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RESEARCH ARTICLE

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A simple method for effective cryopreservation of antlerogenic periosteum of sika deer

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Abstract

Antlerogenic periosteum (AP) is the unique tissue type that gives rise to antlers and their antecedents, the pedicles. Deer antlers are the only mammalian organ that can fully regenerate. Efficient investigation of the mechanism of antler formation and regeneration requires year-round availability of AP, but naturally AP can only be obtained less than two months in a year. In the present study we took the cryopreservation approach to store the sampled AP in ultra-low temperature to overcome the limited period of availability. First, we evaluated the suitability of vitrification and cell cryopreservation method for cryopreservation of AP, cell migration status of the AP tissue pieces confirmed that vitrification methods did not work as the only few AP cells migrated out, whereas migrated cell numbers in the cell-cryo group (conventional method for cryopreservation of cells) were comparable to those of the fresh AP group. To further evaluate the suitability of cell cryopreservation method for AP tissue, AP samples were allocated into three groups based on the different ratios of cryopreservation reagents (dimethyl sulfoxide [DMSO], dulbecco's modified eagle's medium [DMEM] and fetal bovine serum [FBS]): AP-Cell-1 (1:4:5), AP-Cell-2 (1:2:7) and AP-Cell-3 (1:0:9), the results showed that migrated cell number were again comparable to the fresh AP group. There was no significant difference between the cell-cryo groups (AP-Cell-1 and AP-Cell-3) and the fresh group: (1) in viability ($p > 0.05$) through trypan blue staining (91.2%, 90.8%, and 92.4%, respectively); (2) in the attachment day, and all on Day 5 after cell seeding; (3) in cell proliferation rate ($p > 0.05$) through Cell Counting kit 8 (CCK8) measurement; and (4) in number of the formed clones (Clonogenicity). In the in vivo trials, there was no visible difference in temporal differentiation sequence of the formed xenogeneic antlers between the fresh AP and cryopreserved AP (AP-Cell-1 and AP-Cell-3). Overall, we found that the AP tissue was well cryopreserved just using the conventional freezing and thawing methods for cells, and their viability and developmental potential comparable to the fresh AP both in vitro and in vivo. The long-term preservation of the AP tissue is of great significance for the study of the periosteum biology in general and the mechanism underlying xenogeneic generation and regeneration of deer antlers in specific.

KEYWORDS

antlerogenic periosteum, cell proliferation, cell viability, cryopreservation, xenogeneic antler

1 | INTRODUCTION

Deer antlers are the male secondary sexual characters and organs of bone. Each year, antlers are cast and fully regenerate from the pedicles, the permanent bony protuberances (Goss, 1983). Deer are not born with pedicles, which start to grow from the presumptive antlerogenic region (frontal crest) when male deer approach puberty (Li et al., 1988, 2003). It is known now that formation of pedicles and first antlers relies on the presence of the periosteum overlying the frontal crest before pedicle formation, which is called antlerogenic periosteum (AP) (Kierdorf et al., 2009; Li et al., 2008, 2014). Deletion of the AP abrogates future development of the pedicle and first antler; subcutaneous transplantation of the AP elsewhere on the deer body induces ectopic pedicle and antler formation (Goss & Powel, 1985; Hartwig & Schrudde, 1974). Experiment of marker gene (LacZ) labeling to the AP cells further confirmed this finding, as all the cell types in the growing antler tissue were found to be the decedents of the LacZ-labeled AP cells except for the velvet skin (Li & Suttie, 2001).

Interestingly, subcutaneous transplantation of a small piece of AP (less than 1/10 of AP required compared to the deer themselves) can also induce xenogeneic antler formation on a nude mouse head (Li, Gao, et al., 2001; Li et al., 2009). This finding has created new avenues for a number of mechanistic studies. For example, through transplantation of AP to nude mice, we discovered that recipient animals (nude mouse) sustain the development of xenogeneic organs (antler) through chimeric blood vessel network (Wang et al., 2022); AP must interact with skin epidermis to launch antler formation, but this interaction is indirect and relied by the hair follicle dermal papilla cells (Li, Yang, et al., 2009; Sun et al., 2020); and AP has autonomous self-differentiation attributes, which offers AP an ability to build up an organ (antler) at different niches and in different immune-deficient animals (nude mouse, Rag-/- IL2-/-rat, goat and pig; unpublished). To efficiently carry out these studies using the nude model, we need year-round supply of the AP tissue. Unfortunately, we only have less than 2-month narrow window opportunity (each year March and April) to sample AP tissue and only in the first year of a deer's life, as AP is a piece of transient tissue, once the pedicle is initiated from it, AP transforms to pedicle tissue with totally new features, such as sustaining full potential of antler regeneration (Bubenik & Bubenik, 1990; Kierdorf et al., 2003; Li et al., 2005).

To circumvent the problem of seasonal availability (March and April), in this study we endeavored to preserve sampled AP relatively permanently through the approach of cryopreservation. We found that the AP tissue was well cryopreserved just using the conventional freezing and thawing methods for cells, and their viability and developmental potential comparable to the fresh AP both in vitro and in vivo. The long-term preservation of the AP tissue is of great significance for the study of the periosteum biology in general and the mechanism underlying xenogeneic generation and regeneration of deer antlers in specific.

2 | MATERIALS AND METHODS

2.1 | AP and ovary sample collection

Six fresh deer heads before pedicle initiation were collected from a commercial abattoir in Shuangyang District, Changchun City, and immediately transferred to our cell culture preparation room. The antlerogenic regions on each head were thoroughly shaved and sterilized before the heads being shifted into the culture room, the AP was removed from each side of the frontal crests of a deer head (Figure 1a) following the procedure reported in our previous publication (Li & Suttie, 2003). In total, 12 pieces of AP tissue were collected (2/head) and each piece was cut into small pieces (3 × 4 mm/piece) for subsequent use in vitro and in vivo (Figure 1b).

Two ovaries were collected from an 8-week female C57 mouse as a positive control for evaluation of the suitability of vitrification, attached adipose tissue was carefully removed, and rinsed with PBS to remove stained blood, then the ovaries were cut into 1 mm thick slices (Figure 1c).

2.2 | Grouping and cryopreservation

To evaluate the suitability of vitrification method for cryopreservation of AP, samples were allocated into 4 groups: Ovary-Vit, AP-Vit-1, AP-Vit-2, AP-cell-cryo (as a control). In the Ovary group, the ovary tissue slices were washed with PBS for three times, put in frozen equilibrium solution (7.5%EG + 7.5% propyl alcohol [PROH] + 20% FBS + dulbecco's phosphate-buffered saline [DPBS]) for 10 min, then transferred into vitrification solution (15% EG + 15% PROH + 0.5 M Sucrose + 20% FBS + DPBS) for 5 min. Finally, the vitrified ovary slices were frozen in liquid nitrogen, and then transferred into −80°C freezer (Qin, 2018; Shi et al., 2017). The same procedure was adopted for the AP-Vit-1 group, but with some adjustments for the AP-Vit-2 group (PROH was replaced by dimethyl sulfoxide [DMSO]). In the AP-cell-cryo group, the procedure that was used for cryopreservation of AP cells (Li et al., 1999), was used for AP tissue slices.

To further evaluate the suitability of cell cryopreservation method for AP tissue, AP samples were allocated into three groups based on the different ratios of cryopreservation components (DMSO, dulbecco's modified eagle's medium [DMEM], and FBS): AP-Cell-1 (1:4:5[20]), AP-Cell-2 (1:2:7) and AP-Cell-3 (1:0:9). Each small AP piece was transferred into a cryo-tube containing 1 mL cryopreservation solution, precooled in a refrigerator at 4°C for 20 min, then transferred to the programmed gradient cooling polystyrene box in a −80°C freezer (Shen et al., 2003).

One group of small pieces of fresh AP and ovary were placed in the 4°C fridge for 5 h used for positive controls.

2.3 | Thawing procedure

Samples in the vitrification-cryopreservation groups were thawed by sequentially putting in thawing solution I (1.0 M Sucrose+20%

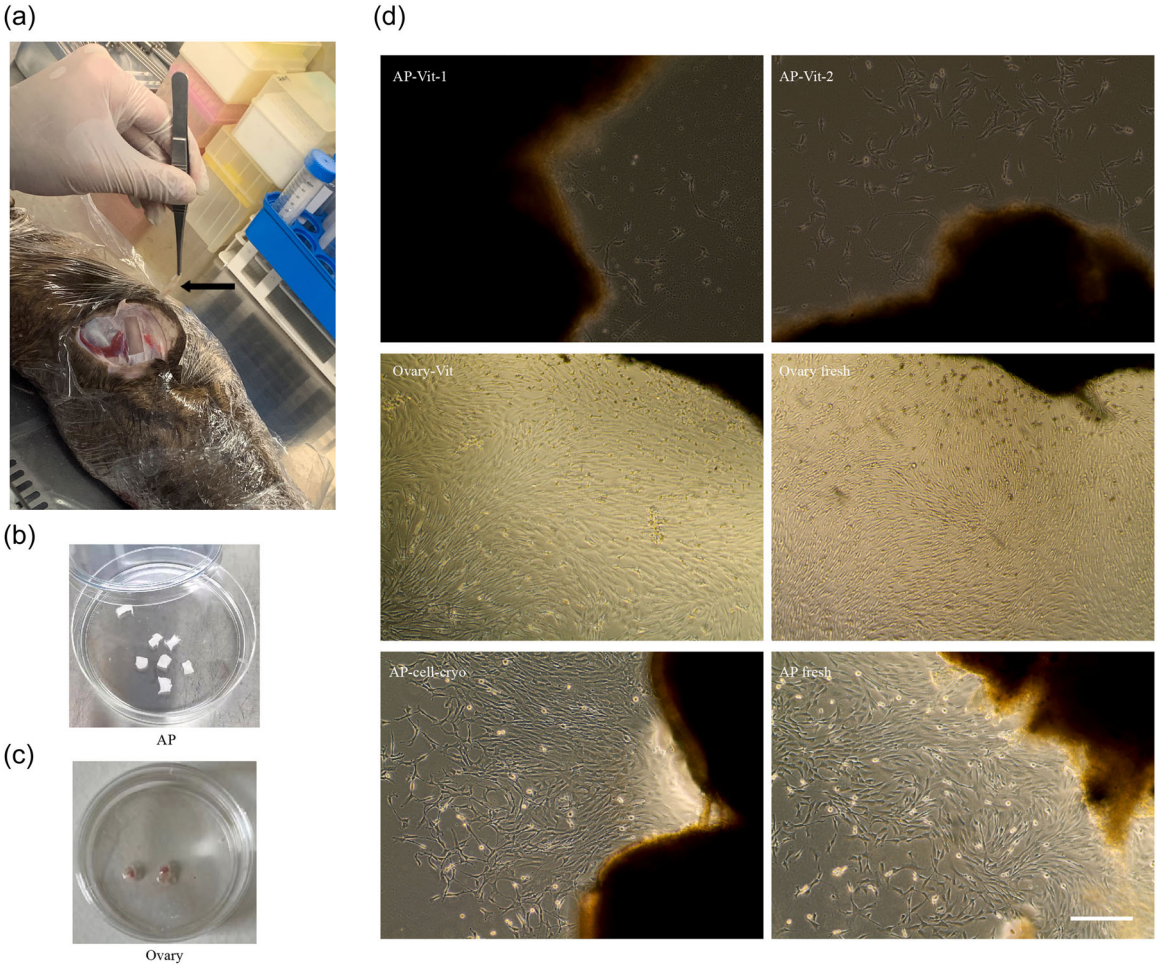

FIGURE 1 Sampling and primary culture of AP and ovary tissues. (a) AP sampling from the frontal crest (arrow); (b, c), Small AP slices and ovarian; (d) Cells migrated out from AP and ovary tissues after cryopreservation and fresh tissues on the 5th day of primary culture, respectively. Bar: 200μm. AP, antlerogenic periosteum.

FBS + DPBS), thawing solution II (0.5 M Sucrose+20% FBS + DPBS + 1% penicillin-streptomycin) and thawing solution III (0.25 M Sucrose +20% FBS + DPBS + 1% penicillin-streptomycin) for 10 min each at 37°C; then washed with cleaning solution (20% FBS + DPBS) twice, 10 min each. Finally, the samples were placed in the complete culture medium for further cell culture.

Samples in the cell-cryopreservation groups after cryopreservation for 5 h were thawed in a water bath at 37°C with gently shaken until the content in the tube was thoroughly thawed. Tissue and solution were decanted to a Petri dish, and the cryopreservation solution was replaced by the complete culture medium for further cell culture and nude mouse transplantation (Qin, 2018), respectively.

2.4 | Cell isolation and in vitro culture

The small pieces of thawed AP and ovary (kept in −80°C for 5 h) and fresh tissue (kept in 4°C for 5 h) were cut into thin sections (0.7 mm) using custom-built tissue cutter respectively, following the procedure reported by us (Li, 2017), then the tissue was digested with collagenase mix at 37°C for 20–25 min, centrifuged at 1000 r/min to remove collagenase, and washed twice using basic medium. The mixture was transferred to a 10 cm culture dish containing 3 mL culture medium, and cultured in a CO_2 incubator at 37°C until the cells started to migrate out.

2.5 | Evaluation of cell viability through CCK8 and clonogenicity

Proliferation rates of the cultured AP and ovary cells were determined using a Cell Counting kit 8 (CCK-8 kit) (APExBIO). The cells were seeded in 96-well-plates at density of 2000 cells/well. When the cells reached around 60% confluence, the medium was replaced by 90 μL new complete medium and 10 μL CCK-8 solution/well for further 2 h. Subsequently, the solution was measured at 450 nm using a microplate reader (Feyond-A300).

Viability of the cultured cells was also assessed using trypan blue staining. The adherent cells were digested with trypsin and diluted to prepare a single cell suspension; The cell suspension

was mixed with 0.4% trypan blue solution at a ratio of 9:1. Living cells and dead cells were counted within 3 min to calculate the viability of each cell type.

Clonogenicity of the cultured cells was measured as follows: the cells were trypsinized and suspended in 10 mL complete culture medium. The cell suspension was aliquoted into a six-well plate at 1000 cells/well, and the plate was gently rotated and then placed in a cell incubator for 2 weeks. Cell clones were examined under a microscope. The culture was fixed in 1000 μL of 4% paraformaldehyde for 15 min. Crystal violet staining solution was added to replace the fixative for 10 min. The clones were washed with water and then dried, observed under the microscope and the number of cell clones was counted in each well.

2.6 | Subcutaneous transplantation of the thawed AP to nude mice

For the transplantation of AP to nude mice, the thawed AP tissues of the cryopreservation method for cell group (kept in −80°C for 5 h) and the fresh AP tissue (kept in 4°C for 5 h) were transferred to a Petri dish containing complete culture medium, respectively.

Nude mice were anesthetized with 10% chloral hydrate (0.03 mL). The detailed procedure of AP transplantation was reported in our previously published papers (Li et al., 2001, 2008). Briefly, the forehead area of each mouse was shaved and sterilized. A coronal incision (around 0.5 cm) was made between the two ears and a pair of scissors was inserted into the incision to make pocket anteriorly. The mouse periosteum inside each pocket was gently scraped using a hand-held scalpel; each small piece of the thawed AP tissue was inserted into each pocket, and then the pocket was sutured. Growth status of AP was observed daily and xenogeneic antler samples were taken 20 days after transplantation.

2.7 | Histology of xenogeneic antlers

The xenogeneic antlers were removed surgically. The samples were fixed in 4% paraformaldehyde, dehydrated, embedded, sagittally sectioned, then counter-stained with Alcian blue and hematoxylin-eosin (HE) and observed under a scanning microscope (PreciPoint M8).

3 | RESULTS

3.1 | Cell viabilities of the tissues in different cryopreservation methods

Five days after culture, AP cells started to migrate out of the tissue blocks, but were highly different in cell number in the different vitrification groups: only few AP cells were migrated out in AP-Vit-1 and AP-Vit-2 groups, which was in sharp contrast to that of the fresh AP group (Figure 1d); whereas, cell migration status of the ovary tissue pieces confirmed that our vitrification methods well worked as the migrated-out cell numbers were comparable to those of the fresh ovary tissue (Figure 1d).

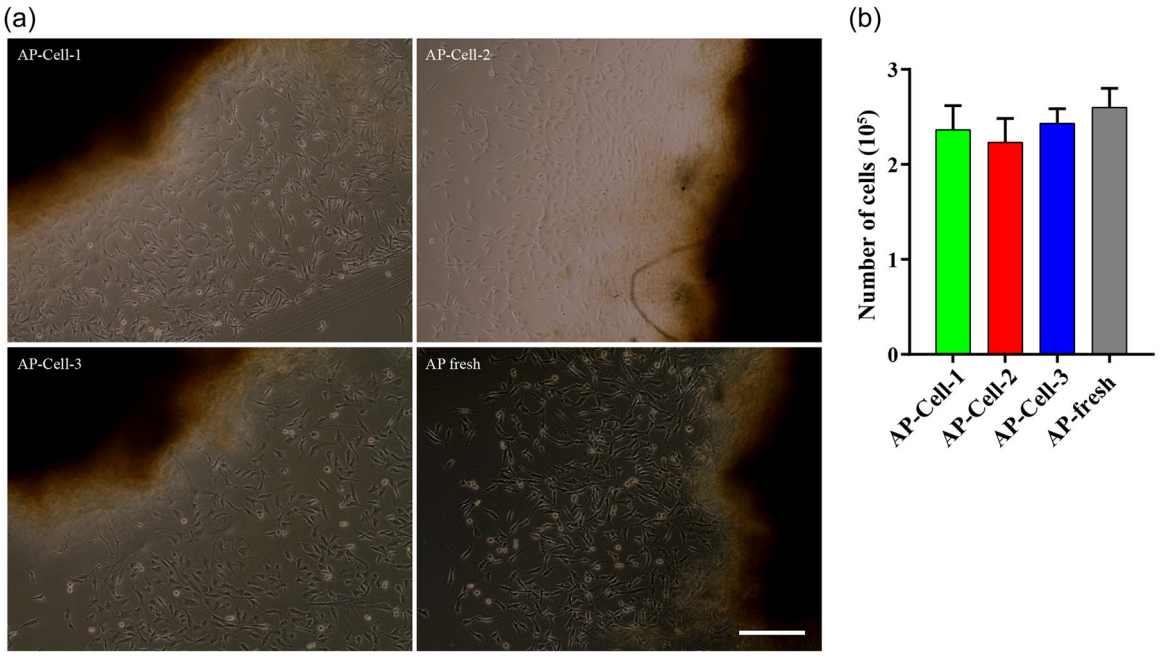

FIGURE 2 Primary culture of AP tissues using cell cryopreservation method. (a) Cells migrated out from the AP tissues with different cryoprotectant ratios on the 5th day of primary culture; (b) migrated cell number of AP tissues using cell cryopreservation method were comparable to those of the fresh AP group. Bar: 200μm. AP, antlerogenic periosteum.

Surprisingly, the migrated cell numbers of the frozen AP tissue in the cell-cryo group were comparable to those of the fresh AP group, that is migrated cells were densely populated around the tissue block (Figure 1d).

3.2 | Migration status of cells from the AP tissues using cell cryopreservation method

For cell culture, thin slices from fresh AP and cell-cryo group AP (AP-Cell-1, AP-Cell-2 and AP-Cell-3 group) were cultured in cell culture dishes for 5 days, AP cells migrated out in appreciable numbers in all three cell-cryo groups (Figure 2a), and migrated cell number were comparable to the fresh AP group (Figure 2b).

3.3 | Proliferation rate, viability and clonogenicity of cells from the AP tissues using cell cryopreservation methods

The cells from the AP cryopreserved using cell cryopreservation methods (AP-Cell-1 and AP-Cell-3 group) and fresh AP were cultured in a 96-well plate (2000 cells per well; Figure 3a), and proliferation rates of these cells were measured using CCK8 at 24, 48, and 72 h, respectively. The results showed that there were no significant differences between the three groups in cell proliferation rate ($p > 0.05$) (Figure 3b). Trypan blue staining was used to determine the cell viability. The results showed that the average number of viable cells of the two groups (91.2% and 90.8%, respectively) had no significant differences ($p > 0.05$) with

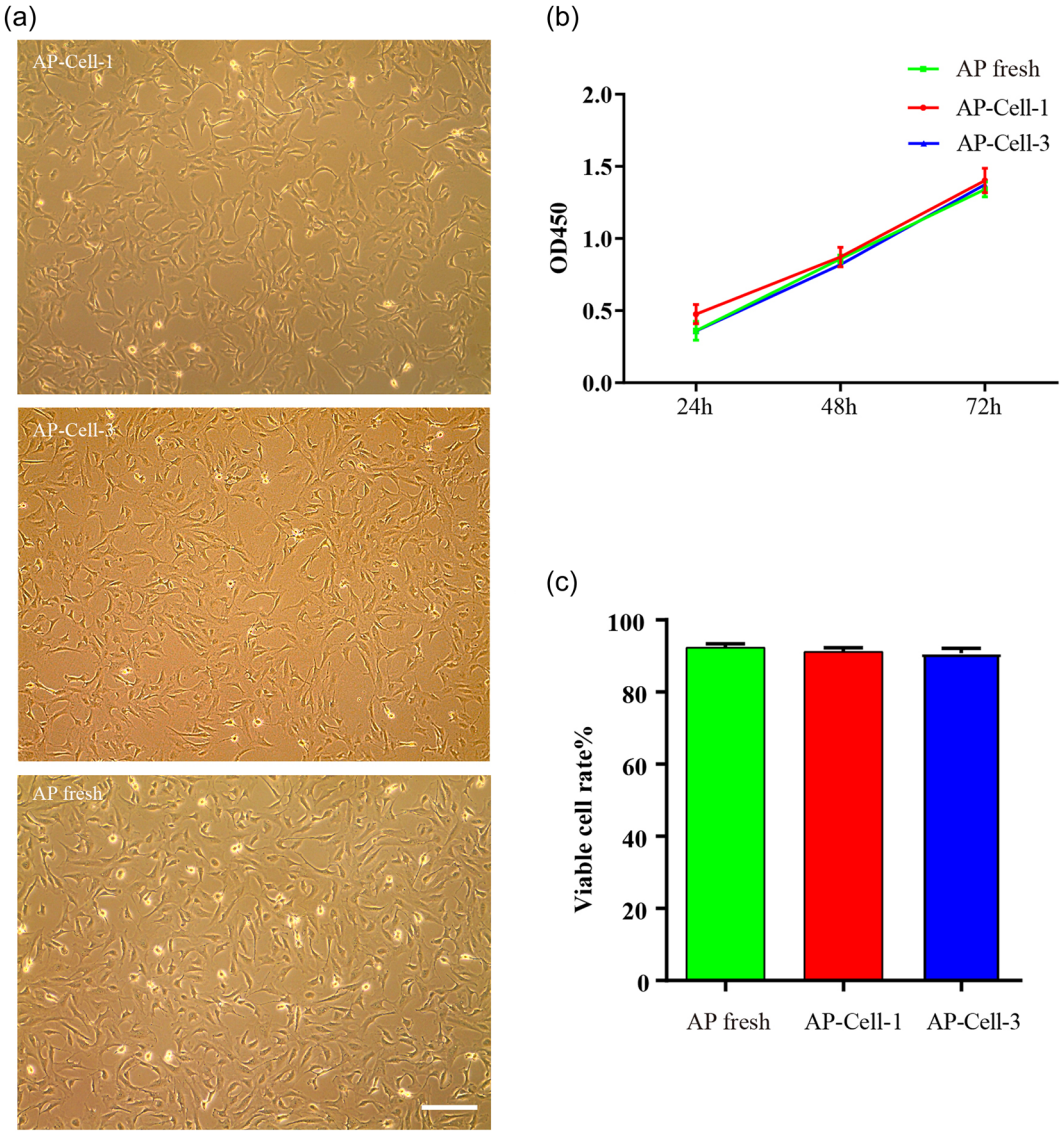

FIGURE 3 Proliferation rate and viability of cells from the cryopreserved AP tissues. (a) Cells from the AP cryopreserved using cell cryopreservation methods (AP-Cell-1 and AP-Cell-3 group) and those from the fresh AP. (b) Proliferation rates of cells from the AP-Cell-1, AP-Cell-3 groups and the fresh AP group were measured using CCK8 at 24, 48, 72 h, respectively. (c) Cell viabilities of the cells from the AP-Cell-1, AP-Cell-3 and the fresh AP group using trypan blue staining. Bar: 200μm. AP, antlerogenic periosteum.

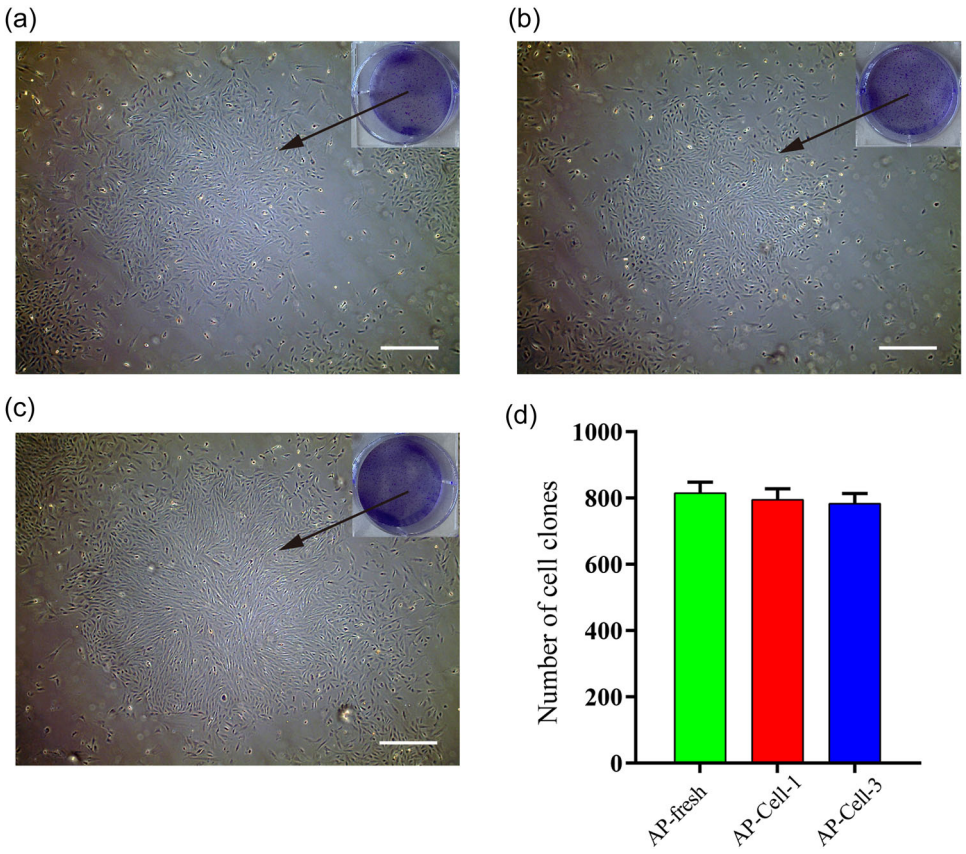

FIGURE 4 Clonogenicity of the cells from the cryopreserved AP tissues and the fresh AP tissue. (a) Clone of cells from the AP-Cell-1 group. (b) Clone of cells from the AP-Cell-3 group. (c) Clone of cells from the fresh AP tissue. (d) Comparation in clone number of cells from the fresh and cryopreserved AP groups. Note that there was no significant difference. AP, antlerogenic periosteum.

those of the fresh AP (92.4%) (Figure 3c). Overall, there was no significant difference between the cell-cryo groups and the fresh group in cell viability ($p > 0.05$).

Cell proliferation rates of the AP-Cell-1 and AP-Cell-3 groups were also compared with the fresh tissue group via clonogenic analysis (Figure 4a–c). There were no significant differences being found in cell proliferation ($p > 0.05$), and in the number of clones (around 800; Figure 4d).

3.4 | Formation of xenogeneic antlers in vivo by the AP tissue cryopreserved using cell-cryo-methods

The cryopreserved AP tissues (AP-Cell-1 and AP-Cell-3 group) and the fresh AP tissues were respectively transplanted subcutaneously to the foreheads of nude mice to test their difference in vivo viability via generating xenogeneic antlers. The AP-Cell-1, AP-Cell-3 groups and fresh AP group grew similar size of xenogeneic antler 20 days after transplantation (Figure 5a–c), xenogeneic antlers of all groups were firmly attached to the skull of nude mice, and the nude mice in each group were all healthy. The results demonstrated that the cryopreserved AP tissue had almost the same potential of xenogeneic antler generation as the fresh AP in vivo. The xenogeneic antlers were removed 20 days after transplantation and counter-stained with HE and Alcian blue. From the results, blue staining were chondrocytes under the antler skin in each group, further down were many irregular shaped bone trabecular structures. Although the number of blue stained chondrocytes was relatively less in AP-Cell-1 group, we believe it was caused by the high degree of ossification during the development of transplanted AP tissue, this phenomenon varied greatly among different individuals, but the positioning of cell types was the same in the three groups, namely fibroblasts, chondrocytes and osteocytes of xenogeneic antlers distoproximally (Figure 5d–f). The results demonstrate that there was no visible difference in temporal differentiation sequence between the fresh AP and cryopreserved AP group (AP-Cell-1 and AP-Cell-3 group).

In conclusion, AP, the tissue that is for initial pedicle and first antler formation, can be cryopreserved successfully via a simple way (general method for cryopreservation of cells) without losing growth potential in vitro and in vivo. The cryoprotective solutions consisting of DMSO, DMEM and FBS (either in a ratio of 1:4:5, 1:2:7 or 1:0:9) can adequately protect the AP tissue from freezing damage after thawing. Adaptation of this method would greatly facilitate biology research of deer antlers, a unique model of mammalian organ regeneration.

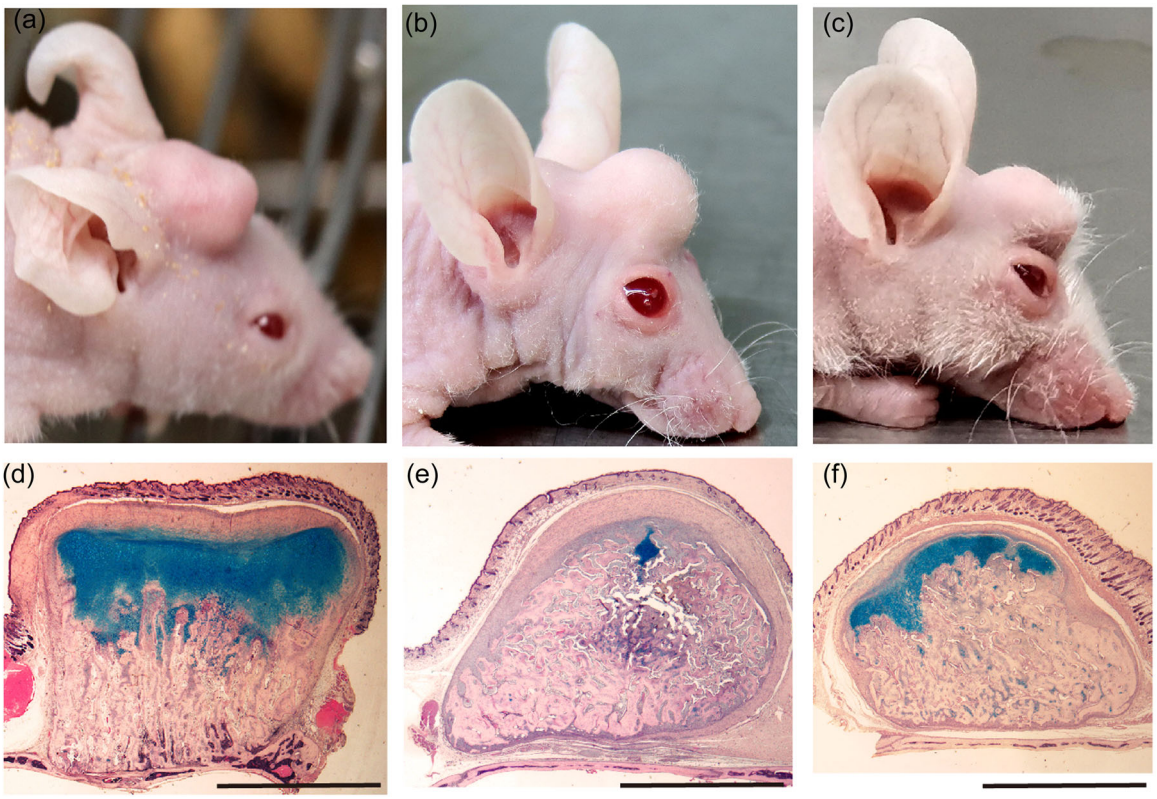

FIGURE 5 Potential of xenogeneic antler generation of the cryopreserved and fresh AP tissues. (a–c) Xenogeneic antlers, (a) generated from the fresh AP tissue. (b) generated from AP-Cell-1 group. (c) generated from AP-Cell-3 group. (d–f) Vertical histological sections of the xenogeneic antlers with HE and Alcian blue staining. (d) generated from the fresh AP tissue. (e) generated from AP-Cell-1 group. (f) generated from AP-Cell-3 group. The positioning of cell types was the same in the three groups, namely fibroblasts, chondrocytes and osteocytes of xenogeneic antlers distoproximally. Note that there was no visible difference in histological makeups among these xenogeneic antlers. Bar: 2 mm. AP, antlerogenic periosteum.

4 | DISCUSSION

The antler offers a unique model for studying organogenesis and epimorphic regeneration (Goss & Powel, 1985; Li, 2013). The AP, which is the key tissue type that gives rise to the deer pedicle and first antler, is essential to study these biological processes (Gao et al., 2012). However, using deer themselves to study the generation and regeneration of antlers is not only extremely expensive, but also time-consuming (deer grow antlers once a year) (Li et al., 2010). So, we established a xenogeneic model using nude mice to study these processes (Li, Gao, et al., 2009). In so doing, statistically viable experiments can be feasibly designed one deer can provide AP tissues for around 20 nude mice (Li et al., 2001, 2008) and the work can be carried out year-round. However, that a limiting factor exists for fully establishment of the nude mouse model is that the narrow-window opportunity for AP sampling (less than 2 months in a year). Therefore, cryopreservation of AP may be one of the effective ways to solve the shortage of year-round availability of the tissue AP.

It has been reported that somatic periosteum of long bone has been successfully cryopreserved (Kreder et al., 1993; Mase et al., 2006), but the AP tissue was 3–4 times thicker than somatic periosteum (such as facial periosteum) (Kierdorf et al., 1994; Li & Suttie, 1994). It is unknown whether it is feasible to freeze AP with the somatic periosteum freezing method. The somatic periosteum freezing method requires an expensive procedural cryometer and a large quantity of liquid nitrogen, which is difficult to meet in general laboratories. Fortunately, it has been reported that vitrification can successfully freeze tissue samples, such as human ovarian tissue (Leonel et al., 2019). Therefore, in the study we tried to determine whether vitrification method could be used to freeze AP tissue, taking conventional freezing methods for cells as a control. The results of both in vitro and in vivo experiments showed that the vitrification procedure failed to successfully cryopreserve AP tissue with significantly losing cell viability, although had the ovarian tissue well preserved. The possible reason for the discrepancy could be the tissue texture: the former is hard and dense but the latter is soft. Unexpectedly, the cells of AP tissue frozen using the conventional cryopreservation method for cells had similar vitality as those of the fresh AP tissue. Therefore, AP tissue seems suitable preservation using the procedure for cryopreservation of cells. Because our established cryopreservation procedure for AP tissue does not require expensive gradient cooling instruments, nor expensive protective agents, we believe we have developed a simple and effective method for cryopreservation of thick periosteum

(such as AP) or likewise. The nude mouse model for xenogeneic antler formation coupled with effective cryopreservation for the AP tissue would greatly facilitate antler biology research, and contribute to the eventual revealing the mechanism underlying full regeneration of antlers, the only mammalian organ capable of doing so.

AUTHOR CONTRIBUTIONS

Conceptualization: Pengfei Hu and Chunyi Li. *Methodology*: Jiping Li. *Validation*: Dongxu Wang, Jing Ren, and Yusu Wang. *Formal analysis*: Pengfei Hu. *Investigation*: Yusu Wang. *Resources*: Jiping Li. *Data curation*: Dongxu Wang. *Writing—original draft preparation*: Jiping Li and Pengfei Hu. *Writing—review and editing*: Chunyi Li. *Visualization*: Pengfei Hu. *Supervision*: Chunyi Li. *Project administration*: Pengfei Hu. *Funding acquisition*: Chunyi Li. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ETHICS STATEMENT

All procedures related with animals were conducted in accordance with the guidelines of care and use of experimental animals established by the Ministry of Agriculture of China, and all protocols were approved by the Animal Ethics Committee of Institute of Antler Science and Product Technology, Changchun Sci-Tech University, Changchun, China (Ethics No.: CKARI202105).

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