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Effects of a dimethyl ether, propane and isobutene solution on conjunctiva and cornea in isolated pig eyes and comparison with standard LN_2 cryosurgery: a pre-clinical study.

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RUNNING TITLE: dimethyl ether, propane and isobutene on cornea

Abstract

Purpose: To evaluate the effect on the cornea and conjunctiva of a new cryosurgical method based on the application of a dimethyl ether, propane and isobutene solution (DMEPI, Histofreezer®), and to compare it to Liquid Nitrogen (LN2; Cry-Ac Cryogun®).

Methods: 52 isolated pig eyes were obtained at the slaughterhouse, preserved for less than one hour in Ringer Lactate and divided in four groups. The first group included eyes treated with two applications of DMEPI in the center of the cornea. The second group included eyes treated with DMEPI on the third eyelid's conjunctiva. The third group included eyes treated with LN_2 on the third eyelid's conjunctiva. The fourth group included eyes treated with LN_2 on the central cornea. Each application of the cryogen (0.8 cm area) was of 40 seconds. Each group included one (not treated) control eye. The eyes were submitted for histopathological evaluation. Histological alterations were recorded and scored with a semiquantitative scoring system.

Results: No tissue alteration was detected on the conjunctiva, in any of the groups. All treated corneas showed various degrees of stromal cleft formation and cytoplasmic vacuolization of epithelial basal cells, but all the alterations were mild without significant differences between the groups.

Conclusions: No significant damage to cornea or conjunctiva was detected and no significant differences between the two methods were recorded. Soft cryosurgery could represent a valid and safe method to treat different corneal and conjunctival diseases. Clinical studies should be performed to verify the effect of this method in vivo.

Key Words: cryosurgery, cornea, conjunctiva, liquid nitrogen, Histofreezer®, histopathology

INTRODUCTION

Cryosurgery is an innovative and effective surgical technique that has been defined as "therapeutic application of cold to freeze biological tissues".[29] It sometimes represents an alternative to conventional surgery, but most of the time it can only act as an adjuvant therapy to a surgical treatment. It offers many advantages, such as: wide range of indications; ease of application; controlled tissue destruction; satisfying pain management; less invasiveness and lower morbidity compared with surgical resection; good hemostasis; no need of anesthesia for superficial operations (particularly suitable for old, high-risk surgical patients); possible autoimmunization with tumor tissue antigens; low incidence of complications as strictures, exuberant granulation tissue and infections, usually resolvable by gentle cleansing. [13,15,61]

In veterinary ophthalmology this technique has found many applications for periocular and intraocular lesions,[13,35,48] particularly for tumors management: papillomatosis,[5,12] squamous cell carcinoma,[5,33,58] limbal melanoma[20] and melanocytoma[44] in dogs; melanomas[7,13,40] and squamous cell carcinomas in horses,[25,26,27,31,32,51] squamous cell carcinoma[8,19,23,34,55] and papillomatosis[17] in cattle; conjunctival-corneal neoplasm in elephants,[21] squamous cell carcinoma[24] and third eyelid hemangiosarcoma[41] in cat. In humans it has been described for the treatment of basal cell carcinoma,[36] uveal[30] and choroidal[53] melanomas, ocular parasitic granulomas,[42] Acanthamoeba keratitis,[1,6] glaucoma,[35,38,48,56,57,59] pigmentary keratitis,[3,5,28] corneal pannus,[28,49] proliferative keratoconjunctivitis,[60] trichiasis,[44,54,56,59,63] corneal ulcers,[38,47,48,56] vitreocorneal contact.[48,56]

Cellular death is due to the formation of intracellular ice crystals, which disrupt organelles and cell membranes. [22] These are more likely to occur when a rapid freezing is performed, while a slow freezing induces the formation of crystals in the extracellular space, without bringing cells to death. [18,48] The maximum amount of cellular destruction results from a rapid freeze-slow thaw cycle. [22,29,52,61] Normally it is advised to produce at least a temperature of

-50°C to obtain destruction of tissue.[22]

Liquid nitrogen is the most widely used cryogen. It reaches a temperature around -196°C and, providing maximal cellular destruction, it represents the most aggressive cryogen available.[9] Its main disadvantages are due to slowness of units defrosting, very short storage life and its considerable high potency, which can lead to a tissue over destruction involving normal tissue or vital structures. [29,56]

A new dermatological cryosurgical device (Histofreezer®) uses an environmentally safe mixture of dimethylether (95%), propane (2%) and isobutene (3%) (DMEPI). It reaches a warmer (-55°C) freezing temperature than liquid nitrogen and appears more easily tolerated by patients, with minimal risk of scarring, blistering or depigmentation.[14,50] It has been tested to be effective on benign skin lesions in human medicine[14,16, 39] and demonstrated its efficacy in the treatment of HPV-related skin lesions in dogs.[37] DMEPI efficacy on skin benign lesions in humans was proven to be totally comparable with liquid nitrogen. The temperature reached by the new DMEPI spray (low freezing cryosurgery) appears adequate for effective tissue destruction and generally only few complications occur, most of which are slight and normally heal spontaneously.[10]

Azoulay (2014) tested the efficacy of soft cryotherapy, with two applications of 50 seconds each using dimethylether, isobutene and propane gas mixture, on 16 canine corneas affected by pigmentary keratitis.[4] A highly targeted ablation of pigmented cells was observed, with a relatively limited effect on the less cold-sensitive corneal cells.[4] However an in-depth analysis is necessary in order to verify the effects of this procedure.

The present study aims to preliminarily verify the effect of the soft cryosurgical system DMEPI on the corneal and conjunctival surface. It is oriented to evaluate the immediate tissue damages caused by DMEPI and liquid nitrogen on cornea and conjunctiva and to

compare them through a semi-quantitative score system. The final objective is to define DMEPI as appropriate to be used in small animal medicine.

MATERIALS AND METHODS

The study was performed on cornea and conjunctiva of isolated porcine eyes. All the cryogenic procedures were performed by the same veterinary ophthalmologist at the Department of Veterinary Medicine, University of Milan, Italy.

Tested substances

Two different cryosurgical products were used in this study: DMEPI Portable System(Histofreezer®) and liquid nitrogen.

DMEPI Portable System is composed by a pressurized aerosol canister, filled with a cryogenic gas mixture (95% dimethylether, 2% propane and 3% isobutene); cotton and foam buds (diameter of 2 mm), with hollow tubes (Fig.1).

Liquid nitrogen was preserved in specific tank. Safety equipment was worn every time. The cryosurgical probes had a flat, circular freezing surface with a diameter of 0,5cm (Fig.2).

Samples collection

Fifty-two porcine eyes were removed immediately at the end of the slaughter process. The eyes, including eyelids and adnexa, were stored in Ringer Lactate solution until the beginning of the study (maximum 30 minutes).

Cryogens application

The samples were divided in four groups. Each group included a control eye, which did not receive any cryogen application.

In the first group sixteen eyes were treated with DMEPI: each eye received two applications (40 seconds each) in the central cornea.

The second group consisted of sixteen eyes treated with DMEPI: each eye received two applications (40 seconds each) on the third eyelid conjunctiva.

The third group included ten eyes treated with two applications of liquid nitrogen (40 seconds each) on the third eyelid's conjunctiva.

In the fourth group fifteen eyes were treated with two applications of liquid nitrogen (40 seconds each) in the central cornea.

After the treatments, all eyes and periocular tissues were fixed in buffered formalin and submitted for histopathological evaluation along with control eyes.

Histopathology

Each eye was transected with a microtome blade. Section was performed in the center of the site of cryogen's application. Both such obtained halves were routinely processed for histology and paraffin embedded. Five-micrometer microtomic sections were cut from paraffin blocks and routinely stained with hematoxylin and eosin.

On histological examination different parameters were considered:

- Presence of epithelial erosion/ulcers;
- Histological signs of epithelial cell degeneration (i.e. variation in shape, cytoplasmic vacuolization);
- Corneal stromal cleft formation. The deepness of the clefts within the stroma (i.e. upper, medium and deep corneal stroma) was also recorded.

The severity of histological alterations (compared with control eye) was scored with a semi-quantitative scoring system and four classes of increasing severity were created: 0, 1+, 2+, 3+ (Table 1).

Specifically, stromal clefts were scored based on the extent of stromal tissue affected by cleft formation (0: few single scattered clefts; 1: up to 30% of the layer filled by clefts; 2: up to 50% of the layer filled; 3: 70% or more of the layer filled by cleft).

Evaluating epithelial cells intracytoplasmic vacuolization, intensity of the lesion was defined as the extent of cytoplasm affected by vacuoles (0: no vacuolization; 1: up to 30% of the cytoplasm affected, 2: up to 50% of the cytoplasm affected; 3: vacuoles filling 70% or more of the cytoplasmic area). Diffusion of vacuolization was defined as the number of cells affected by the lesion, considering the whole corneal section (0: single to few scattered vacuolized cells; 1: small groups or single segments of vacuolized cells; 2: up to 50% of epithelial cells contained vacuoles; 3: more than 50% of epithelial cells contained vacuoles). Moreover, variation in shape of epithelial cells was considered and scored as follows: 0: single to few scattered altered cells; 1: up to 30% of cells in the corneal section showed altered shape; 2: up to 50% of corneal cells were altered in shape; more than 50% of cells were altered in shape.

Data analysis and statistical methods

Collected data were elaborated with the statistical software MINITAB® 17 and the nonparametric Mann-Whitney test was used to compare the medians of the two not normally distributed groups. Null hypothesis was set as no existing difference between two medians of treatments and the alternative one as the presence of differences.

A significance level of 0.05 (α=5%) has been used during every statistical test.

RESULTS

Conjunctiva

When compared with control eyes, the conjunctiva of treated eyes did not show any histological alteration, neither in those treated with DMEPI nor in those treated with LN_2. Therefore, conjunctival tests (group 2 and 3) were not included in the statistical analysis.

Cornea

Corneal alterations were overall mild, independently of which cryogenic method was applied. They consisted in intracytoplasmic vacuolization, variation of epithelial cells' shape and presence of clefts within the stromal layers. Erosion or ulceration of corneal epithelium were never observed. Control eyes did not present any of the post-treatment abnormalities observed in eyes that underwent cryotherapy.

Intracytoplasmic vacuolization of corneal epithelial cells was observed as tiny, round, regular, smooth contoured intracytoplasmic vacuoles within basal epithelial cell layers (fig. 6). Vacuolization varied in the extent of cytoplasmic involvement (intensity) and in the number of cells involved (diffusion). Detailed results are reported in table 2 and 3.

No statistically significant differences between DMEPI and LN_2 groups were detected as far as cytoplasmic vacuolization intensity and diffusion were considered, (Table 5). This consideration is also confirmed by descriptive analysis, which showed a comparable data trend and a very small distance between means values (Fig. 7).

Scattered or small groups of corneal basal epithelial cells showed marked alteration of shape and tinctorial affinity: cells were elongated, with basally located dense nuclei and spindle shaped, "stretched" apical, intensely eosinophilic cytoplasm (Fig. 8). When the severity of this parameter was semiquantitatively evaluated, as detailed in Tables 2 and 3, no statistically significant difference was observed between DMEPI and LN_2 groups (Table 6). This consideration emerged also from the descriptive analysis that showed a comparable data trend and a very small distance between means values (Fig. 9).

Corneal stroma was characterized by cleft formation. Corneal stromal clefts were defined as elongated, fusiform empty spaces splitting stromal fibers (Fig. 3). They were detected throughout all stromal layers as detailed in Tables 2 and 3. No statistically significant difference was detected between DMEPI and LN_2 groups (Table 4) as far as stromal cleft

formation was concerned. This consideration emerged also from the descriptive analysis that showed a comparable data trend between means values (Fig. 4).

DISCUSSION

DMEPI Portable Spray is a new cryosurgical method that uses an environmentally safe mixture of dimethylether (95%), propane (2%) and isobutene (3) that is currently considered more easily tolerated by patients, with minimal risk of scarring, blistering or depigmentation.[14,50]

In order to test DMEPI gas mixture on ocular tissues we decide to compare its immediate effects with those of a well-known cryosurgical agent such as liquid nitrogen.

Clinical trials potentially give the best information concerning the in vivo effect of these compounds, mostly in a medium-long term response involving tissue reaction, inflammation and repair. However, current limitation on experimental testing but also care of animal welfare should prevent from using new compounds on eyes ignoring completely their possible effects.

Recently, the use of isolated chicken eye has been validated as an alternative method for the identification of severe irritants and histopathology of the cornea has been shown to be an additional useful tool that can strengthen evidence of eye irritation. [4,11,45,46]

In the present study, pig eyes were preferred to chicken eyes due to their larger size, offering a wider surface for cryogens application, and their histological architecture that is more similar to the target species.

In the present study, neither liquid nitrogen nor DMEPI caused any significant histological alteration on conjunctival tissue. Based on literature data, conjunctiva seems resistant and not easily damageable by cryosurgery, even when a powerful cryogen as liquid nitrogen is applied.[60] Wheeler *et al.* (1989), for example, tested LN_2 cryosurgery on proliferative

keratoconjunctivitis in five dogs and observed neither tissue loss nor apparent damage to the cartilage on the third eyelid conjunctiva, nor a decrease in tear production.[60]

Based on these results, it seems conceivable that both the aforementioned treatments could be used safely for several diseases involving conjunctiva, such as ciliary disorders[56,59] and eyelid tumors (as adjuvant therapy to surgical excision).[2,12,34,41] Given the tested effects of DMEPI on conjunctiva, it seems also reasonable to consider this dimethylether-propane-isobutene-based cryosurgical system a good alternative to LN_2.

As far as the cornea is concerned, subtle stromal and epithelial abnormalities were observed in treated eyes when compared with control eyes.

In the stroma, clefts were present throughout all corneal thickness both in eyes treated with LN_2 and DMEPI. Clefts gathered particularly in the superficial layer with a progressive decrement in medium and deep layer in both groups. Cleft formation is a well-known corneal histological alteration that most of the time is supposed to be a mere consequence of fixation (i.e. processing artifact).[11] However, since clefts were absent or minimal in control eyes, we investigated their possible relation with LN_2 and DMEPI application, hypothesizing that their presence could be related to the freezing temperature, determining the separation of stromal fibers. Statistical analysis did not detect any significant difference in cleft formation between treated groups. This indicates a similar result from the application of the two cryogens, leading to consider them as comparable.

Mild to moderate intracytoplasmic vacuolization was observed in epithelial cells of the basal layer of corneal epithelium. Cytoplasmic vacuolization can be interpreted as histological feature of cell degeneration and it can be hypothesized that vacuolization was determined by exposure to low temperature, i.e. to cryogen application. Intracytoplasmic vacuolization of corneal epithelial basal cells was observed in all samples independently from the type of treatment applied, while it was absent in control eyes. Again, no statistically significant

difference was found when LN_2 and DMEPI groups were compared, suggesting a high similarity between the effect of the two cryogens used.

Eventually, no significant difference appeared from statistical analysis of elongated basal cells observed in corneal epithelium after DMEPI and LN_2 application. In swine species, corneal epithelial cells in the *stratum basale* have been described to be columnar with pencil-shaped nuclei.[43] This feature was confirmed in the control eyes examined in the present study, however in treated corneas markedly elongated and shrunken, intensely eosinophilic cells, single or in small bundles, were also observed. These cells were not present in control corneas and were therefore hypothesized to be due to the effect of freezing. Their presence did not vary, however, between the two considered treatments, presenting an additional evidence of the remarkable similarity detected between DMEPI and LN_2 application.

CONCLUSION

This preliminary experimental study demonstrated that the soft cryosurgery did not cause any severe histologically detectable damage of cornea and conjunctiva in isolated pig eyes. Moreover, no significant difference was observed when liquid nitrogen and DMEPI treatments were compared. Further studies, specifically in vivo, should be performed in order to verify any possible delayed effect and the real efficacy of DMEPI cryosurgery towards specific corneal and/or conjunctival diseases.

CONFLICT OF INTEREST

None of the authors of this article has affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this paper.

	0	1+	2+	3+
CLEFTS				
Upper	Few single clefts in the superficial layer	Up to 30% of the superficial layer is occupied by clefts	Up to 50% of the superficial layer is occupied by clefts	Up to 70% of the superficial layer is occupied by clefts
Medium	Few single clefts in the medium layer	Up to 30% of the medium layer is occupied by clefts	Up to 50% of the medium layer is occupied by clefts	Up to 70% of the medium layer is occupied by clefts
Deep	Few single clefts in the deep layer	Up to 30% of the deep layer is occupied by clefts	Up to 50% of the deep layer is occupied by clefts	Up to 70% of the deep layer is occupied by clefts
DEGENERATION				
Intensity	No evidence of cellular degeneration	Up to 30% of the cytoplasm is degenerated	Up to 50% of the cytoplasm is degenerated	Up to 70% of the cytoplasm is degenerated
Diffusion	Single to few scattered cells	Groups or single strings of degenerated cells	Up to 50% of epithelial cells are degenerated	50-100% of epithelial cells are degenerated
SHAPE ALTERATION	Single to few scattered cells	Up to 30% of cells present altered shape	Up to 50% of cells present altered shape	50-100% of cells present altered shape

Table 1. Semi-quantitative evaluation isolated pig eyes

EYE	STROMAL CLEFTS			EPITHELIAL CELL VACUOLIZATION		SPINDLE BASAL CELLS
	Upper	Medium	Deep	Intensity	Diffusion	
A	3+	2+	1+	1+	1+	1+
B	2+	1+	0	1+	1+	1+
C	1+	1+	0	2+	2+	1+
D	1+	1+	0	1+	3+	1+
E	2+	2+	1+	2+	2+	1+
F	2+	2+	0	0	0	1+
G	2+	1+	0	1+	2+	1+
H	1+	1+	1+	1+	2+	0
I	2+	1+	1+	2+	2+	0
J	2+	2+	0	1+	1+	0
K	2+	1+	1+	2+	2+	1+
L	1+	1+	0	2+	1+	0
M	2+	2+	1+	2+	2+	1+
N	2+	1+	0	2+	2+	1+
O	2+	1+	0	2+	2+	1+
P	1+	1+	0	1+	1+	2+

Table 2. Collected data during histopathological analysis of the cornea of eyes treated with DMEPI.

EYE	STROMAL CLEFTS			EPITHELIAL CELL VACUOLIZATION		SPINDLE BASAL CELLS
	Upper	Medium	Deep	Intensity	Diffusion	
A	0	0	0	0	0	0
B	3+	3+	3+	2+	3+	1+
C	1+	1+	0	2+	2+	1+
D	2+	2+	2+	2+	1+	1+
E	1+	1+	0	1+	1+	0
F	1+	0	0	0	0	0
G	1+	1+	1+	2+	1+	2+
H	2+	1+	1+	2+	2+	2+
I	3+	2+	2+	1+	2+	2+
J	1+	1+	1+	3+	2+	2+
K	2+	1+	1+	0	0	2+
L	2+	1+	0	2+	1+	1+
M	1+	0	0	0	0	2+
N	2+	1+	0	0	0	1+
O	1+	1+	0	3+	2+	1+

Table 3. Collected data during histopathological analysis of the cornea of eyes treated with LN_2.

CLEFTS	p-value	p-value adj	U	different?
Upper	0,4065	0,3618	277,5	NO
Medium	0,2859	0,2084	283,5	NO
Deep	0,4408	0,3801	236,0	NO

Table 4. Clefts' medians comparison according to Mann-Whitney test. Significance level set at 0.05. No significant difference detected.

VACUOLIZATION	p-value	p-value adj	U	different?
Intensity	0,8744	0,8659	260,5	NO
Diffusion	0,1665	0,1395	291,5	NO

Table 5. Vacuolization's medians comparison according to Mann-Whitney test. Significance level set at 0.05. No significant difference detected.

	p-value	p-value adj	U	different?
BASAL CELLS	0,1605	0,1196	220,0	NO

Table 6. Basal cells' medians comparison according to Mann-Whitney test. Significance level set at 0.05. No significant difference detected.

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Figure 1. Histofreezer® Portable System.

827x983mm (72 x 72 DPI)

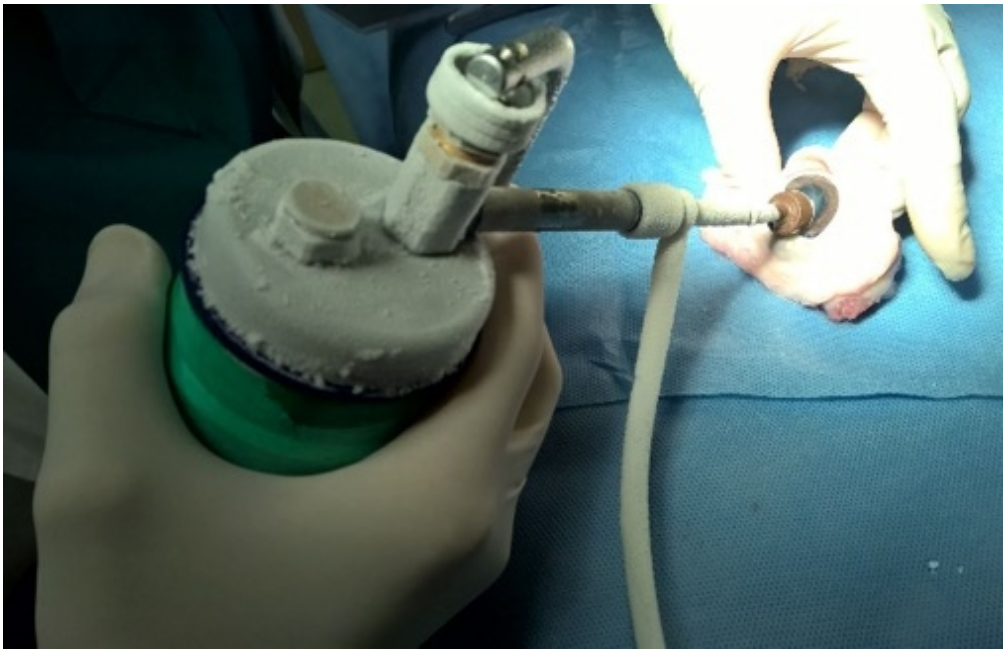

Figure 2. Liquid nitrogen application on cornea in isolated pig eye.

178x115mm (72 x 72 DPI)

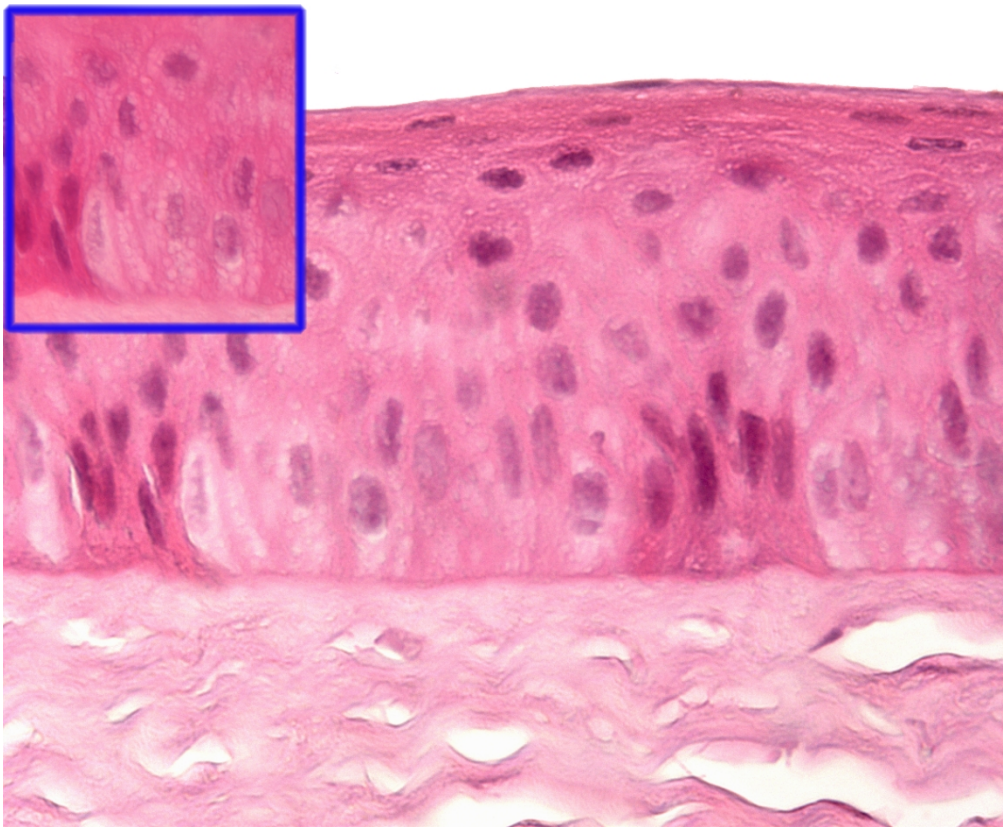

Figure 3. Cornea. Small groups of elongated epithelial cells with hyperchromatic nuclei, and moderate intracytoplasmic vacuolization (see also inset) are evident in corneal epithelium basal layers. Small clefts are detectable within superficial cornea Hematoxylin and eosin, 40x

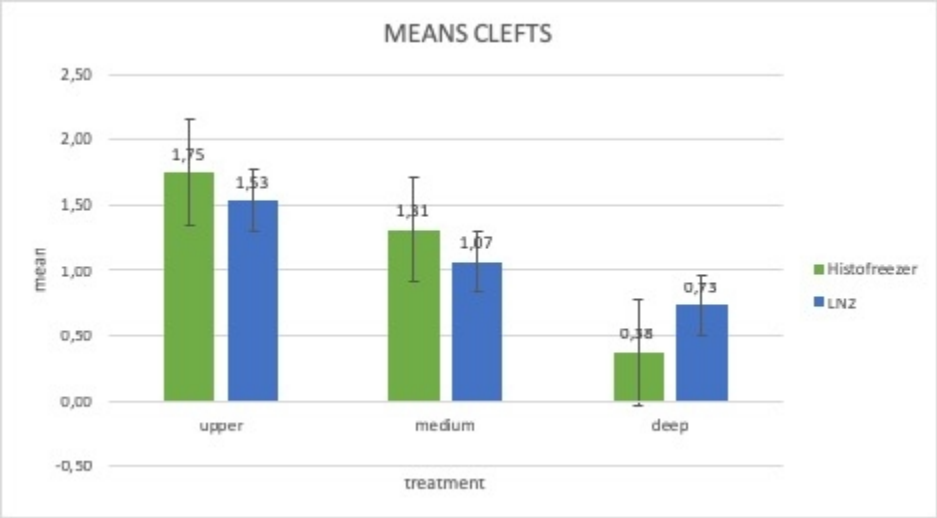

Figure 4. Comparison between means of scores of corneal clefts in upper, medium and deep stromal layer in two treatments groups.

165x91mm (72 x 72 DPI)

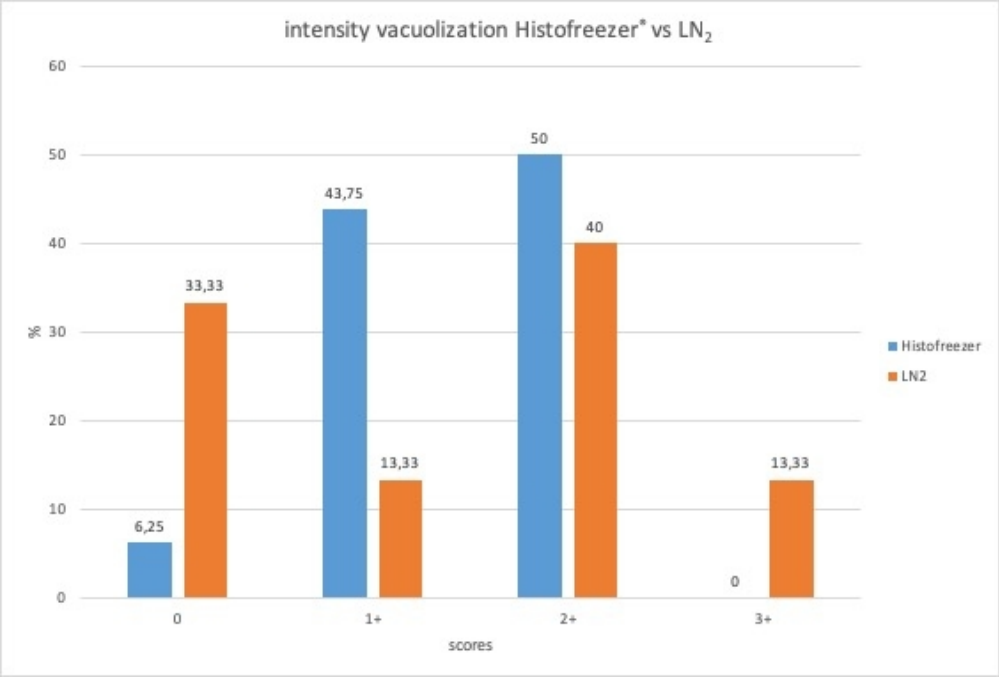

Figure 5. Percentage of eyes per each score, in both of the treatment groups. Intensity of vacuolization.

198x134mm (72 x 72 DPI)

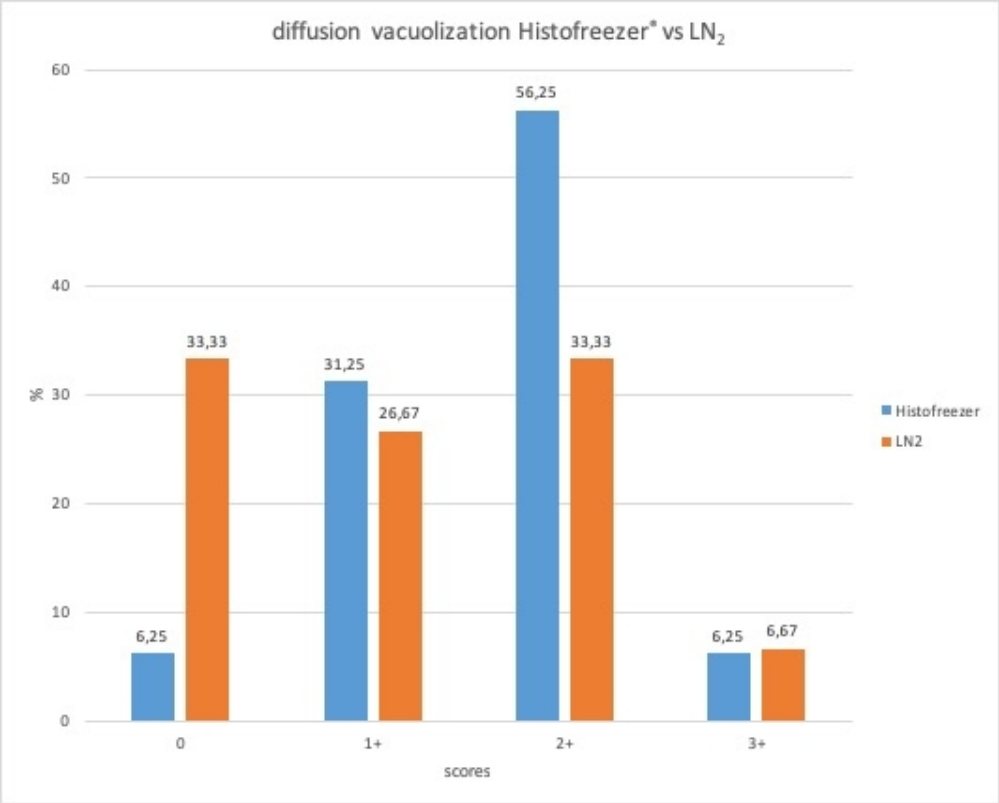

Figure 6. Percentage of eyes per each score, in both of the treatment groups. Diffusion of vacuolization.

188x151mm (72 x 72 DPI)

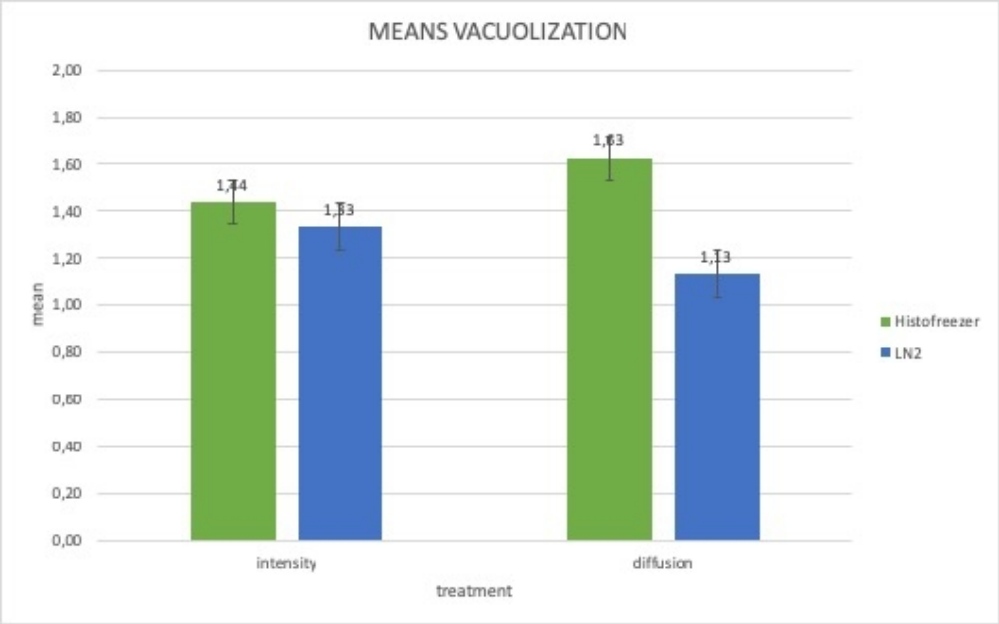

Figure 7. Comparison between means of scores vacuolization intensity and diffusion in two treatments groups.

186x116mm (72 x 72 DPI)

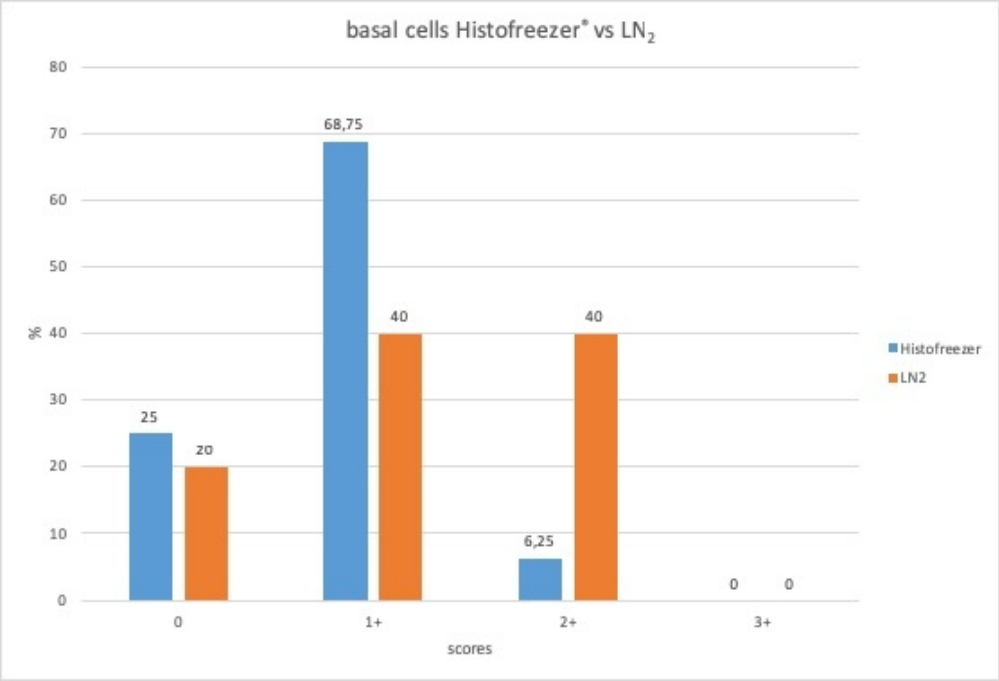

Figure 8. Percentage of eyes per each score, in both of the treatment groups, regarding basal cells elongation.

196x133mm (72 x 72 DPI)

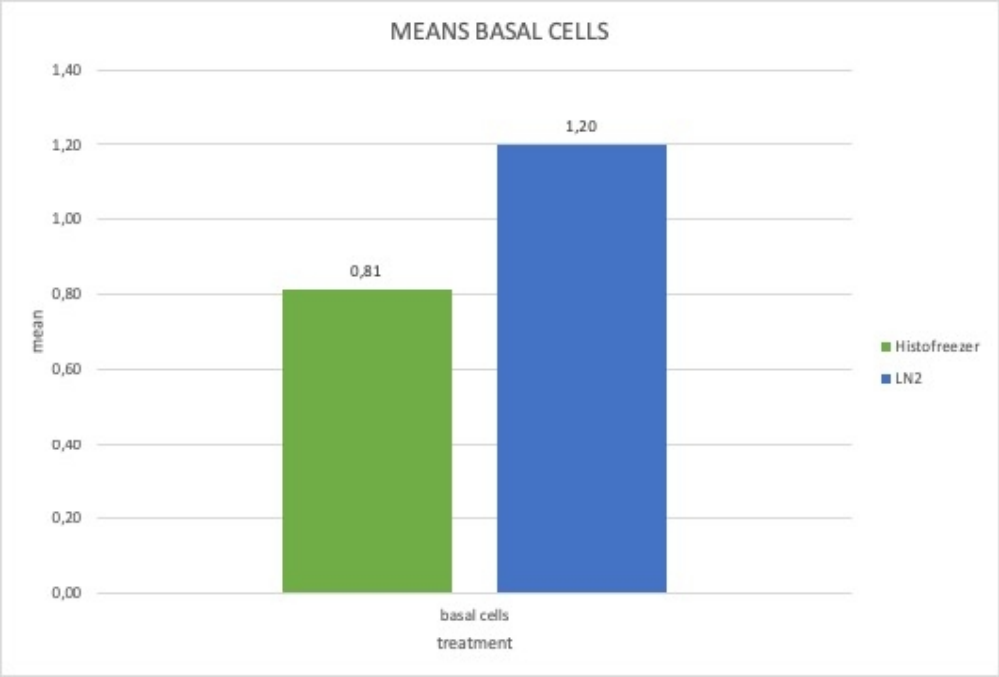

Figure 9. Comparison between means of basal cells elongation scores in two treatments groups.

187x127mm (72 x 72 DPI)