Histol Histopathol (2015) 30: 1477-1485

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Chondrocyte differentiation for auricular cartilage reconstruction using a chitosan based hydrogel

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Summary. Tissue engineering with the use of biodegradable and biocompatible scaffolds is an interesting option for ear repair. Chitosan-Polyvinyl alcohol-Epichlorohydrine hydrogel (CS-PVA-ECH) is biocompatible and displays appropriate mechanical properties to be used as a scaffold. The present work, studies the potential of CS-PVA-ECH scaffolds seeded with chondrocytes to develop elastic cartilage engineered-neotissues. Chondrocytes isolated from rabbit and swine elastic cartilage were independently cultured onto CS-PVA-ECH scaffolds for 20 days to form the appropriate constructs. Then, *in vitro* cell viability and morphology were evaluated by calcein AM and EthD-1 assays and Scanning Electron Microscopy (SEM) respectively, and the constructs were implanted in *nu/nu* mice for four months, in order to evaluate the neotissue formation. Histological analysis of the formed neotissues was performed by Safranin O, Toluidine blue (GAG's), Verhoeff-Van Gieson (elastic fibers), Masson's trichrome (collagen) and Von Kossa (Calcium salts) stains and SEM. Results indicate appropriate cell viability, seeded with rabbit or swine chondrocyte constructs; nevertheless, upon implantation the constructs developed neotissues with different characteristics depending on the animal species from which the seeded chondrocytes came from. Neotissues developed from swine chondrocytes were similar to auricular cartilage, while neotissues from rabbit chondrocytes were similar to hyaline cartilage and eventually they differentiate to bone. This result suggests that neotissue characteristics may be influenced by the animal species source of the chondrocytes isolated.

Key words: Auricular cartilage, Microtia, Chondrocyte differentiation, Chitosan hydrogel, Endochondral ossification

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DOI: 10.14670/HH-11-642

Introduction

Different conditions may cause deformities or absence of external ear, such as congenital diseases like microtia, or trauma (Jiamei et al., 2008; Alasti and Van Camp, 2009), and this is why it is very important to produce biomaterials similar in structure and function to auricular cartilage. Pinna reconstruction involves a big challenge due to the difficulty in recreating the anatomical structure (a different anatomical structure for each patient) and physiological and biomechanical properties of the ear. Autologous transplantation from synchondrosis of the costal cartilage for ear reconstruction has been used for a long time (Chin et al., 2009; Yanaga et al., 2009; Chauhan and Guruprasad, 2012; Cho et al., 2012); however, this technique for the treatment of diseases such as microtia has some drawbacks, e.g.in cell harvesting or shaping the cartilage

framework. Several surgeries may be required for a better cosmetic result (often the aesthetic result is unsatisfactory), which implies more treatment time (Han and Son, 2002; Romo et al., 2006). Furthermore, this method may cause postoperative complications such as pneumonia or thorax deformation due to costal cartilage usage as a framework to build the ear shape.

In addition, biological and mechanical properties of hyaline are different from those of elastic cartilages, due to specific molecular components of the extracellular matrix (ECM) and growth factors of each tissue type (Daley et al., 2008). Auricular cartilage is rich in elastin content and its chondrocytes do not form bone (Eyre and Muir, 1975). On the other hand, hyaline cartilage may form bone via endochondral ossification (Staines et al., 2013), which implies high expression of collagen type X (Alvarez et al., 2001) and type I (Roach et al., 1995) produced by hypertrophic chondrocytes.

When biomaterials are used to recreate the structural and physiological properties of auricular cartilage, it is also important for the biomaterial to eventually degrade and allow cells to synthesize their characteristic ECM, and thus, to maintain or resume the corresponding tissue cell phenotype. In a previous study we demonstrated that chemical and biomechanical properties of the chitosan-polyvinyl alcohol hydrogel crosslinked with epichlorohydrin, and we proposed it as a potential biomaterial to be used in tissue engineering applications (Garnica-Palafox et al., 2014). In this paper, we analyze whether or not auricular chondrocytes isolated from cartilage of rabbit and swine resume or modify their differentiated phenotype when implanted, immersed in a chitosan-based hydrogel.

Materials and methods

Reagents and animal management

Auricular chondrocytes were cultured in DMEM-F12, Serum Bovine Fetal (SBF) and penicillin-streptomycin, all from Life Technologies (Carlsbad, CA). Viability was assessed with a Live/Dead viability/cytotoxicity kit for mammalian cells from Molecular Probes (Eugene, OR).

All animals were treated in accordance to the Public Health Service Policy on Humane Care and Use of Laboratory Animals (August, 2002), implemented by the Office of Laboratory Animal Welfare, Harvard Medical School IACUC.

Animals were kindly donated by the Facultad de Medicina Veterinaria y Zootecnia, UNAM; Mexico City, Mexico. Carbon dioxide (CO_2) euthanasia was performed in newborn swine and rabbits.

Three rabbits were used to develop constructs implanted and analyze neotissues at one month, three rabbits to analyze neotissues at four months; and three swine were used to produce neotissues and analyze it at four months. Three neotissues were developed per animal.

Isolation and expansion of chondrocytes

Elastic cartilage was obtained from ears of 5 week old New Zealand rabbits and newborn swine. Cartilage was washed in Phosphate Buffer Saline (PBS) solution with 10% penicillin-streptomycin, connective tissue was removed and cartilage was cut into ≈1 mm^3 pieces and digested with 3 mg/ml collagenase I. Isolated chondrocytes were passed through a 100 μm nylon cell strainer and centrifuged to obtain a pellet. Swine and rabbit auricular chondrocytes were plated at $2x10^4$ cells/cm^2 for cell expansion in DMEM-F12 with 10% FBS and 1% penicillin-streptomycin, and incubated at 37°C with 5% CO_2. Culture medium was changed every 36 hrs.

Generation of the CS-PVA-ECH construct

After 8 days, chondrocyte cultures reached about 90% confluence; then, chondrocytes were trypsinized and cell expansion continued until first passage before they were seeded onto CS-PVA-ECH films. Constructs were formed seeding $7.5x10^5$ chondrocytes/cm^2 on CS-PVA-ECH disc-shaped copolymers and kept under standard culture conditions for 20 days. On day 15, constructs were encapsulated according to the methodology previously described (Masri et al., 2007), with 2-3 chondrocyte monolayers grown separately. Monolayers were carefully detached from the culture flask surface using tweezers. After 5 days in culture, swine and rabbit constructs were encapsulated with chondrocytes monolayers and subcutaneously implanted in 6 week old *nu/nu* athymic mice for either 1 month or 4 months to allow neotissue formation.

Viability assay

Viability of monolayer culture as well as constructs of swine and rabbit auricular chondrocytes was evaluated with a Live/Dead viability/cytotoxicity kit for mammalian cells (Molecular Probes), according to the manufacturer's instructions. Fluorescent calcein green signal is positive for viable cells and EthD-1 red signal is positive for dead cells. Images were captured and analyzed by fluorescence microscopy (Axiovision Observer A.1 microscope, Zeiss).

Scanning electron microscopy

Neotissues and constructs were washed twice with PBS buffer, fixed in a 2.5% glutaraldehyde/0.1 M PBS buffer (pH=7.4) and placed over an aluminum holder, secured with carbon tape and analyzed with a Scanning Electron Microscope, SEM (XL-30 Phillips Electronics, Holland) in a low vacuum environment. Energy-Dispersive X-ray spectrometry (EDAX-New XL-30, USA) was also performed. SEM working conditions were 25 kV, Spot 5, 10% dead time, 200 sec live time, 10 mm working distance (WD), 2200 cpm and 0.5 Torr.

Histological analysis

Neotissues were fixed with paraformaldehyde (PFA) 4% and embedded in paraffin. Microtome sections of 5 μm thick were stained with Safranin-O for proteoglycan detection, toluidine blue for polysaccharides, Verhoeff-Van Gieson for elastic fibers, Masson's trichrome for collagen fibers and Von Kossa for calcium precipitates.

Statistical analysis

For chondrocyte viability, calcein positive cells and EthD-1 positive cells were counted individually and the percentage of viability was obtained from all the cells seeded. At least three independent assays were performed. Graph values are expressed as means ± SD (mean standard deviation). Each group of data passed a normality test before it was analyzed with parametric statistics. Comparison between groups was performed using Student's t test and $p<0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism 5 software.

For histochemistry analysis, photographs of neotissues for each dye (Safranin O, Verhoeff's elastic fibers and Von Kossa) were analyzed. Analysis of the stained area percentage was performed using ImageJ software, and data were statistically analyzed with GraphPad Prism software using a 1 way ANOVA methodology, Bonferroni's Multiple Comparison Test; $p<0.05$ was considered statistically significant.

Results

Swine and rabbit auricular chondrocytes maintain cell morphology when expanded in vitro

Auricular chondrocytes from neonatal swine and rabbit were cultured and expanded *in vitro* in order to increase cell number and to determine their morphological characteristics. Both swine and rabbit chondrocytes were able to attach, spread and proliferate over the surface of the culture flask. At the beginning of primary culture, auricular chondrocytes maintained their typical polygonal morphology, reaching confluence 8 days after culture started. There was no difference in cell types during the *in vitro* expansion, and cell morphology was consistent (Fig. 1A,B). Swine and rabbit auricular chondrocyte viability was evaluated according to the methodology previously mentioned (Fig. 1E,F).

Calcein AM is a cell-permeable molecule, which is converted into fluorescent calcein by esterase activity within live cells. After counting individual cells in different fields, viability of swine and rabbit auricular chondrocytes in monolayer was found to be about 90% and there were no significant differences between both species. However, a tendency indicating a slightly higher number of EthD-1 positive cells in rabbit than in swine chondrocytes (Fig. 1G) was observed. Although chondrocyte's viability is high in both species, this small difference may result in a wrong development of the auricular cartilage.

CS-PVA-ECH hydrogel is cytocompatible with swine and rabbit auricular chondrocytes

We have previously reported the potential use of chitosan hydrogels as a scaffold in tissue engineering techniques (Garnica-Palafox et al., 2014). To determine the CS-PVA-ECH hydrogel potential for neotissue formation, swine and rabbit auricular chondrocytes were seeded onto disc-shaped hydrogels (Fig. 2A). Both swine and rabbit auricular chondrocytes attached and grew onto the surface of the CS-PVA-ECH hydrogel (Fig. 1C,D). Cytocompatibility was proven since chondrocytes remained viable after 20 days of culture onto the hydrogel (Fig. 2B). Scanning Electron Microscopy revealed that both swine and rabbit auricular chondrocytes formed a monolayer over the hydrogel surface and started to secrete a surrounding ECM (Fig. 2C,D).

Swine and rabbit auricular chondrocytes cultured onto CS-PVA-ECH hydrogels formed neotissues with different characteristics

Either 1 or 4 months after implantation, mice were ethically sacrificed and neotissues obtained. Both cell types generated neotissues with different characteristics; four months after construct implantation, rabbit chondrocytes developed stiff and yellowish neotissues with an external vascularized layer penetrating the tissue (Fig. 2E). Meanwhile, swine neotissues presented a white coloration and were soft, resembling native elastic cartilage. There was no vascularized layer covering swine neotissues (Fig. 2F).

In order to determine the structure of neotissues, Scanning Electron Microscopy was performed. Analysis revealed that the rabbit engineered-tissue was porous and had architecture similar to trabecular bone; space between rabbit chondrocytes was reduced and the tissue presented a more dense composition, suggesting the presence of mineralized tissue (Fig. 2G,I). On the other hand, swine engineered-tissue displayed a less dense ECM with auricular chondrocyte isogenic groups organized in lacunae and surrounded by an extracellular matrix. Most of these chondrocytes were separated from each other and the space among them was filled by abundant ECM (Fig. 2H,J). Chondrocytes in rabbit tissue-engineered cartilage were slightly bigger than those found in swine tissue-engineered cartilage.

Rabbit and swine auricular chondrocytes cultured onto CS-PVA-ECH hydrogel form different types of cartilage

Auricular chondrocytes generally do not reach hypertrophy and do not form bone; this distinguishes them from chondrocytes of hyaline cartilage; ECM and growth factors trapped inside it regulate these processes.

Both types of cartilage have large amounts of glycosaminoglycan regulating the action of TGF-β and BMPs; however, they may also act as binding domains for cell receptors in chondrocytes. The CS-PVA-ECH hydrogel contains large amounts of glycosaminoglycan (GAG's), so we thought this might encourage these processes. Initially, we wanted to test these mechanisms in two animal models, rabbit and swine, thinking we would obtain similar results; however, results varied depending on the source of cells species, cell density and post-implantation time.

Constructs using rabbit or swine cells at a density of 750,000 cell/cm^2 formed cartilage; this can be observed by the Safranin-O staining (Fig. 3A-C) and Toluidine staining (Fig. 3D-F), which indicates cartilage formation due to its affinity with the GAG's sulfated groups. Four months after implantation (Fig. 3C), swine cells formed cartilage at an equivalent of 70.06±13.07% the total area of the implant tissue section (Fig. 4), while rabbit cells showed a cartilage formation covering only 2.14±0.36% of the implant tissue section total area (Fig. 3B). However, after 1 month of implantation, neotissue from rabbit cells showed cartilage covering 31.56±4.4% of the total area (Fig. 3A); the remaining area (at 1 and 4 months after implantation) presented a phenotype similar to that of bone tissue, indicating that chondrocytes continued their differentiation for endochondral bone formation, while swine chondrocytes maintained the auricular cartilage phenotype for the whole implantation period.

The challenge for pinna reconstruction is to form a tissue with similar characteristics to auricular cartilage

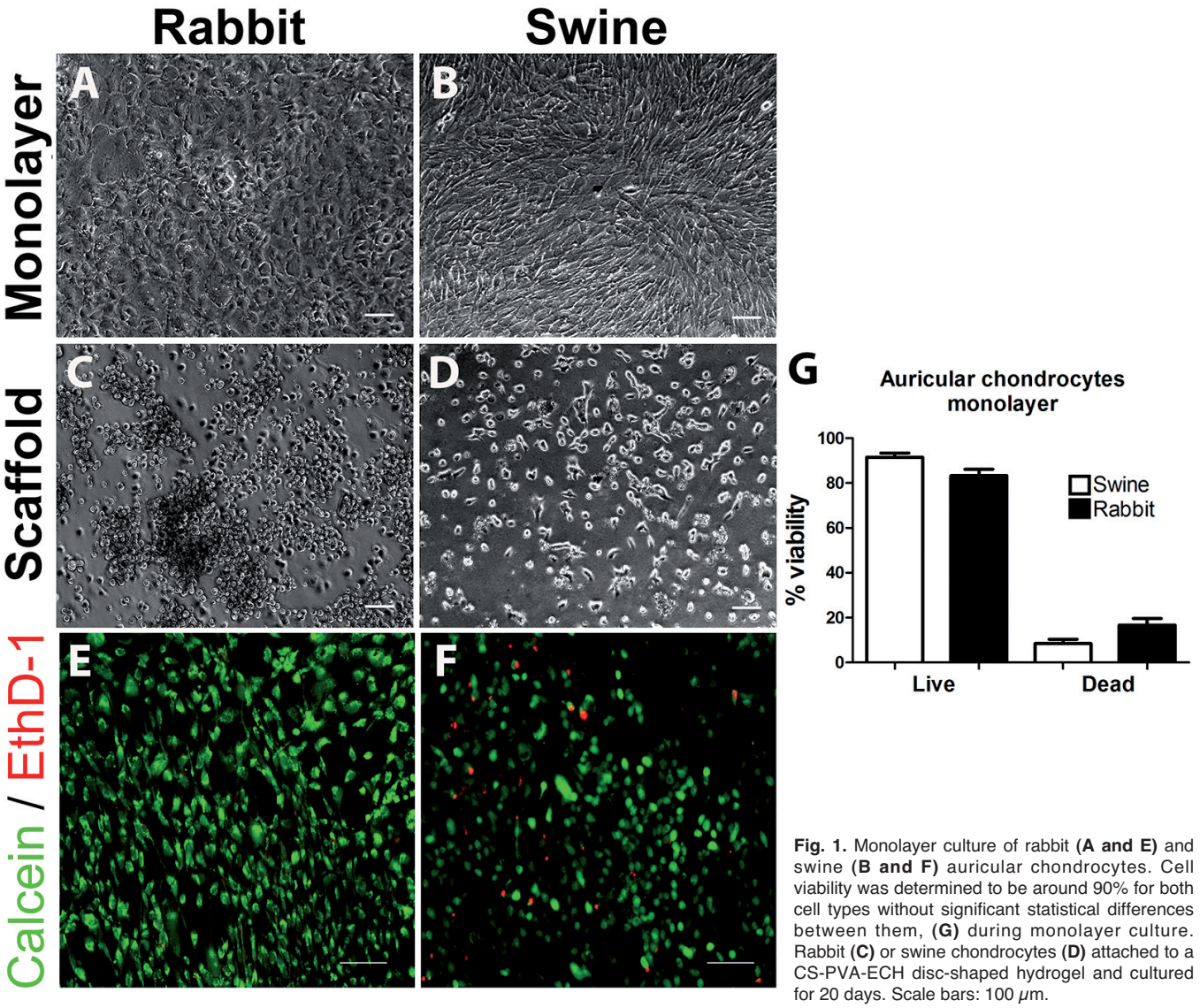

Fig. 1. Monolayer culture of rabbit **(A and E)** and swine **(B and F)** auricular chondrocytes. Cell viability was determined to be around 90% for both cell types without significant statistical differences between them, **(G)** during monolayer culture. Rabbit **(C)** or swine chondrocytes **(D)** attached to a CS-PVA-ECH disc-shaped hydrogel and cultured for 20 days. Scale bars: 100 μm.

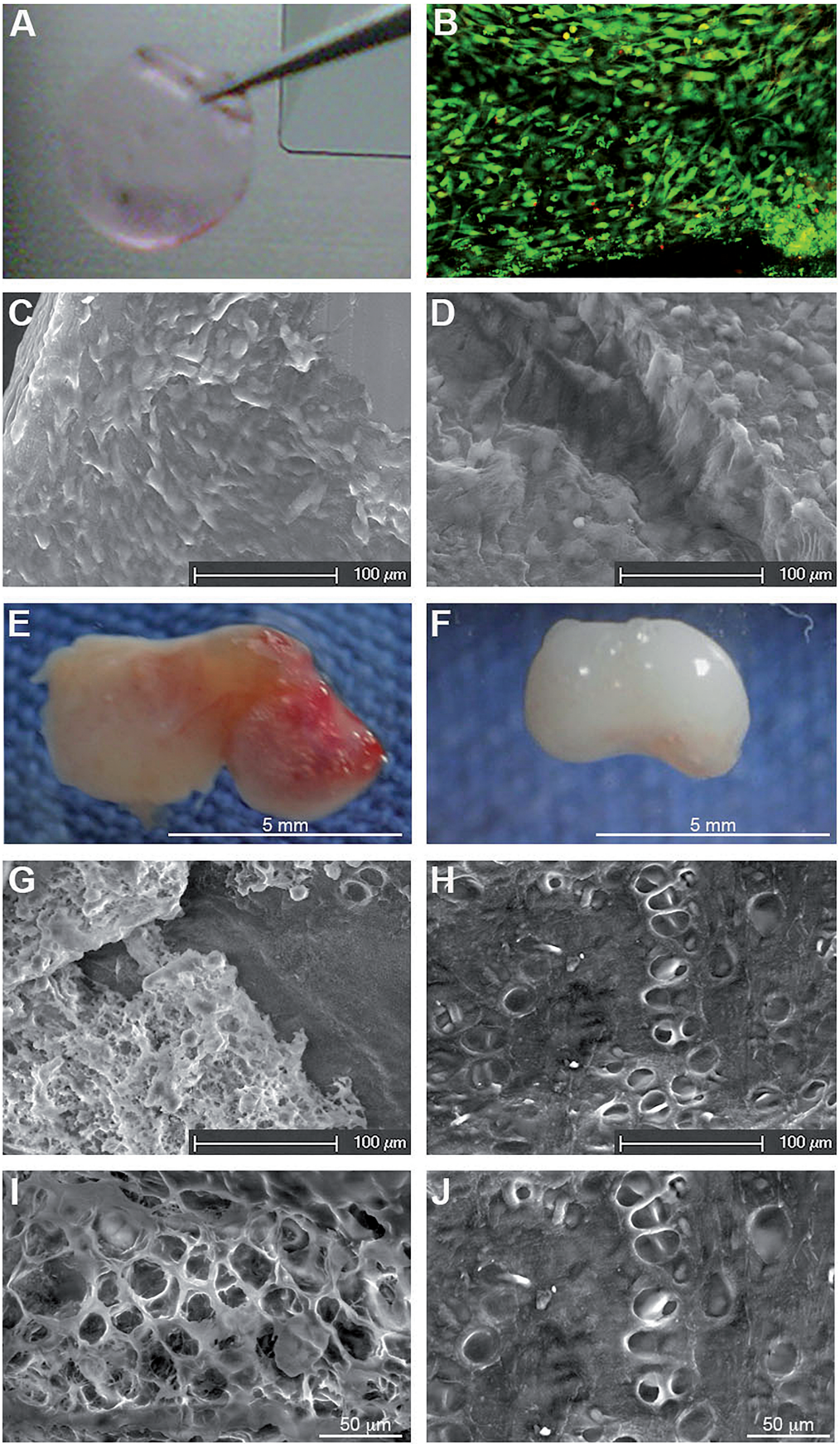

Fig. 2. Auricular chondrocytes and CS-PVA-ECH hydrogel constructs previous to implantation **(A)**. Rabbit and swine auricular chondrocytes were viable after a 20 days culture onto hydrogels **(B)**. SEM analysis of rabbit and swine chondrocyte constructs after a 20 days culture **(C and D)**. Gross morphology of tissue-engineered cartilage after 4 months of implantation showing a yellowish vascularized tissue (rabbit chondrocytes, **(E)** and a white and soft elastic-like tissue (swine chondrocytes, **(F)**. Rabbit neotissues had a mineralized and trabecular composition similar to bone tissue **(G and I)**. Swine neotissues contained chondrocytes distributed in lacunae with a dense ECM **(H and I)**.

(elastic cartilage); the Verhoeff/Van Gieson staining (Fig. 3G-I) mainly stains elastic fibers. This staining revealed a 49±10.39% positive staining, indicating the presence of elastic fibers in the neotissue with swine cells (Fig. 3I), whereas the neotissues formed from rabbit cells was negative for this stain at 0.08±0.07% (Fig. 3G,H), indicating a clear difference between the types of neocartilage formed, depending on the animal species (Fig. 4). Despite the fact that cells from the two different animal species were auricular chondrocytes, rabbit chondrocytes retook an endochondral differentiation pattern, while swine chondrocytes maintained the elastic cartilage phenotype.

The presence of mature collagen, evidenced by intense Masson's staining (Fig. 3J-L), was observed in all neotissues with rabbit cells in the bone matrix at 1 month after implantation (Fig. 3J) and 4 months after implantation (Fig. 3K), while mature collagen in

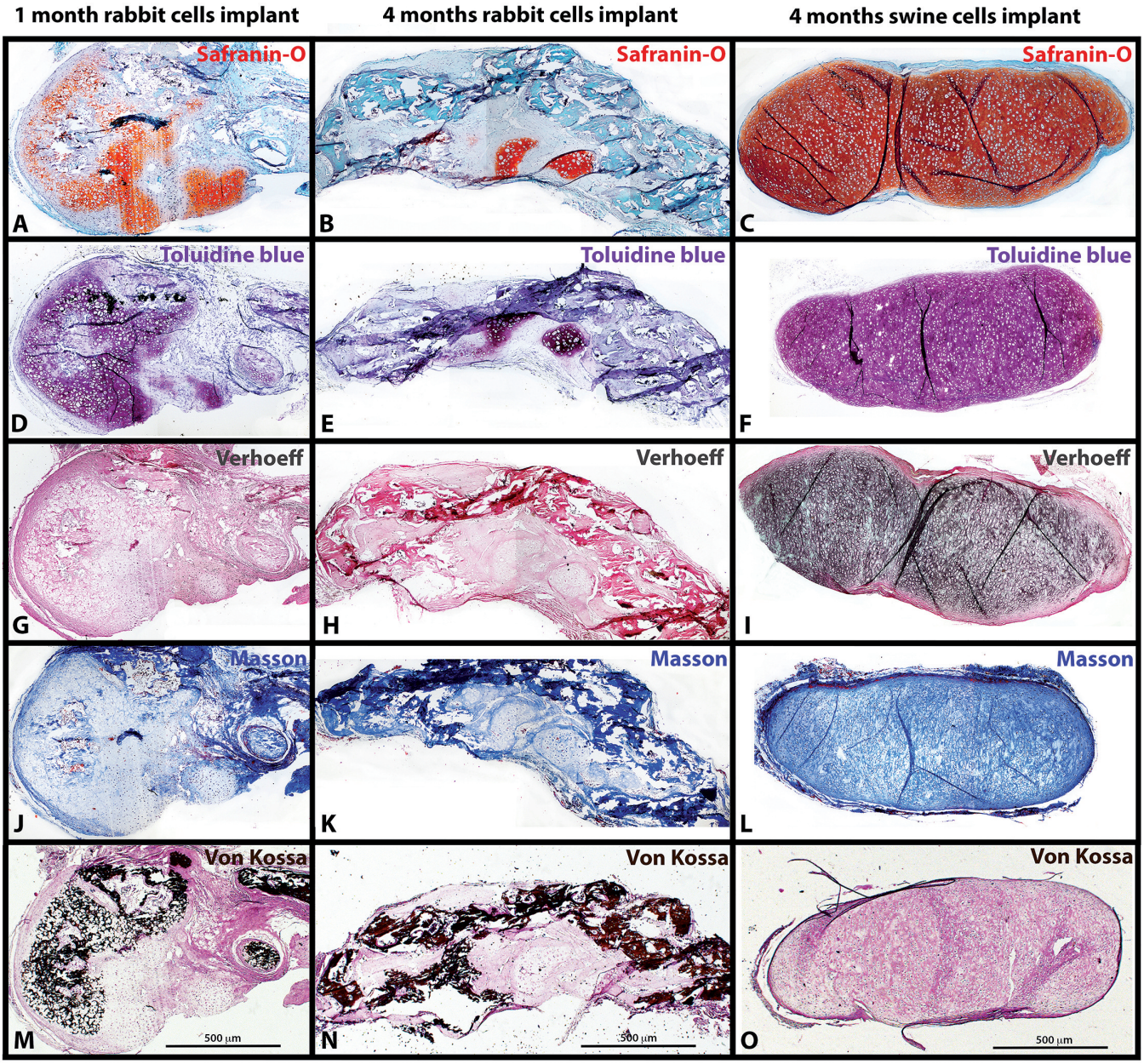

Fig. 3. Safranin-O **(A-C)**, Toluidine blue **(D-F)**, Verhoeff/Van Gieson **(G-I)**, Masson's trichrome **(J-L)** and Von Kossa **(M-O)**. Staining for constructs after 1 month **(A, D, G, J and M)** and 4 months **(B, C, E, F, H, I, K, L, N and O)** of implantation with rabbit and swine cells seeded at a density of 750,000 cell/cm^2. Scale bar: 500 μm.

implants with swine cells is only present in the perichondrium of the cartilage formed (Fig. 3L). Finally, with Von Kossa stain (Fig. 3M-O) we determined about 13.98±0.85% (Fig. 4) of the total area to be bone in neotissues with rabbit cells after 1month of implantation (Fig. 3M) and 36.71±6.8% after 4 months (Fig. 3N). Opposite to this, mature neotissues with swine cells after 4 months of implantation were completely negative for calcium precipitates and bone formation (Fig. 3O).

Discussion

In this work, the potential of a CS-PVA cross-linked with ECH hydrogel to generate auricular cartilage-engineered neotissues was evaluated. Crosslinking of a chitosan-PVA hydrogel by ECH increases its stability and slows down the degradation rate. We first isolated and expanded *in vitro* rabbit and swine auricular chondrocytes from ear cartilage to increase cell number. Both cell types were able to proliferate in culture conditions and reached confluence after 1 week of culture.

It has been reported that chondrocytes change their morphology after serial passages when cultured in a monolayer, this change being accompanied by a change in the ECM protein expression (Ruszymah et al., 2007). However, in the present study, rabbit chondrocytes maintained their typical polygonal-rounded shape morphology during monolayer culture, while swine chondrocytes moderately turned into a spindle-shape after 8 days of culture, reaching approximately 90% of viability.

These results show similar phenotype and viability for chondrocytes cultured in a plastic dish or onto a scaffold. Although there were no significant statistical differences between swine and rabbit cell viability, cultures from rabbit cells presented a few more dead cells than those from swine cells, and so,it is being planned to analyze the effect of cell death on auricular chondrocyte differentiation in rabbit chondrocytes. After seeding both chondrocytes onto CS-PVA-ECH hydrogels and culturing them for 20 days, the rounded shape was regained and chondrocytes were able to survive, as demonstrated through calcein assay (Fig. 2B).

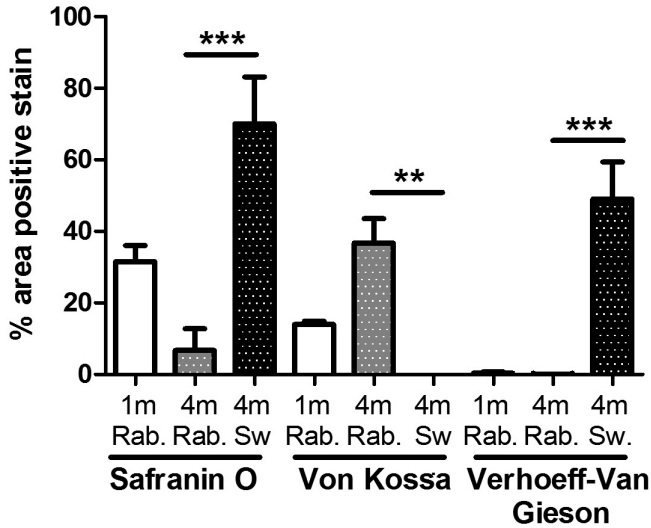

Fig. 4. Statistical evaluation of histochemistry data. Percentage of positive area for Safranin-O, Von Kossa and Verhoeff-Van Gieson stains in neotissues with rabbit cells at 1 and 4 months, and swine cells at 4 months; mean values are shown with standard deviations (n=3 independent experiments). (ANOVA P<0.0001).

Cytocompatibility is one of the main features of a successful biomaterial and it is closely related to its chemical composition and structure. The biomaterial surface will interact with chondrocyte membrane proteins promoting cell attachment, as shown by SEM analysis (Fig. 2C,D). Chitosan is a linear, semi-crystalline polysaccharide composed of N-acetyl D-glucosamine and D-glucosamine units (Croisier and Jérôme, 2013). Rabbit and swine auricular chondrocytes were able to attach and survive onto the hydrogel after 20 days of culture, conserving their typical morphological characteristics; they also remained viable as depicted previously. It has been described that CS possess structural similarities to glycosaminoglycans (GAG's) (Ravi Kumar, 2000), which may promote chondrocyte attachment and hence cell survival.

We have previously described rabbit chondrocyte viability and proliferation onto CS-PVA-ECH hydrogel (Garnica-Palafox et al., 2014). In this work we wanted to test, besides viability, the potential of this material for future applications in the tissue engineering field as a model for pinna reconstruction. Cell attachment is given by the physical characteristics and chemical composition of chitosan during the neutralization process (Noriega and Subramanian, 2011). In the present work, Scanning Electron Microscopy analysis demonstrated that rabbit and swine auricular chondrocytes, attached to a hydrogel surface, were able to form a monolayer over that surface. An explanation for this result could be the interaction of chondrocyte membrane proteins (i.e. integrins) with positively charged amino groups along the chitosan chains, which in turn may activate signaling pathways leading to ECM deposition, and cell-cell and cell-ECM interactions (Fig. 2C,D).

Rabbit and swine auricular chondrocytes presented the same behavior in culture conditions, after constructs were formed and before implantation in *nu/nu* athymic mice model. However, 4 months after implantation, constructs from rabbit or swine cells generated engineered tissues with dissimilar and particular characteristics.

Rabbit chondrocytes formed neotissues with a vascularized layer covering them; they had a yellowish coloration and were stiff to contact, like bone tissue. This indicates that chondrocytes may have undergone a differentiation process leading to hypertrophy and bone formation as the final step. This hypothesis was

supported after analyzing neotissues by SEM. Bone ECM consists of mineralized matrix with collagen fibers producing a rigid tissue (Hassenkam et al., 2004); rabbit auricular chondrocytes formed an engineered neotissues with a dense ECM, similar in composition and architecture to trabecular bone. The ECM showed small hollow spaces that may have been once occupied by chondrocytes.

Instead, swine auricular chondrocytes formed engineered neotissues of white coloration and without an exterior vascularized layer; neotissues were soft and resembled native elastic cartilage. SEM analysis revealed a less dense ECM with chondrocytes organized in lacunae and forming isogenic groups. Spaces between chondrocytes were wider, and abundant ECM surrounded the cells. This arrangement could be related to phenotype preservation, since it has been described that cell-ECM interactions activate important signaling pathways involved in cartilage ECM protein expression. The way in which activation of some of these pathways occurs is determinant for maintaining a prehypertrophic state and forming cartilage tissue, or promoting hypertrophy and generating bone tissue. Since we found big differences in neotissue formation between two different animal species, it would be interesting to analyze the capability of our scaffold to maintain human chondrocyte phenotype.

Rabbit and swine auricular chondrocytes generated different types of cartilage when seeded onto chitosan scaffolds and implanted in athymic mice. Although both chondrocytes types form cartilage, their differentiation programs are different. Rabbit chondrocytes continued their differentiation into hypertrophic chondrocytes, which could be observed by safranin-O stain at one month and four months of implantation (Fig. 3A,B).

In a previous work, scaffold-free elastic cartilaginous constructs with rabbit auricular chondrocytes formed a similar structure to auricular cartilage; however, type X collagen was expressed without calcium salts deposited. Though that work was developed for only four weeks in a bioreactor, the chondrocytes reached the hypertrophy but did not form bone (Giardini-Rosa et al., 2014).

Nevertheless, in the present work it was also possible to observe regions where bone began to form a mineralized matrix, detected by Von Kossa staining (Fig. 3M), indicating that the rabbit chondrocytes continued the differentiation program to form endochondral bone, as they do in the hyaline cartilage of the long bones and growth plate; these implants were almost completely ossified four months after implantation (Fig. 3N).

Swine auricular chondrocytes under the same conditions did not undergo a cartilage differentiation program to form endochondral bone. These chondrocytes maintained the auricular phenotype; even four months after implantation the ECM presented a rich content of elastic fibers, as evidenced by Verhoeff staining (Fig. 3I), and proteoglycans, as evidenced by the safranin-O staining, and was completely negative for bone formation, evidenced by the Von Kossa staining (Fig. 3C,O). This is characteristic of auricular cartilage (particularly the presence of elastin).

Still, we do not know the signals or mechanisms of the cell differentiation programs producing the differences found in rabbit and swine chondrocytes. It would be interesting to find out which characteristics of swine chondrocytes allow them to form exclusively auricular cartilage, in order to modify the cell differentiation program with rabbit chondrocytes and direct their differentiation phenotype exclusively to the auricular phenotype. This may allow development of neotissues with the necessary mechanical and biological features for a better implant.

The present study also shows that swine chondrocytes are better for ear tissue engineering than rabbit chondrocytes. If results with human chondrocytes were similar to those of the swine cells CS-PVA-ECH hydrogel constructs, then the CS-PVA-ECH hydrogel could be a promising material for the treatment of microtia patients.

Acknowledgements. The authors thank MVZ Hugo Lecona Butrón for their technical assistance. This work was supported by grants CONACyT Sectoriales: 114359 (to C. Velasquillo) and CONACyT Ciencia Básica: 84683 (to D. Garciadiego) from Consejo Nacional de Ciencia y Tecnología, México.

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Accepted June 29, 2015