RESEARCH ARTICLE

# Antler Development Was Inhibited or Stimulated by Cryosurgery to Periosteum or Skin in a Central Antlerogenic Region Respectively

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FUHE YANG[1,4], WENYING WANG[2], JUNDE LI[3], STEPHEN HAINES[2], GEOFF ASHER[2], AND CHUNYI LI[2,4]*

[1]Institute of Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, Beijing, P. R. China
[2]AgResearch Invermay Agricultural Centre, Mosgiel, New Zealand
[3]Institute of Materia Medica, China Academy of Chinese Medical Sciences, Beijing, P. R. China
[4]State Key Laboratory for Molecular Biology of Special Economic Animals, Beijing, P. R. China

## ABSTRACT

Antler development is triggered by interactions between antler stem cells resident in the antlerogenic periosteum (AP) and the niche cells in the upper portion of overlying skin mediated by diffusible molecules. These interactive cell populations are interposed by the lower portion of the skin and the subcutaneous loose connective tissue (SLCT). It is known that mechanical deletion of just the central AP (having an area equivalent to the size of a pedicle base) by cutting through the skin and SLCT effectively stimulates the marginal AP to initiate antler development. This study was designed to investigate whether the SLCT layer plays a role in antler development by acting as a physical barrier. The results showed that the marginal AP failed to give rise to an antler after the central AP was cryosurgically destroyed with the preservation of the collagen structure of the SLCT. Furthermore, antler development was significantly advanced when the collagen structures of the skin and SLCT layers were substantially attenuated by repeated sprays with liquid nitrogen while keeping the central AP intact. Therefore, we conclude that the interposing SLCT layer acts as a physical barrier between antler stem cells and the niche cell types, and that timing of antler development is primarily controlled by the permeability of the SLCT layer to the putative interactive diffusible molecules. *J. Exp. Zool. (Mol. Dev. Evol.) 316:359–370, 2011.* © 2011 Wiley-Liss, Inc.

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Deer antlers offer a prime opportunity for studying the role of stem cells in mammalian organ development and regeneration, including stem cell renewal and multipotency, fate determination, positional information, and interactions between stem cells and their niche (Goss, '90; Kierdorf et al., 2009; Li et al., 2009b). It is known that the potential to grow a pedicle (antecedent of an antler) and an antler is exclusively held in the periosteum overlying a frontal crest (immediately behind an eye socket) of a prepubertal deer. This tissue is thus known as "antlerogenic periosteum" (AP). Removal of AP abolishes the future pedicle and antler growth, whereas transplantation of AP elsewhere on the deer body induces formation of ectopic antlers (Hartwig and Schrudde, '74; Goss and Powel, '85; Li and Suttie, 2001). Further research has demonstrated that the AP cells express key embryonic stem cell markers (October 4, Nanog, Sox2 and CD9) and can be induced in vitro to differentiate into chondrocytes, osteoblasts, adipocytes, myoblasts, and neuronal-like cells

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*Correspondence to: Chunyi Li, AgResearch Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand. E-mail: chunyi.li@agresearch.co.nz
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(Harper et al., 2009; Li et al., 2009b). Therefore, these AP cells are regarded as antler stem cells.

As male secondary sexual characters, pedicle and antler development are under the control of androgen hormones (Bubenik, '82; Suttie et al., '95; Li et al., 2003). When male deer approach puberty, rising circulating androgen hormones trigger the proliferation and differentiation of antler stem cells. Gradually, a pedicle with species-specific thickness (around 34 mm in diameter in red deer (Li, '97) and 30 mm in sika deer (Li et al., '88)) and height (45–50 mm in both species) is built up and antler transformation takes place subsequently from its apex (Li and Suttie, '94a, 2000; Li, '97, 2000). Interestingly, the antlerogenic region is widespread over a deer frontal bone and antlerogenic potential is not confined to the AP in a central area (central AP/antler stem cells) of the region covering a pedicle base or a pedicle itself. Instead, periosteum (marginal AP/antler stem cells) that surrounds the central AP can also initiate antler growth if the central AP (for prepubertal deer) or pedicle (for post-pubertal deer) is removed/destroyed mechanically (Bubenik and Pavlansky, '65; Jaczewski, '67; Li and Suttie, '94b) or chemically (Robbins, '81). It has been postulated that antlerogenic potential of AP (either central or marginal) is unleashed by sheer mechanical traumatization (Jaczewski, '82) or by the establishment of the connections between AP and the putative antler growth center in the central nervous system through mechanical traumatization (Bubenik, '82).

When carrying out AP transplantation experiments, Goss ('87) noticed that ectopic antlers could not form unless the AP-derived tissue became intimately adhered to the overlying skin. This led him to conclude that close association between AP-derived tissue and skin is the prerequisite for the establishment of interactions between AP-derived tissue and the skin epidermis, and that it is these interactions which trigger subsequent antler development (Goss, '90). Systematic histological examination of pedicle and first antler development (Li and Suttie, 2000) strongly support this view, as antler transformation from a growing pedicle does not take place until apical pedicle tissue, the derivative of AP, becomes intimately associated with the overlying skin. Furthermore, the interactions between AP and skin epidermis may be mediated by dermal papilla cells (DPC) of hair follicles. This is because only skin that is adorned with hair follicles is competent to interact with transplanted AP to initiate ectopic antler formation (Goss, '87), and epidermis and its associated partial DPC-containing dermis are found to be sufficient to interact with AP to initiate ectopic antler formation using a nude mouse model (Li et al., 2009a). Therefore, DPC and epidermal cells have been considered as essential niche cell types in the antler stem cell system (Li, 2010).

In order to functionally test the tissue interaction theory, Li et al. (2008) carried out a membrane insertion experiment. The results convincingly demonstrated that interactions between AP-derived tissue and the overlying skin are indispensible for initial antler formation and are mediated through diffusible molecules, because insertion of an impermeable membrane completely stopped antler growth, whereas a semi-permeable membrane (0.45 m pore size) only delayed (by a year) but did not abrogate antler transformation. Consequently, antler development results from the interactions between antler stem cells and the niche cell types via exchange of diffusible molecules.

Based on these aforementioned findings, Li et al. (2003) put forward a new explanation of why mechanical wounding is so effective for stimulating AP to grow antlers. That is, mechanical wounding can effectively break the interposing tissue layers between antler stem cells (AP cells) and the niche cell types (DPC and epidermal cells) and create an opportunity for their direct contact, thus bypassing the requirement for the close tissue association that leads to establishment of interactions between these cell types and initiation of antler formation. These interposing layers, including subcutaneous loose connective tissue (SLCT) and its associated partial dermal tissue just below the bulbs of hair follicles (Li et al., 2009a), act as a physical barrier for controlling the timing of antler development. However, thus far this hypothesis has not been experimentally tested.

One experimental approach to test this hypothesis would be to use cryosurgery. Cryosurgery is the most widely used method for destroying abnormal or diseased tissue, and currently the preferred cryogen is liquid nitrogen ($LN_2$). Interestingly, $LN_2$ spray applied for less than 30 sec does not result in scarring, because of the preservation of the collagen layer of the dermis including SLCT, which allows for in-migration of the cellular components in the healing process and normal integrity of the skin layers (Andrews, 2004). Because appropriate $LN_2$ treatment can effectively decellularize the treated tissue, and at the same time keep extracellular collagen components intact, cryosurgery offers an opportunity to directly test the hypothesis advanced by Li et al. (2003) that breaking the interposing physical barrier is the main reason why mechanical wounding can stimulate AP either from the central or marginal region to grow antlers.

The aims of this study were to determine (1) whether marginal AP could initiate antler formation following the destruction/decellularization of the central counterparts through cryosurgery while leaving the interposing collagen layers (physical barrier) intact by applying $LN_2$ spray for less than 30 sec; and (2) whether selectively repeated cryosurgery of the skin overlying the central AP in an antlerogenic region could significantly advance initiation of antler formation, i.e. whether antler transformation would take place before a pedicle reaching its final species-specific height, as repeated $LN_2$ spray would attenuate the interposing collagen layers, if not completely destroy them, and hence would be expected to promote molecular signaling between antler stem cells and their niche cell types.

## MATERIALS AND METHODS

### Experiments and Animals

This study consisted of three experiments (Table 1). Experiment 1: To establish the minimum duration of $LN_2$ spray for totally destroying central antler stem cells while preserving the interposing physical barrier (within the limits of spray duration, i.e. $<30$ sec) using female red deer (*Cervus elaphus*) heads immediately after slaughter (Southern hemisphere). The reason for the selection of female deer heads rather male is because scientifically it makes no difference for the purpose of the experiment, and because male deer heads at the suitable stage were not available from the local slaughtering plant at that particular sampling time. Female deer also possess AP at the corresponding presumptive antler growth regions, and do not grow antlers under the normal condition solely because their circulating androgen level is low (Bubenik, '82). Experiment 2: To determine whether the marginal AP could initiate antler growth following extermination of the central AP through cryosurgery (while keeping the extracellular collagen component intact) using living red deer stag calves (Southern hemisphere). Experiment 3: To find out whether antler development could be advanced by selectively repeated $LN_2$ spray to the skin (attenuating the interposing physical barrier) overlying the central AP; and at the same time to ascertain that the failure of antler formation from a sprayed antlerogenic region (skin+AP) was owing to preservation of the interposing physical barrier, rather than cryosurgery of the overlying skin using living sika deer (*Cervus nippon*) stag calves (Northern hemisphere, for the purpose of speeding up the study).

A CRY-AC 500 mL cryogun fitted with a size "A" nozzle (Brymill, Ellington, CT) was used to deliver $LN_2$ to the presumptive antlerogenic region of the deer.

### Methods

Two types of tissue cryosurgery were included in the study: skin-only and skin+AP. For skin-only cryosurgery, the skin over the AP was moved back and forth during the intervals of $LN_2$ spraying to avoid cryodamage to the underlying AP. Spray duration was 15 sec after the icefield filled in the central region (directly overlying a frontal crest), an area roughly equivalent to the correspondent deer pedicle base. For skin+AP cryosurgery, spray duration was counted from the time when skin had frozen to the underlying AP (the skin could no longer be moved over AP by fingers). Before each cryosurgery, the hair over each antlerogenic region was thoroughly shaved and the nozzle of the cryogun was positioned 2–4 cm above the deer skin surface.

Experiment 1: The aim of the experiment was to establish the minimum duration of $LN_2$ spray for totally destroying central antler stem cells. Three 10 month old female red deer heads were recovered immediately after slaughter from a local abattoir, and both presumptive antlerogenic regions of each head were

Table 1. Experimental design.

| | Deer | | | | Pedicle at spray | | $LN_2$ spray | |
|---|---|---|---|---|---|---|---|---|
| Experiment | Species | Sex | Status | N | Height (mm) | Side | Design Left/right | Diameter (mm) |
| 1 | Red | F | Dead | 3 | $<5$ | Spray duration/side | 5, 10, 20, 30, 40 sec **or** 3-FT-C | 33 |
| 2 | Red | M | Live | 13 | $\leq 15$ | Left or right | Skin+AP **or** NT | 33 |
| | | | | 1 | 22 | Left/right | NT/Skin+AP | Apex, shoulder, and shaft |
| 3 | Sika | M | Live | 1 | $<5$ | Left/right | NT/Skin+AP | 28 |
| | | | | 4 | 5–15 | Left or right | Skin-only $\times$ 4 **or** Skin-only $\times$ 3+Skin+AP | 28 |
| | | | | 1 | 22 | Left/right | Skin-only $\times$ 4 **or** Skin-only $\times$ 3+Skin+AP | Apex, shoulder, and shaft |

F, female; M, male; N, number; $LN_2$, liquid nitrogen; AP, antlerogenic periosteum; NT, no treatment; 3-FT-C, 3 freeze and thaw cycles (15 sec spray and 60 sec thaw time).

thoroughly shaved and sterilized using 70% alcohol and 5% iodine tincture. Each region was allocated to one of the six treatments (region/treatment), five of which involved different spray durations (skin+AP): 5, 10, 20, 30, and 40 sec, respectively, whereas in the final treatment, three freeze and thaw (FT) cycles were used with 15 sec spray and 60 sec thaw per cycle (Table 1). Selection of these spray durations were based on previous reports (Gage and Baust, '98; Andrews, 2004), which showed that destruction of benign lesions requires temperatures from −20 to −30°C, and that tissue temperatures from −25 to −50°C can be achieved within 30 sec if a sufficient amount of $LN_2$ is applied by spray. The sprayed area in this experiment was around 33 mm in diameter (Fig. 1A), an area that is slightly smaller than an average pedicle base (35 mm in diameter) for red deer (Li, '97). When properly thawed to room temperature after $LN_2$ spray treatment, a piece of AP (around 5 × 10 mm) was sampled from the center of each sprayed region (Fig. 1B) following removal of the overlying skin under aseptic precaution. Each piece of sampled AP was transferred under aseptic conditions into a 50 mL centrifuge tube containing 20 mL of sterile 2% penicillin/streptomycin DMEM medium (Invitrogen, New Zealand), and was then cut into small explants (each around 1 $mm^2$) in a laminar flow hood. Following established AP cell culture procedures (Li et al., '99), the sampled AP explants were enzymatically digested to release antler stem cells, which were then cultured in T25 flasks (Nunc, New Zealand, flask/AP piece) in a 5% $CO_2$ incubator. The cultures were observed daily after an initial 5-day incubation period, and the date of initial cell attachment and cell abundance were recorded.

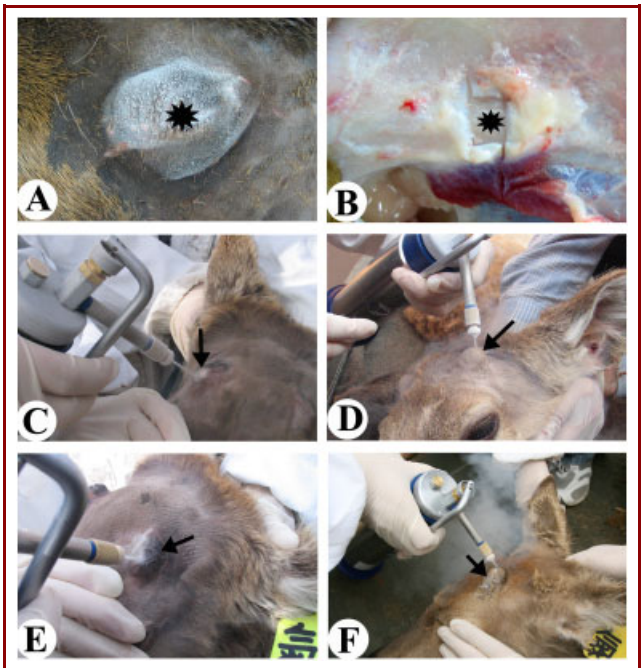


Figure 1. Cryosurgery of the antlerogenic regions using LN2 spray: **(A)** A 33-mm-in-diameter-ice-field formed in the central area of an antlerogenic region (asterisk) in a female red deer. **(B)** The area (asterisk) to show the sampled size of AP from the centre of sprayed antlerogenic region in a female red deer. **(C)** LN2 spray to an antlerogenic region at crest stage (arrow) in a red deer. **(D)** LN2 spray to an antlerogenic region at incipient pedicle (22 mm high) stage (arrow) in a red deer. **(E)** Third time of LN2 spray to an early stage pedicle (>15 mm high) in a sika deer. Note that skin blisters (arrow) had formed following previous LN2 spray. **(F)** Fourth time of LN2 spray to an incipient pedicle (22 mm high) in a sika deer. Note that the pedicle was covered by scab (arrow), which was formed from skin blisters.

Experiment 2: The aim of the experiment was to determine whether the marginal AP could initiate antler growth following extermination of the central AP through cryosurgery. Fourteen red deer stag calves were selected during the pedicle initiation period (around 10 months old, Southern hemisphere), when pedicle heights ranged from 4 to 22 mm (Table 1). The antlerogenic region on one side was randomly selected for skin+AP cryosurgery and the other was left untreated as a control. A single 3-FT-cycle spray (as per Experiment 1) was applied to each treated region. For an antlerogenic region that had a frontal crest or a very early stage growing pedicle (≤15 mm in height; Fig. 1C), the area sprayed was similar to that in Experiment 1 (around 33 mm in diameter); and for the only incipient pedicle (22 mm in height) in the experiment, the spray was applied to its apex, shoulder, and shaft regions (Fig. 1D). Observation of the scalp skin reaction to the spray and pedicle/antler growth status was conducted daily for 2 weeks after the treatment and then once a week.

Experiment 3: The aim of the experiment was to find out whether antler development could be advanced by selectively repeated $LN_2$ spray to the skin. Six sika deer stag calves were selected during the pedicle initiation period (around 10 months old, Northern hemisphere), when pedicle heights ranged from 4 to 22 mm (Table 1). For Deer 1, the antlerogenic region on right side was randomly selected for skin+AP cryosurgery and the other left untreated as a control. A single 3-FT-cycle spray was applied to the skin+AP of this deer to determine whether there was any difference in reaction of antlerogenic region to $LN_2$ spray between red deer and sika deer. For Deer 2–6, the antlerogenic regions on one side were randomly selected for skin+AP cryosurgery and on the other side for skin-only cryosurgery. Before the final spray, three 3-FT-cycle sprays for skin-only were applied to each region assigned for either skin+AP or skin-only at the final spray. For an antlerogenic region that had a frontal crest or a very early pedicle (≤15 mm in height), the area sprayed was around 28 mm in diameter, which is slightly smaller than the pedicle base area (around 30 mm in diameter) of this species (Li et al., '88). For the only incipient pedicle (22 mm in height) in the experiment, the spray was applied to its apex, shoulder, and shaft regions, as in Experiment 2. Apart from deer species, the

key difference in design between this experiment and Experiment 2 was that before carrying out skin+AP or skin-only cryosurgery, three additional 3-FT-cycle sprays had already been applied to the skin at 1 week intervals, to attenuate the interposing physical barrier overlying the central AP/incipient pedicles (Fig. 1E and F). Deer in this experiment were kept for two antler growth seasons, and any antlers that grew during the first growth season were cut off above the pedicle using standard procedures at the normal time of antler removal.

The 3-FT-cycle spray treatment was selected for both Experiment 2 and 3 because, although both the 40 sec continuous spray and the 3-FT-cycle spray treatments were found equally effective to decellularize AP tissue (refer to the results of Experiment 1), the 40 sec spray was likely to cause extracellular component damage (i.e. disruption of integrity of the physical barrier). In contrast, the 3-FT-cycle spray treatment was less likely to cause this problem as each spray remained within the 30 sec limit shown to preserve the extracellular component (Andrews, 2004). Furthermore, repetition of the FT cycles is known to be a more effective way of destroying cells than a continuous spray of the same duration (Andrews, 2004).

## RESULTS

### Experiment 1

The aim of Experiment 1 was to establish the minimum duration of $LN_2$ spray for totally destroying central antler stem cells. Cells were enzymatically released from the $LN_2$-sprayed AP tissue and cultured in vitro. The first sign of AP cell attachment with identifiable cell colonies (around 6–10 cells/colony) was observed in the flask of the 5 sec spray treatment on day 7 after seeding. For the 10, 20, and 30 sec spray treatments, cell attachment was first observed on days 9, 14, and 18, respectively. No cell attachment was detected in either of the 40 sec spray or the 3-FT-cycle treatment flasks by day 25, when the cell culture experiment was terminated. The results of Experiment 1 are summarized in Table 2.

### Experiment 2

The aim of Experiment 2 was to determine whether the marginal AP could initiate antler growth following extermination of the central AP through cryosurgery. Within 1–2 days after the spray treatments, reddish edema of varying severity (Fig. 2A) occurred in the sprayed regions of all deer. After 2 weeks, the edema had completely disappeared and a demarcation line was evident at the boundary between the sprayed and unsprayed areas (Fig. 2B). The superficial layer (epidermis possibly associated with a very thin layer of dermis) of the sprayed skin was shed around 3 weeks after the spray to expose the underlying dermal tissue, the outmost surface of which at the time had already dried without signs of bleeding (Fig. 2C). These exposed areas were rapidly covered by healing epidermis with hypopigmented hair within about 2 weeks (Fig. 2D). The hypopigmented hair regions in most cases reduced in area during the period of pedicle and antler growth from the control sides, but never completely disappeared (Fig. 2E and F). By the end of the first antler growth season (January, Southern hemisphere), 12 (having pedicle height all under 15 mm at spray) out of 14 stags had not grown pedicles and/or antlers from the $LN_2$-sprayed (skin+AP) antlerogenic regions, although the control sides all formed normal pedicles with the extension of either spike (Fig. 2G) or branched (Fig. 2H) antlers. Interestingly, $LN_2$ spray failed to inhibit the growth of antler (which reached a final height of 40 mm; Fig. 2I) from one antlerogenic region that had a 5 mm crest when sprayed. Likewise, the spray treatment did not stop the 22 mm high incipient pedicle from growing further and subsequently undergoing antler transformation (Fig. 2J), although this was delayed compared with the control side.

### Experiment 3

The aim of Experiment 3 was to find out whether antler development could be advanced by selectively repeated $LN_2$ spray to the skin. A single 3-FT-cycle spray treatment to the right side of the antlerogenic region (skin+AP) of Deer 1 caused a skin reaction with reddish edema, which then turned into a superficial scab in the sprayed region (Fig. 3A). The scab was eventually shed and the area was quickly covered by healing epidermis with hypopigmented hairs (Fig. 3B). For Deer 2–6, the skin reaction to the 3-FT-cycle spray treatments, either four times of skin-only (control) or three times of skin-only plus one time of skin+AP (treated), was much more severe than that of the single 3-FT-cylce spray given to Deer 1. The initial reaction to these sprays was the formation of reddish edema (Fig. 3C), which developed into blisters (Fig. 3D) on the apex of each incipient pedicle/crest 4–5 days after the final spray. These blisters were then dried and exfoliated from the control sides owing to pedicle growth taking place (skin-only cryosurgery side; Fig. 3E, F and G) around

Table 2. Effects of $LN_2$ spray duration on AP cell survival rate.

| Duration (sec) | 5 | 10 | 20 | 30 | 40 | 3-FT-cycle |
|---|---|---|---|---|---|---|
| Commencement of cell attachment (days) | 7 | 9 | 14 | 18 | – | – |
| Final cell abundance | ++++ | ++++ | ++ | + | 0 | 0 |

+: ≤2 cell colonies; ++: 3–5 cell colonies; and ++++: >15 cell colonies. 3-FT-cycle: 3 freeze and thaw cycles

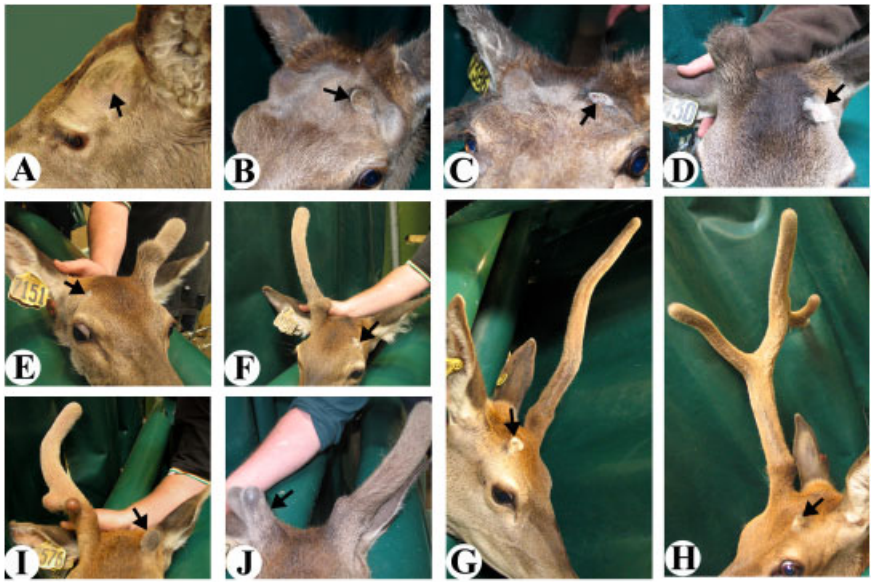


Figure 2. Effects of a single 3-FT-cycle LN2 spray to the antlerogenic regions on subsequent pedicle and antler development in red deer: **(A)** Reddish edema (arrow) was formed in the antlerogenic region 2 days following LN2 spray. **(B)** A suture line (arrow) formed along the boundary between the sprayed and unsprayed areas on an antlerogenic region. **(C)** Outmost surface (arrow) of the sprayed area after the scab shedding. Note that the surface was dry and no bleeding occurred. **(D)** The exposed area after the scab shedding was healed by skin having hypopigmented hairs (arrow). **(E)** and **(F)** Hypopigmented-hair-area (arrows) significantly reduced at early (2E) or mid (2F) antler growth stage. **(G)** and **(H)**. No pedicle or antler was formed from the LN2 sprayed regions (arrows), although each control region formed a spike (2G) or a branched (2H) antler. **(I)** Antler (arrow, final length reached 40 mm) growth occurred from the LN2 sprayed area of the antlerogenic region without obvious pedicle development at the spray. **(J)** Pedicle and antler formed (arrow) after LN2 spray from the antlerogenic region that had developed to 22 mm high pedicle stage at the spray.

10 days after the final spray. Similar to Experiment 2, the cryosurgery of skin+AP, although causing a delay compared with the control side, did not stop the further growth of the 22 mm high pedicle of Deer 6. Therefore, in this deer, dried scab (formed from blisters) was also shed from the treated side (Fig. 3H).

By the end of the first antler growth season, 5 out of 6 deer (Deer 1–5, having pedicle heights: 4–15 mm at spray) failed to grow pedicles and/or antlers from the treated sides, although the control (skin-only cryosurgery) sides all developed pedicles and spike antlers (Fig. 4A, E–H, I, J, M, N, Q, R). The remaining deer, Deer 6 (which had 22 mm high pedicles) formed a pedicle and subsequent antler not only from the control side, but also from the treated side (Fig. 4U and V).

Selectively repeated (four times) $LN_2$ spray to skin-only resulted in significant advancement of the skin transformation from scalp to antler velvet (mean length from pedicle base to antler velvet: 22.5 mm) and the coronet formation (mean length from pedicle base to coronet: 40.8 mm) during a pedicle formation (Table 3), compared with the average pedicle height (45–50 mm, including both the skin change point and the coronet height) in this species.

All deer except Deer 2 survived to the second antler growth season, and antler remnants (leftover after antler removal in the first growth season) were all properly calcified (Fig. 4B, K, O, S and W) and dropped off at normal casting time, which was immediately followed by antler regeneration. The average pedicle length of these deer was 35 mm (Table 3), and all regenerated antlers, but one (Deer 4), had two branches (Fig. 4D, L, P, T and X).

## DISCUSSION

The discovery of antler stem cells has opened a new avenue for the study of mammalian organogenesis and regeneration. To be qualified as stem cells, cells must reside and interact with their niche. In this case, AP cells must interact with the overlying dermal papilla and epidermal cells for the activation of proliferation and differentiation to form a pedicle and subsequent antler (Li et al., 2009b). This study clearly demonstrates that an interposing physical barrier effectively blocks the interactions between antler stem cells and the niche cell types during the early pedicle developmental stage to control the timing of antler initiation. Because cryosurgery (<30 sec spray) would have maintained the integrity of the interposing collagen tissue while decellularizing the central AP, marginal AP failed to initiate antler formation in this study. In contrast, the outcome of

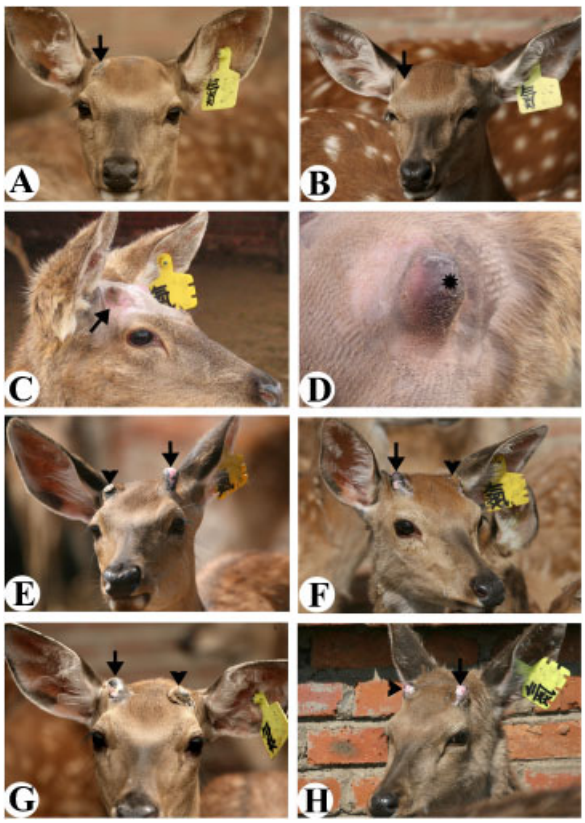


Figure 3. Reaction of the antlerogenic regions to repeated LN2 spray (4 times of 3-FT-cycles) in sika deer. **(A)** A superficial scab (arrow) formed in the sprayed area of Deer 1. **(B)** The scab was shed and the exposed area was healed with epidermis having hypopigmented hairs (arrow). **(C)** Reddish-edema (arrow) was formed after the 2nd time spray in Deer 2. **(D)** Skin blister (arrow) was developed from the reddish-edema in Deer 2. **(E-G)**. Scabs that were developed from skin blisters were exfoliated from growing pedicles on the control (skin-only) sides (arrows), but still remained on the treated (skin+AP) sides (arrowheads) as no pedicle growth occurred on those treated sides. **(H)** Scabs were exfoliated from both control (arrow) and treated (arrowhead) sides, as a pedicle growth also occurred from the treated side.

mechanical resection/destruction of the central AP (cutting through the physical barrier) is the promotion of antler formation (Jaczewski, '82; Li and Suttie, '94b). Moreover, repeated $LN_2$ spray selectively to skin (skin-only) overlying the central AP significantly advanced antler development (this study), i.e. antler transformation took place from the precocious pedicles (at 18–35 mm high, c.f. the species-specific height of 50–60 mm). This advancement must result from early establishment of the interactions between antler stem cells and the niche cell types, which is facilitated by attenuation of the interposing physical barrier.

### Androgen-Responsive vs. Androgen-Unresponsive AP Cells

Antler development can take place from a region outside the pedicle growth area (Wislocki, '52; Nellis, '65) or following destruction/resection of central AP/pedicle (Bubenik and Pavlansky, '65; Jaczewski, '90; Li and Suttie, '94b), suggesting that antlerogenic potential is widespread on a deer head. However, normally only the central area of an antlerogenic region gives rise to a pedicle and an antler. Li ('97) proposed that only AP cells resident in a future pedicle growth area are capable of receiving androgen stimulation to build up a pedicle, whereas those in the surrounding area cannot; therefore, he divided a future antlerogenic region into an androgen-responsive (central AP) and an androgen-unresponsive (marginal AP) subregions. The outmost boundary of marginal AP is not known, as the demarcation between antlerogenic and nonantlerogenic regions has thus far not been clearly delineated (Goss, '83).

Furthermore, Li ('97) hypothesized that under the stimulation of elevated androgen hormones when a deer approaches puberty, cells in the central androgen-responsive subregion start to proliferate and differentiate, whereas those in the surrounding androgen-unresponsive subregion remain dormant. However, both prepubertal central AP (before androgen stimulation) and dormant marginal AP can be stimulated to grow antlers by mechanical wounding (a strong nonphysiological stimulus). Therefore, the antlerogenic potential of androgen-responsive AP cells can be unleashed by both physiological and non-physiological stimuli; whereas that of androgen-unresponsive AP cells can only be activated via means of a nonphysiological stimulus. Based on this assumption, in this study, the cells that migrated from the marginal AP to repopulate the decellularized central AP would not be able to receive androgen hormone stimulation to build up tissue mass, as required to form the close association that is for establishing interactions with the overlying skin. Consequently, no antler growth occurred from the central region AP that had been subjected to cryosurgery (skin+AP).

### Mechanical Wounding vs. Cryosurgery

The reason why removal/decellularization of central AP (before androgen stimulation) by different means can give out completely opposite results, i.e. mechanical wounding stimulates (Bubenik and Pavlansky, '65; Jaczewski, '82; Li and Suttie, '94b) and cryosurgery inhibits (this study) marginal AP to initiate antler development, is open to conjecture. By careful comparison between these two techniques, we can conclude that it is the presence (intact) or absence (broken) of an effective interposing physical barrier that causes this difference.

By mechanically removing/destroying the central region skin and the underling AP (Fig. 5A), the integrity of the interposing physical barrier between antler stem cells and the niche cell types will inevitably be disrupted (Fig. 5B). This disruption will create an opportunity for centripetally migrating healing cells (antler stem cells and niche cell populations) from the marginal


Figure 4. Effects of repeated LN2 spray (4 times of 3-FT-cycles) to the antlerogenic regions on subsequent pedicle and antler formation in sika deer. Note that antlers formed from the sprayed area had very short pedicles. **(A–D)**. Deer 1. (A) No pedicle and/or antler was developed from the sprayed side (arrow). (B) A pedicle and antler was properly formed and antler calcified (arrow) from the control side (no treatment) in the 1st antler growth season. (C) Hard antler (remnant) was dropped off from the control side pedicle and new antler regeneration (arrow) was immediately followed at the normal antler regeneration season. (D) A two branched antler (arrow) was regenerated in the 2nd antler growth season. **(E–H)** Deer 2. (E and F) Pedicle and/or antler growth was occurred at the control (skin-only) sides (arrows), but inhibited at the treated sides (arrowheads). (G) A reasonable large coronet (arrow) was formed between the pedicle and the antler. (H) Pedicle and antler became shrunk and lost shiny surface (arrow) toward end of the 1st antler growth season. **(I–L)** Deer 3. (I and J) Pedicle and/or antler growth was occurred at the control (skin-only) sides (arrows), but inhibited at the treated sides (arrowheads). (K) Control side antler (remnant) was properly calcified (arrow) by the end of the 1st antler growth season. (L) A two branched antler (arrow) was regenerated in the 2nd antler growth season. **(M–P)** Deer 4. (M and N) Pedicle and/or antler growth was occurred at the control (skin-only) sides (arrows), but inhibited at the treated sides (arrowheads). (O) Control side antler (remnant) was properly calcified (arrow) by the end of the 1st antler growth season. (P) A spike antler (arrow) was regenerated in the 2nd antler growth season. **(Q–T)** Deer 5. (Q and R) Pedicle and/or antler growth was occurred at the control (skin-only) side (arrow), but inhibited at the treated side (arrowhead). (S) Control side antler (remnant) was properly calcified (arrow) by the end of the 1st antler growth season. (T) A two-branch-antler (arrow) was regenerated from the control side pedicle in the 2nd antler growth season. **(U–X)** Deer 6. (U and V) Pedicle and antler growth occurred from both control (arrows, skin-only) and treated (arrowheads) sides. (W) Both side antlers (remnant) were properly calcified (arrow) by the end of the 1st antler growth season. (X) A two-branch-antler (arrows) was regenerated from each side pedicle in the 2nd antler growth season.

periosteum and skin to come into direct contact, which will effectively facilitate establishment of the interactions between these cell types, and hence induce antler development without going through the close tissue association step. This conclusion is strongly supported by the fact that, in most cases, antlers formed from the mechanically traumatized AP sites (Bubenik and Pavlansky, '65; Li and Suttie, '94b) only had very short or invisible pedicles (Li and Suttie, '94b). This is because establishment of the interactions between the interactive cell populations and hence antler initiation can be triggered through direct contact, without requiring the growth of a pedicle to establish the usual close tissue association. Therefore, the whole antler development process triggered by mechanical wounding includes only a short transient pedicle growth stage. In contrast, cryosurgery (a single 3-FT-cycle; with limited spray duration) in this study only decellularized skin and the underlying AP but left all the interposing extracellular collagen components intact (Fig. 5C). In due course, the skin and AP would have been repopulated by the cells through in-migration from the marginal skin and AP (Fig. 5D). However, because the repopulated AP and skin cells were still well separated by an intact physical barrier (collagen structure; Fig. 5D) and because the repopulated AP cells (from the androgen-unresponsive region) would be incapable of receiving androgen stimulation to expand their mass, which is required for the tissue close association and interaction with the overlying skin, no antler growth occurred from these $LN_2$-sprayed sites (Skin+AP) in this study.

Here, one may argue that the failure to grow antlers from the marginal antler stem cells in this study may have been caused by the destruction of the majority of these cells or significant impairment of their antlerogenic potential that might have occurred while spraying the central AP, rather than preservation

**Table 3.** Final pedicle length (left/right; mm) in Experiment 3.

| | In the first season | | |
|---|---|---|---|
| Deer | Based on skin type | Based on coronet | In the second season |
| 1 | 42/0 | 45/0 | 40/0 |
| 2 | 0/35 | 0/40 | x |
| 3 | 27/0 | 33/0 | 25/0 |
| 4 | 0/20 | 0/40 | 0/20 |
| 5 | 0/18 | 0/42 | 0/15 |
| 6 | 25/10 | 50/40 | 30/20 |

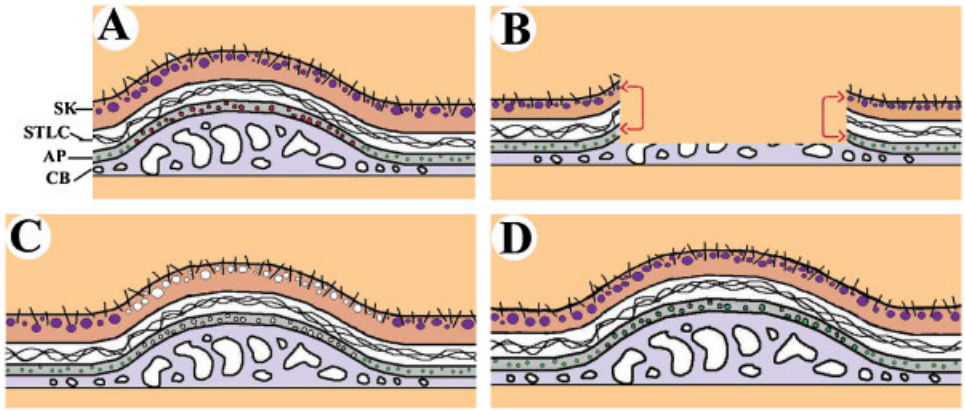


**Figure 5.** Schematic drawing to illustrate how mechanic wounding stimulates but cryosurgery inhibits antler formation based on the physical barrier theory. Labels in 5A also apply to the rest of figures in Figure 5. **(A)** A pre-pubertal antlerogenic region. SK, skin consists of dermis (dermal papilla cells in purple) and epidermis; STLC, subcutaneous loose connective tissue; AP (androgen responsive antler stem cells (red) are located in the centre, and unresponsive stem cells (green) located peripherally); CB, crest bone; Purple dots, dermal papilla cells; red dots, androgen-responsive AP cells; green dots, androgen-unresponsive AP cells. **(B)** Mechanical deletion of androgen-responsive-region (equivalent to an average size of pedicle base) tissues. Note that the tissue resection stimulates androgen-unresponsive antler stem cells and the overlying skin cells (red arrows) to migrate centripetally, which would create the opportunity for these cells to directly contact. Red arrows point to the broken surfaces of AP and the overlying skin after the resection of central area of an antlerogenic region. **(C)** Cryosurgery of a pre-pubertal antlerogenic region. Note that following LN2 spray to the central area of an antlerogenic region, androgen-responsive antler stem cells and the overlying skin cells are destroyed, but the marginally located androgen-unresponsive stem cells are still intact. White dots, empty spaces after the cells destruction. **(D)** AP and the overlying skin in the decellurised central area are repopulated in due time by in-migrating marginal antler stem cells (androgen-unresponsive) and skin cells respectively. Note that the antler stem cells in the AP and the skin cells are separated by a wide and loose collagen layer.

of an effective interposing physical barrier. Because, although the area sprayed was roughly equivalent to that of an average pedicle base, the area affected (transition zone between the sprayed and unsprayed) may be more extensive. However, this explanation is unlikely, because it has been reported that the transition zone is less than a couple of millimetres (Andrews, 2004). In this study, the sprayed area (around 32 mm in diameter) plus the expected transition zone would have been still smaller than the area (>35 mm in diameter) from which skin and AP were removed through mechanical means (our unpublished data). Interestingly, the latter not only initiated, but also significantly advanced antler formation from the marginal periosteal cells (Bubenik and Pavlansky, '65; Jaczewski, '82). Besides, antler formation occurred in most cases from the marginal periosteal cells surrounding the chemically destroyed central area (including skin and AP) that was up to 50 mm in diameter (Robbins, '81). Likewise, the phenomenon that no antler growth occurred from the sprayed central region (skin+AP) cannot be attributed to the possibility of the overlying skin damage either, because repeated sprays selectively to skin over the central AP region in this study did not prevent antler growth from happening, so long as the underlying AP was protected from cryodamage. Consequently, we conclude that it was the presence of an interposing physical barrier that effectively blocked the interactions between antler stem cells and the niche cell types.

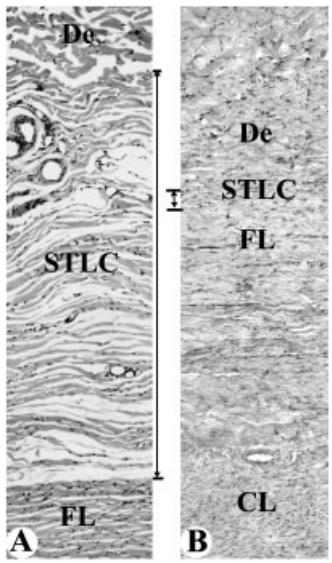


Figure 6. Interposing subcutaneous loose connective (STLC) tissue layer (Reproduced with the permission from Li and Suttie, 2000; Anat Rec 260:62-71). **(A)** Subcutaneous loose connective tissue layer in a pre-pubertal antlerogenic region. Note the layer is wide and loose. STLC, subcutaneous loose connective tissue; De, dermis; FL, fibrous layer of AP. The width of the STLC was indicated at the right side of the figure. **(B)** STLC in the apex of a late growing pedicle or early antler. Note that the layer has been compressed into a 3-4 cell strip. CL, cellular layer of AP. The width of the STLC was indicated at the left side of the figure.

### Cell Interactions vs. Physical Barrier Permeability

As a stem cell-based process, antler development relies on interactions between stem cells and the niche cell types, i.e. AP cells and the overlying skin (dermal papilla and epidermal) cells (Goss, '90; Li et al., 2009b). These interactions can only be triggered when the wide and loose interposing collagen tissue (Fig. 6A) is fully compressed and thinned into a 3–4 cell thick strip (Fig. 6B; Li and Suttie, 2000), and can be inhibited or impaired by inserting an impermeable or a semi-permeable membrane, respectively (Li et al., 2008). The results from these experiments clearly indicate that these cell interactions are mediated by diffusible molecules, and the timing of their establishment would reasonably be expected to be controlled by the permeability of the interposing physical barrier. The effectiveness of the barrier would be proportional to the distance between the two interactive cell types and the barrier's density/permeability.

Under the stimulation of elevated androgen hormones, the central region AP (having an area equivalent to the size of a pedicle base) starts to build up pedicle tissue mass (Bubenik, '82; Suttie et al., '95; Li et al., 2003), which gradually creates mechanical pressure to the overlying SLCT and the skin. To accommodate this expanding tissue mass, the former becomes totally compressed and then substantially stretched (thinned) and the latter starts to form new skin to release the tension (Li and Suttie, 2000). The consequence of the continuously thinning of the SLCT layer interposing the AP-derived tissue (stem cells) and the overlying skin (niche cells) would be the steady increase in permeability of the barrier, and this would eventually reach to the point at which these two tissue types can effectively establish the interactions by exchanging diffusible molecules through the barrier. This probably explains why there is a critical AP mass below which antler growth does not occur at a ectopic grafting site (Goss and Powel, '85), as the tissue mass formed from an insufficient quantity of AP would not be able to bring the interactive cell types as close as required for the onset of the interactions. The reason why mechanical disruption of the interposing barrier integrity not only can totally substitute the tissue close association step (i.e. can directly stimulate a prepubertal antlerogenic region to grow an antler), but also generate a much stronger stimulus (e.g. can initiate antler growth outside the antler growth season) for the induction of antler formation (Bubenik and Pavlansky, '65; Li and Suttie, '94b), is because barrier disruption would create complete permeability for exchanging diffusible molecules between the interactive cell populations. Consequently, permeability of the interposing barrier must play a critical role in controlling the cell interactions, and hence timing of antler growth.

If the theory of barrier permeability is correct, the phenomenon of significant advancement of antler transformation from a pedicle (23 mm in this study vs. 50 mm in species specific) after repeated $LN_2$ spray to the central region skin in this study can be explained as follows. Repeated FT cycles, during which no single spray exceeded 30 sec (the minimum duration for collagen structural damage) and hence cannot cause complete destruction of collagen structure, would have significantly impaired skin integrity (as evidenced by the serious skin blisters that formed) and attenuated the SLCT (interposing barrier). The outcome of these consequences would be the increase in barrier permeability and precocious establishment of cell interactions. Based on this assumption, the failure to inhibit antler formation from the two 22 mm high pedicles (one in Experiment 2 and the other in Experiment 3) in this study can be explained as follows. When a pedicle reaches more than 20 mm, a height that approaches the close tissue association stage in both red deer (Li and Suttie, 2000) and sika (our unpublished data), even if the apical antler stem cells are destroyed by cryosurgery at this stage, the repopulated cells from the shoulder of the incipient pedicles can still establish the interactions with the overlying niche cells because (1) antler stem cells and the niche cell populations are already very close to each other; and (2) the permeability of the interposing barrier would be significantly increased after repeated $LN_2$ spray cycles (attenuation). By the same token, formation of a rudimentary antler from a frontal crest in Experiment 2 after $LN_2$ spray (skin+AP) may be owing to the $LN_2$ spray being too strong, the interposing physical barrier in the deer too fragile, or both.

Overall, this study is the first to report the following findings: (1) preservation of the interposing physical barrier (extracellular collagen component) between antler stem cells and the niche cell types while decellularizing the central region AP effectively inhibits antler formation from the viable marginal AP; and (2) cryosurgery to the central region skin via repeated controlled $LN_2$ spray while keeping the underlying AP intact significantly advanced antler formation, which is clearly achieved through attenuation of the interposing physical barrier. Consequently, the interposing physical barrier between AP and the overlying skin is an integral part of the antler stem cell system and plays a critical role in controlling timing of antler development. Further research into the antler stem cell system may have important implications for understanding how stem cells interact with their niche in general.

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