and the modified Ashworth scale (Ashworth, 1964)

3.6.5. Autonomic dysreflexia

Autonomic dysreflexia usually results from increased BP that cannot be compensated for due to dysfunction of the autonomic nervous system. It usually appears in high lesions of the spinal cord and presents with episodes of BP that fluctuates from very high to very low. Animal model studies have been conducted to determine the effects of dysreflexia as well as the changes in the spinal cord that contribute to the development of this disorder. However, the mechanism underlying autonomic dysreflexia is poorly understood (Weaver et al., 2006; Alan et al., 2010).

In our experience, with SCI at a relatively low spinal level, we found no cases of severe autonomic dysreflexia. However, this complication can appear as a consequence of urinary retention, infection or even a change of position, as has been described by some authors (Jacob et al., 2001; Hagen et al., 2011). In the presence of this complication, the animal's head must be raised using pillows, and the noxious stimulus must be removed (usually bladder distention). If the animal has a bladder catheter, its permeability must be confirmed. Persistent dysreflexia can be treated with antihypertensive agents such as nifedipine at a dose of 0.2 mg/kg oral, three times a day (Campagnolo and Merli, 2002).

3.6.6. Neuropathic pain

Neuropathic pain can occur within hours, days, weeks or even many months after SCI and is variable in intensity. It is not common in complete thoracic lesions but is more frequent after incomplete injuries (Bockenek and Stewart, 2002). This complication must be addressed in a preventive manner, so our animals received analgesia in the first days after the injury. We subsequently carried out extensive monitoring to look for clinical signs of pain. If we observed any signs of pain (changes in behavior or vocalizations), we quickly administered analgesic treatment (Henke and Erhardt, 2004). The drugs used were as follows: morphine intravenous, 0.1 mg/kg every 4 h; buprenorphine 0.01–0.02 mg/kg intravenous or intramuscular every 8–12 h (treatment of choice); tramadol hydrochloride 1–3 mg/kg/h for 2 h intravenous; meloxican, 0.1 mg/kg subcutaneous or intravenous every 24 h. This can cause loss of appetite and vomiting. Therefore, when it is used it should be administered with a protector of the gastric mucosa.

The signs that an animal is in pain are diverse and may manifest as follows: cessation of eating or movement, avoidance of contact with caretakers, aggression or even self-mutilation. There may be vocalization, loud exhalations, or grinding of theeths. To assess the

degree of pain in animals, different scales may be used. We use the scale described by Haskins (Haskins, 1992a,b) which divides the degrees of pain as follows: (a) mild pain is a tolerable pain that does not involve changes in animal behavior and that is only expressed as a certain defensiveness when handling body parts; (b) moderate pain is a more intense pain that can be produced by disease and can lead to significant changes in behavior, such as reduced activity, or appetite; and (c) severe pain is a pain that involves major changes in behavior and is accompanied by vocalizations (moaning, grunting, etc.), aggressive or self-mutilation.

In our series of 20 animals we did not find any animal in a phase of chronic paraplegia showing signs of pain. The extensive rehabilitation that takes place in these animals may prevent pain, but when in doubt, analgesics should always be administered.

3.7. Housing conditions in the chronic phase

During maintenance of the animals in the chronic phase, the pigs only remain in their cribs to sleep for 12 h per night. The minipigs, for reasons of hygiene, remain with incontinence pads at all times. As in the acute phase, incontinence pads are changed every 4 h for grooming to keep the skin clean, followed by administration of hyper-oxygenated fatty acids for protection and hydration. At this phase, except when our animals are resting or undergoing rehabilitation, they remain in wheelchairs to allow for independent movement and so that they can play and relax.

3.8. Neurological examination of the animals in the chronic phase

During the first days after SCI we find a total anesthesia and hypotonia below the injury site, but muscle tone and reflexes gradually and moderately increase from the second week. Neurological examination of animals at least once a week is necessary to assess possible changes.

The scales used to evaluate animals with SCI are as follows: (a) Motor function scale (Zurita et al., 2008), which refers to purely motor aspects (Table 3); (b) Modified SCI Neurological scale (Kuluz et al., 2010), which not only assesses the sensory and motor function of the lower extremities but also emphasizes bladder and bowel function (Table 4); and (c) Walking porcine scale (Kuluz et al., 2010), which is an adaptation of the BBB scale (Basso et al., 1995) from rodents to minipigs, to assess the locomotor function of animals (Table 5).

To assess the degree of muscle atrophy and the state of joints, it is necessary to measure muscle mass every week and to measure the degree of joint motion with a goniometer. To assess muscle mass, thigh circumference is measured at the following three levels: (a) greater trochanter; (b) zone sesamoid, and (c) 2/3 distal. The measurement should always be conducted in the same place and values for both limbs should be recorded. The degree of joint motion (ROM, or range of motion) is measured by placing the central point of the goniometer in the center of the range of joint motion, in both flexion and extension, and measuring the angle. In the normal state, the angles measured by the goniometer are (flexion/extension): hip: 50/162; knee: 42/162; tarsal: 39/164.

Table 3
Modified Tarlov scale for assessment of motor function of hind limbs (Zurita et al., 2008).

0. Complete paraplegia, with absence of movements in hindlimbs
1. Beginning of movement in the one or the other of the hindlimbs
2. Scarce spontaneous movement in both extremities
3. Important movements in both extremities, but pigs cannot get up
4. Pigs get up with assistance sometimes, but they cannot stay standing
5. Pigs get up without any help sometimes, but they cannot stay standing
6. They stand up without assistance, but they are not able to start hiking
7. Pigs commence standing and they can start hiking with some type of help
8. Pigs begin hiking and they take some steps without necessity of help
9. Pigs can walk without assistance, but with obvious difficulty
10. Constantly useful hike

3.9. Physiotherapy and rehabilitation

Numerous studies have described the beneficial effects of intensive rehabilitation treatment in the maintenance and recovery of patients who have suffered SCI, maximizing preservation of patients capabilities after injury (Wernig and Müller, 1992; Wernig et al., 1995; Rodríguez-Boto and Vaquero, 2009). It seems clear that similar approaches should be applied to experimental animals with SCI because it can facilitate the acquisition of better functional results (Kunkel-Bagden et al., 1993; Gazula et al., 2004; Norrie et al., 2005; Carvalho et al., 2008; Macias et al., 2009; Kuerzi et al., 2010; Robert et al., 2010). Therefore, in our laboratory, animals with SCI are subjected to a series of daily exercises designed to keep their joints and musculature in good condition (Figs. 6 and 7). Although the mechanism of locomotion is very complex (Grillner, 1975; Grillner and Wallen, 1985; Pearson, 1993; Jordan, 1998), animals undergoing intense rehabilitation sessions were able to create useful gait patterns.

3.10. Kinesitherapy: Passive rehabilitation of sublesional joints

The care and affection given to animals must ensure that they behave as pets (see Supplementary Video 1). This is essential for proper rehabilitation.

Daily treatment by passive movement of the lower limbs in paraplegic animals allowed us to accomplish the following: (a)

Table 4
Scale for neurological examination of minipigs with SCI. It is based on the scale used by Kuluz et al. (2010), and provides an index of overall neurological status, with special emphasis on bladder and bowel function, motor and sensitivity function, and perineal sensation.

Bladder function		0 – not spontaneous	1 – helped with massage	2 – overflow	3 – spontaneous
Intestinal function		0 – no noise	1 – noise	2 – constipation	3 – normal bowel
Get up		0 – can not	1 – need help	2 – itself but with difficulty	3 – independent
Crawl	RHL, LHL	0 – can not	1 – some step	2 – many steps	3 – normal
Walk steps	RHL, LHL	0 – can not	1 – partial support	2 – partial without support	3 – normal
Spasticity	RHL, LHL	0 – disabling	1 – partially disabling	2 – nondisabling	3 – without spasticity
Paresis	RHL, LHL	0 – complete paraplegia	1 – severe paraparesis	2 – mild paraparesis	3 – no paresis
Propioceptive sensitivity (gait coordination)		0 – absent	1 – severe ataxia	2 – mild ataxia	3 – normal
Sensation below injury					
Light touch	RHL, LHL	0 – no response	1 – weak	2 – increase	3 – normal
Puncture (heat)	RHL, LHL	0 – no response	1 – weak	2 – increase	3 – normal
Perineal sensitivity	RT	0 – absent	1 – poor	2 – normal	3 – normal
	S4/S5 sensitivity	0 – absent	1 – poor	2 – normal	3 – normal

RHL: right hindlimb. LHL: left hindlimb. RT: rectal tone. Total Score: (0–54).

Table 5
Scale for assessment of locomotor function in minipigs with SCI. It is an adaptation of the scale used by Kuluz et al. (2010). It is useful for assessing limited to the spontaneous movements in the hindlimbs. The animal is placed on the floor for 20 min, two times a day for the assessment.

1.	No motion
2.	Move only the hips
3.	Movement of hips and knees
4.	Rhytmic flexion extension of all joints, without weight-bearing itself
5.	Attempt to maintain body weight, but cannot stand unaided
6.	Occasionally bear weight on the hindlimbs but does not start up and drag the limbs
7.	It attempts to walk but does not alter either the movement on the hindlimbs (poor coordination)
8.	Start up with 3 or 5 steps, with some alteration of the hindlimbs, but showing poor coordination between forelimbs and hindlimbs during walking
9.	Walk 5 or more steps with alternating forelimbs and hindlimbs, but showing limited flexion of the knee and sometimes dragging hooves
10.	Walk giving 5 or more steps, alternating forelimbs and hindlimbs, with good flexion of the knee
11.	Normal run

maintain joint tours; (b) prevent the onset of circulatory problems, favoring venous return; (c) prevent contractures and deformities; (d) preserve muscle-tendon elasticity; (e) prevent the onset of pathological ossifications; and (f) reduce the extent of possible spasticity.

Passive motion of all joints of the lower limb should be performed on all animals at least twice a day in sessions of about 30 min each. If possible, this should be performed more often in the acute phase to promote better circulation and to prevent contractures (Guttmann, 1981). However, passive movements should be performed based on the following rules: all of the joints of the affected members must be moved smoothly and slowly, without straining the joints, and joints must be mobilized in all planes and axes of movement, reaching the maximum amplitude of the joint. It is important that the mobilization is performed very carefully, because animals have no feeling in these areas, which can lead to tissue damage. One should keep in mind that a possible cause of pathological ossification is careless mobilization that traumatizes periarticular tissues and muscles. If we observe edema, reddish-cyanotic engorgement of superficial veins, or increased temperature in the hind legs, suggesting thrombophlebitis, kinesitherapy must be suspended.

In our present series we observed no cases of pathological ossification, but in the early periods of our model, we had a case with severe spasticity after SCI that developed pathologic ossification in the right knee, preventing effective rehabilitation.

The program of passive exercise that is performed daily on the lower limbs (first with one limb, then the other) is based on the work of Alcántara et al. (1995) and consists of the following: (a) flexion–extension of the hoof (bending and stretching with the

Fig. 6. Different aspects of rehabilitation. (a and b) Kinesitherapy with therapeutic ball. (c) Hydrotherapy in the pool. (d) Exercise of gait in treadmill. (e) Exercise of gait in the walker. (f) Exercise of gait with a harness.

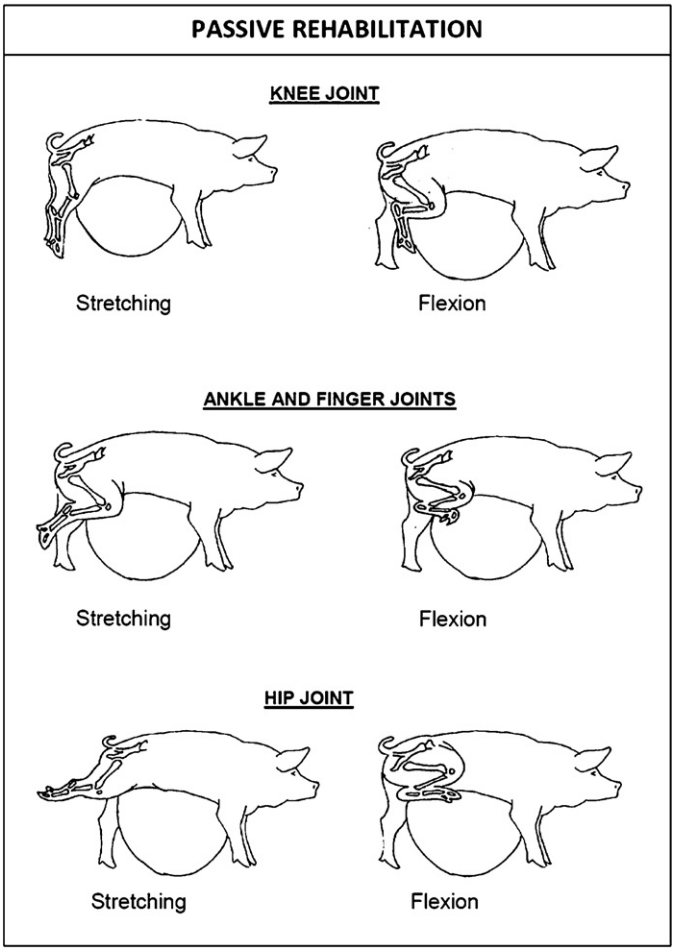

Fig. 7. Passive rehabilitation of the different joints. See also Supplementary Video 2.

fingers); (b) flexion of the ankle so that the hoof is brought toward the knee, never the reverse: (c) flexion–extension of the knee and hip (bending and straightening the knee and hip); (d) circumduction of the hip (making circles in both directions of the hip with the knee bent); (e) rotation of the hip; (f) abduction and adduction; and (g) stretching of muscles in the back of the thigh (Figs. 6a, b, 7 and Supplementary Video 2).

3.11. Hydrotherapy

Hydrotherapy is a technique that is widely used in rehabilitation after SCI, and its effectiveness has been proven not only in humans but also in experimental models (Hutchinson et al., 2004; Smith et al., 2006; Magnuson et al., 2009; Robert et al., 2010). This therapy is useful for strengthening the muscles that have been affected by the injury; stabilization and flotation in water facilitates exercises that could not be done on the ground. The body supports less weight in water and this reduces the load on the joints, allowing for more comfortable exercise. The use of hydrotherapy in the injured animals has been very useful for the following: (a) reducing spasticity; (b) strengthening muscles; (c) improving the balance of the trunk, and (d) providing play time in the water.

The pool is maintained with chlorinated water with a temperature between 27 and 30 °C, with bacteriological testing to ensure sterility. Animals should have a period of adaptation to exercising in the water. Initially, the animals must use life jackets and only passive rehabilitation should be performed (Fig. 6c and Supplementary Video 2).

We allow the animal to get into the water and always monitor stress and fatigue. It is important to increase the intensity of exercise gradually. At the start, two or three sessions per week are sufficient, but this can become a daily routine. When the animals treated after the SCI begin to regain motor and sensory function, as a result of cell therapy in our studies (Zurita et al., 2008; Vaquero and Zurita, 2011), the water exercises are eventually performed with the following different water levels daily: 60 cm for 60 min per day, 40 cm and 25 cm.

3.12. Gait training: Treadmill

Several studies have demonstrated the usefulness of treadmill gait training in patients with SCI (Wernig and Müller, 1992; Dietz et al., 1998; Dietz, 2003; Dobkin et al., 2006). Moreover, experimental studies have shown positive effects not only on gait but also on the control of neuropathic pain and autonomic dysfunction (Vilensky and O'Connor, 1998; Thota et al., 2001; Laird et al., 2009). Studies in rodent models with incomplete injuries confirmed that daily treadmill exercise allows for a significant recovery of walking ability (Multon et al., 2003).

In our pig model of SCI, daily exercise on the treadmill allows us to establish intensive gait training, helping to strengthen the muscles of the affected limbs and improving the balance of the trunk (Fig. 6d). To perform this exercise it is necessary to use a harness system to keep the animal suspended, as after SCI at the thoracic level, animals can no longer support their body weight and cannot generate an automatic locomotion pattern. Daily sessions of 30 min should be performed, in which all of the joints are passively mobilized following the gait pattern.

3.13. Walker and wheelchair

Recent advances in neurorehabilitation therapy in humans have shown that daily standing, along with other exercises, improves muscle, cardiovascular and pulmonary function (Vidal, 2009). In our experimental model, pigs support all limbs daily using wheelchairs designed specifically for each animal (Fig. 5). This is conducted in combination with a program of gait training with a walker that was also designed in our laboratory (Fig. 6e).

3.14. Electrostimulation

Electrostimulation has few indications in our experimental model. However, it has occasionally been used in attempts to reduce spasticity, but has met with little success (Fig. 8).

Electrostimulation in animals with complete SCI should be applied only to major muscles, such as the tibialis anterior and

Fig. 8. Electrical stimulation of muscle mass in a paraplegic animal.

quadriceps. Opinions on the effectiveness of electrical stimulation on denervated muscle are contradictory, and although the goal is to maintain muscle tropism (Herbison et al., 1973), some authors believe that it is generally ineffective. We could not obtain clear conclusions about its usefulness, but our experience in chronically paraplegic pigs suggests that electrostimulation delays muscular atrophy without improvement when atrophy is already present. In two cases of our series we have used electrostimulation to treat spasticity or reduce muscle atrophy. We have used an intensity of 80 mA, pulse duration between 200 and 250 μs, and a frequency range between 30 and 50 Hz. Electrostimulation was applied 20 min/day for 3 months.

3.15. Neuroimaging studies

In the course of various research protocols, our paraplegic animals have been subjected to neuroimaging studies, usually under general anesthesia, including isotope scans to study the distribution of labeled autologous stem cells after systemic administration (De Haro et al., 2005) and magnetic resonance imaging (MRI) to study the morphological evolution of spinal cord lesion.

In our series, paraplegic pigs were studied by MRI 3 months after SCI, using a 1.5-T superconducting imager (Siemens SA, Madrid, Spain), and at this time point, MRI characteristically showed a cystic centromedullary lesion that was hypointense on T1 and hyperintense on T2-weighted images. Spinal cord atrophy and myelomalacia were common along with persistent centromedullary cysts. The average values for the volume of the posttraumatic centromedullary cavity, according to the image reading software (Siemens Imaging software, Siemens AG Medical Solutions, Erlangen, Germany) were $135 \pm 38\ mm^3$ (Fig. 9).

3.16. Electrophysiological studies

Electrophysiological studies in our experimental model allowed us to obtain objective data to confirm the potential efficacy of our therapeutic strategies. To assess the integrity of the ascending and descending tracts of the spinal cord, somatosensory evoked potentials (SSEPs) and motor evoked potentials (MEPs) should be performed. To conduct both types of tests, the animals must be anesthetized by induction with sevorane and placed on the operating table in the prone (sternal) position. PowerLab system (ADInstruments, Inc., Colorado Springs, CO, USA) was used.

MEPs were recorded using needle monopolar recording electrodes located in the anterior tibial muscle and plant (at the third metatarsal space). The stimulus electrodes were located

Fig. 9. Imaging studies after SCI. (a) Minipig in the course of a MRI study. (b and c) Examples of MRI after SCI, showing a hyperintense centromedullary cavity on T2-weighted images. (d) Minipig in the course of a scintigraphic study.

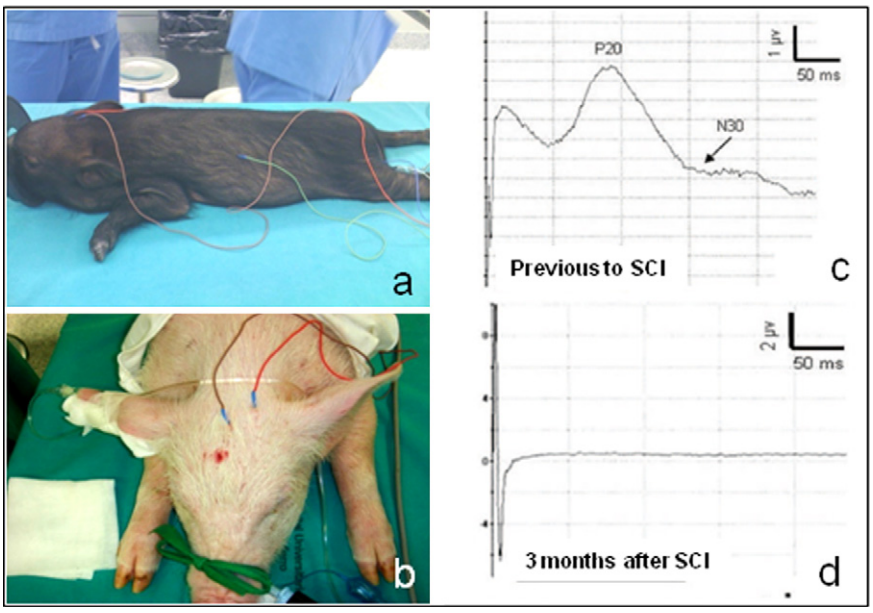

Fig. 10. (a and b) Animals in the course of a neurophysiological study for recording SSEP. (c) Normal SSEP, previously to SCI. (d) Absence of SSEP, 3 months after SCI.

subcutaneously in the contralateral sensorimotor cortex (the cathode) and on the skin of the nose (anode). A stimulation train (3 pulses, 200–300 V, 0.1 ms pulse duration, and 2 ms interstimulus interval) was used (Strauch et al., 2004).

To record SSEPs in response to stimulation of the tibial nerve, tibial nerve stimulation was performed using AgCl electrodes (2.17 Hz) 15 mm in length. Electrodes were placed on the tibial nerve subcutaneously and were separated by 2 cm. For recording, electrodes were inserted in the contralateral somatosensory cortical area. Electrical stimulation (0.2 ms duration and 19 mA intensity) was performed with a constant current range of 2-fold threshold responses to stimulation of muscles. The signals of the evoked potentials were amplified (10 μV), filtered (1–3 kHz bandpass) and fed to a compatible PC to be averaged using Scope software (Scope Software Ltd., Co. Dun Laoghaire, Dublin, Ireland). Each measurement consists of an SSEP average of 512 single sweep epochs. The signals were amplified and filtered (band-pass 1 Hz/5 kHz for MEPs and 10 Hz/1 kHz for SSEPs) (Fig. 10).

4. Conclusion

In this paper we have described the conditions and procedures that we used to maintain paraplegic minipigs in good health for long periods of time after SCI.

Our present results demonstrate that adult paraplegic pigs can be maintained in good clinical condition for long periods of time if given proper care. This animal model may help to optimize new experimental strategies that need sufficient follow-up time to assess their efficacy, providing an important resource for studies of regenerative techniques applied to paraplegic patients.

Conflict of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or material discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pneurobio.2012.04.005.

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