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I. Subject of Research

A preclinical experiment research which confirms the safety and effectiveness of the dermal penetration by HIFU (High Intensity Focused Ultrasound).

II. Research Background

The ultrasound has been adopted as a diagnostic equipment for years. Since it is being recognized as one of the safest diagnostic tools, the obstetrics and gynecology especially well utilizes the method in general practices such as the fetal condition observation check-up.

The HIFU (High Intensity Focused Ultrasound) is a consistent pattern of energy distribution obtained when the ultrasound generating ceramic is shaped as a concave so that the ultrasound is focused around on the center of the sphere.

The center of the sphere in which the ultrasound is focused is called the focal point and the energy distribution of the ultrasound is termed as the size of the focal point.

When the ultrasound generated from the ceramic is focused, the heat is produced from the focal point due to the focused high intensity ultrasound energy. The frequency of the ultrasound initiates vibration between molecules in tissues, which becomes the source of frictional heat by the vibrating molecules. The tissue coagulation is then occurred at the ultrasound’s focal point by the heat. The size of the tissue coagulation is determined by the energy intensity as well as the size of the focal point.

Based on the principle, the HIFU surgical unit uses such a thermal method in treatment and provides a selective cure for local tumors, leaving no wound or adverse effect and pain. Currently, it is used in the treatment of breast cancer, uterine cancer, uterine myoma and liver cancer. The demand is increasing due to the faster recovery rate and the positive perception about safety in comparison with a surgical operation.

It has recently been confirmed that the HIFU method can also be applied to the skin care field related to skin elevation. Such medical devices utilizing the technology have been developed and imported to domestic hospitals and clinics. For the skin elevation treatment, the high intensity focused ultrasound does not harm the epidermis at all and induces the thermal coagulation points within the SMAS layer in the innermost dermal layer. A couple of clinical cases have been reported abroad that the thermal coagulation is effective in the wrinkle reduction as well as the antiaging effects.

In the field of skin care, the non-invasive methods – peeling, microdermabrasion and laser have been used for the facial wrinkle care. However, such treatments cannot overcome the restriction in permeability but focus only on the epidermis level.

The non-invasive facial skin rejuvenation mainly uses the CO₂ (Carbon Dioxide) laser. The laser has an extensive use in both the skin epidermis reproduction and wrinkle reduction care.
The facial skin rejuvenation using the CO\textsubscript{2} laser can be broadly classified as follows:

(i) Removes and drops the skin epidermis.
(ii) Delivers the energy down to the deep superficial papillary dermis to induce collagen damage. Then fibroblast is stimulated by cytokinesis for healing the damage and reproduces collagen (the process of the collagen remodeling is one of the essential steps in the facial skin rejuvenation).

The treatment stage of a complete epidermis removal remains for 7-10 days and the scale-like skin and erythema does for a couple of months after being treated with the CO\textsubscript{2} laser. However, the CO\textsubscript{2} laser peeling is not advisable as it causes an inflammation reaction after operation, although its effectiveness for a non-surgical wrinkle care has been approved.

Under the circumstances, another method of regenerating and remodeling collagen with the minimized epidermis damage is developed using RF (Radio Frequency).

HIFU occurs a thermal damage as the fractional laser does, however, it is totally unlikely to the laser considering that the thermal damage by HIFU is generated under the skin epidermis in various types of forms.

The performance-testing research uses the swine skin, as it has the most similar tissues and structure to the human’s, forms the thermal coagulation point by the HIFU surgical unit and discusses the result to expect the safety and effectiveness when applying the HIFU unit to the human skin tissue.
III. Research Objectives

The objectives are to observe the ultrasound by irradiating the HIFU surgical unit to the swine skin which has the most similar skin structure to the human’s; and to study the thermal coagulation generated within the dermal tissue to be able to assess the HIFU’s safety and effectiveness for the human skin tissue.

IV. Executing Organization and Period of Research

In order to provide the most similar condition to the human skin tissue irradiation, the experiment is designed to irradiate the ultrasound to a living pig. The only domestic facility which provides the in vivo Micro-Pig® experiment is selected and another organization is included to monitor the samples collected after the ultrasound observation. Moreover, the experiment is also designed to incorporate two more organizations to observe the resultant irradiation and to further study the observed result, respectively. The executing organizations, their duties and each laboratory are suggested as follows:

1. Period of Research
   • Total period of Research: 12 March 2014 ~ 13 June 2014 (90 days)
   • 12 March ~ 13 April – Establishment of the experiment design and made agreement with executing organizations.
   • 22 April ~ 30 April – Execution of the miniature pig experiment and collecting samples.
   • 1 May ~ 13 June – Observation and report of samples from a pathology organization.

2. Executing Organizations and Responsibilities
   2-1. Eulji University Regional Innovation Center (http://ric.eulji.ac.kr) (ERIC – Eulji University Bio-MediTech Industrial Regional Innovation Center)
      • Responsibility: Comprehensive consultation and supervision in the progression of the whole experiment.
      • Period of Participation: 12 March 2014 ~ 13 June 2014
      • Responsibility: Provision of Micro-Pig® for experiment and of clean facility. Sample collection and clearing of the bodies of experimental subjects.
      • Period of Participation: 22 April ~ 31 April
   2-3. Eulji University Bio-MediTech Industrial Regional Innovation Center (Animal Experiment Histopathology Laboratory)
      • Responsibility: Histopathology testing and sample drug treatment and control. Making a connection to an institution for the sample observation.
      • Period of Participation: 1 May ~ 13 June
   2-4. Eulji University Clinical Laboratory
      • Responsibility: Sample slicing and microscope observation. Provision of the photomicrograph data.
      • Period of Participation: 25 May ~ 13 Jun
V. Sample, Methodology and Progress in Process

1. Sample
   It is reported that the foreign research case has used the swine muscle and skin tissue after defrosting to 25°C right before the experiment. They were provided from IRB in a frozen form and kept in a freezer. However, for this experiment a living swine tissue is irradiated to make the most similar condition to the human tissue irradiation. The Micro-Pig® is selected as the sample.
   The HIFU surgical unit is designed to deliver the ultrasound into the skin tissue (Model name: sono-Queen, Manufacturer: Bistos Co., Ltd.). Its hand piece embeds a probe that visualizes the cross-section in which the ultrasound is to be irradiated. A transducer can be equipped for the ultrasound irradiation.
   The thermal coagulation points can be regulated to arrange in line to the fixed depth. The intensity, the interval between the thermal coagulation points and the length of the point formation section can also be setup. In this experiment, two cartridges with different ultrasound frequency and the length of point are being used.

< Equipment and the Two Cartridges Used in the Experiment >

1-1. Micro-Pig®
   It is a species that is bred within the strict SPF system in a sanitary environment according to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care). The reason of choosing Micro-Pig® for the study is because, compared to other species, the species is less hairy and has a similar mechanism of drug action and the structure of epithelial cell to the human, which leads to the most similar conditions to the human in the aspect of the skin absorption, allergic reaction and the dermal administration. In particular, the females are chosen as their surface of skin is thinner.

< Micro-Pig® _ from the MediKinetics homepage >
### Biological Characteristics of Micro-Pig®

<table>
<thead>
<tr>
<th>Type</th>
<th>Extra T-Type</th>
<th>T-Type</th>
<th>M-Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth Weight</td>
<td>0.18 ~ 0.3kg</td>
<td>0.2 ~ 0.4kg</td>
<td>0.4 ~ 0.6kg</td>
</tr>
<tr>
<td>Weight at 24-month</td>
<td>18 ~ 23kg</td>
<td>25 ~ 35kg</td>
<td>45 ~ 55kg</td>
</tr>
<tr>
<td>Life Expectancy</td>
<td></td>
<td>10 ~ 15 years</td>
<td></td>
</tr>
<tr>
<td>Pregnancy Period</td>
<td></td>
<td>111 ~ 114 days</td>
<td></td>
</tr>
<tr>
<td>Litter Size</td>
<td>5~6</td>
<td>6~7</td>
<td>7~9</td>
</tr>
<tr>
<td>Weaning Age</td>
<td>30 ~ 36 days</td>
<td></td>
<td>28 ~ 35 days</td>
</tr>
<tr>
<td>Puberty Age</td>
<td></td>
<td>4 ~ 6 months</td>
<td></td>
</tr>
<tr>
<td>Reproductive Age</td>
<td></td>
<td>6 ~ 8 months</td>
<td></td>
</tr>
</tbody>
</table>

1-2. HIFU Surgical Unit

A) Body

(1) Product name and manufacturer
   (a) Product name: sono Queen
   (b) Manufacturer: Bistos Co., Ltd.

(2) Electrical Rating
   (a) Rated voltage and frequency: AC 220V, 60HZ
   (b) Power consumption: Below 300VA
   (c) Type and extension of protection by electric shock: First-rated, B-type equipment

(3) Generator of Treatment Ultrasound
   (a) Maximum distance of treatment setup: 25mm±10%
   (b) Generation interval: 1.5mm ~ 3mm±20%
   (c) Treatment time: 30ms ~ 60ms±10% (5msec/step)

B) Cartridge

<table>
<thead>
<tr>
<th>Type</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of Exterior</td>
<td>70x37.64x78.9 (±1.0mm)</td>
<td></td>
</tr>
<tr>
<td>Treatment Frequency</td>
<td>7MHz±10%</td>
<td>4MHz±10%</td>
</tr>
<tr>
<td>Focal Point Distance (From the surface)</td>
<td>3.0mm±1.0mm</td>
<td>4.5mm±1.0mm</td>
</tr>
<tr>
<td>Maximum Output of Ultrasound</td>
<td>Below 1.2J/cm²±25%</td>
<td></td>
</tr>
<tr>
<td>Peripheral Temperature around Ultrasound Focal Point</td>
<td>Maximum temperature rise gap before and after the treatment is below 10°C</td>
<td></td>
</tr>
</tbody>
</table>
2. Sample
As mentioned above, in order to irradiate the ultrasound to a living pig, the Micro-Pig® has been chosen. After conducting the premedication and deep anesthesia with the method mentioned below, the hair on the back is removed using a clipper then the area of 60x24mm is marked for irradiation. Before irradiating to the subject, a performance test using an acrylic plate of the HIFU surgical unit is carried out as a means of establishing the final reliability test of the equipment.

As described in the ultrasound irradiation test table (page 9), two types of cartridges – 3mm and 4.5mm – are prepared and 4 times of irradiation of the frequently used energy value are applied to each. The irradiated parts are excised and treated with the 4% formalin fixing solution after the biopsy for preparing the skin substitute sample.

After completing the irradiation and sample collection processes, the body is removed by the Micro-Pig® provider. The samples are delivered to the tissue pathology organization and observed by a 400 magnification microscope after staining with H&E and slicing into a 6/1,000mm thickness.

2-1. Place of Experiment
The clean facility (operation room) of the Micro-Pig® provider is selected as the place of experiment. Firstly, the Micro-Pig® is anesthetized and prepared in the operation room; a technician in a sterility suit carries the HIFU surgical unit into the operation room through the sterilization gate. The experiment is conducted in a totally isolated operation room in order to prevent any reaction by contaminants from outside.

2-2. Sample Preparation
The Micro-Pig® is premedicated (ketamine: 1mL/10kg (Yuhan Corp.), xylazine: 1mL/10kg (Intervet Korea)) and deep anesthetized (isofluorane: oxygen = 2.5: 2.5 (JW Pharmaceutical)). The area for irradiation of 40x20mm is marked from the backside with the thinner epidermis after removing hair with a clipper. The preparation process for the ultrasound irradiation is completed by spreading the gel for ultrasound on the area for irradiation.
Experimental Subject (Micro-Pig®) on the Operation Table after Anesthesia and Hair Removal

Processes of Marking the Area for Irradiation and Gel Spreading

The HIFU surgical unit is carried inside through the sterilization gate and a final performance reliability is tested on an acrylic plate and through the phantom testing. The experiment is continued once no mechanical problems that may occur during transportation of the equipment from the manufacturer to the place of experiment are confirmed.

Irradiation Process with the HIFU Unit on an Acrylic Plate
For a simple performance test, an acrylic plate and gelatin phantom are used. A cartridge is placed on the acrylic plate and the plate is irradiated by the HIFU then whether or not the thermal coagulation points are formed can be confirmed. It is likely for the gelatin phantom, the thermal coagulation points can be formed in the gelatin phantom after irradiation. Those two methods are actually performed as a simple way to check the operation of an equipment before irradiating ultrasound.
2-3. Irradiation of Ultrasound
Using the HIFU surgical unit, the ultrasound irradiation is conducted on the area of mark for each cartridge as below. By replacing the hand piece, each cartridge is irradiated with 0.7J, 1.0J and 1.2J in the 1.5mm mode. After irradiating more than four times for each energy, the most representable sample is selected. In order to prevent that the breathing of the subject influences on the depth of irradiation, the ultrasound is irradiated, by being closely adhered to the subject, at the exhalation.

<table>
<thead>
<tr>
<th>Type of Cartridge</th>
<th>Energy (J)</th>
<th>Time of Irradiation (ms)</th>
<th>Frequency (MHz)</th>
<th>Focal Point Distance (mm)</th>
<th>Repetition (N)</th>
<th>Confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7</td>
<td>30</td>
<td>7</td>
<td>3.0</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>50</td>
<td>7</td>
<td>3.0</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>30</td>
<td>4</td>
<td>4.5</td>
<td>4</td>
<td>- Influence on the surrounding tissue</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>50</td>
<td>4</td>
<td>4.5</td>
<td>4</td>
<td>- Accuracy in the penetration depth.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Size of the coagulation part</td>
</tr>
</tbody>
</table>

< Ultrasound Irradiation>

< Ultrasound Irradiation with the HIFU Unit to a Subject >
2-4. Sample Collection and Body Handling

The marked part for irradiation is excised by 60x24x10mm and treated with the 4% formalin fixing solution after the biopsy for preparing the skin substitute sample. After completing the sample collection process, the body is handled by the sample (Micro-Pig®) provider.
2-5. Sample Staining and Observation

The samples are delivered to the tissue pathology organization and the histological study is conducted. As described below, the samples are observed by an optical microscope system (Olympus BX-51, a 40 ~ 1,000 magnification) after staining with H&E and slicing into a 6/1,000mm thickness.

Any changes in the tissue around the area of ultrasound irradiation are observed with a 40 magnification microscope. The formation of thermal coagulation and its size and depth are measured and any damages in the surrounding tissue are detected. The slice with a significant observation is photomicrographed and preserved for a further result discussion.

2-5-1. H&E Staining

A. Reagent

1) Harris Hematoxylin Solution
   Hematoxylin (C.I. 75290), 5.0g
   95% ethanol, 50mL
   Potassium Alum (AlK(SO$_4$)$_2$·12H$_2$O)
   Or Ammonium Alum (AlNH$_4$(SO$_4$)$_2$·12H$_2$O)
   dH$_2$O, 1,000mL
   Mercuric Oxide R(HgO) 2.5g

2) 1% Eosin-alcohol Solution
   Eosin Y (C.I. 45380), 1g
   dH$_2$O, 20mL
   95% Ethanol, 80mL

3) 1% HCl-alcohol Solution
   70% Ethanol, 99mL
   Concentrated HCl 1mL

4) 0.5~1% Ammonia Water
   Ammonia Water (NH$_4$OH), 0.5~1mL
   80% Ethanol, 100mL

B. Fixation: 10% NBF (Neutral Buffered Formalin), 10% Formalin

C. Microtomy: 6 μm
VI. Result of Preclinical Experiment

Photomicrograph of the test area of the sample

- Scale: 40 times
- Focal Point Distance: 3.1mm from the surface
- Size of Coagulation: 0.5x0.8mm
- Change in Surrounding Tissue: None
- Dissolution of the adipose tissue around the focal point due to the thermal effect is observed.
< Photograph 2. 2 Sample Slice >

- Scale : 40 times
- Focal Point Distance : 3.1mm from the surface
- Size of Coagulation : 0.5x0.8mm
- Change in Surrounding Tissue : None

- Dissolution of the adipose tissue around the focal point due to the thermal effect is observed.
< Photograph 3. 3 Sample Slice >

- Scale : 40 times
- Focal Point Distance : 4.6mm from the surface
- Size of Coagulation : 0.5x0.7mm
- Change in Surrounding Tissue : None
- Dissolution of the adipose tissue around the focal point due to the thermal effect is observed.
Photograph 4. 4 Sample Slice

- Scale: 40 times
- Focal Point Distance: 4.6mm from the surface
- Size of Coagulation: 0.5x0.9mm
- Change in Surrounding Tissue: None
- Dissolution of the adipose tissue around the focal point due to the thermal effect is observed.
VII. Discussion and Supervisory Opinion

For the cartridge 1, the irradiation with a 0.7J energy is repeated four times on the four different points of the marking area for 30ms and with a 7MHz frequency and the distance of focal point of 3.0mm. From the observation by the 40 magnification microscope, an approximate size of a 0.5x0.8mm coagulation (dissolution) to the depth of 3.1mm from the epidermis is detected and no specific change is occurred at the surrounding tissue (photograph 1).

For the cartridge 1, the irradiation with a 1.0J energy is repeated four times on the four different points of the marking area for 50ms and with a 7MHz frequency and the distance of focal point of 3.0mm. From the observation by the 40 magnification microscope, an approximate size of a 0.5x0.7mm coagulation (dissolution) to the depth of 3.1mm from the epidermis is detected and no specific change is occurred at the surrounding tissue (photograph 2).

For the cartridge 2, the irradiation with a 0.7J energy is repeated four times on the four different points of the marking area for 30ms and with a 7MHz frequency and the distance of focal point of 4.5mm. From the observation by the 40 magnification microscope, an approximate size of a 0.5x0.7mm coagulation (dissolution) to the depth of 4.6mm from the epidermis is detected and no specific change is occurred at the surrounding tissue (photograph 3).

For the cartridge 2, the irradiation with a 1.2J energy is repeated four times on the four different points of the marking area for 50ms and with a 7MHz frequency and the distance of focal point of 4.5mm. From the observation by the 40 magnification microscope, an approximate size of a 0.5x0.9mm coagulation (dissolution) to the depth of 4.6mm from the epidermis is detected and no specific change is occurred at the surrounding tissue as the other cartridges (photograph 3).

Unlike the description in the foreign paper on the performance, it is strongly meaningful that the result of this research is derived by arranging the condition of experiment as similar as possible to the human’s by irradiating the ultrasound to a living pig’s tissue but not a frozen swine tissue.

The dominant result is corresponding to the case that the layers of fat of the experimental subject, the Micro-Pig®, a 3.0mm and 4.5mm deep from the epidermis are dissolved by the heat generated from the ultrasound irradiated. No alteration is detected in the layer between the epidermis and the thermal coagulation (dissolution) point and in the surrounding tissue of the coagulation (dissolution) point.

It is indicated that the thermal coagulation caused by the ultrasound controlled and irradiated from the HIFU surgical unit does not generate an unexpected damage within the living swine skin tissue and that the occurrence of a thermal coagulation (dissolution) is significant. Since the coagulation (dissolution) is observed at the controlled area, a reproduction stage for the tissue is expected to follow to cure the TIZs (Thermal Injury Zones) based on the reported clinical results proving the skin regeneration after a thermal injury.
Discussion

As shown in the photomicrographs of the sample no epidermis injury in the swine skin of the area of the HIFU irradiation has been observed. No certain alteration of the surrounding tissue of the thermal coagulation has been detected either. Through the skin layers between the epidermis and the coagulation point, no sign of influence has been found.

Supervisory Opinion

It is considered that the thermal coagulation (dissolution) shown in the photomicrographs of the sample has been generated by 55-60°C of heat at the focal point; and it is the technical characteristics of the HIFU surgical unit that does not induce any change through the layers from the epidermis to the coagulation point. The safety of the HIFU surgical unit can be assured as a therapeutic equipment since there was no detection of tissue injury around the irradiation area.

Animal Laboratory No. 182 appointed by the Ministry of Food and Drug Safety, Eulji University, Professor of Clinical Pathology / Head of Animal Experiment

Jae-Ho Shin

RIC-07-06-05 appointed by the Ministry of Trade, Industry & Energy, Eulji University Bio-Medi Tech Industrial Regional Innovation Center, Director of Centor

Woo-Cheol Lee
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)
