PATHLICON NV

FINAL STUDY REPORT

Device: EKSIMO (working name)

Report date: August 20, 2014

Title: Safety, efficacy and usability study with the Eksimo device in the rabbit

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Exp. No.: OYS001

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SUMMARY

The efficacy as well as safety of the Eksimo device was evaluated after dermal application in male and female albino New-Zealand-White rabbits. This study aimed at comparing the effects of the Eksimo device with those of the Wartner and Cryopen devices when adopted on the rabbit skin.

In the current study, a prototype of the Eksimo device was used.
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No mortality or affected general clinical signs were seen in any of the animals throughout the study.
Local clinical alterations were related to swelling/tumor and redness, only to a minimal to slight extent, in particular in the Eksimo and Wartner treated-skin, in the majority of animals, without any significant difference between the 20 and 40 seconds time points. Any sign of inconvenience or pain was absent in each rabbit.

Clinical signs were in correlation with the histological findings: i) the use of the Eksimo device is save when used under the experimental conditions in the current study, ii) the use of the Eksimo device is most effective in inducing acute epidermal cell death (100%) when compared to the Cryopen (17%) and the Wartner (66%) devices, iii) the Eksimo device can be repetitively used after respecting a minimum time interval of 7 days wound healing under the experimental conditions in the current study and iv) the Eksimo device is equally effective in inducing epidermal cell death when applied for 20 seconds compared to the application for 40 seconds in the current rabbit experiment.

It is concluded that the Eksimo device is safe in rabbits at both 20 and 40 sec. Depending on the animal, no, or transient minimal to slight local signs of redness and/or swelling were recorded, without any signs of inconvenience or pain. These findings were confirmed by the histopathological evaluation.

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1 INTRODUCTION

General

Title:	Safety, efficacy and usability study with the Eksimo device in the rabbit		
Project number (Pathlicon):	OYS001		
Device name:	Eksimo (project name)		
Batch number:	P 3.4.2 (prototype)		
Major action mechanism:	Freezing (N_2O)		
Working mechanism device:	Hold on skin		
Test system:	Albino New-Zealand-White rabbit		
	(3 females and 3 males, 6 rabbits in total) (see amendment 2)		
	aged 12-16 weeks at start treatment; weight range 3010-3420 g		
Devices:	Eksimo	Wartner	Cryopen
Treatment duration:	20'	20'	10'
	40'	40'	30'
Number of treatments:	2	2	2
Days:	0-6	0-6	0-6
Route of administration:	Dermal		
Protocol date:	April 23, 2014		
Experimental starting date:	May 5, 2014 (see amendment 1)		
Experimental completion date:	May 22, 2014		
Dates of treatment:	May 9 and 15, 2014		
Variables studied:	Mortality		
	Clinical observations (local and systemic)		
	Body weight and weight gain		
	Food consumption		
	Water uptake		

1.1 Purpose of the study

The Eksimo device system is designed to deliver nitrous oxide at very low temperature transdermally at a constant rate to injure and kill HPV infected cells for treatment of cutaneous warts. This study was carried out to investigate its safety and to compare its efficacy with the currently at the market available Wartner device (using Dimethyl Ether and Propane) and the "gold standard", Cryopen (using N_2O). Therefore, each medical device was applied to the rabbit skin for a specified time on two independent time points. Six rabbits were euthanized 24 hours after the second treatment. Safety was evaluated during the *in vivo* phase by clinical examination, as well as post-mortem by histopathological analyses which also were adopted for evaluating the efficacy of the treatment with the Eksimo device compared to the competitive systems.

1.2 Sponsor and test facility

Sponsor: Oystershell Laboratories nv
Booiebos 24
9031 Drongen, Belgium

Test facility: Pathlicon nv
Noorwegenstraat 4
9940 Evergem, Belgium

Tests were performed at:
Reibroekstraat13,
9940 Evergem, Belgium

1.3 Regulatory Compliance

The study was performed to the best available scientific principles, in a Belac-ISO15189 accredited environment (Ref. 1), but was not intended to be in full compliance with GLP guidelines.

1.4 Animal welfare

The *in vivo* study was conducted at the Faculty of Veterinary Medicine, Merelbeke, Belgium and was approved by the Ethics Committee before the onset of the study.

It is the policy of Pathlicon nv to use animals for laboratory research to the minimum extent necessary to assess the safety and efficacy of products to be tested for use in humans and animals. Consistent with this policy, all animals are treated humanely and cared for in accordance with the European (Ref. 2) and Belgian (Ref. 3) guidelines, and with the principles of euthanasia as stated in the Report of the American Veterinary Medical Association Panel (Ref. 4).

2 MATERIALS AND METHODS

2.1 Test article

2.1.1 Test device identity

EKSIMO device

Eksimo device designed by Oystershell delivering N_2O at a temperature of -87 to -89°C (batch No. P 3.4.2) was used.

Competitive devices

Two instruments intended for destruction of warts and verruca by application of (extreme) cold using liquefied nitrogen (Cryopen) or an aerosol of Dimethyl Ether and Propane (Wart and Verruca remover, Wartner) were included in the study for comparison.

Cryopen cartridges: ref: S-HO-NOCX-12-S24; Ghislenghien, Belgium; batch: Pr0184464
Wartner: Wartner, Omega Teknika, Dublin, Ireland (distributor: Omega Pharma Belgium nv; batch: 00000803

2.2 Test system and husbandry

2.2.1 Justification for selection of the test system

The New Zealand White rabbit was selected because this animal species reveals a general physiology that is similar to that of humans, which makes the rabbit a good model for the research of human disease. New Zealand whites are key to many aspects of medical research including skin conditions.

The rabbit has often been used for research on skin permeability and irritation studies, and is also an animal model which is regularly adopted in drug development safety studies (refs 5,6,7,8). Using rabbit skin in this study provides additional benefits to evaluate the use of the new medical Eksimo device. As rabbit skin contains many hair follicles, as these follicles together with their hair sheets are located throughout the skin and as the epithelium of these follicles is continuous renewing itself, this model is very useful to evaluate objectively the effect of Eksimo device on skin.

2.2.2 Description of the test system

Three young and healthy non-pregnant female and three male NZW (New Zealand White) SPF (with exception for an apathogenic strain of *Bordetella bronchiseptica,* which was documented by the vendor's facility) albino rabbits were ordered at Carfil (Turnhout, Belgium).

At the vendor's facility the animals were tattooed with a unique identification number and they had not been subjected to any previous experimental procedure. The animals arrived at the Faculty of Veterinary Medicine on May 05, 2014. They were 12-16 weeks old and in the weight range of 3010-3420 g at the start of treatment.

A health certificate was transmitted at delivery. Upon arrival the animals were weighed, checked for physical condition and housed individually.

2.2.3 Housing and environmental enrichment

The rabbits were housed in a limited access rabbit facility (in the experimental stable n° 5 at the faculty of Veterinary Medicine, Salisburylaan 133, Merelbeke, room n° 101). The animal facility has its own supply of filtered, fresh air that is not recirculated (overpressure). The temperature and relative humidity in the animal room were recorded daily.

Temperature range was 20,3°C – 21,0°C, while relative humidity was ranging from 38% to 59,4% during acclimatisation and in-life study phase. Relative humidity was once out of limits, but this was considered to have no impact on the test system.

Animals were exposed to light-dark cycles of 12h per 12h.

The rabbits were kept individually in plastic cages (90x60 cm surface x 60 cm height) with solid floors and bars in the front side, the latter allowing the animals to have visual, olfactory and auditory contact. The cage floor was covered with irradiated wood shavings (Brandenburg Grade 5, delivered by Bio-Services, Uden, The Netherlands), changed on a regular basis. Each rabbit was provided with a platform area to sit on, made out of an upside down placed plastic box (35 x 60 cm surface and 30cm height) from which parts of two walls were removed, to allow the rabbit hiding underneath.

The rabbits were individually housed to prevent them from fighting, which could damage the treated areas on each other's skin and subsequently blur the experimental results.

Rabbits received hay in a hopper and wooden gnawing material.

2.2.4 Diet and water supply

A commercially available pelleted rabbit food, administered in hoppers, was administered *ad libitum* throughout the study. The diet, type "maintenance" diet, Batch n°: KO-14-03-11-89, produced by Trouw Nutrion (Ghent, Belgium) and delivered by Carfil (Turnhout, Belgium), met all the nutritional requirements of the test system and was certified, i.e. an extensive analysis and quality control was performed on this diet before release by the manufacturer.

Bottle water was administered to the animals via a plastic bottle.

2.3 Study design

2.3.1 Justification for selection of treatment route and duration

The test device was used through dermal application. This route is the route of human exposure to the test device. The selection of both treatment durations (10s, 20s, 30s and/or 40s) was based on literature as well as on the application time during treatment with the Wartner and Cryopen devices in human patients (ref. 9,10). In addition, the duration of treatment selection with regard to the test device in the present study was based upon results from previously conducted *in vitro* experiments by the sponsor (ref. 11).

The sampling time point one day after treatment was included to evaluate direct effects and safety issues, while samples taken seven days after application, were used to provide more information about long-term therapeutic effects and efficacy.

Both sampling time points have been chosen to have additional quantitative measures for early cell death (using Ki67 staining), next to the routinely used staining which enables a robust characterization of cell morphology, level of cell death, inflammation etc. The turnover time of the epidermis from basal layer to granular layer is around 7 days in the rabbit which explains the 7-Day post-treatment sampling time point (ref.12).

2.3.2 Acclimatisation period

The animals had an acclimatisation period of 4 days.

2.3.3 Application of the test device

At first, animals were treated IM in the m. quadriceps with Buprenorfine hydrochloride as analgesic (Vetergesic Multidosis 0,3 mg/ml solution, Ecuphar, Batch: 013093) using a G26 needle (see amendment 2).

Fur was then removed from the dorsal area of the trunk (caudal and lateral on the dorsal midline) of the rabbits by shaving, without abrading the skin, on Day 0. Afterwards, Emla creme 5% (25 mg, Astrazeneca; Ref.: 0428-474; Batch n°: 5951), containing a mixture of lidocain and prilocain, was applied on the back of each animal to induce local anesthesia. Afterwards (at least 15 min), potential remaining crème was removed using physiological solution (Mini-Plasco NaCl 0.9%, B. Braun; Ref.: 0819-094).

Every rabbit was then handled twice with each device for a specified duration (10, 20, 30 or 40 s) on a different spot on the skin on the prepared area of the back (lateral on the dorsal midline).The first Cryopen application in rabbit 5 had a duration of 8 sec instead of 10 sec on Day 0.

Consequently, each rabbit had in total 12 application spots (see annex 1 attached to the protocol for the location of every spot).

Before application of the treatment on Day 6, animals were treated with buprenorphine hydrochloride and fur was shaved again without abrading the skin. The same application procedure as on Day 0, was followed (Batch n° Emla: 5951).

Application of the test and competitive devices were performed as given in the table below, based on Sponsor's expertise and leaflets of the competitive systems.

Every treated spot was marked by circumambulation using a black permanent marker.

Six rabbits were arbitrarily allocated and treated as follows:

Rabbit n° (tattoo)	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Day of study
1 (FA04004)	Wartner 20’	Wartner 40’	Cryopen 10’	Cryopen 30’	Eksimo 20’	Eksimo 40’	0 and 6
2 (MC08006)	Wartner 20’	Wartner 40’	Cryopen 10’	Cryopen 30’	Eksimo 20’	Eksimo 40’	0 and 6
3 (FA04002)	Wartner 20’	Wartner 40’	Cryopen 10’	Cryopen 30’	Eksimo 20’	Eksimo 40’	0 and 6
4 (MC08001)	Wartner 20’	Wartner 40’	Cryopen 10’	Cryopen 30’	Eksimo 20’	Eksimo 40’	0 and 6
5 (FA04003)	Wartner 20’	Wartner 40’	Cryopen 10’	Cryopen 30’	Eksimo 20’	Eksimo 40’	0 and 6
6 (MC08007)	Wartner 20’	Wartner 40’	Cryopen 10’	Cryopen 30’	Eksimo 20’	Eksimo 40’	0 and 6

2.3.4 Duration of the study

The in-life phase lasted for 8 days.

2.4 Clinical observations and measurements

Data on mortality, clinical observations, body weight, and food and water consumption were recorded manually (see raw data).

2.4.1 Mortality and clinical observations

The rabbits were observed at 0.08, 1 hour (locally) and at 4, 7 and 24 hours (locally, generally) after treatment and once (locally) or twice (generally) a day on Days 2,3,4 and 5. Body temperature, water- and food uptake were only measured once a day.

The animals were observed for signs of ill health (heart rhythm and frequency, respiratory rate), abnormal behaviour or appearance, alertness, occurrence of untoward clinical effects (calor, rubor, tumor, dolor), moribund state and mortality. Individual records were maintained for each animal.

2.4.2 Body weight

Individual body weights were recorded prior to the start of treatment and daily from the start of treatment until Day 7.

The measurements were carried out by an automated weighing procedure by use of an appropriate balance (Tefal, +/- 10g) and were recorded on paper.

2.4.3 Food consumption

Food hoppers were filled daily in the morning (400 g/hopper). The hoppers were not weighted by an automated weighing procedure. Food take-up was estimated (400g – remaining volume) and was documented on paper.

2.4.4 Water consumption

Bottles were filled till the bar of 600 ml daily in the morning. The water uptake was calculated daily (600ml – remaining volume) and was recorded on paper.

2.5 Histopathology

See Contributing Scientist Report.

2.6 Data analysis

Analysis regarding Ki67 expression was done using the Virtuoso software (Roche Diagnostics).

2.7 Usability evaluation of the Eksimo device

The sponsor provided written information/training and explanation on the use of the devices and application before the beginning of the present study. Several usability parameters of the test device were evaluated by the applicator by giving a score as depicted in the study protocol. Every malfunction, difficulty or defect was communicated as soon as possible to the sponsor and was documented. Importantly to mention, in the current study, a prototype of the device, with limited finish grade and ergonomics, was used.

3 RESULTS AND CONCLUSIONS

3.1 Mortality

The results are presented as follows:

Detailed results: see Table of Annexes (individual animal data)

Discussion and conclusion

There were no deaths or sacrifices in any of the animals during the study.

3.2 Clinical observations

The results are presented as follows:

Detailed results: see Table of Annexes (individual animal data)

3.2.1 General clinical observations

Discussion

In general, the applications did not have any effect on general activity, general appearance nor alertness, heart frequency, heart rhythm and breathing frequency. Breathing frequency was moderately increased (tachnypnea) throughout the study, but was correlated to excitation due to handling, other external stimuli and curiosity. Following treatment with buprenorphine, one could notice a lower, but within normal range, respiratory rate, due to its correlation to the analgesic, that was hence considered clinically not relevant. Faecal output, although not measured as a clinical parameter as such, was daily checked and considered normal; a finding that was in line with food uptake.

Conclusion

No clinically relevant changes in general activity or general appearance were noted in any of the rabbits.

3.2.2 Local clinical observations

In general, the rabbit skin turned white during and just following application with the Eksimo device. The whitish-colour faded away after removing the device, as was also the case for Wartner, and mainly Cryopen.

Local pain, wound tenderness and local heat were absent on every treatment spot in all animals during the entire in-life phase.

On Day 0, and in some cases onwards, it was noted that some minimal to slight alterations regarding local redness and swelling were present in four rabbits out of the six. In rabbit 1, minimal redness was seen after treatment with the Eksimo and Cryopen device, while rabbit 2 revealed minimal or slight redness and oedema on the Eksimo as well as Wartner-treated skin. In the latter, redness on the Wartner-treated skin was not correlated to the application since the surrounding tissue was reddish too, also before application. This finding was in correlation with the histopathological findings.
Rabbit 5 also showed some (very) slight changes with regard to redness and swelling at the Wartner and Eksimo treated skin (20 and 40 sec).
Clinical signs in these animals were not recorded anymore from Day 3 onwards, partly because of hair growth masking the treated spots.
In animal 3, one could note minimal to slight local redness on the Wartner-treated spots from Day 1 onwards (score 1-2).

On Day 6 and 7, before sacrifice, local clinical signs (swelling and/or redness) were noted in all animals and were most pronounced in skin treated with the Wartner and Eksimo device, as was the case on Day 0.
In general, alterations were minimally to slightly present, except for rabbit 5 after 40 sec-treatment with the Wartner device inducing moderate local swelling. None of these observations were recorded on the control spot.

None of the clinical local signs were associated with pain or inconvenience, at any moment during the experiment. General condition was neither affected (see general clinical signs).

See tables with individual data of each animal for more detailed data per rabbit (annexes).
All clinical findings were correlated to the histopathological observations.

It is clear that mainly the observed changes related to redness and swelling, although often barely to slightly noticeable (score 1-2), were induced by the Wartner and Eksimo device, without any differences in gender. Bot instruments are “hold on skin” devices (pressure-contact with skin), which may be a potential explanation for the local swelling and local inflammation (redness). In none of the cases, surrounding tissue was affected. One may therefore presume that, taken the clinical signs into account, that the Eksimo device is safe to use in a natural biological environment, when adopted for 20 sec as well as 40 sec, which is confirmed by the histopathological examination.

3.3 Body weight and weight gain

The detailed results are presented in the Table of Annexes (individual animal data).

Discussion

Body weight was not affected in any of the animals. No weight loss was noted. Total weight gain ranged from 30 g to 250 g and was correlated with food uptake.

Conclusion

It is concluded that the applications did not affect body weight or weight gain in any of the six rabbits.

3.4 Food consumption

The detailed results are presented in the Table of Annexes (individual animal data).

Discussion

Daily and total food uptake were affected in none of the animals, a finding that was correlated to body weight and weight gain.

Conclusion

Food consumption was not affected by any treatment in male and female rabbits.

3.5 Water consumption

The detailed results are presented in the Table of Annexes (individual animal data).

Discussion

Daily water uptake was ranging between 150 and 300 ml. On Day 3, however, water uptake in animal 2 was only 100 ml, due to the detachment of the drinking bottle. Hence, water consumption was not directly affected by the dermal applications in rabbit 2. One was not able to measure water uptake in animal 3 on Days 4, 5 and 6 because the bottle was found empty or was found on the floor. Food uptake and general as well as hydration conditions in this rabbit were normal, so the lower water intake was not considered critical for the animal, nor having any impact on the animal welfare.

Conclusion

Water uptake was not directly affected by any treatment in male and female animals.

3.6 Histopathology

See attached contributing scientist report (pathologist).

3.7 Usability evaluation of the Eksimo device

In the current study, a prototype of the device, with limited finish grade and ergonomics, was used. On Day 0, when loading the Eksimo device, it happened that gas started suddenly to escape on top, resulting in an empty device which could not be used anymore. This issue mainly occurred during the beginning of experiment. It is important to note that the device was not used if loading did not take place as described in the manual or as explained during the training. The device was only used when activation was unconditionally possible and when gas did not escape on top. Once the device was loaded correctly, no issues were reported by the applicator. To overcome the issue regarding the loading of the device, the project leader of the Sponsor performed the loading and activation of the device on Day 6. The same applicator as on Day 0 treated the animals, in a same way as done on Day 0. Importantly, the loading step had to be carried out in this experiment because of the use of a prototype of the device. The activation step itself was easy to perform.

4 RECORDS AND ARCHIVES

At the end of the study, the protocol and its amendments, deviations, digital images, raw data, pictures and the originally signed final report are stored in the archives of Oystershell Laboratories nv. Digital images and specimens (tissues, paraffin blocks and slides) are stored at Pathlicon nv.

5 REFERENCES

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6 TABLE OF ANNEXES

Annex General clinical parameters

Annex Calor (local heat)

Annex Dolor (local pain)/Wound tenderness

Annex Rubor (local redness)

Annex Tumor (local swelling)

Annex Body weight

Annex Pictures treatment spots

Annex Histopathology: see attached Contributing Scientist Report

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