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VIII. References
I. Title of the Preclinical Study

Preclinical Study of the Safety and Effectiveness of High-Intensity Focused Ultrasound in Skin Penetration

II. Background

Ultrasound equipment has been used for years for diagnostic purposes. Diagnostic ultrasound equipment, regarded as the safest system among its kind, is used for general medical care in obstetrics and gynecology such as observations of the fetus.

In case the ceramic generating ultrasonic waves is in a cave form, the ultrasonic waves become focused at the center point and have a constant energy distribution. This is referred to as high-intensity focused ultrasound (HIFU).

The center point at which the ultrasonic waves become focused is called a focal point, and the energy distribution of the ultrasonic waves is referred to as the focal point size.

When the ultrasonic waves generated by the ceramic become focused, heat is produced at the focal point due to the energy of the HIFU. To be more specific, the ultrasonic waves cause vibrations between tissue molecules, resulting in friction that becomes the source of heat, and coagulation in the tissue occurs due to the heat at the focal point. The size of the coagulated tissue is determined by the magnitude of the ultrasound energy and the focal point size.

HIFU surgical unit is a heat treatment system that uses these principles. It is a selective localized cancer treatment method that does not cause pain or side effects or leave any scarring. For this reason, it is presently used in treating breast cancer, uterine cancer and myoma, and liver cancer among others. It is high in demand as patients recover more quickly compared to other surgical procedures.

Recently, it has been confirmed that it can be applied to aesthetic treatments in relation to skin lifting treatments. Internationally speaking, related medical devices have been developed and are being used at hospitals and clinics. As for the principles applied in the skin lifting treatment field, HIFU is used to induce a thermal injury zone (TIZ) in the SMAS layer in the dermis without damaging the epidermis, and the thermal coagulation has an anti-aging effect such as reduction of wrinkles and fine lines in the epidermis, according to the clinical results reported overseas.

In the skin aesthetics field, a wide range of non-invasive methods have been employed to reduce or eliminate wrinkles on the face and they include peel, microdermabrasion and laser treatments. However, due to the limitations of skin penetration, they are primarily treating the epidermis layer of the skin.

The most common type of non-invasive facial skin rejuvenation methods is using C02 laser. C02 laser has been mainly used for skin regeneration and wrinkle reduction purposes, focusing on the epidermis layer in the face.
The facial skin rejuvenation treatments using CO2 laser can largely be classified as follows:

(i) The method of dissociating and removing the epidermis layer

(ii) The method of transmitting energy to the superficial papillary dermis to cause damage to the collagens and stimulate the fibroblasts through cytokinesis for the healing process to produce new collagens (the collagen remodeling process is an important step in skin regeneration.)

The treatment procedure for complete removal of the epidermis layer must last 7 to 10 days, and after the CO2 laser treatment, red spots and “scale-like skin” will occur for months. While the CO2 laser peel treatment has been proven to be an effective non-surgical anti-wrinkle treatment, it is not the most ideal method as it results in inflammation.

For this reason, radio frequency (RF) treatment methods have been attempted, and methods have been developed to regenerate and remodel collagens while minimizing damage after surgery.

HIFU is similar to fractional laser in that it causes heat damage. However, HIFU differs from its counterpart as it causes heat damage below the epithelial layer and it is in various forms.

This study was conducted with the aim to derive predictions regarding the safety and effectiveness of using an HIFU system on human skin tissue based on a performance test by analyzing the results of creating a TIZ using HIFU on pig skin, which is most similar to the tissues of the human skin.
III. The Purpose of the Performance Study

The purpose of the performance study is to assess the safety and effectiveness of high-intensity focused ultrasound (HIFU) by irradiating the skin tissues of pigs, which are most similar to the tissues of the human skin, with HIFU and analyzing the thermal coagulation that occurred in the skin tissues.

IV. Implementing Organizations and Research Periods

In order to make the conditions as similar as possible to the actual cases of irradiating the human skin, the study was designed to have a live pig irradiated with ultrasound waves. For this purpose, the sole organization in Korea that can perform biological tests on micro-pigs was selected as the testing institution, and another organization was chosen for observations of the samples collected after the irradiation. Also, an organization for observing the irradiation results and an organization for examining the observation results were chosen. The implementing organizations and their roles as well as the research periods are specified hereunder.

1. Research Periods
   - Total research period: April 1 to June 30, 2015 (90 days)
   - March 12 to April 13, 2015 – Establishment of the experiment protocol and signing of agreements by the relevant implementing organizations
   - April 23 to April 30, 2015 – Experiments on mini pigs and sample collection
   - May 1 to June 30, 2015 – Observation of samples and reporting (pathology institution)

2. Implementing Organizations and Their Roles

2-1. Eulji University Bio-MediTech Industrial Regional Innovation Center (ERIC) (http://ric.eulhi.ac.kr)
   - Role: Advise on and supervise the testing
   - Participating period: April 1 to June 30, 2015

   - Role: Provide micro-pigs for testing and aseptic lab, collect samples, and dispose of the carcass
   - Participating period: April 23 to 30, 2015

2-3. Eulji University Bio-MediTech Industrial Regional Innovation Center (Animal Testing Pathology and Biopsy Lab)
   - Role: Run histopathological examinations, drug treatment and management of samples, and exchange information with the sample observing organization
   - Participating period: May 1 to June 13, 2015

2-4. Eulji University Dept. of Bio Medical Laboratory Science
   - Role: Slice the samples, observe under microscope and provide microscopic imaging
   - Participating period: May 1 to June 30, 2015
V. Test Subject and Testing Method

1. Test Subject

In the overseas research cases, the muscles and skin of refrigerated pigs were obtained from IRB, and the refrigerated samples were defrosted to 25°C before performing the experiments. On the other hand, in this test, the skin tissues of a live pig were irradiated directly so that the condition is the same as irradiating the human skin for aesthetic purposes. To this end, a micro-pig was chosen as the test subject.

The HIFU surgical unit (Model name: HFR-1000A (ULVERIN) Manufacturer: RoboMax) used in this study was manufactured to deliver ultrasound waves into the skin tissues, and the hand piece of this device has a probe for a cross-sectional view of the areas to be irradiated with ultrasound waves. Also, a transducer can be mounted on this device for ultrasound irradiation.

Using this device, TIZs can be formed serially in the skin tissues at constant depth, and the magnitude, intervals between the TIZs, and the length of the TIZs can be specified in the settings. In this test, two transducers with different settings for the ultrasound frequency and the focal length were used.

< The equipment and two transducers used in the test >
1-1. Micro-Pig®

The test subject was reared in a sanitary environment and in accordance with the strict SPF system according to the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) standards. Compared to other species of pig, micro-pigs have little body hair and their skin is similar to that of humans in terms of drug delivery mechanisms and epithelial cell structure. Thus, their skin tissues are most similar to the human skin in the aspects of absorption, allergic reactions and dermal administration, which is the reason they were chosen as the test subject. To note, a female micro-pig, with a thin skin surface, was used.

1-2. HIFU Treatment Device

A) Main device
   (1) Product name and manufacturer
       (a) Product name: ULVERIN
       (b) Manufacturer: RoboMax
   (2) Electric rating
       (a) Rated voltage and frequency: AC 220V, 60HZ
       (b) Service power: 200VA and below
   (c) Type of protection against electric shock: Class 1 device, BF-type mounting
   (3) Ultrasound wave generation device
       (a) Max. treatment length: 25mm ±10%
       (b) Treatment intervals: 1mm ~ 3mm±20%
       (c) Treatment time: 30ms ±10%

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<table>
<thead>
<tr>
<th>Category</th>
<th>Extra T-Type</th>
<th>T-Type</th>
<th>M-Type</th>
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</thead>
<tbody>
<tr>
<td>Body weight at birth</td>
<td>0.18 ~ 0.3 kg</td>
<td>0.2 ~ 0.4 kg</td>
<td>0.4 ~ 0.6 kg</td>
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<tr>
<td>Body weight at 24 months</td>
<td>18 ~ 23 kg</td>
<td>25 ~ 35 kg</td>
<td>45 ~ 55 kg</td>
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<td>Life span</td>
<td>10 ~ 15 years</td>
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<td>Pregnancy period</td>
<td>111 ~ 114 days</td>
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<td>Litter size</td>
<td>5 ~ 6</td>
<td>6 ~ 7</td>
<td>7 ~ 9</td>
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<td>Breastfeeding period</td>
<td>30 ~ 36 days</td>
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<td>28 ~ 35 days</td>
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<td>Age of sexual maturity</td>
<td>4 ~ 6 months</td>
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<tr>
<td>Reproductive age</td>
<td>6 ~ 8 months</td>
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< Photos of Micro-Pig® (from Medi Kinetics website) >

< Biological characteristics of Micro-Pig® >
B) Transducers

<table>
<thead>
<tr>
<th>Category</th>
<th>1</th>
<th>2</th>
</tr>
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<tbody>
<tr>
<td>External dimensions</td>
<td>90mm x 60mm x 25mm(±1.0mm)</td>
<td>90mm x 60mm x 25mm(±1.0mm)</td>
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<tr>
<td>Treatment frequency</td>
<td>4.3MHz ±10%</td>
<td>4.3MHz ±10%</td>
</tr>
<tr>
<td>Focal distance(from the surface)</td>
<td>3.0mm ±10%</td>
<td>4.5mm ±10%</td>
</tr>
<tr>
<td>Max. ultrasound output</td>
<td>1.8 J/cm² ±10% and below</td>
<td>1.8 J/cm² ±10% and below</td>
</tr>
<tr>
<td>Temperature around the focal point</td>
<td>Max. 10°C increase from the original temperature</td>
<td>Max. 10°C increase from the original temperature</td>
</tr>
</tbody>
</table>

2. Testing Method and Process

As mentioned above, in order to irradiate a live pig with ultrasound waves, we chose micro-pigs as the test subject. It was placed under general anesthesia and cardiac anesthesia using the method described hereunder, and a clipper was used to remove the hair on its back, on which a 60 x 24(mm) irradiation area were marked.

Before the irradiation, an acrylic plate was used in a reliability test on the HIFU surgical unit.

As shown in the table below, the typical energy values of the two transducers (3mm and 4.5mm) were used to irradiate the pigs four times each. Then, the irradiated areas were resected, and after a preparation of skin substitute samples and biopsy, the samples were treated with 4% formalin for preservation.

After the ultrasound irradiation and sample collection, the carcasses were disposed of by the organization who provided the micro-pigs, while the samples were sent to the histopathological observation organization for H&E staining before being sliced to a thickness of 4/1,000mm and observed under a microscope at 40x magnification.

2-1. Testing Location

The experiments were carried out in the aseptic lab (surgical room) of the organization that provided the micro-pigs. The test subject was administered anesthetics and prepared for the experiment in the surgical room. Then, the investigators in sterilized labwear transported the HIFU surgical unit through the sterilized gate. The experiment was carried out in a lab that was completely isolated from the external environment to prevent contamination.
2-2. Preparation of the Test Subject

The micro-pig transported into the aseptic lab (surgical room) was placed under general anesthesia (ketamine: 1 ml/10 kg (Yuhan Corp.), xylazine: 1 ml/10 kg (Inter Vet Korea) and cardiac anesthesia (isofluorane : oxygen = 2.5 : 2.5 (JW Pharma)). A clipper was used to remove the hair from the back, with a thin epidermis layer, and the 40 x 20(mm) areas to be irradiated was indicated with a marker. Ultrasound gel was applied on the marked area to complete the preparation for the ultrasound irradiation.

[Test subject (Micro-Pig®) on the surgical table after anesthetics administration and hair removal>

<Ultrasound irradiation areas and gel application>
The HIFU surgical unit was transported in through the aseptic gate, and an acrylic plate test was performed for final testing of the reliability of system operation to check for the presence of any defects that could have potentially occurred during its transport from the manufacturing site to the testing location. After confirming normal operation, the experiment was carried out.

An acrylic plate was used for a simple performance test. A transducer was placed on top of the acrylic plate for HIRU irradiation, and it was checked whether it resulted in the formation of TIZs. This testing method is used as a simple measure to check for normal operation of an ultrasound surgical device before its actual use.
2-3. Ultrasound Irradiation Test

Using the HIFU surgical unit, the hairless and marked areas on the back of ultrasound waves were irradiated using the transducers. The hand piece was replaced to irradiate the subject with 1J and 1.8J of energy and in 3mm and 4.5mm modes for more than four times for each condition. The most representative samples were found afterwards. Taking into account that the respiration of the micro-pig may affect the depth of irradiation, the device was placed on the skin surface, and ultrasound waves were generated during exhalation.

<table>
<thead>
<tr>
<th>Transducer Type</th>
<th>Energy (J)</th>
<th>Irradiation Time (ms)</th>
<th>Frequency (MHz)</th>
<th>Focal distance (mm)</th>
<th>Repetition (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (3mm)</td>
<td>1</td>
<td>30</td>
<td>4.3</td>
<td>3.0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>30</td>
<td>4.3</td>
<td>3.0</td>
<td>4</td>
</tr>
<tr>
<td>2 (4.5mm)</td>
<td>1</td>
<td>30</td>
<td>4.3</td>
<td>4.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>30</td>
<td>4.3</td>
<td>4.5</td>
<td>4</td>
</tr>
</tbody>
</table>

Checklist

- Impact on the surrounding tissues
- Accuracy of the penetration depth
- Size of the coagulation

<Ultrasound irradiation test>
<Irradiating the test subject with ultrasound waves using the HIFU device (#1)>

<Irradiating the test subject with ultrasound waves using the HIFU device (#2)>
2-4. Post-Irradiation Sampling and Carcass Disposal

A 60x24x10mm piece of the skin was resected from the irradiated area and after the preparation of skin substitute samples and biopsy, 4% formalin fixing solution was used for preservation purposes. The carcass was disposed of by the organization that provided the test subject (micro-pig).

2-5. Sample Staining and Observation

The collected samples were sent to the histopathological observation organization for a histology study. As shown below, the samples underwent H&E staining before being sliced to a thickness of 4/1,000mm, and they were observed under an optical microscope (Olympus BX-51, 40 ~1,000x magnification).

By observing the changes in the tissues of the irradiation area using a microscope at 40x magnification, we checked for the occurrence of TIZs around the focal distance and measured the depths and sizes thereof. Also, the surrounding tissues were checked for damages. In the case of slices with significance, they were photographed using a microscopic camera and were retained as observation results.

2-5-1. H&E staining

A. Reagent

1) Harris Hematoxylin solution
Hematoxylin (C.I. 75290) 5.0 g
95% ethanol 50ml
Potassium alum (A1K(SO4)2 -12H2O)
or ammonium alum (A1NH 4(SO4)2 -12H2O) 100g
dH2O 1,000ml
Mercuric oxide R (HgO) 2.5g

2) 1% eosin-alcohol solution
Eosin Y (C.I. 45380) lg
dH2O 20ml
95% ethanol 80ml

3) 1% HCl-alcohol solution
70% ethanol 99ml
Cone. HC1 1ml
4) 0.5-1% Ammonia water
Ammonia water (NH₄OH) 0.5~1ml
80% ethanol 100ml

B. Fixing solution: 10% Neutral buffered formalin (NBF), 10% formalin

C. Microsectioning: 6 um
VI. Results of the Preclinical Study

Microscopic image of a sample taken from the tested area

- Scale: 40x magnification
- Focal distance: 3.1mm from the surface
- Size of the coagulation: 0.5 x 0.8mm
- Changes in the surrounding tissues: None
- Fat tissues were found to have become dissolved due to the heating effect in the focal area.
- Scale: 40x magnification
- Focal distance: 3.1 mm from the surface
- Size of the coagulation: 0.6 x 0.9 mm
- Changes in the surrounding tissues: None
- Fat tissues were found to have become dissolved due to the heating effect in the focal area.
- Scale: 40x magnification
- Focal distance: 4.6mm from the surface
- Size of the coagulation: 0.5 x 0.7mm
- Changes in the surrounding tissues: None
- Fat tissues were found to have become dissolved due to the heating effect in the focal area.
- Scale: 40x magnification
- Focal distance: 4.6mm from the surface
- Size of the coagulation: 0.6x0.9mm
- Changes in the surrounding tissues: None
- Fat tissues were found to have become dissolved due to the heating effect in the focal area.
VII. Discussion of the Results and Comments by the Supervising Organization

In the case of Transducer #1, the test subject was irradiated on the four marked areas with ultrasound waves with 1J of energy at 4.3MHz for 30ms at a focal distance of 3.0mm, and the samples thereof were observed under a microscope at 40x magnification. As shown in Fig. 1, the results indicated that coagulation (melting) occurred in a 0.5x0.8mm area at a depth of 3.1mm from the epidermis, while no notable changes occurred in the surrounding tissues.

In the case of Transducer #1, the test subject was irradiated on the four marked areas with ultrasound waves with 1.8J of energy at 4.3MHz for 30ms at a focal distance of 3.0mm, and the samples thereof were observed under a microscope at 40x magnification. As shown in Fig. 2, the results indicated that coagulation (melting) occurred in a 0.6x0.9mm area at a depth of 3.1mm from the epidermis, while no notable changes occurred in the surrounding tissues.

In the case of Transducer #2, the test subject was irradiated on the four marked areas with ultrasound waves with 1J of energy at 4.3MHz for 30ms at a focal distance of 4.6mm, and the samples thereof were observed under a microscope at 40x magnification. As shown in Fig. 3, the results indicated that coagulation (melting) occurred in a 0.5x0.7mm area at a depth of 3.1mm from the epidermis, while no notable changes occurred in the surrounding tissues.

In the case of Transducer #2, the test subject was irradiated on the four marked areas with ultrasound waves with 1.8J of energy at 4.3MHz for 30ms at a focal distance of 4.6mm, and the samples thereof were observed under a microscope at 40x magnification. As shown in Fig. 4, the results indicated that coagulation (melting) occurred in a 0.6x0.9mm area at a depth of 3.1mm from the epidermis, while no notable changes occurred in the surrounding tissues.

This study has significance in that it was conducted on the skin tissues of a live pig, unlike most of the performance tests performed overseas, in which refrigerated pig skin tissues were irradiated with ultrasound waves. Thus, the experimental conditions were as close as possible to the actual conditions, i.e. irradiating the human skin with ultrasound waves.

Observation of the skin tissues of the micro-pig used as the test subject in this study mainly showed that the adipose tissues at a depth of 3.0mm and 4.5mm were dissolved by the heat generated as a result of the ultrasound irradiation. There were no changes observed in the skin layer at and around the site of thermal coagulation (melting). This shows that controlled ultrasound irradiation using high-intensity focused ultrasound (HIFU) surgical unit is safe as it did not cause any expected damages to the skin tissues of the pig, and that thermal coagulation (melting) occurred effectively. Because thermal coagulation (melting) occurred in the controlled areas, one can expect the tissue regeneration process to occur to heal the thermal injury zone (TIZ) based on the reports of prior clinical studies regarding skin reservation following thermal coagulation.
Conclusion

As shown in the above microscopic images of the samples, there were no damages in the pig skin tissues that were irradiated with HIFU. There were no notable changes found in the tissues around the sites of thermal coagulation. An observation of the tissues from the skin surface to the sites concerned showed that there were no areas that were affected.

Supervisor Comments

The thermal coagulation (melting) observed in the microscopic images of the samples appear to have occurred due to heat with a temperature of 55-60°C at the focal point concerned. Due to the technological characteristics of the HIFU surgical unit used in this study, no changes occurred between the skin surface layer that was irradiated and the focal areas concerned and no damages occurred in the surrounding tissues, as shown in the results. Accordingly, it is deemed that the HIFU surgical unit used in this study would be safe to use as a treatment device.
VIII. References


5. Clinical Pilot Study of Intense Ultrasound Therapy to Deep Dermal Facial Skin and Subcutaneous Tissues (Arch Facial Plast Surg. 2007;9:88-95)
