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The effect of direct cell injury inflicted by cryotherapy on eyelid sebaceous gland carcinoma cells: An ex-vivo experimental study

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Purpose: To evaluate the effect of direct cell injury of cryotherapy on eyelid sebaceous gland carcinoma cells by an *ex vivo* cryotherapy experiment. **Methods:** It was a prospective interventional case series. Six patients with biopsy-proven nodular sebaceous gland carcinoma were included. After excision of the mass, a thin slice of the mass resembling the thickness of the conjunctiva was shaved off and was oriented over the broad end of a tissue forceps. Cryotherapy was applied to both its anterior and posterior aspects by the triple freeze-thaw technique. The mass was then labeled and sent separately for histopathological evaluation by fixation and staining. **Results:** A total of six patients with a mean age of 58.2 ± 15.5 years were included. There were four females and two males. The mean duration of the lesion was 21.6 ± 17.51 months. All patients had involvement of the upper eyelid. The patients were clinically staged as T2b (*n*=2), T1a (*n*=2), T2c (*n*=1), and T3a (*n*=1) respectively. There was no regional lymphadenopathy or metastasis in any of the cases. The experimental cryo-tissue containing the cryo-treated lesion revealed the presence of viable tumor cells (>50%) in all six specimens. **Conclusion:** The direct cell injury caused by cryotherapy may not be sufficient to kill all the residual sebaceous gland carcinoma cells on the tumor bed.

Key words: Cryotherapy, *ex vivo*, intraoperative, sebaceous gland carcinoma

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The origin of cancer cryotherapy can be traced back to the 1850s when James Arnott used salt solutions containing crushed ice for advanced breast and uterine cervix carcinomas. With the advent of the modern era of cryosurgery in the 1860s, the procedure of cryotherapy was laid down as rapid freezing, slow thawing, and repetition of the freeze-thaw cycle.[1-4]

Initially, cryotherapy was used exclusively for basal cell carcinoma (BCC).[5] The first application of cryotherapy in sebaceous gland carcinoma (SGC) in English literature was reported by Lisman R *et al*.,[6] where they treated the pagetoid variant of SGC with cryotherapy. The application of cryotherapy on the palpebral and bulbar conjunctival edges following excision of the lesion to completely eliminate the residual tumor cells has become a preferred practice pattern these days.[7] Cryotherapy inflicts twofold damage to the tumor cells; the first one is immediate and is caused by direct cell injury while the second one is delayed and is caused by vascular damage.[2-5]

It is extremely difficult to demonstrate the delayed vascular injury caused by cryotherapy.[2] The tumor cells should be in contact with viable tissues to study the effect of microcirculation failure and that would be ethically challenging. The present study was aimed to evaluate the effectiveness of direct cell injury inflicted upon by cryotherapy in eliminating the SGC cells by an *ex vivo* experiment.

Methods

This was a prospective interventional case series carried out between April 2018 and March 2020. All biopsy-proven cases of eyelid SGC were included in the study. The study was conducted after obtaining clearance from the institutional review board and it adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all the patients regarding publication of their photographs and clinical details. The authors confirm that the data supporting the findings of the study are present within the article [Table 1].

Detailed history including the duration of the mass, past surgical intervention, and systemic associations was taken. A comprehensive ophthalmic evaluation comprising visual acuity, ocular motility, anterior segment examination, and fundoscopy was done. The location, size, appearance, consistency, depth of the mass lesion, and presence of regional lymphadenopathy were assessed, and clinical staging (cTNM) was done according to the American Joint Committee on Cancer (AJCC) 8th edition staging system following which the patients were posted for surgical excision.[8]

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Experimental steps

Surgical excision of all six cases was accomplished by the same oculoplastic surgeon (MSA). The mass on the lid was marked. The surgical site was infiltrated with 0.2% lignocaine with adrenaline (1:200000). The mass lesion was excised with 4-mm clear margins under frozen section. Cryotherapy was applied to the residual conjunctival edges using the triple freeze-thaw technique.

After excision, a very thin slice of tissue resembling the thickness of the conjunctiva was removed from the main mass using a sharp 15 number blade [Fig. 1a]. The mass was then oriented over the broad holding edge of a tissue forceps and cryotherapy was applied using the triple freeze-thaw technique. Freezing was stopped after the formation of snowball and then it was left to thaw spontaneously. Three such cycles were applied on both the anterior and posterior surface of the sliced mass [Fig. 1b]. The slice of tissue was transferred to the formalin bottle within 30 minutes and sent for histopathological examination where it was studied after 24 hours.

Results

Six patients (4 females and 2 males) were enrolled in this experimental study. The mean age of the study population was 58.2 ± 15.5 years. All patients had unilateral mass lesions with the right eye being involved in 4 cases (67%) and left eye in 2 cases (33%) respectively. The mass lesion was in the lateral part of the upper eyelid in all the cases except in one case where it was located centrally. All were well-defined nodular lesions. The mean duration of the mass lesion was 21.6 ± 17.51 months. One of the cases had undergone excision 2 years ago and had a recurrent mass. Two patients were clinically staged as T2b (40%), 2 patients as T1a, while the rest two as T2c and T3a, respectively [Table 1]. There was no regional lymphadenopathy or metastasis in any of the cases. Histopathology confirmed the diagnosis of SGC in all the cases. Two cases had poorly differentiated tumor cells belonging to the histological grade G3 (AJCC 8th edition), while three cases had moderately differentiated tumor cells and were classified as histological grade G2; the remaining one case demonstrated well-differentiated tumor cells belonging to the histological grade G1. None of the patients demonstrated signs of pagetoid spread. None of the tumor masses showed significant necrosis on histopathology.

The experimental cryo-tissue containing the cryo-treated lesion revealed the presence of tumor cells in all six specimens with more than 50% viable tumor cells along with areas of extensive necrosis [Fig. 1c and d].

Discussion

In our experimental *ex vivo* cryotherapy study, we tested the effect of the direct cell injury induced by cryotherapy on sebaceous gland carcinoma cells of the eyelid. It is standard practice to apply cryotherapy to the residual conjunctival edges after excision of SGC.[7] We observed that the direct cell injury inflicted by the freeze-thaw cycle was insufficient to kill the tumor cells completely, though extensive necrosis was present suggesting the cryo effect.

Char reported the application of cryotherapy in basal cell carcinoma in 1980.[5] Lisman *et al*.[6] highlighted the use of adjunctive cryotherapy for pagetoid growth in SGC in 1989. He reported a series of six patients with pagetoid invasion who were reluctant for exenteration and thus, cryotherapy was used as an alternative treatment. Surgical excision and cryotherapy were performed at separate sessions and the patients were followed up at regular intervals with conjunctival biopsy. They reported good outcomes in their study. However, they could not attribute the good clinical outcome solely to cryotherapy, as pointed out by Kass in his letter against the article.[9] However, Lisman paved the way for the application of cryotherapy in SGC and gradually surgical resection of the lid tumor along with cryotherapy became the protocol in the management of eyelid SGC. It is believed that in cases without any clinical evidence of pagetoid invasion, supplementary cryotherapy on the residual palpebral and bulbar conjunctival edges might kill any microscopic tumor residue and prevent the intraepithelial spread.[7,9-15]

Cryotherapy inflicts twofold damage on the neoplastic cells, that is, direct cell injury and vascular injury. While the hypothermia produced by the direct cooling causes degeneration of the structural proteins and lipids, with the further drop in temperature, there is the formation of intracellular ice crystals, which disrupts cellular organelles and membranes. During thawing, multiple ice crystals fuse to form a large crystal, which has a lethal effect on the cell membrane. Slow thawing allows the ice to melt; the hypotonic environment of the extracellular space leads to inflow of water into the cell, resulting in the rupture of the cell membrane and consequently cell death. This is direct cell injury that occurs immediately following cryotherapy, and its effects can be noted on histopathology in the form of extensive tissue necrosis.[2-5]

The vascular injury, which is delayed, damages the tumor cells by producing coagulative necrosis resulting from ischemia and vascular stasis. Gage stated that *in vitro* studies cannot demonstrate the vascular mode of injury produced by cryotherapy.[2] The microcirculation failure can only be studied with the tumor still intact within the eyelid and that would

Table 1: Demographical, clinical, and histopathological findings

Case	Age	Gender	Past history	Location	Staging (AJCC 8th edition)	Morphology	Cell type	Viable tumor cells	Follow up (months)	Recurrence
1	65	F	Surgical excision of SGC 2 years ago	Upper lid, lateral part	T2b	Lobular, peripheral palisading	Baso-squammous	>50%	12	No
2	72	F	Nil	Upper Lid, Lateral part	T2b	Lobular	Basaloid	>50%	12	Yes
3	69	M	Nil	Upper lid, lateral part	T2c	Lobular	Basaloid	>50%	6	No
4	52	F	Nil	Upper lid, central part	T1a	Lobular	Basaloid	>50%	6	No
5	61	M	Nil	Upper lid, lateral part	T3a	Lobular	Basaloid	>50%	6	No
6	30	F	Nil	Upper lid, Lateral part	T1a	Lobular	Basaloid	>50%	6	No

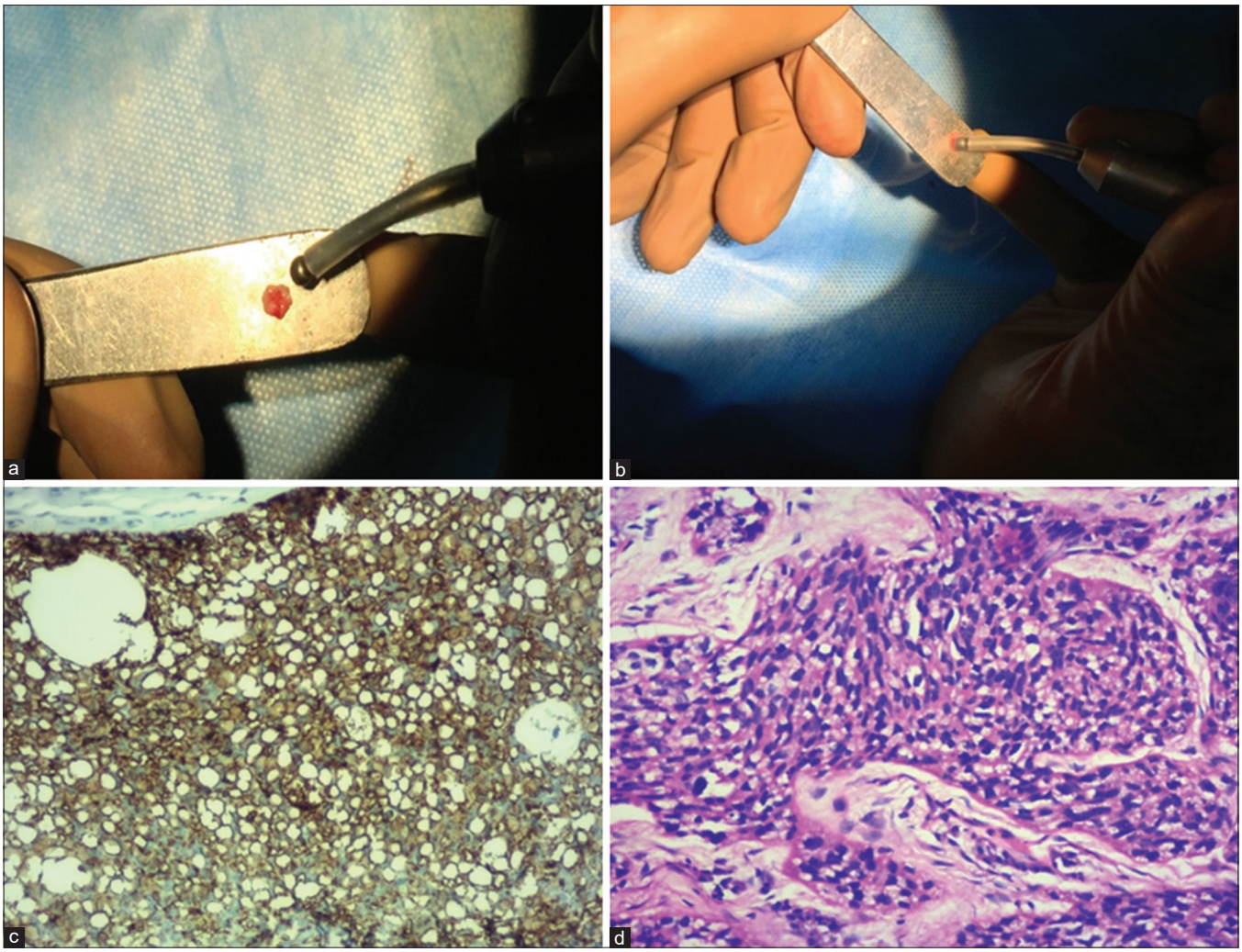

Figure 1: (a) Thin tissue from the main mass kept for *ex vivo* cryotherapy. (b) *Ex vivo* cryotherapy procedure. (c) Microphotograph showing adipophilin stained SGC cells. (d) Microphotograph (Hematoxylin and Eosin) showing viable tumor cells in the cryo-specimen

be very challenging to do on ethical grounds. Our study too has this inherent limitation as it only suggests that the direct cell injury inflicted by cryotherapy may not be sufficient to completely destroy the sebaceous gland carcinoma cells.

The factors that influence the efficiency of cryotherapy are i) tissue temperature, ii) the cooling rate, iii) the duration of freezing, iv) thaw rate, v) the number of freeze-thaw cycles, and vi) the interval between each cycle. The tissue temperature at which intracellular ice formation takes place is –50°C. Rapid cooling has been reported to be lethal to the cell. Moreover, holding the tissue at a temperature above –40°C for a long time promotes recrystallization and brings about massive destruction.[2,3,16-18] A shortcoming of cryotherapy is that only the tissue in contact with the cryoprobe attains the freezing temperature at a rapid freezing rate. Tissues located at more than 1 cm from the cryoprobe do not exhibit intracellular ice formation as the freezing temperature is not reached.[2,3] However, in our series, we had exposed all the margins of the experimental mass to cryotherapy as is clear from the picture. As we were interested in knowing the effect of cryotherapy on residual tumor cells in the conjunctiva, we tried to simulate the thickness of the sliced mass as close to conjunctiva as possible.

We used the triple freeze-thaw cycle with slow and complete thawing. Despite all the measures of successful cryotherapy, the procedure failed to kill all the tumor cells by direct cell injury as evident from the histopathological outcomes. Cryotherapy inflicts direct cell injury immediately, which is evident by the necrosis seen in samples undergoing cryotherapy.

The presence of more than 50% viable cells in all the cases following cryotherapy indicates that direct cell injury alone is not effective in completely eliminating the SGC cells. As microcirculation failure (vascular injury) is secondary to the direct cell injury inflicted by cryotherapy, we believe that this mode would also not be sufficient when the direct effect has failed. The finding has significant clinical implications as far as the protocol of intraoperative cryotherapy during SGC excision is concerned, and surgeons should be aware that this might not be sufficient to achieve a completely tumor-free area for their patients. The present study paves way for further research in this particular area for arriving at some definite conclusions.

The present study is limited by its small sample size. As the samples after cryotherapy were placed in formalin within 30 min, any further effect of direct cell injury would not have

happened because of the fixation effects of formalin. However, the transfer into formalin cannot be delayed as there will be autolysis of tissues confounding the effects.

Conclusion

Cryotherapy works by the mechanism of direct cell injury and microcirculation failure, which is secondary to direct cell damage. However, the cell damage generated by the direct effect of cryotherapy may not be sufficient in ensuring a tumor-free residual tissue bed.

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Conflicts of interest

There are no conflicts of interest.

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