Effect of electric current stimulation in combination with external fixator on bone healing in a sheep fracture model

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Summary
Biophysical stimulations with electric and electromagnetic fields have been demonstrated to accelerate the bone-healing rate. This study has been designed to investigate the effects of electricity directly connected with the central pins of an external fixator in an experimental osteotomy model in sheep. Thirty mg/kg of tetracycline chloride were administered on the 30th and on the 45th day after surgery for histomorphometric studies. Plain radiographs were obtained in standard projections every 15 days after surgery and were analyzed with a software program (Corel Photo-Paint Pro X2, Corel Corporation, Ottawa, Canada). The specimens obtained after 60 days were examined with histological analysis. The results show that biophysical treatment with alternating electricity in combination with external fixator enhances new-bone formation. The translational value of this study, due to the similarities between ovine and human species, suggests that this treatment could be useful in speeding the bone-healing rate both in animals and humans.

Effetto della stimolazione elettrica associata a fissatore esterno nella guarigione ossea in un modello di frattura nella pecora

Parole chiave
Corrente elettrica,
Fissatore esterno,
Guarigione ossea,
Stimolazione biofisica,
Trattamento ortopedico.

Riassunto
La stimolazione biofisica con campi elettrici ed elettromagnetici è efficace nell'accelerare la guarigione del tessuto osseo. Scopo del presente studio è stato quello di investigare gli effetti dei campi elettrici in un sistema direttamente collegato alla vite del fissatore esterno in un modello sperimentale animale (pecora) di osteotomia. Dopo 30 e 45 giorni dall'intervento sono stati somministrati trenta mg/kg di tetraciclina per eseguire studi di istomorfometria e sono state eseguiti esami radiografici secondo modalità standard ogni 15 giorni, poi valutati mediante *ad hoc* software. Infine i campioni ottenuti dopo 60 giorni sono stati esaminati mediante esame istologico. I nostri risultati indicano che il trattamento biofisico con correnti elettriche alternate e l'utilizzo di un fissatore esterno, favorendo la formazione di nuovo tessuto osseo, accelera il processo di guarigione tissutale. Il valore traslazionale di questo studio suggerisce che questo trattamento possa essere utilizzato con successo in medicina umana.

Introduction

A long-standing interest exists about the use of various forms of biophysical stimulation that could positively affect the growth, strength and remodeling of tissues. Since Fukada and Yasuda (1957) first reported electrical phenomena in bone, many reports have been published about the effects of electricity on bone and cartilage (Akai and Hayashi 2002, Massari *et al.* 2007). It has been stated that, as the stimulation modalities are different in their physics and biochemistry, each modality produces a variety of biological responses in a wide range of animal models (Black 1985). Early references showed that various modalities of direct and pulsatile currents are effective enhancers of bone healing (Hassler *et al.* 1977). More recently it has been demonstrated that electric and electromagnetic fields can increase gene expression for - and synthesis of - growth factors, including several bone morphogenetic proteins (BMPs) and transforming growth factors (TGF), mainly TGF-ß1 (Aaron *et al.* 2004), which may enhance endochondral bone formation. Biophysical stimulation techniques, including Capacitively Coupled Electric Field (CCEF) and Pulsed Electro Magnetic Field (PEMF), have been used in clinical trials to treat fresh fractures and osteotomies, spine fusions, and delayed and non-union fractures (Aaron *et al.* 2004). Capacitively Coupled Electric Field has also been demonstrated to accelerate osteoblast like cells proliferation and to increase bone extracellular matrix (Hartig *et al.* 2000). Although an experimental comparative (untreated controls) trial in dogs suggests that CCEF can delay the recovery of bone strength during distraction osteogenesis (Pepper *et al.* 1996), several experimental and clinical studies indicate that CCEF can accelerate the bone-healing rate in various situations including stress fractures (Brighton *et al.* 1985 and 1989, Beck *et al.* 2008, Benazzo *et al.* 1995), lumbar spinal fusions (Goodwin *et al.* 1999) and non-union (Brighton and Pollack 1985, Impagliazzo *et al.* 2006, Scott and King 1994). In 2008, a novel external fixator coupled with an alternating electric current stimulation device has been developed for the treatment of distal radius fractures (Itoh *et al.* 2008). This system exploits a capacitive setting since the fixation pins are utilized as electrodes. However, the fracture location was in a non-weight bearing bone and the stability of the device was not a challenge factor. Various methods have been described to evaluate the newly formed bone in experimentally stimulated tissue, including radiographic analysis, biomechanical testing (Hantes *et al.* 2004, Malizos *et al.* 2006), quantitative computerized tomography, and ultrasound system, histological (Canè *et al.* 1991) and biochemical analysis (Lorich *et al.* 1998). To our knowledge, there has been no previous attempt to conduct an experimental study evaluating the effects of CCEF on an osteotomy gap in a large animal model using an histological analysis. On the basis of these premises, the present study has been designed to determine the efficacy of an alternating electric current derived from a CCEF stimulator directly connected with the pins of an external fixation device in the healing of a large osteotomy created in the tibial midshaft of adult sheep.

Materials and methods

Animals

The study was conducted in compliance with the Italian Animal Welfare guidelines[1]. It was performed on 8 adults, 2-year old, appenninica breed sheep, 62-70 kg of weight. The sheep were bred according to the European community guidelines[2]. To investigate the efficacy of an alternating electricity derived from a CCEF stimulator on fracture healing, animals were randomly divided in 2 groups: the stimulation group (SG) and the control group (CG).

External fixator

The device for external fixation was custom made and was developed by Citieffe s.r.l. (Calderara di Reno, Bologna Italy). Briefly, in the configuration used for this study, it is composed of 6 transcortical stainless steel bone screws, a radiolucent rod, and 6 clamps (for pin/rod connection). The radiolucent rod, made up in polyether ether ketone (PEEK), measured 12 mm in diameter and 160 mm in length and allowed radiographic examinations. The bone screws came with a self-drilling and self-tapping tip and do not require pre-drilling, except in case of extremely hard bone cortex. Besides the bone screws have a 2 diameter profile, which allows for better balancing stress distribution. They are made of AISI 316L stainless steel and are 5/6 mm in diameter and 120 mm in length. The clamps, made of in Avional 2024, allow for the insertion, for each segment of the rod, of 2 screws in parallel position and a third one with an angle of about 30°.

Surgical procedure

After 2 weeks of animal quarantine, surgical procedures were conducted in an authorized

[1] Request of authorisation - Prot. n. 407 on 24.09.2008. European Commission (EC). 1986. Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Off J*, **L 358**, 18/12/1986.

[2] Decreto Legislativo 27 January 1992 n. 116. Attuazione della direttiva n. 86/609/CEE in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici. *Off J*, **40**, 18.02.1992 (Suppl Ordinario n. 33).

veterinary hospital. Animals did not receive food for 12 hours prior to surgery. Anaesthesia was accomplished as follows: sedation with xylazine (0.2 mg/ kg i.m; Rompun®; Bayer), diazepam (0.2 mg/ kg i.v.; Diazepam® 0.5; Intervet), and atropine sulfate (6 mg i.m.; atropine sulfate; Fort Dodge); induction with ketamine (10 mg/kg i.m.; Ketavet® 100; Intervet) and, after endotracheal intubation, maintenance by inhalation of 2.5 % halothane (Halotane®; Merial) in oxygen. Saline was also administered intravenously throughout the procedure. A gastric tube was inserted and maintained for the entire procedure. The left pelvic limb was prepared under sterile conditions. As previously described for experimental sheep osteotomy models (Malizos *et al*. 2006), an unilateral, 1-plane external fixation device was implanted on the lateral surface of the tibia, followed by a midshaft osteotomy on the medial side. Four pins were inserted, through stab incisions of the skin, in the proximal and distal third of the tibia (approximately 30 mm distal to the knee and 30 mm proximal to the tarsus) on its antero-lateral surface. Rarely, when the bone cortex was too hard to drill through, the insertion of the pin was preceded by a pre-drilling with a 3.5 mm drill bit under constant cooling with sterile saline. A suture stitch with non-absorbable suture tape was applied, when indicated, to prevent the incoming of infection. To increase the stability, 1 pin was then inserted between the 2 pins of the proximal pair and 1 pin was inserted between the 2 pins of the distal pair, obtaining a unilateral 2-plane configuration (Figure 1). The pins were firmly secured to the rod by the clamps. The limb was then elevated and the osteotomy was realized on the medial side of the tibia. Briefly, a skin incision was made between the 2 central pins through a 3.5 cm-long straight longitudinal incision. The fascia was incised and the periosteum incised and carefully stripped medially and laterally. A complete transverse osteotomy was then performed. With the help of Homan levers soft tissues were divaricated and some 3.5 mm holes were drilled circumferentially in the tibial diaphisis; each hole encompassed proximal and distal bone cortex. The drillings were cooled with sterile saline to avoid heating of the bone tissue. When necessary, the osteotomy was completed with an osteotome. Immediately after the completion of the osteotomy a visible gap formed between the bony fragments. Fascia and skin were routinely closed. A soft well padded bandage was finally applied encompassing the external fixator and leaving the extremities of the pins uncovered for electric stimulator connection. The animals were allowed unrestricted activity in the in sheep pen after the recovery from the anaesthesia, and were observed daily for fitness, wound-healing, and development of pin-track infection. The pins and the skin around the penetration points were daily cleaned and treated with povidone iodine (Betadine® 10% Gel, Mundipharma Basilea, Switzerland). Animals were euthanized at 60 days after surgery.

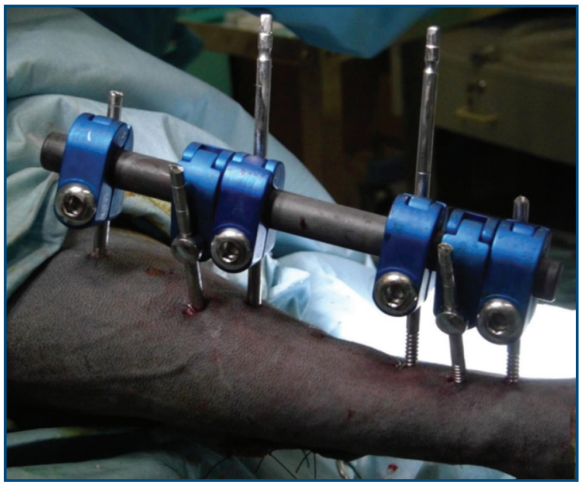

Figure 1. *Unilateral, two-plane configuration of the external fixator assembled on the antero-lateral suface of the left tibia of 1 of the on 8 adults, 2-year old, appenninica breed sheep considered in this study.*

Technical development

Electrical stimulation was delivered through a portable medical device. It was a properly modified CCEF stimulator for bone growth (OsteoBit BS1, IGEA, Carpi, Italy); the generator was able to deliver an alternating electricity of 1500μA in the region of interest. The electric signal consists of electrical pulses of 12.5 Hz with a duty cycle of 50% (40 msec). The active part of the burst is a sine wave at 60kHz with an amplitude adjusted by a microprocessor according to the impedance of the body interposed between the electrodes. The stimulation device has been used in conjunction with the Citieffe external fixator and it was attached to it with a dedicated adapter. The central pins of external fixator were employed as electrodes to deliver the alternating current in the fracture site.

Electrical stimulation

Four animals were randomly selected and assigned to SG, while 4 animals were assigned to CG. On the second post-operative day (POD), the stimulation device was connected to the central pins (proximal and distal, nearest to the osteotomy), by means of electric leads connected to the stimulator that was secured to the sheep thorax with an elastic bandage (caudal to the elbow to allow free movements and avoiding

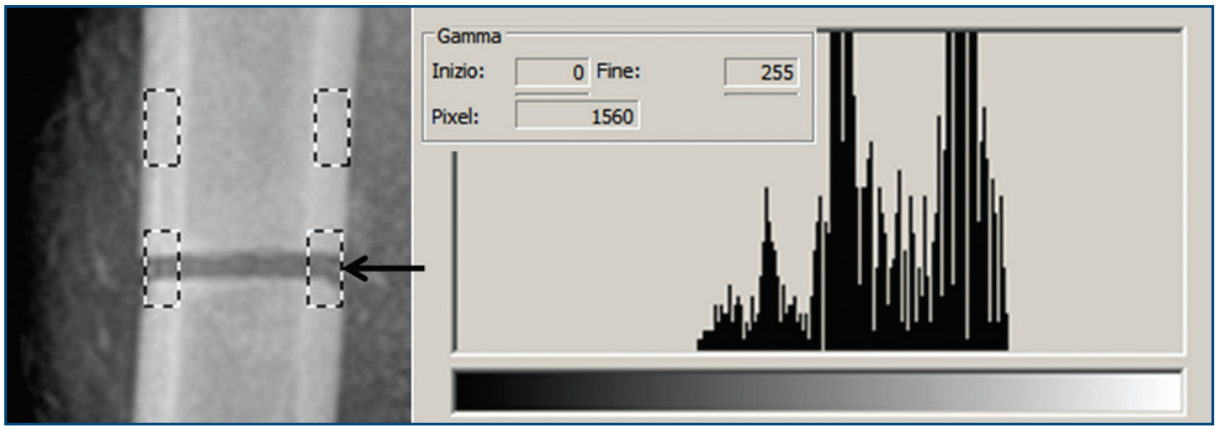

Figure 2. *Radiological study performed by comparing 2 identical sampling areas at the level of the osteotomy and normal cortical bone of the 8 adults, 2-year old, appenninica breed sheep.*

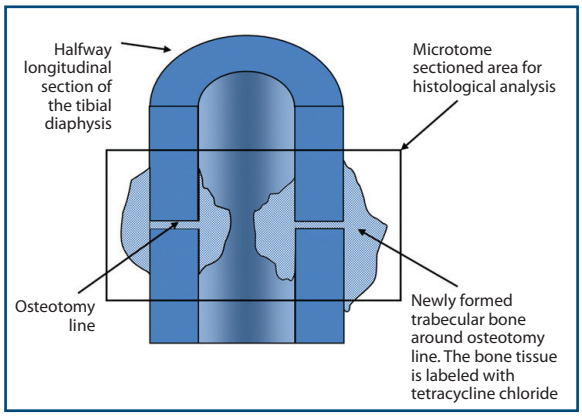

Figure 3. *Schematic representation of the specimen cut for histological analysis.*

stimulator dislodgement). Electrical stimulation was delivered to SG for 12 hours daily (from 8 am to 8 pm). The sheep of CG had the same assembly but the stimulator was always switched off.

Rx examination

Radiological evaluation was carried out at POD 1, 15, 30, 45 and 60. The radiographs have been obtained with a Computer Radiography System (AGFA Solo CR Digitizer, Agfa HealthCare NVB-2640 Mortsel, Belgium). All the images have been taken in standard conditions to objectively compare analysed parameters, as previously described (Petrizzi *et al.* 2007). Briefly, data were expressed as ratio between radiodensity of normal cortical bone and radiodensity of a tract of bone including osteotomy site. A sampling area of 1560 pixel (image at 300 dpi resolution) was calculated on the mean value (lateral and medial) in normal cortical bone, and an identical sampling area was evaluated at the level of the osteotomy (Figure 2). By this way each bone segment, including the osteotomy site, was compared with a normal segment of the same animal, thus avoiding experimental error due to the difference in physiological bone radiodensity among different animals. For each area, the number of pixels with each of the 256 levels of grey, ranging from white (255) to black (0), was measured and the ratio was calculated as follows: ratio = mean levels of grey of treated bone/mean levels of grey of normal bone.

Bone tissue labeling, histology and histomorphometry

To perform dynamic histomorphometry, sheep were given an intravenous injection of 30 mg/kg of tetracycline chloride at 30 and 45 POD (Tam and Anderson 1980). Euthanasia was performed according to European Guidelines on POD 60 (range from 62 to 65 days). Left tibiae were carefully harvested and stripped of soft tissues. The external fixator was removed, and the specimens were collected. The bone segments comprising the osteotomy site were removed and fixed in 4% buffered paraformaldehyde for 72 hours, dehydrated in a graded series of ethanol and embedded in poly-methylmetacrilate (Sigma Aldrich, Milan, Italy). The specimens were then cut in half according to a longitudinal plane as shown in Figure 3. Longitudinal serial 200-µm thick sections were then obtained by means of a Leica SP 1600 microtome (Leica, Wetzlar, Germany), from each half specimen. Four of these sections were randomly selected, glued to a methacrylate support and cut with a Reichert-Jung Autocut 1150 microtome (Microsystems, Nussloch, Germany) to obtain a series of 7-µm thick sections. These sections were analysed with a UV light microscope (Zeiss Axiophot; Jena, Germany) equipped with an image analysis system (Nikon DS-5Mc camera connected to a personal computer – NIS Elements AR 2.20 Nikon software). Histomorphometric evaluations were performed only in the newly formed bone around, or within, the

osteotomy line where osteon-like structures were recognizable and 2 parallel tetracycline labels were present. The interlabel distance was measured at regular intervals and averaged to calculate the Mineral Apposition Rate (MAR) (Parfitt *et al.* 1987, Schilling *et al.* 1992), MAR values have been obtained by dividing the distance (in micron) between the tetracycline chloride labels (Figure 4) for the time interval between the 2 tetracycline chloride administrations (15 days in this study). The mineral apposition rate has been determined to evaluate the bone deposition due to osteoblasts activity both in SG and in CG.

Statistical analysis

The data were assessed for normalcy by using the D'Agostino and Pearson omnibus normality test and were compared by a 2-way ANOVA model (the variables considered were time and treatment) for repeated measures (radiograph evaluation) or by one-way ANOVA (histological data). The data were considered different for $p<0.05$, and were reported as mean ± standard deviation.

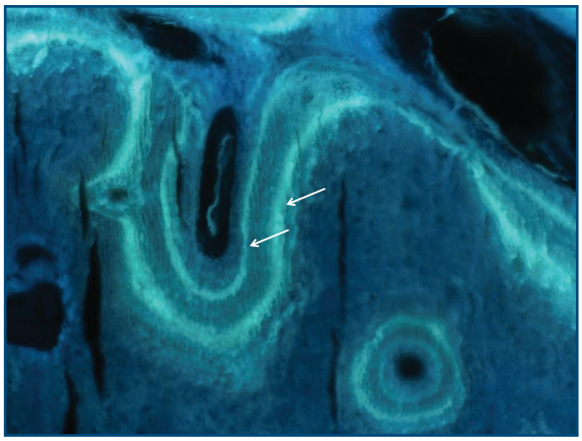

Figure 4. *Histomorphometry evaluation.* Tetracycline chloride labeled bone tissue (arrows) of the 8 adults, 2-year old, appenninica breed sheep.

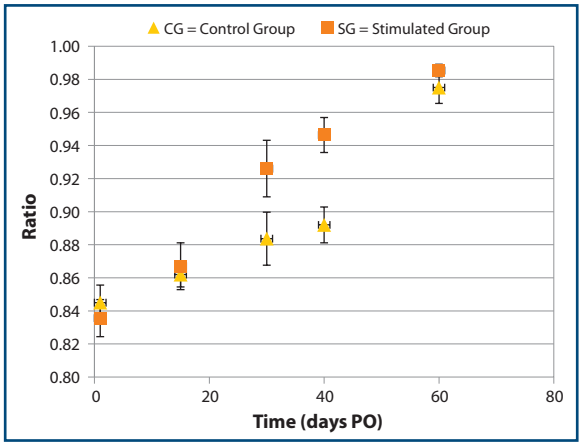

Figure 5. *Graph showing the values of radiodensity (ratio treated bone gray level/normal bone gray level).* Different superscript denote statistically difference among data sets ($p<0.05$). The different superscripts denote the statistical difference between control and treated samples. For difference among different time, and for interaction of time and treatment refer to the text.

Results

Clinical results

Surgical procedure was well tolerated by all animals and standing position was resumed 2-5 hours after surgery. A severe lameness was evident for some days, then all sheep progressively resumed the use of the operated limb. At 45 POD all animals recovered the almost complete use of the left pelvic limb. They never showed any sign of discomfort for the activation of the electrical stimulation. In some cases a light discharge was noted around the pin entrance that was easily controlled by daily dressing.

Rx examination

Statistical analysis of radiodensity showed that the values differ as a function of time after surgery ($p=0.0008$, 3.96% of total variance) and in control versus treated animals ($p<0.0001$, 87.24% of total variance); interestingly it was found that the interaction between the 2 analysed variables (time and treatment) has a statistically significant effect ($p<0.0043$, 5.36% of total variance), see Figure 5.

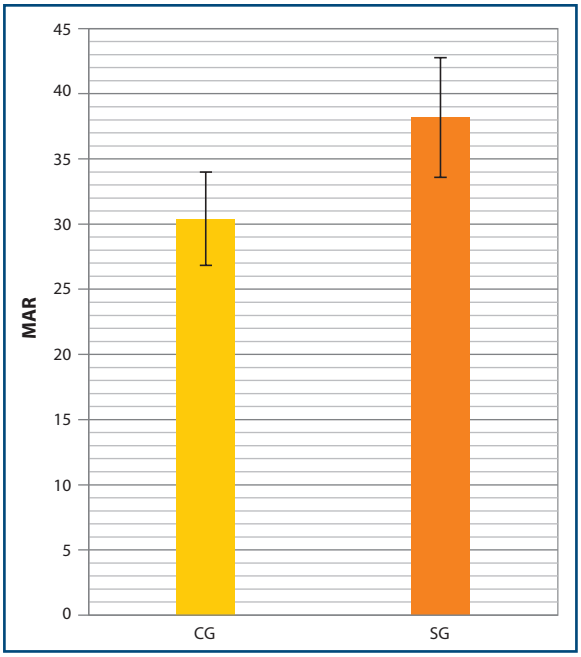

Figure 6. *Graph showing the interlabel distance (micron) values of control (CG) and stimulated (SG) samples, different superscripts denote statistically significative difference between the groups ($p<0.05$).*

Histology and histomorphometry

Mean values of the interlabel distance in 4 sections in each animal of both groups are summarized in Figure 6. Statistical analysis displays a significant difference ($p<0.001$) between control and treated samples. Moreover the mean value of MAR in SG is 2.54 ± 0.09 micron/day, while it is 2.02 ± 0.14 micron/day in CG. On the basis of these data, MAR has been increased by 25.73% in SG in comparison to CG.

Discussion

Although limited to a small number of animals, this study demonstrates that biophysical stimulation with alternating electricity in combination with external fixator can increase the rate of callus maturation. Our results are in agreement with those observed in the clinical study by Itoh and colleagues (Itoh *et al.* 2008) for distal radius in human and add new finding supporting the thesis that positive effects are not impaired or modified by the weight bearing that started to act approximately 30 days after surgery. The evaluation of bone formation in this investigation was carried out by radiography and MAR; the latter is a dynamic histomorphometric index and can be defined as the distance between the midpoints or between the corresponding edges of 2 consecutive tissue labels, divided by the time of the labelling period (Parfitt *et al.* 1987). It is commonly used for the characterization of bone formation (Schilling *et al.* 1992). In the past, this system was effectively used in similar experimental studies in horses to evaluate the effects of pulsed electromagnetic field on bone repair (Canè *et al.* 1992). Our data demonstrate that, in treated animals, the bone healing is improved in the window of time between 30 and 45 POD. In particular, it is evident that the bone radiodensity increases more in treated animal than in control ones, and that the effect of biophysical stimulation is the most important variable in determining bone healing (about 87% of total variance is due to the treatment, and the interaction of treatment with time has a well detectable statistic effect on data). Besides, the method used for the radiographic study, based on the ratio between the bone segment subjected to the osteotomy and a segment of intact bone of identical dimensions, can be considered extremely objective since the comparison is between different tracts of bone in the same animal, thus avoiding the differences between different subjects (Figure 7). The statistical analysis of obtained data confirms the increase of radiodensity of SG *versus* CG at the level of osteotomy gap at 30 and 45 POD. Since previous investigation, using identical experimental setting, showed that during the time range between 30 and 45 POD, the bone callus formation reached the

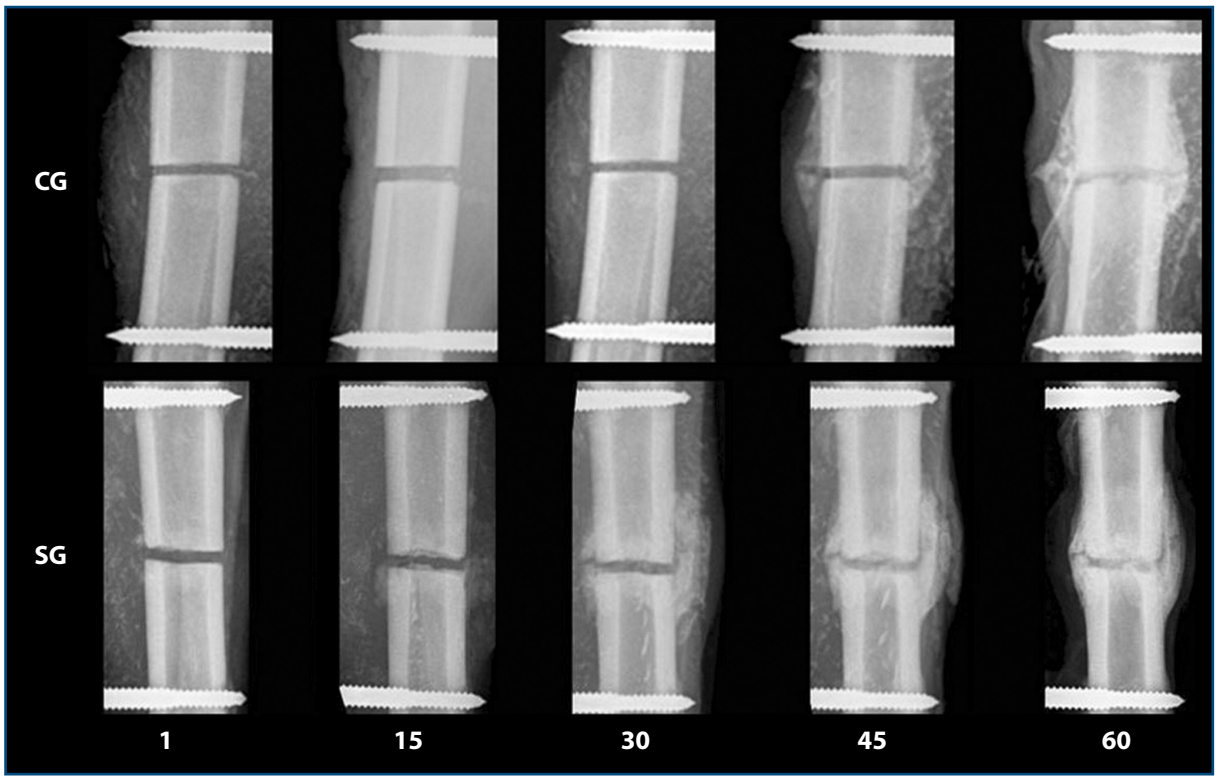

Figure 7. *Radiographic study showing the comparison between a sheep of Stimulated Group (SG) and a sheep of Control Group (CG) of the 8 adults, 2-year old, appenninica breed sheep considered in this study at different time (1, 15, 30, 45 and 60 POD).*

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highest level, the bone labelling was conducted at 30 and 45 POD (data not showed). Accordingly, this window of time is characterized by a significant increase of new bone formation, as showed by MAR that is higher in SG than in CG. When dealing with external fixation, device acceleration of bone formation is of paramount importance, since it might counteract the decrease of stability due to pin alisteresis. Another important problem to address, when dealing with bone callus formation, is the selection of an appropriate method to deliver the correct amount of electricity at the fracture/pathological site. In fact, it is well known that the field applied by plate capacitors can determine a voltage drop across the insulating layers so that only weak electric fields are active in the selected site (Hartig *et al.* 2000). To avoid this, the stimulation device used in this study was able to detect the body impedance and to adapt the voltage delivered to the electrodes, during the whole period of bone healing process. Moreover, in complex situations, *i.e.* severely comminuted and/or contaminated fractures, the possibility to couple an effective method to enhance callus formation with an external fixation to stabilize bone fragments would represent an effective strategy of treatment. In fact, as reported by Frost (Frost 1989), in 50% of cases pseudoarthrosis is due to a mechanical failure, 20% is due to a biological failure; while in the remaining 30% of cases the failed union is accounted for by combined problems of mechanical and biological order. It is well known that CCEF is able to achieve healing of non-union fractures only in presence of mechanical stability, fracture alignment and bone loss (less than half of the diameter of the treated bone) (Impagliazzo *et al.* 2006). Even if the results of this study lack the confirmation of biomechanical testing, due to the limited number of animals, the obtained data are in agreement with the results reported in a similar experimental model, with low intensity pulsed ultrasound (Hantes *et al.* 2004). Thus, it is possible to hypothesize that the enhancement of callus formation could be achieved irrespective of the kind of energy supplied. There are various mechanisms of action by which osteogenesis is enhanced through application of biophysical stimuli (Brighton *et al.* 2001). It has been claimed that fracture (bone) repair must be considered a regenerative process rather than a healing process, because the discontinuity is replaced by the formation of new bone tissue instead of scar tissue (Mora *et al.* 2006). Indeed the stimulation of bone progenitor cells is crucial in the treatment of bone discontinuity. Thus, it is quite obvious that every method for the stimulation of bone callus formation without any adverse effect, would offer a useful strategy of treatment both in normal situations and in case of particular fractures (*i.e.* stress fracture) or callus disorders. In the extant literature, there are mainly 4 methods to enhance new bone formation: electrical current directly applied to the fracture site, pulsed electromagnetic fields, capacitive coupling electric fields, and the use of ultrasound to produce mechanical stimulation. Nevertheless, it is uncontroversial that alternating electric current stimulation may accelerate the maturation of a callus with increase of the volume of callus itself (Kawamoto *et al.* 2005).

In vitro studies demonstrated that CCEF enhances the proliferation maturation and extracellular matrix protein synthesis of osteoblasts (Hartig *et al.* 2000). In particular, cell proliferation results from CCEF induced increase in TGF-ß1mRNA in osteoblastic cells by a mechanism involving the cytosolic Ca2+/calmodulin pathway (Brighton *et al.* 2001, Carl *et al.* 2001). Additionally, electric currents act promoting the differentiation of mesenchymal cells within the callus (Itoh *et al.* 2008).

In conclusion, since the treatment of experimental osteotomy was conducted only with external fixation without the insertion of cells and/or growth factors, this study confirms that electrical stimulation applied to a long bone fracture model accelerate callus formation presumably stimulating resident osteogenic cells. The number of animals for this study has been limited to 8 subjects, since economic and ethical reasons prevent the use of larger numbers (Ferdowsian and Beck 2011). Further studies, investigating biomechanical properties of stimulated callus versus non stimulated ones would add relevant information concerning the mechanical properties of developing callus. The present study suggests that trans-osseous application of electric current, derived from a properly modified CCEF device, and delivered through the central pin of the external fixator, enhances the formation of bone tissue al the level of the experimental osteotomy site. This method also demonstrated to be simple and devoid of complications. The translational value of these observations can be emphasized considering that sheep is recognized as an optimal experimental model for *in vivo* studies on bone tissue due to the similarities with humans in terms of weight, bone structure and regeneration (Sakar *et al.* 2001, Nuss *et al.* 2006).

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