

Dead, frozen and revived: how xenotransplantation can contribute to species conservation

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Abstract

Wildlife-vehicle collisions represent a severe threat to the genetic diversity of the jaguar (*Panthera onca*). Rescuing high-quality genetic material from vehicular casualties is often limited by the prolonged post-mortem interval (PMI), which leads to advanced tissue degradation. This study evaluates an innovative pipeline combining solid-surface vitrification and xenotransplantation into immunodeficient mice to revitalize compromised jaguar tissues. Auricular cartilage collected approximately 24 h post-mortem was vitrified, warmed, and grafted into subcutaneous (XenoSC) and intraperitoneal (XenoIP) sites of NOD scid gamma (NSG) mice for seven days. Both grafting sites successfully promoted neovascularization and tissue-level revitalization. Notably, the XenoSC group exhibited superior structural recovery, characterized by enhanced fibroblast proliferation and lacunar filling ($p < 0.05$). Fibroblasts recovered from xenotransplanted tissues maintained $> 80\%$ viability and successfully reached the third passage, whereas ungrafted post-warming controls failed to yield somatic cell outgrowth. These findings demonstrate that xenotransplantation effectively restores the biological competence of tissues undergoing post-mortem decomposition. This methodology provides a robust tool for enriching germplasm biobanks and supporting advanced assisted reproduction programs for the conservation of threatened felids.

Introduction

The accelerated loss of biological diversity represents one of the most pressing global environmental challenges, indicating an ongoing sixth mass extinction¹. Among the anthropogenic factors driving this decline, road infrastructure expansion stands out as a primary cause of wildlife population reduction². Annually, billions of animals are killed in wildlife- vehicle collisions worldwide³. Estimates suggest that over two million mammals perish on Brazilian roads alone^{2,4}. Medium and large mammals exhibit greater susceptibility to these accidents due to their body mass, dispersal patterns, and low population densities^{5,6}.

The jaguar (*Panthera onca*), the largest carnivore in the Americas and a keystone species across its ecosystems, has experienced severe population declines⁷. Within this context, vehicle collisions represent a critical manifestation of human-wildlife conflict. Wildlife road crossings pose a recurrent threat, adversely impacting both animal populations and road safety⁸. For instance, in Brazil, at least six jaguar-vehicle collision casualties were recorded during the first half of 2023: four in the Pantanal (Miranda, Mato Grosso do Sul) and two in the Atlantic Forest (Teodoro Sampaio, São Paulo). This critical situation remains ongoing, with at least five additional fatalities documented across Mato Grosso do Sul highways in 2025 alone (three on BR-262, one on BR-267, and one on BR-163). The 2023 mortalities in the Atlantic Forest occurred near Morro do Diabo State Park, where the local jaguar population is estimated at fewer than 20 individuals⁹. Given that the Atlantic Forest jaguar population is critically endangered, comprising fewer than 300 individuals distributed across fragmented subpopulations¹⁰,

and that only a fraction of wildlife-vehicle collision casualties are detected, such mortalities constitute a severe threat to the species' conservation.

Given the jaguar's ecological and scientific significance, implementing effective tools to maintain its genetic variability is crucial, particularly concerning the utilization of biological material from deceased individuals^{11–13}. Consequently, biobanks serve as an essential strategy for *ex situ* conservation, aligning with the "One Conservation" framework¹⁴. Biobanking enables the cryopreservation of gametes, embryos, and, notably, viable somatic tissues. These resources can be employed in advanced assisted reproduction programs, such as somatic cell nuclear transfer (SCNT) protocols, thereby providing a valuable asset for genetic rescue^{11,15–17}.

Although several studies have advanced jaguar somatic cell conservation—addressing tissue selection¹⁸, *in vitro* culture optimization¹⁹, and cryoprotectant evaluation²⁰—maintaining the viability of genetic material from deceased individuals, particularly wildlife-vehicle collision casualties, remains a persistent challenge. These samples frequently exhibit advanced deterioration due to prolonged post-mortem intervals (PMI), compromising their suitability for biobanking and reproductive programs. To overcome this limitation and maximize the utilization of this genetic heritage, tissue revitalization strategies are imperative. Xenotransplantation into immunodeficient mouse models has emerged as a promising tool in assisted reproduction and conservation, demonstrating the capacity to promote neovascularization and restore the viability and biological competence of vitrified or severely compromised tissues^{21,22}.

Therefore, this study evaluates xenotransplantation in immunodeficient mice as an innovative methodology for tissue revitalization—emphasizing neovascularization and cellular viability—and for the qualification of cultured somatic cells derived from jaguar vehicular casualties. Ultimately, this approach aims to expand and enhance biobanking efforts for the species' conservation.

Results

Histological analyses

Auricular cartilage samples were obtained from three jaguars (Jaguar#01, Jaguar#02, and Jaguar#03), which were fatally injured in vehicular collisions on Highway BR-262, and subsequently transported to Reprogen/UFMS for processing (Fig. 1). For purposes of traceability and reference, these specimens were registered in Reprobank.org under the following unique identifiers: 4CGXQ-A0087 (Jaguar#01), 4CGXQ-A0088 (Jaguar#02), and 4CGXQ-A0089 (Jaguar#03).

Upon receipt at the laboratory, the cartilaginous tissue fragments exhibited a paler coloration than expected, likely due to post-mortem ischemia. Seven days post-xenotransplantation, macroscopic observation revealed explant encapsulation and a pinker hue, indicative of successful reperfusion. This visual change was more pronounced in intraperitoneal explants (XenoIP) compared to subcutaneous explants (XenoSC).

Jaguar#01

Quantitative histological analysis of Jaguar#01 (Table 1) revealed that subcutaneously xenotransplanted explants (XenoSC) contained a significantly higher number of fibroblasts ($p = 0.0334$) compared to the Post-warming group, and a significantly higher count ($p < 0.001$) than the intraperitoneally xenotransplanted explants (XenoIP).

Additionally, both the XenoIP and XenoSC groups exhibited a significantly higher number of normal chondrocytes ($p = 0.0013$ and $p = 0.0008$, respectively) relative to the Post-warming group. Conversely, the XenoSC group demonstrated a lower incidence of degenerated chondrocytes compared to the Post-warming group ($p = 0.0011$). Regarding cartilaginous lacunae, the highest percentage of filled lacunae was identified in the XenoIP group ($p = 0.0199$ compared to Post-warming; $p = 0.0065$ compared to XenoSC). Correspondingly, empty lacunae were significantly more prominent in the XenoIP group ($p < 0.001$) relative to both other groups.

Table 1

Consolidated cellular quantification in the auricular cartilage of three jaguars across Fresh, Post-warming, and Xenotransplantation (Intraperitoneal and Subcutaneous) treatment groups.

Animal	Group	Fibroblasts	Chondrocytes		Lacunae	
			Normal	Degenerated	Filled	Empty
Jaguar#01	Post-warming	20.6 ± 7.2	7.5 ± 3.3	20.1 ± 4.3	39.3 ± 4.3	6.5 ± 2.1
Jaguar#01	XenoIP	11.1 ± 5.5	15.8 ± 5.5	16.0 ± 7.0	46.7 ± 7.0	25.6 ± 5.2
Jaguar#01	XenoSC	28.4 ± 9.5	15.0 ± 5.1	11.3 ± 5.9	39.3 ± 5.9	13.6 ± 7.1
Jaguar#02	Fresh	25.1 ± 12.6	17.1 ± 4.7	26.3 ± 9.8	43.8 ± 9.0	0.8 ± 0.9
Jaguar#02	Post-warming	14.6 ± 6.8	11.9 ± 3.5	38.5 ± 6.5	50.8 ± 9.5	2.2 ± 1.1
Jaguar#02	XenoIP	5.7 ± 4.7	22.3 ± 8.1	53.7 ± 14.5	76.0 ± 16.5	7.0 ± 6.9
Jaguar#02	XenoSC	17.2 ± 9.0	20.8 ± 12.9	19.6 ± 9.8	40.4 ± 15.1	4.2 ± 4.4
Jaguar#03	Post-warming	6.5 ± 3.5	24.6 ± 3.5	43.5 ± 7.1	68.1 ± 10.4	2.1 ± 3.0
Jaguar#03	XenoIP	1.8 ± 1.9	15.2 ± 9.4	47.4 ± 16.1	62.6 ± 17.2	13.6 ± 9.5
Jaguar#03	XenoSC	7.4 ± 4.7	21.0 ± 8.1	39.8 ± 9.8	60.8 ± 12.0	8.65 ± 5.7

Data are presented as Mean $\pm$ Standard Deviation (SD). Superscript letters (a, b, c) denote significant differences ($p < 0.05$) between treatment groups **within the same animal**.

Qualitative histological evaluation (Fig. 2) revealed that, post-warming, the post-warming explant (Fig. 2A, B) displayed active and disorganized loose connective tissue, characterized by degenerating fibers and increased intercellular spacing. The XenoSC explant (Fig. 2E, F) demonstrated mild to moderate neovascularization near the perichondrial region, alongside abundant lacunae filled with viable chondrocytes and numerous chondroblasts. In contrast, the XenoIP explant (Fig. 2C, D) exhibited degenerating elastic cartilaginous tissue, characterized by fibroblasts with normal morphology but minimal proliferation.

Jaguar#02

Histological evaluation of Jaguar#02 further elucidated the tissue-level effects of xenotransplantation. Fresh tissue (Fig. 3a) exhibited a normal morphology, featuring viable chondrocytes and organized, active loose connective tissue occupying most of the perichondrial region, alongside rare inflammatory cells. Conversely, post-warming tissue (Fig. 3b) displayed a perichondrium with disorganized collagen fibers and wide intercellular spaces. A marked, diffuse presence of degenerated fibrocytes and fibroblasts was observed, alongside moderately empty lacunae or lacunae containing degenerated chondrocytes. Mild to moderate eosinophilic clumping was also noted within the extracellular matrix, indicating degeneration. However, following subcutaneous xenotransplantation (Fig. 3c), highly organized proliferation of active loose connective tissue was observed surrounding the elastic cartilaginous matrix. The cartilaginous region featured numerous lacunae filled with viable chondrocytes and abundant chondroblasts. Mild to moderate neovascularization occurred in the subcutaneous region adjacent to the perichondrium, and the extracellular matrix appeared homogeneous and well-distributed.

In the XenoIP explant from this animal (Fig. 3d), a degenerative process was evident, characterized by a granular, eosinophilic extracellular matrix. Lacunar walls were thickened, and lacunae were predominantly occupied by degenerated chondrocytes and moderate multifocal necrotic chondrocytes. The perichondrium was mildly thickened, containing moderate fibrocytes, normal fibroblasts, and rare proliferating fibroblasts.

Cellular quantification (Table 1) revealed that, among the post-warming treatments, only the XenoSC group showed no significant difference ($p = 0.8375$) compared to the Post-warming group, although it trended toward a difference when compared to the Fresh group ($p = 0.0556$). The XenoIP treatment exhibited the lowest fibroblast count compared to all other groups ($p < 0.001$ vs. Fresh; $p = 0.0245$ vs. Post-warming; $p = 0.0001$ vs. XenoSC). However, the number of normal chondrocytes in the XenoIP group was significantly higher than in the Post-warming group ($p = 0.0247$). Furthermore, the number of filled lacunae in the XenoIP group was significantly higher than in the other treatments ($p < 0.001$).

Jaguar#03

Histopathological analysis of Jaguar#03 (Fig. 4a) revealed that the post-warming tissue exhibited an irregularly distributed perichondrium and degenerating collagen fibers within the connective tissue, accompanied by widened intercellular spaces. Active fibroblasts were observed moderately and diffusely, alongside rare multifocal proliferative fibroblasts. The cartilaginous tissue underwent degeneration, characterized by lacunae almost entirely occupied by chondrocytes in various stages of degeneration. In some instances, swollen, basophilic nuclei were noted, displaying prominent nucleoli and/or clumped chromatin, alongside several empty lacunae.

In the XenoIP tissues (Fig. 4b), encapsulation by fibroblasts was evident, accompanied by the presence of inflammatory cells (lymphocytes, plasma cells, and macrophages) and cellular debris. One extremity of the capsule was more adherent and exhibited neovascularization. The perichondrium contained densely and regularly distributed collagen fibers, with mildly degenerated cytoplasm. Moderate to severe fibrocytes were also diffusely observed. The cartilaginous tissue displayed a moderate loss of extracellular matrix, featuring lacunae with irregular and thickened margins, as well as mild, multifocally distributed empty lacunae.

Cellular quantification (Table 1) revealed a significantly higher number of fibroblasts in the XenoSC and Post-warming groups compared to the XenoIP group ($p < 0.001$ and $p = 0.0033$, respectively). Similarly, the XenoIP treatment exhibited a lower count of normal chondrocytes ($p = 0.0112$) and a higher number of empty lacunae ($p = 0.0004$) relative to the Post-warming group.

Cellular Culture

Following in vitro culture, fresh cells required 14 and seven days to detach from the explants of Jaguar#02 and Jaguar#03, respectively. For post-warming tissues, this process took over 60 days for Jaguar#02 and 39 days for Jaguar#03. In contrast, cells from xenotransplantation treatments detached in 13 and five days, respectively. Consequently, the Post-warming group explants failed to yield somatic cell outgrowth by the time the XenoIP and XenoSC cultures reached their third passage (Fig. 5); therefore, the post-warming treatment was excluded from the cellular viability analysis.

Viability data obtained via Trypan Blue exclusion (Vbat), flow cytometry (Vbcit), and acridine orange staining (Vblacr) for the XenoIP and XenoSC treatments across all animals showed no significant differences ($p > 0.05$) between them, and, notably, did not differ significantly from the Fresh treatment (Table 2).

Table 2
Viability analysis of jaguar fibroblasts using Trypan Blue exclusion (Vbat), Flow Cytometry (Vbcit), and Acridine Orange (Vblacr) assays across Fresh, Intraperitoneal Xenotransplantation (XenoIP), and Subcutaneous Xenotransplantation (XenoSC) treatments. Data are presented as medians.

Animal	Group	Vbat (%)	Vbcit (%)	Vblacr (%)
Jaguar#01	Fresh	90.8 ^a	97.9 ^a	74.0 ^a
	XenoIP	92.0 ^a	87.9 ^a	88.9 ^a
	XenoSC	94.8 ^a	94.6 ^a	91.0 ^a
Jaguar#02	Fresh	97.6 ^a	84.2 ^a	64.0 ^a
	XenoIP	97.6 ^a	86.5 ^a	70.5 ^a
	XenoSC	99.7 ^a	96.4 ^a	87.0 ^a
Jaguar#03	Fresh	96.4 ^a	87.7 ^a	77.0 ^a
	XenoIP	85.0 ^a	96.0 ^a	74.0 ^a
	XenoSC	83.3 ^a	90.7 ^a	70.0 ^a

Identical letters in the same column indicate no significant differences between groups ($p > 0.05$).

Genotyping

In the genotyping analysis, successful DNA amplification was exclusively observed in the wells containing jaguar-specific primers. This was consistent for fresh fibroblasts, jaguar blood, and somatic cells derived from both subcutaneous and intraperitoneal explants (Fig. 6).

Fibroblast Authentication via Immunofluorescence

To definitively authenticate the cellular lineage and evaluate the metabolic status of the cultured cells recovered from the NSG mice, immunofluorescence microscopy was performed. Cells isolated from both subcutaneous (Fig. 7) and intraperitoneal (Fig. 8) xenotransplants exhibited robust positive immunoreactivity for vimentin, a canonical mesenchymal marker, thereby confirming their identity as true jaguar fibroblasts. Furthermore, functional staining with MitoTracker revealed a dense, widely distributed active mitochondrial network across the cytoplasm in the vast majority of cells. Complete nuclear integrity was also confirmed via DAPI counterstaining. These findings collectively corroborate the high cellular viability and structural competence of the somatic cells rescued from the xenotransplanted tissues.

Figure 7. Microscopic immunofluorescence of fibroblasts isolated from jaguar (*Panthera onca*) auricular cartilage xenotransplanted into immunodeficient NSG mice. Panels (1a–1d) represent cells recovered from the intraperitoneal region, and panels (2a–2d) represent cells recovered from the subcutaneous region. (1a, 2a) Merged images displaying overlapping nuclear, mitochondrial, and cytoskeletal labeling. (1b, 2b) Active mitochondrial networks robustly labeled with MitoTracker (red). (1c, 2c) Positive immunoreactivity against vimentin, confirming the definitive fibroblastic lineage of the isolated cells (green). (1d, 2d) Nuclear counterstaining with DAPI preserving structural chromatin integrity (blue). Scale bars = 75 μ m.

Discussion

Implementing reproductive biotechnologies for wildlife is frequently hampered by restricted access to individuals, which impedes the development of consistent and reproducible protocols¹⁶. Furthermore, advancing assisted reproductive technologies (ARTs) requires specialized personnel and equipment for gamete collection and processing. Consequently, somatic cell-based technologies emerge as valuable alternatives, particularly when obtaining viable and functional gametes is challenging or unfeasible using traditional techniques. These technologies not only complement traditional ARTs but also expand the possibilities for conserving genetic diversity, an essential element for the long-term genetic viability of populations. In a conservation scenario exacerbated by anthropogenic pressures, as observed with jaguars, utilizing somatic cells derived from deceased animals offers the potential to preserve and even enhance genetic diversity within threatened populations.

This study reports the first successful production of somatic cells from free-ranging jaguars using material collected extended periods post-mortem. Previous studies have compared the viability of tissues maintained at 4 °C for varying periods and the resulting fibroblast quality²³, as well as the viability of tissue collected shortly after death and stored for seven days at 4 °C prior to vitrification¹⁸. However, this is the first study to evaluate the revitalization of tissues from animals discovered many hours post-mortem, exhibiting advanced signs of decomposition. By employing a protocol that facilitates the revitalization of biological material post-warming, the proposed technique broadens the possibilities for utilizing and preserving genetic material within germplasm biobanks, representing a crucial tool for the genetic conservation of jaguars. A major challenge in utilizing biological material from wildlife-vehicle collision casualties for biobanking is the post-mortem interval (PMI) prior to tissue collection and culture. Most wildlife collisions occur between dusk and midnight, or between dawn and 9:00 AM, correlating with both species activity patterns and traffic volume²⁴. In the Pantanal, jaguar activity peaks occur before dawn (between 03:30 and 06:00) and after dusk (from 18:30 to 21:00)²⁵. In this study, the estimated time between death and tissue receipt at the laboratory was approximately 24 hours. Although the exact time of death cannot be determined, reports from the Mato Grosso do Sul Environmental Police, who recovered the fatally injured animals, indicated that all specimens exhibited signs of decomposition, substantiating our hypothesis regarding the prolonged PMI.

Tissue selection is therefore crucial: because blood circulation ceases post-mortem, highly vascularized tissues rapidly succumb to hypoxia. Consequently, auricular cartilaginous tissue presents a viable option due to its lower metabolism and resistance to ischemia²⁶. Furthermore,¹⁸ demonstrated that auricular tissue can be successfully utilized in biobanks following solid-surface vitrification (SSV), making this the tissue of choice for our research group when culturing jaguar fibroblasts.

The ability to vitrify tissues from vehicular casualties facilitates genetic rescue across diverse geographic regions. This technique enables the subsequent shipment of samples for fibroblast culture and conservation in specialized laboratories. These advancements open new perspectives for species conservation and conservation biology research. Additionally, they expand the possibilities for enriching biobanks with high-quality genetic material, enabling future applications in advanced assisted reproductive techniques, such as somatic cell nuclear transfer (SCNT).

In this study, evaluation of the tissue post-warming (Post-warming Control) demonstrated that the SSV method maintained sufficient structural integrity to permit cellular rescue and subsequent revitalization, successfully preserving fibroblasts in the perichondrium and chondrocytes, albeit with signs of degeneration in some samples. SSV is the preferred method for cryopreserving jaguar skin samples, maintaining the high quality of cultured fibroblasts²⁷. The more pronounced decline in explant quality post-warming observed in our work is directly linked to the initial state of the tissue, which was already compromised by decomposition. Given that culturing fresh tissue from these casualties is frequently unfeasible, tissue vitrification becomes a practical and cost-effective option to utilize this material. In this context, our results demonstrate that xenotransplantation effectively revitalizes the tissue, yielding fibroblast quality that is comparable to that of fresh explants.

Xenotransplantation is commonly employed for ovarian and testicular tissue grafts, yielding satisfactory results^{28,29}. Explants can be transplanted into various sites, such as the peritoneum, ovarian bursa (for ovarian tissues), the subcutaneous space, the renal capsule, or intramuscularly^{28,29}. In this study, we opted to graft the explants into the subcutaneous and peritoneal regions due to their procedural simplicity and high potential for tissue neovascularization, respectively. Considering that the post-mortem interval significantly affects tissue viability and varied among the studied animals, we compared treatments within each individual to effectively evaluate the efficacy of xenotransplantation in revitalizing the warmed tissues.

Our results demonstrate that both intraperitoneal (XenoIP) and subcutaneous (XenoSC) xenotransplantation successfully generate viable fibroblasts from vitrified casualty tissues. However, the XenoSC group exhibited superior performance regarding tissue-level revitalization. This was characterized by enhanced fibroblast proliferation, lacunar filling, and a higher number of normal chondrocytes (alongside a reduction in degenerated cells), indicating that the subcutaneous environment more effectively sustained metabolic activity. Although the XenoIP group did not promote as robust a structural recovery as the XenoSC group, it still permitted efficient cell culture and the recovery of cells with comparable viability (Table 2). Furthermore, it also significantly improved the

number of normal chondrocytes (Jaguar#01) and filled lacunae (Jaguar#02) relative to the strictly in vitro ungrafted post-warming explants, confirming that both routes effectively rescue compromised material.

Viability data obtained via Trypan Blue exclusion and flow cytometry for the Fresh group were consistent with those reported by ¹⁹; however, our viability analyses for XenoIP and XenoSC surpassed those of the post-warming group evaluated in their study. Thus, the somatic cell viability data indicate that it is feasible to obtain high-quality cells from decomposing, deceased animals that are comparable to those derived from living animals. Xenotransplantation of jaguar tissues into the subcutaneous and intraperitoneal regions of NOD scid gamma (NSG) mice facilitated the recovery of cells with quality comparable to that of tissue cultured from live animals. In this regard, although both sites are viable for cartilage transplantation, yielding high-quality somatic cells, the technical simplicity, reduced procedural invasiveness, and superior capacity to stimulate tissue activity lead us to recommend the subcutaneous site as the optimal location for xenotransplantation.

In evaluating the cell viability of explants from vehicular casualties, the Post-warming Control group was excluded because it failed to yield somatic cell outgrowth by the time of assessment, whereas the XenoIP and XenoSC groups had already reached the third passage. This observation reinforces the superior cellular quality of the Fresh and xenotransplanted groups compared to the ungrafted group.

Ultimately, our results indicate that the xenotransplantation of jaguar auricular cartilage into immunodeficient mice is a highly viable approach for revitalizing tissues from vehicular collision casualties. We successfully rescued somatic cells from jaguars already undergoing post-mortem decomposition. Xenotransplantation of vitrified tissues significantly enhanced their biological competence, resulting in greater culture efficiency and cellular quality comparable to that of fresh tissue.

Despite inherent logistical challenges, this innovative revitalization of somatic tissues from deceased jaguars holds the potential to overcome the strict limitations of conventional ARTs, driving significant advancements in conservation protocols. The routine integration of these biobanking technologies into wildlife management programs offers practical solutions for achieving biodiversity targets, ultimately ensuring that vulnerable species possess enhanced chances of survival in a rapidly changing world.

Methods

This study was approved by the Ethics Committee on Animal Use of the Federal University of Mato Grosso do Sul (CEUA-UFMS, Protocol No. 1278/2023) and authorized by the Chico Mendes Institute for Biodiversity Conservation (SISBIO, Protocol No. 75762-1) for the collection of biological material from free-ranging jaguars. All experiments were performed in accordance with relevant named guidelines and regulations.

Animals

Auricular cartilage tissues were obtained from three adult jaguars fatally injured in wildlife-vehicle collisions on Highway BR-262, between Mirada and Corumbá, in the Pantanal of Mato Grosso do Sul. The specimens included two males weighing 108 kg (Jaguar#01, collected on 28/01/2023 at 19°38'40.9"S 57°02'07.2"W) and 103 kg (Jaguar#02, collected on 22/03/2023 at 19°39'25.2"S 57°01'20.2"W), and one female weighing 65 kg (Jaguar#03, collected on 29/04/2023 at 19°49'11.9"S 56°54'20.9"W). Tissue fragments were recovered by the Mato Grosso do Sul Environmental Police and transported to the Laboratory of Genetic Editing and Conservation at UFMS (Reprogen/UFMS; Campo Grande, MS, Brazil; 20°30'22.6"S 54°36'53.6"W). Based on the rigor mortis and the stage of decomposition reported by the recovering officers, the collisions likely occurred during the crepuscular period of the preceding day. Consequently, the estimated post-mortem interval (PMI) prior to laboratory receipt was at least 24 hours, although variations may have existed between individuals.

Individual age was classified as 'Adult' based on a combination of physical indicators assessed during the post-mortem examination, including body mass, dental wear patterns, and pelage characteristics. These assessments were initially conducted by the Environmental Police officers and subsequently confirmed by the laboratory team upon receipt of the specimens.

Six immunodeficient NOD scid gamma (NSG) mice, bred at the UFMS Central Animal Facility, were utilized. The animals were housed in individually ventilated cages (IVC) within a controlled environment maintained at a temperature of 22 ± 2 °C and 50% humidity, under a 12h light/12h dark cycle. To promote animal welfare, cages were furnished with irradiated pine wood shavings (Flocos de Pinus) and environmental enrichment, including autoclaved paper for nesting and shelters. Mice were provided ad libitum access to a sterile pelleted diet (CR1, Quimtia, Brazil) and filtered water.

Experimental Design

All reagents and media, unless otherwise specified in the text, were purchased from Sigma-Aldrich and Gibco.

An auricular cartilaginous tissue sample measuring approximately 3 cm² was extracted from each animal. Each cartilaginous tissue sample was sectioned into 16 smaller fragments (explants; approximately 9 mm²). A portion was used for fresh evaluations (Fresh Control), and the remainder was vitrified to be evaluated immediately post-warming (Post-warming Control) or implanted into mice (Xenotransplantation) (Supplementary Information). Two mice were assigned per jaguar, each receiving two subcutaneous and two intraperitoneal explants (n = 6 mice total). To minimize the impact of inter-individual biological variation, a paired experimental design was employed, where each mouse served as its own internal control by receiving both treatment sites simultaneously. This approach ensured that any observed differences in tissue revitalization were attributable to the transplantation environment rather than idiosyncratic physiological differences between individual mice.

- Fresh Control: Four fragments analyzed fresh; two cultured for evaluating somatic cell quality and two analyzed histologically.

- Post-warming Control: Four vitrified and warmed fragments; two cultured for evaluating somatic cell quality and two analyzed histologically.
- Intraperitoneal Xenotransplantation (XenoIP): Four vitrified and warmed fragments, transplanted into the peritoneum of mice for seven days. Subsequently, two were cultured for evaluating somatic cell quality and two analyzed histologically.
- Subcutaneous Xenotransplantation (XenoSC): Four vitrified and warmed fragments, transplanted subcutaneously into mice for seven days. Subsequently, two were cultured for evaluating somatic cell quality and two analyzed histologically.

Due to the limited amount of available cartilaginous tissue, histological analyses for the Fresh Control group were exclusively performed on Jaguar#02.

Tissue collection and processing

Jaguar ear samples were transported to Reprogen/UFMS submerged in flasks containing physiological saline. In the laboratory, this material underwent surface decontamination with 70% alcohol for 1 minute and was subsequently washed in a biosafety cabinet with PBS for 1 minute. The samples were then transferred to 50 mL Falcon tubes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% Fetal Bovine Serum (FBS) and 5% antibiotic-antimycotic solution until dissection, following the protocol described by ³⁰. Following dissection, the cartilages were fragmented for use in the experimental groups.

Tissue vitrification

For vitrification, the solid-surface method was employed, according to an adapted protocol from ²⁷. Explants were transferred to 24-well plates containing 2 mL of cryoprotectant medium (DMEM supplemented with 10% DMSO, 10% FBS, and 2% antibiotic-antimycotic solution) and maintained at 25 °C for 4 minutes. Subsequently, they were placed onto a metallic surface suspended over liquid nitrogen and then transferred into cryovials for direct storage in liquid nitrogen at -196 °C.

After one week, the cryovials were removed from the liquid nitrogen and held for one minute at 25°C in room air, followed by immersion in a 37°C water bath. To remove the cryoprotectant medium, the explants were washed three times for 5 minutes each in DMEM + 10% FBS solutions supplemented with decreasing concentrations of sucrose (0.5 M, 0.25 M, and sucrose-free) ²⁷.

Xenotransplantation

Mice were anesthetized via intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (10 mg/kg) combination. For analgesia, meloxicam (2 mg/kg) and tramadol hydrochloride (20 mg/kg) were administered subcutaneously 30 minutes prior to surgery. Following analgesia and confirmation of the anesthetic plane, the animal was placed in dorsal recumbency. The surgical site was prepared by fur

clipping, followed by aseptic scrubbing with chlorhexidine. The skin was incised and bluntly dissected, followed by incision of the musculature and peritoneum.

Using ophthalmic forceps, the musculature was manipulated to facilitate visualization of the peritoneum. The xenotransplantation was performed using the previously warmed explants maintained in a sterile Petri dish. Using a swaged suture needle, each explant was transfixed and secured within the intraperitoneal region of the mouse using simple interrupted sutures. Subsequently, a small volume of physiological saline was applied to prevent local adhesions; the musculature was then returned to its anatomical position, and closure was achieved using 4-0 catgut suture in a continuous interlocking pattern, encompassing the peritoneum, fascia, and regional muscles.

The subcutaneous explants were inserted without requiring suture fixation. Following the procedure, the skin was closed with simple interrupted sutures using 4-0 nylon. At the conclusion of the surgical procedure, the animal was placed in ventral recumbency on a 37 °C heating pad for observation until anesthetic recovery. Post-operative analgesia consisting of meloxicam (2 mg/kg) and tramadol hydrochloride (20 mg/kg) was administered once daily for two days.

After one week, the mice were euthanized via an overdose of inhalant anesthesia. Animals were exposed to isoflurane in oxygen (5% induction, 2 L/min) within an induction chamber until death was confirmed, evidenced by the absence of pedal and caudal reflexes, as well as the cessation of vital signs (respiratory movements and heart rate).

Somatic cell culture

All explants were cultured until the third passage in DMEM containing 15% FBS and 2% penicillin-streptomycin-amphotericin B solution, maintained in a cell culture incubator at 38.5°C and 5% CO₂. The culture medium was replaced every 24 hours, and passages were performed when fibroblasts reached 70-80% confluence. Following the third passage, upon each plate reaching 80% confluence, fibroblast viability assessments were conducted. Post-culture, cells were evaluated for morphology, viability, and apoptosis throughout the primary cultures and subcultures.

Assessments

Histological analysis: Explants were fixed in 4% paraformaldehyde and embedded in paraffin.

Subsequently, they were sectioned at a 5 µm thickness, as recommended by ³¹. Samples were stained with hematoxylin and eosin (H&E) for the evaluation of morphological features. Using a fluorescence light microscope (Nikon Eclipse Ni, Japan) equipped with a camera, 20 photomicrographs were captured in random fields at 40x magnification. Cellular quantification was performed via standard histological evaluation on auricular cartilage fragments, counting fibroblasts within the perichondrium, filled and empty lacunae, and chondrocytes. All histological and morphometric assessments were performed by a board-certified veterinary pathologist blinded to the treatment groups (Fresh, Post-warming, XenolP, or XenoSC) at the time of evaluation, thereby ensuring unbiased data collection.

Cell viability assessment: For cell viability analysis, Trypan Blue dye and the fluorescent probes acridine orange (AO), propidium iodide (PI), and Hoechst 33342 were utilized. The analysis was conducted prior to cell cryopreservation, post-warming, and upon reaching confluence.

- Trypan Blue exclusion assay: In a conical tube, 20 µL of cell suspension was combined with 20 µL of 0.4% Trypan Blue. Cells were counted using a Neubauer chamber and considered viable when excluding the blue dye. The viability percentage was calculated by dividing the number of viable cells by the total number of counted cells, according to ³².
- Necrosis and Apoptosis Assay: In a dark conical tube, 20 µL of cell suspension, 20 µL of acridine orange (100 µg/mL), and 4 µL of propidium iodide (2 mg/mL) were mixed. Subsequently, an aliquot of this mixture was placed on a slide, covered with a coverslip, and analyzed under an inverted fluorescence microscope (Nikon). A total of 100 cells were counted and categorized as orange (apoptotic), red (necrotic), or green (viable).
- Flow Cytometry: Viability analysis using Hoechst 33342 was performed on a flow cytometer (CytoFLEX, Beckman Coulter). For this purpose, 40 µL of cell suspension was incubated for 15 minutes with 20 µL of Hoechst 33342 (40 µg/mL) and 4 µL of PI (2 mg/mL). The viable percentage was determined based on the PI-negative (PI-) population.

Somatic cell genotyping: To confirm the origin of the somatic cells derived from the xenotransplanted explants, genotyping was performed using jaguar and mouse primers, referenced against the complete genomes of these species in GenBank. Jaguar somatic cells, jaguar blood, and a mouse ear fragment were used as positive controls. DNA extraction was conducted using the phenol-chloroform method, followed by 2% agarose gel electrophoresis for 1 hour at 100 volts in the presence of GelRed. The primers used were as follows:

- Jaguar:
 1. EA 01: 5' CACACTAGCCATCATCCTCTTATC 3' (Sense)
 2. EA 02: 5' CTGCTGCGTACTCTACGTAAA 3' (AntiSense)
- Mouse:
 1. EA03: 5' CCATTTGCAGACGCCATAAA 3' (Sense)
 2. EA04: 5' TCAGGCTGGCAGAAGTAATC 3' (AntiSense)

Immunofluorescence Staining: Cultured cells derived from the subcutaneous and intraperitoneal xenotransplants were seeded in 96-well glass-bottom plates in biological triplicates for each animal and grafting site. Upon reaching 70% confluence, the wells were gently washed with PBS and incubated with 100 µL of MitoTracker™ Red CMXRos (100 nM) for 15–30 minutes at 37 °C in a 5% CO₂ atmosphere to label active mitochondria. Following incubation, the cells were washed once with PBS and fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT). Post-fixation, the wells were washed three times with PBS. Cellular permeabilization was performed using 0.1% Triton X-100 for 15 minutes at

RT, followed by three additional PBS washes. Non-specific binding sites were blocked using 2% Bovine Serum Albumin (BSA) for 1 hour at RT. Subsequently, cells were incubated overnight at 4 °C in the dark with an Alexa Fluor™ 488-conjugated anti-vimentin primary antibody (MA5-11883-A488; 10 µg/mL diluted in 0.1% BSA). After primary incubation, the wells were washed three times with PBS. Nuclear counterstaining was performed using DAPI (1 µg/mL) for 10 minutes. Finally, image acquisition was conducted using an EVOS M5000 Imaging System (Thermo Fisher Scientific) equipped with DAPI, GFP, and RFP fluorescence filters.

Statistical analyses: For histological quantification, data are expressed as Mean ± Standard Deviation (SD). Due to the biological specificities and varying post-mortem intervals (PMI) of the individual jaguars, each animal was analyzed as a distinct experimental unit. The observational units were defined as the random microscopic fields (photomicrographs) evaluated per treatment group. Consequently, the reported means and variances reflect the cellular distribution across these fields within each specific individual, ensuring that the intrinsic variability of the compromised tissue was accurately captured. For parametric data, a paired Student's t-test was performed to compare means between groups, and exact p-values are reported throughout the text. For non-parametric data, the Kruskal-Wallis test was employed. Somatic cell viability data were non-parametric; therefore, comparisons were also made among individual animals using the Kruskal-Wallis test. All statistical analyses were conducted using R software.

Declarations

Competing interests

The authors declare no competing interests

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Author Contribution

Conceptualization: G.R.A., S.R.P., P.N.J.N., M.C.C.S., A.F.P., T.D.S.; Data curation: G.R.A., S.R.P., M.C.C.S.; Formal analysis: G.R.A., S.R.P., P.N.J.N., E.V.C.S., R.Z.; Funding acquisition: G.R.A.; Investigation: G.R.A.,

S.R.P., P.N.J.N., M.C.C.S., I.N., B.R.A., L.S.F.R.M., S.L.O., L.S.U., H.P.K.C., E.A.C.A., T.D.S.; Methodology: G.R.A., S.R.P., P.N.J.N., A.F.P.; Project administration: G.R.A., S.R.P.; Resources: G.R.A., T.D.S.; Software: E.V.C.S., R.Z.; Supervision: G.R.A., T.D.S.; Validation: G.R.A., P.N.J.N.; Visualization: G.R.A., S.R.P., P.N.J.N., M.C.C.S., T.D.S.; Writing – original draft: G.R.A., S.R.P., P.N.J.N., M.C.C.S., T.D.S.; Writing – review & editing: G.R.A., P.N.J.N., M.C.C.S., C.S.P., T.D.S.

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Data Availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

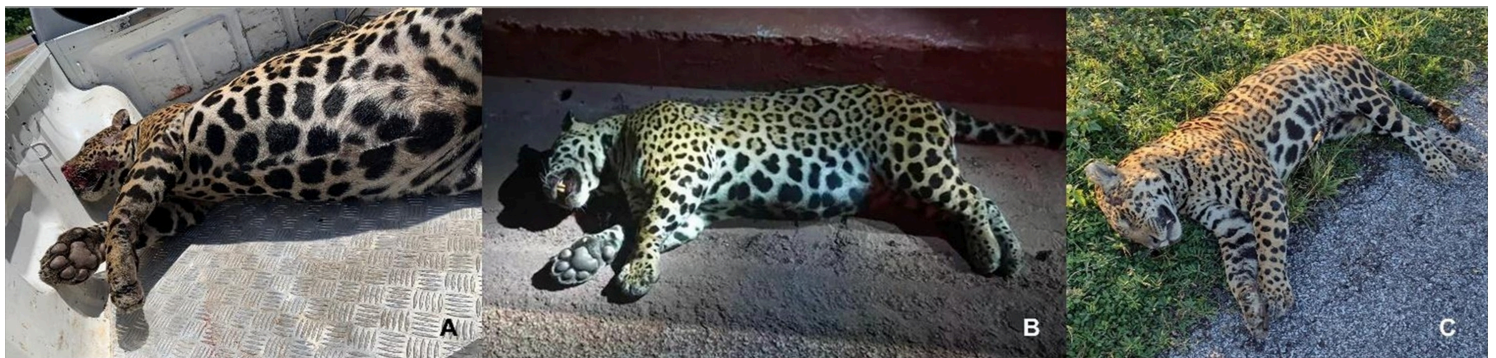


Figure 1

Fatally injured jaguars following vehicular collisions in Mato Grosso do Sul, Brazil. (A) 108 kg male, (B) 103 kg male, (C) 65 kg female.

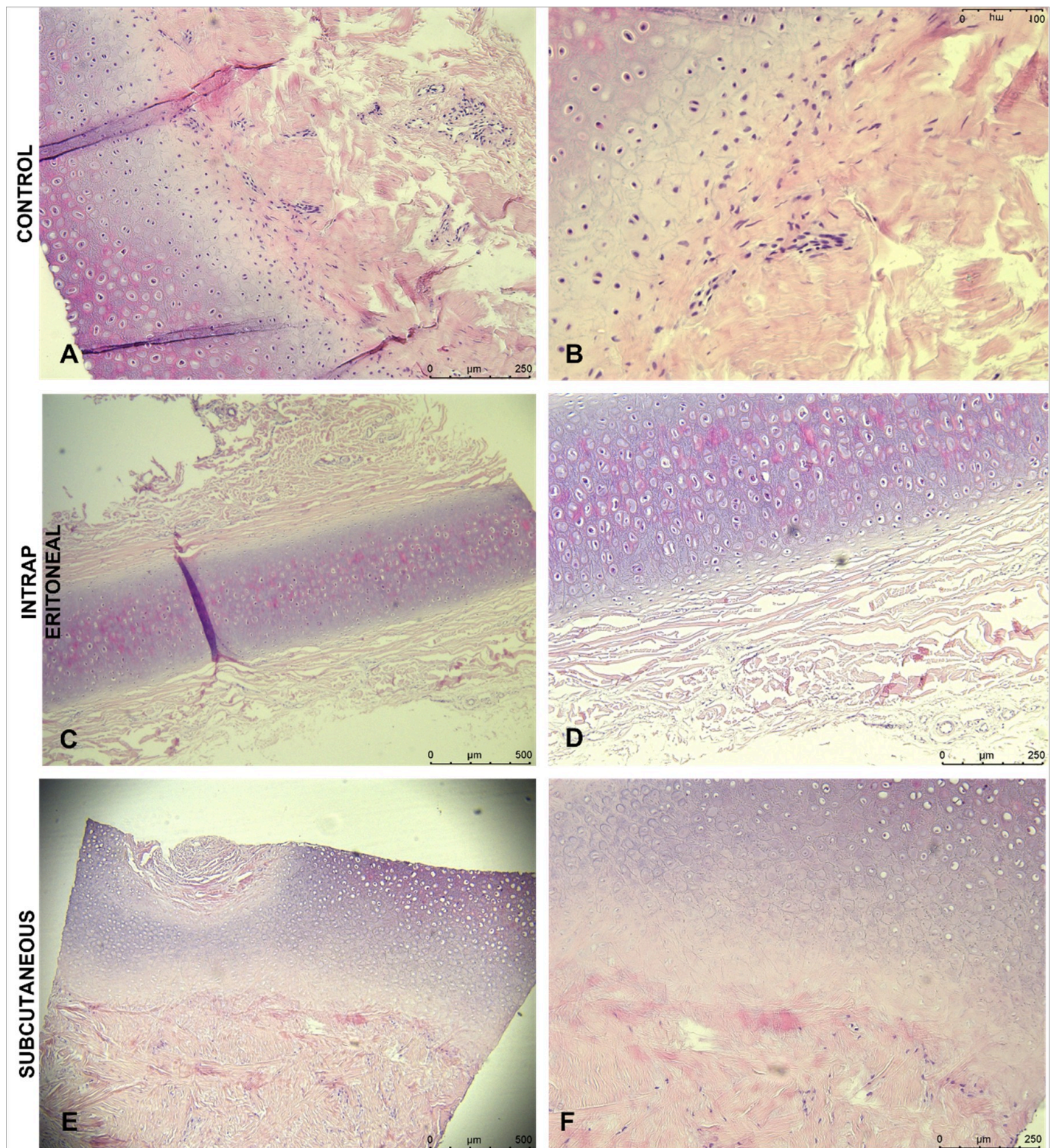


Figure 2

Photomicrographs of histological sections of jaguar (Jaguar#01) auricular cartilage following vitrification and xenotransplantation. H&E stain, magnification 5x (C, E), 10x (A, D, F), and 20x (B). (A, B) Post-warming control fragment, exhibiting active and disorganized loose connective tissue, degenerating fibers, and widened intercellular spaces. (C, D) Intraperitoneal xenotransplant fragment: degenerating elastic cartilaginous tissue, featuring normal fibroblasts and rare proliferative fibroblasts. Loose

connective tissue fibers undergoing degeneration with increased intercellular spacing. (E, F) Subcutaneous xenotransplant fragment: high proliferation of active loose connective tissue disorganizedly surrounding the elastic cartilaginous matrix, occupying the entire extent. The cartilage region exhibits abundant lacunae filled with viable chondrocytes and numerous chondroblasts. Mild to moderate neovascularization is observed near the perichondrial region.

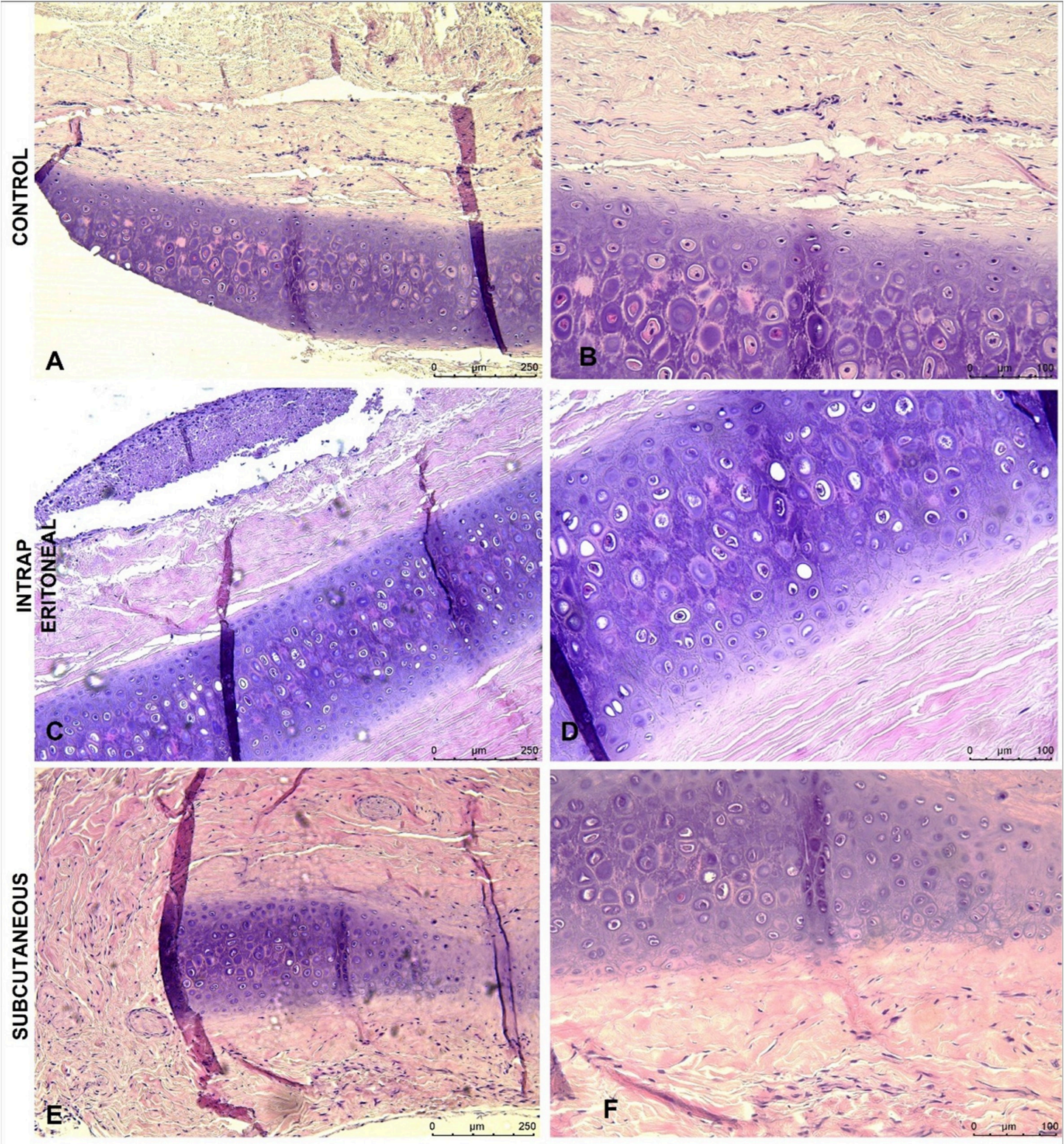


Figure 3

Photomicrographs of histological sections of jaguar (Jaguar#02) auricular cartilage following vitrification and xenotransplantation. H&E stain, magnification 10x (A, C, E) and 20x (B, D, F). (A, B) Control fragment displaying cartilaginous tissue without significant alterations, organized and active loose connective tissue, and rare inflammatory cells. (C, D) Intraperitoneal xenotransplant fragment: degenerating elastic cartilaginous tissue, with mildly thickened perichondrium, moderate fibrocytes, normal fibroblasts, and rare proliferative fibroblasts. Explant encapsulation by mixed inflammatory cells and fibroblasts is evident. (E, F) Subcutaneous xenotransplant fragment: high proliferation of active loose connective tissue organizedly surrounding the elastic cartilaginous matrix. The cartilage region features abundant lacunae filled with viable chondrocytes and numerous chondroblasts. Mild to moderate neovascularization adjacent to the perichondrial region, with a well-distributed and homogeneous extracellular matrix.

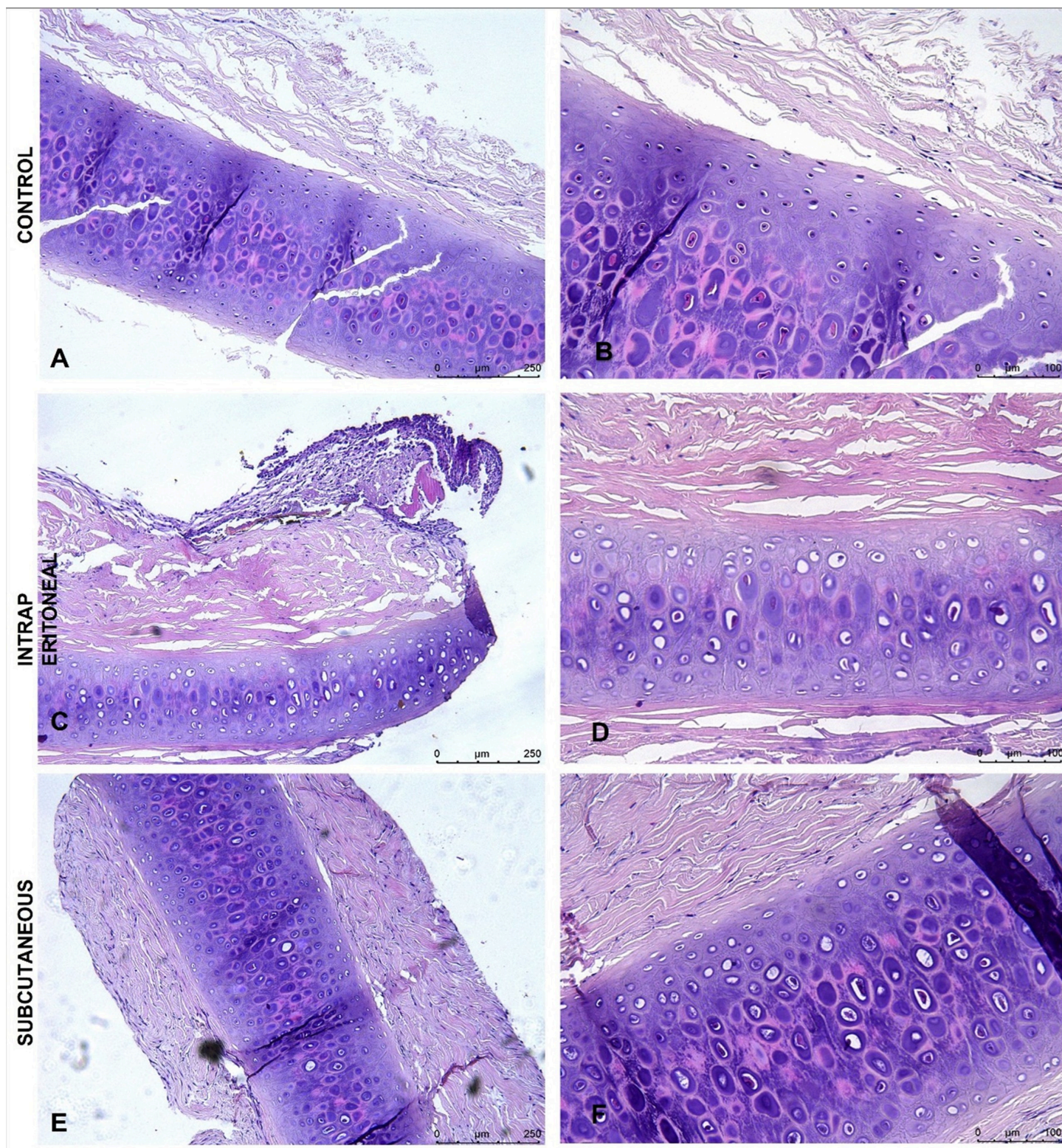


Figure 4

Photomicrographs of histological sections of jaguar (Jaguar#03) auricular cartilage following vitrification and xenotransplantation. H&E stain, magnification 10x (A, C, E) and 20x (B, D, F). (A, B) The perichondrium exhibits irregular distribution, featuring degenerating connective tissue collagen fibers and widened intercellular spaces. Moderate diffuse active fibroblasts alongside rare multifocal proliferative fibroblasts. Chondroblasts predominantly display pyknotic nuclei. Cartilaginous tissue

undergoing degeneration, with lacunae almost entirely filled by chondrocytes in varying stages of degeneration, sometimes exhibiting swollen, basophilic nuclei with prominent nucleoli and/or clumped chromatin. Mild unfilled lacunae are present. (C, D) Throughout the section, the perichondrium displays regular distribution, occupying all spaces with collagen fibers and active fibroblasts. Moderate viable chondroblasts. Cartilaginous tissue undergoing degeneration, with loss of extracellular matrix; however, moderate lacunae containing viable chondrocytes remain visible. The remaining chondrocytes exhibit varying degrees of cellular degeneration. Mild multifocal unfilled lacunae. (E, F) The explant is encapsulated by fibroblasts, predominantly mononuclear inflammatory cells (lymphocytes, plasma cells, and macrophages), and cellular debris; at one extremity, this capsule is more adherent and exhibits neovascularization. The perichondrium contains regularly distributed, dense collagen fibers and mildly degenerated cytoplasm. Moderate to severe diffuse fibrocytes are also observed within the perichondrium. Cartilaginous tissue displays moderate extracellular matrix loss, with some lacunae exhibiting irregular and thickened margins. Mild multifocal empty lacunae.

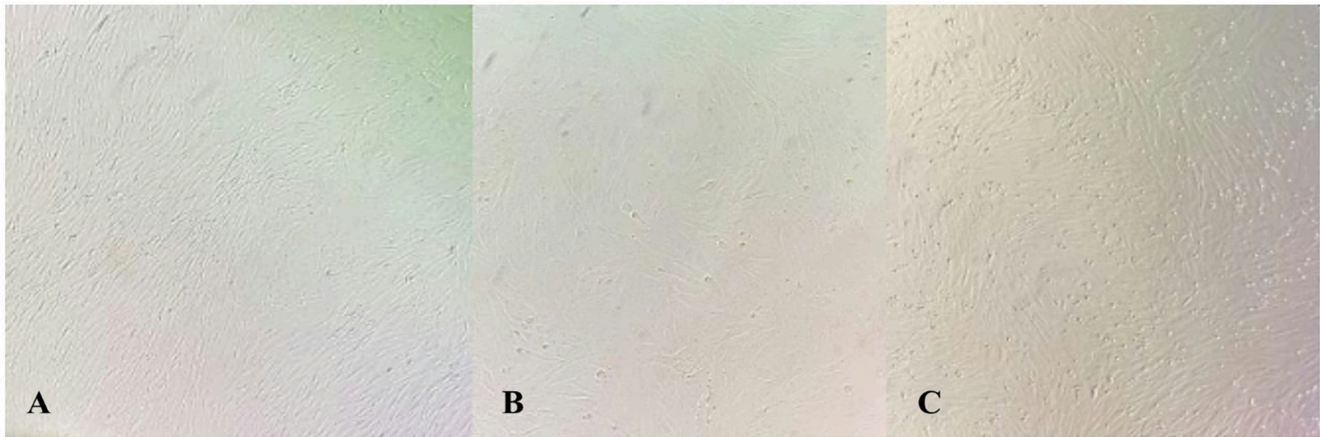


Figure 5

Somatic cell culture following xenotransplantation – 80-90% confluence. (A) Fresh cultured tissue cells – 8 days of cultivation, (B) cells derived from vitrified tissue, cultured following subcutaneous xenotransplantation – 8 days of cultivation, (C) cells derived from vitrified tissue, cultured following intraperitoneal xenotransplantation – 12 days of cultivation.

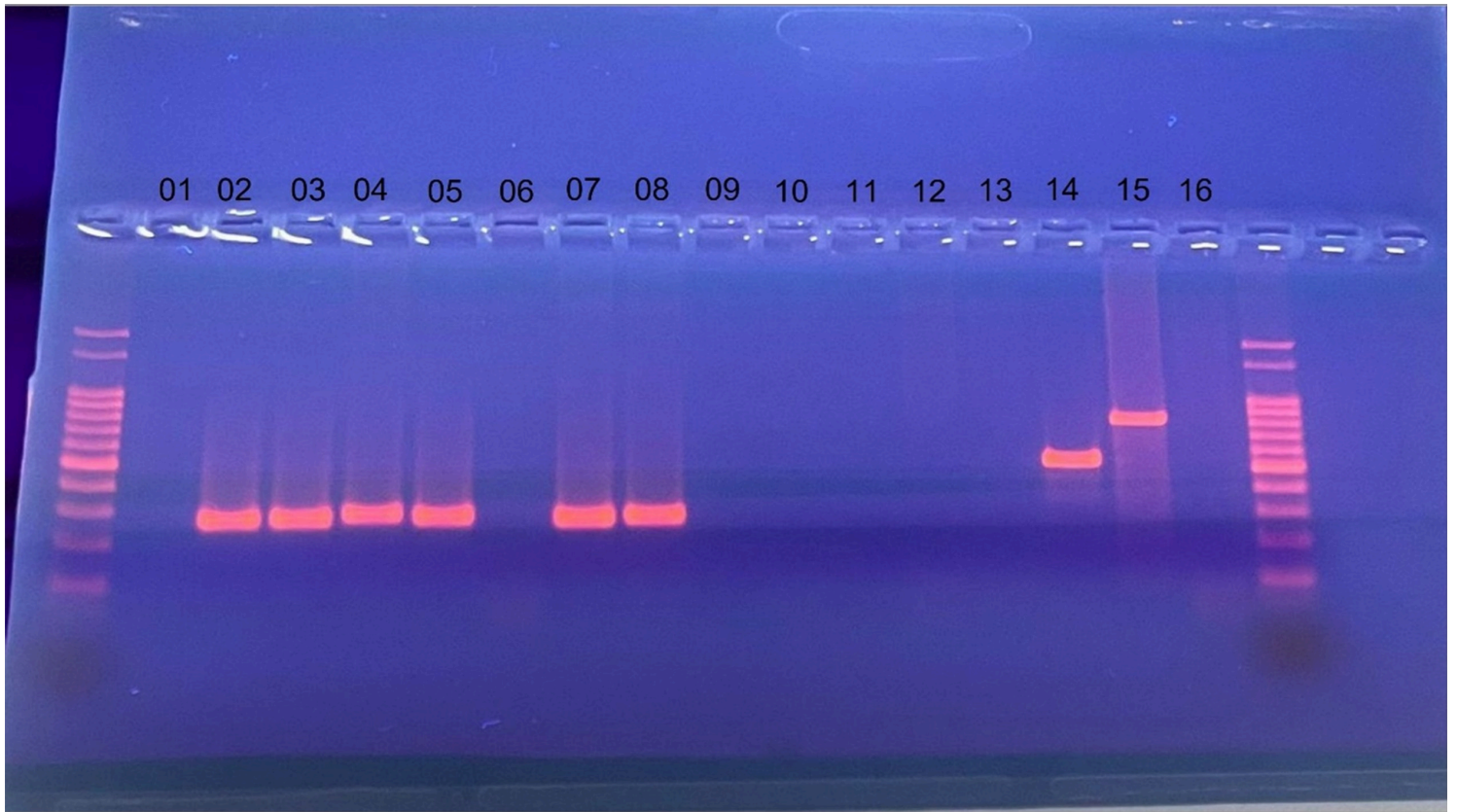


Figure 6

Agarose gel electrophoresis illustrating PCR amplification. Wells 1 to 8: jaguar primer (Well 1: negative control; Well 2: Xeno-intraperitoneal somatic cells; Well 3: Xeno-subcutaneous somatic cells; Well 4: jaguar fibroblasts; Well 5: second-passage Xeno-subcutaneous somatic cells; Well 6: Swiss mouse ear; Well 7: jaguar blood; Well 8: jaguar fibroblast). Wells 9 to 16: mouse primer (Well 9: negative control; Well 10: Xeno-intraperitoneal somatic cells; Well 11: Xeno-subcutaneous somatic cells; Well 12: jaguar fibroblasts; Well 13: second-passage Xeno-subcutaneous fibroblast; Well 14: Swiss mouse ear; Well 15: jaguar blood; Well 16: jaguar fibroblast).

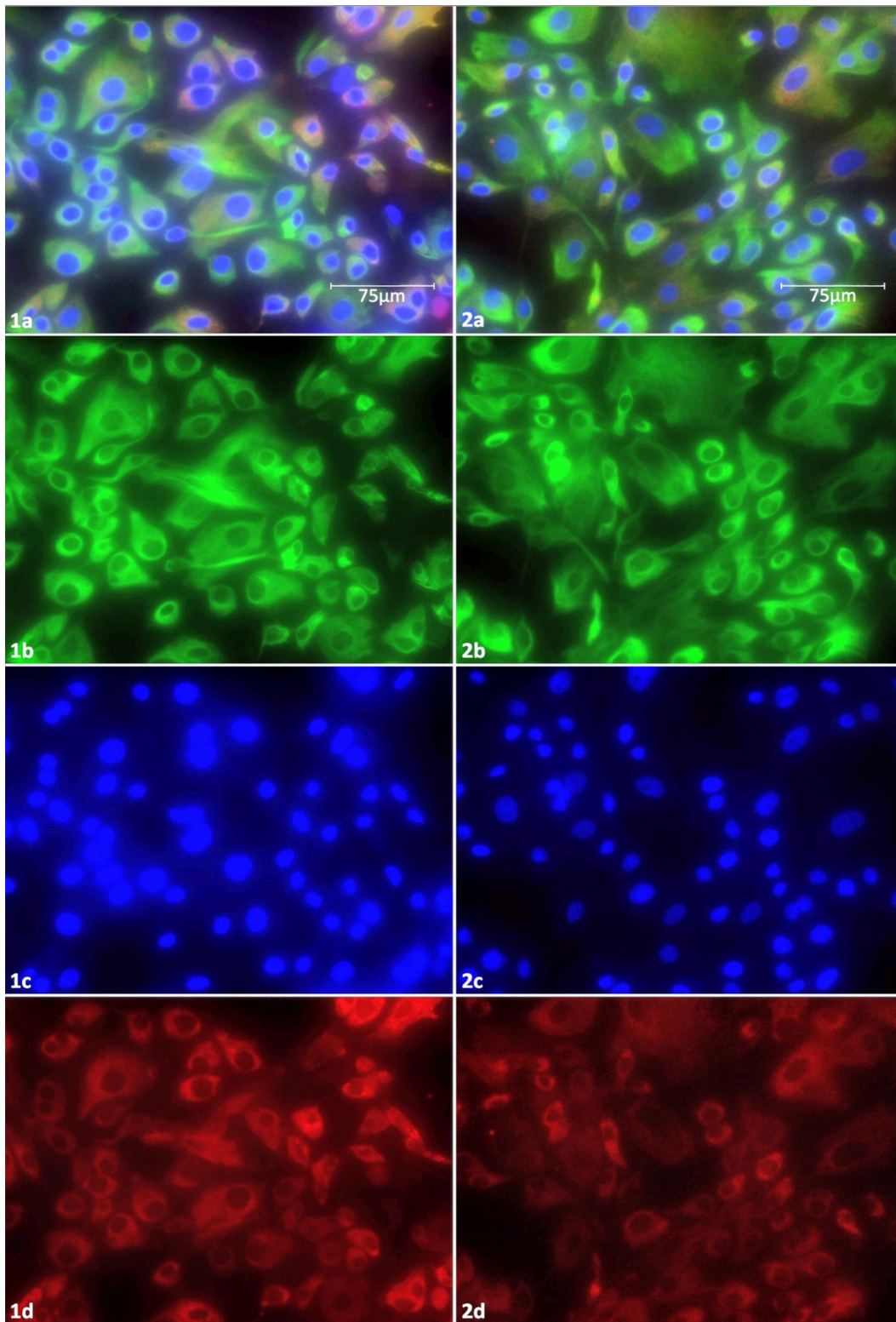


Figure 7

Microscopic immunofluorescence of fibroblasts isolated from jaguar (*Panthera onca*) auricular cartilage xenotransplanted into immunodeficient NSG mice. Panels (1a–1d) represent cells recovered from the intraperitoneal region, and panels (2a–2d) represent cells recovered from the subcutaneous region. (1a, 2a) Merged images displaying overlapping nuclear, mitochondrial, and cytoskeletal labeling. (1b, 2b) Active mitochondrial networks robustly labeled with MitoTracker (red). (1c, 2c) Positive immunoreactivity

against vimentin, confirming the definitive fibroblastic lineage of the isolated cells (green). (1d, 2d)
Nuclear counterstaining with DAPI preserving structural chromatin integrity (blue). Scale bars = 75 µm.

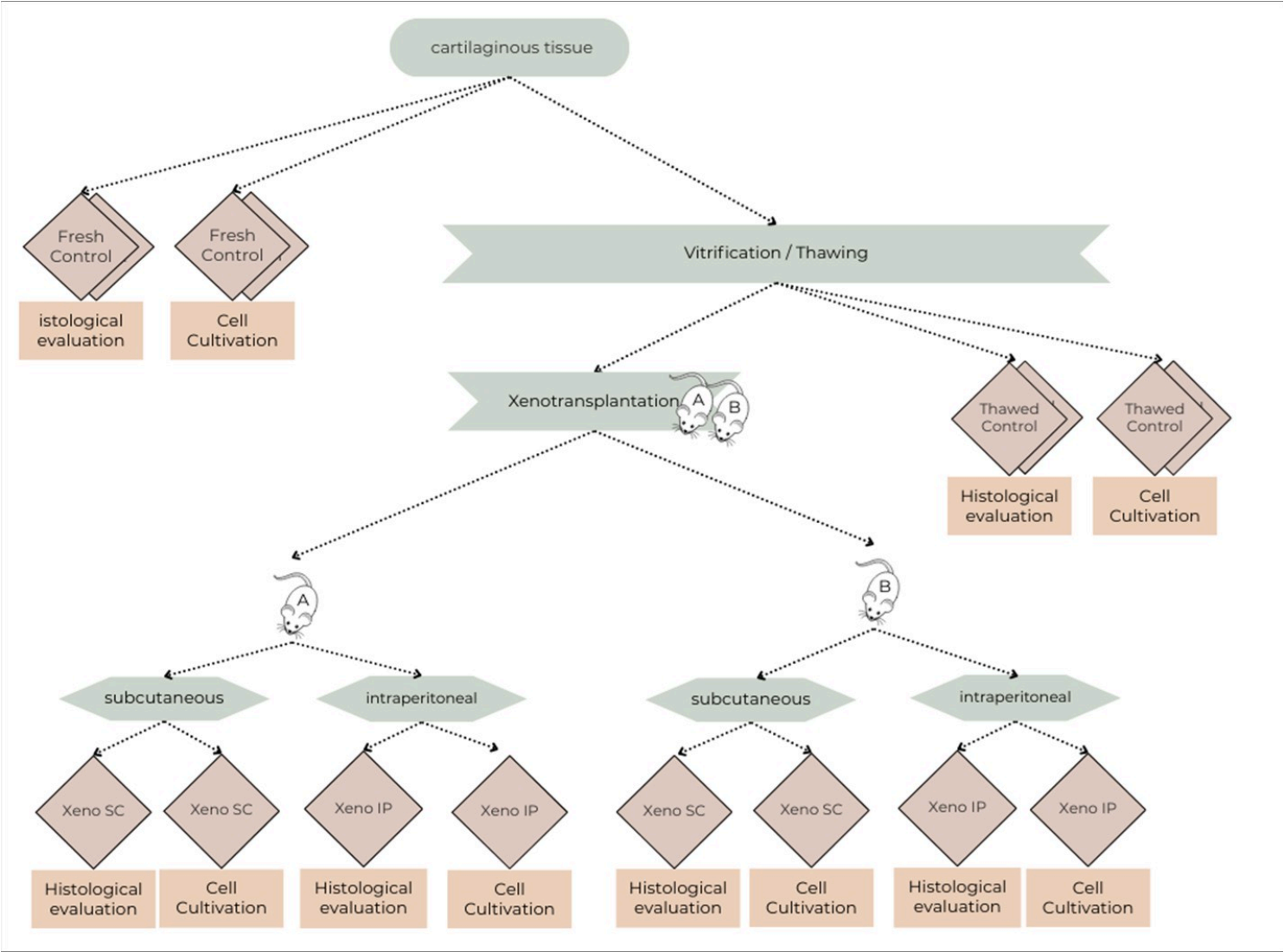


Figure 8

Schematic representation of the ear tissue processing workflow for each animal.

Supplementary Files

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