The Effect of Negative electric field using charged PTFE membrane on Bone Healing of Rabbit Long Bone

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I. Introduction

The biological principles of guided bone regeneration have been successfully applied for regeneration of bone both in experimental animal studies[1-3] and in clinical studies[4,5]. Also, various procedures such as improving the barriers mechanical properties[6] and peripheral sealing[7] and using grafting materials[8] have been tested to enhance, both qualitatively and quantitatively, bone repair in membrane-protected defects. This concept implies that a membrane barrier is placed to prevent non-desirable soft tissue cells from entering the wound area, thereby giving preference to bone-forming cells to repopulate the defect.

Factors which stimulate bone repair are subjects for research in osseous regenerative therapy. Effects of cytokines, or growth factors, on bone repair[9] are examples of such subjects. Another subject is electrical stimulation which naturally occurs in bone, as such bone may be particularly susceptible to electrical therapy[10].

Fukada and Yasuda[11], on the piezoelectric effect of bone, gave impetus for research in medicine and implied that application of external forces in bone results in electric signals. Yasuda[12] implanted electrodes into rabbit femurs and observed new bone in the vicinity of the cathods. Bassett et al.[13] described in vivo osteogenesis in response to electric currents, and Friedenberg et al.[14] reported on the clinical use of electricity to heal nonunions. Thus, these studies demonstrated that if endogenous stress-generated potentials effected bone remodeling, perhaps electric signals applied externally without the simultaneous application of stress could lead to a response resulting in bone formation[12-14].

The exact action mechanism of these electrical stimulation for osteogenesis are currently unclear, but investigators have reported various possible modes, such as decreased pO_2 and increased pH in the vicinity of the cathode[15]; accumulation of positive charges[16]; differentiation of mesenchymal cells into osteoblast and osteocytes[17]; increased alkaline phosphatase activity[18]; elevation of cyclic

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nucleotides(cAMP, cGMP) and prostaglandins[19,20]; increased IGF-II[21,22]; increased intracellular free calcium ion[23,24].

Applied EMFs for biologic systems were of three forms: direct current[10,25,26], inductive coupling[27-29], and capacitive coupling[30,31]. Three forms have increased osteogenesis. However, these forms are partially invasive, and require long, exact patient compliance and additional devices. Therefore, contemporary EMF research has moved towards non-invasive, simple application. Kubota et al[32]. suggested that bone regeneration is enhanced by non-invasive electromagnetic fields(EMFs) stimulation when using guided tissue regeneration procedures or bone replacement grafts. In this concept, Chierico et al.[33] demonstrated the additive effect obtained by negatively charged polytetrafluoroethylene(PTFE) membrane in experimental rabbit calvarial defects.

To clearly define the polarity of charge for osteogenesis, various studies have been conducted. Shandler et al.[10] demonstrated considerably more osteoblastic activity on the electrically stimulated side, with maximal growth nearest to the negative electrode. Ferrier et al.[34] suggested that the osteoclasts migrated rapidly toward the positive electrode, and osteoblast- like cells migrated in the opposite direction. These studies have shown that negative electrical stimulation may have contributed to the acceleration of osteogenesis.

The principles of guided bone regeneration have also been applied to the healing of challenging segmental long-bone defects. However, placement of membrane alone does not significantly promote healing of long bone defects[35-37]. So, based on the additive effect obtained by negative charged PTFE membrane in experimental rabbit calvarial defects[33], it was considered that such a combination would also promote the healing of defects in long bones. The purpose of this study was to evaluate the effects of negatively electric field on bone healing in rabbit segmental long bone defects, using negatively charged PTFE membrane.

II. Materials and Methods

1. Management of Animals

Eight adult male New Zealand white rabbits weighing 3.0 to 3.5 kg were used in this study. They were kept in standard laboratory conditions of a light-dark schedule and relative humidity, were fed a standard rabbit diet and were maintained in separative cages. After anesthesia with an intramuscular injection of ketamine hydrochloride 44 mg/kg of body weight (Ketara®, Yuhan Corporation, Seoul Korea) and xylazine 7 mg of body weight (Rumpun®, Bayer Korea, Seoul Korea), two animals were sacrificed by a heart perfusion at 2 week, two at 4 weeks, two at 6 weeks and two at 8 weeks after surgery.

2. Experimental materials

PTFE membrane (TefGen-FD™, Lifecore Biomedical Inc., U.S.A.) were used in the study. In test group, all membranes were then charge injected using a corona-charging apparatus (Figure 1). Sixteen membranes were cut with 1.6×2.0 cm². Eight membranes were placed on the grounded aluminum template 3.5 cm below a single needle point brass electrode connected to a low current/high voltage direct current (DC) power supply. A nickel mesh grid assembly connected to the low current DC power supply was centered below the needle electrode, 1.5 cm above the membrane. The upper electrode was biased at -15 kV against the grounded PTFE membrane. The nickel mesh was biased to the desired surface voltage (-2 kV initially to obtain -
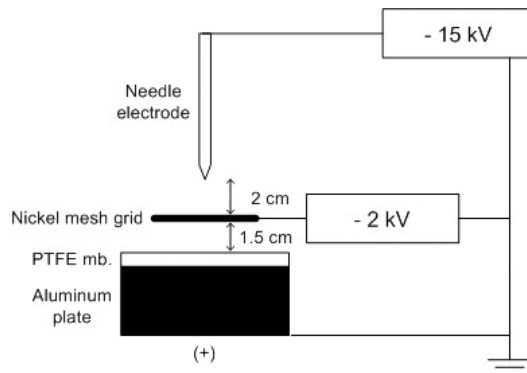
Figure 1. Schematic illustration of corona charging apparatus.

1000 V membrane charge) and the membrane was exposed to this field for 15 minutes. The injected charges were measured with an noncontacting electrostatic voltmeter (Electrostatic meter-9000®, Electrostatics Inc., U.S.A). Additionally, four membranes were charge injected and the injected charges were measured with the above device for three months in order to evaluate charge retention in vitro.

The distribution of the sixteen PTFE membranes according to the charge was as follows: eight negatively charged membranes ; eight noncharged membranes.

Following charge injection, the membranes were sterilized using gamma-radiation.

3. Surgical procedure

The animals were anesthetized preoperatively with an intramuscular injection of ketamine hydrochloride 44 mg/kg of body weight (Ketara®, Yuhan Corporation, Seoul Korea) and xylazine 7 mg of body weight (Rumpun®, Bayer Korea, Seoul Korea). Forelimbs were shaved and prepared aseptically for surgery, and 2% Lidocaine (contained epinephrine 1:80,000, Yuhan, Pharm, Korea) was injected for local anesthesia and bleeding control in the forelimbs. Under aseptic conditions, a direct anterolateral incision was made over the distal radius. The periosteum was incised transversely about 2 cm from the radiocarpal joint, and elevated proximally with a small periosteal elevator. A fine metal band was inserted to make a gap between the radius and the ulna. With the metal band underneath the radius, a distal transverse osteotomy was performed with a disk under continuous physiological saline irrigation. Ten mm length from the distal cut was proximally measured with a ruler, and the proximal transverse osteotomy was completed. After the bone segment was removed, the area was irrigated with physiological saline solution. The left defect was covered with non charged membranes as control groups, whereas the right defect was covered with negatively charged membranes as test group. PTFE membranes were extended approximately 3 mm outside the defect at each end. The membrane was placed between the radius and the ulna, wrapped around the radius, and held in place with ePTFE suture material (GORE-TEX® SUTURE, W. L. Gore & Associates, Inc. U.S.A.) at each end. The overlying tissues were carefully repositioned and sutured in layers. After the surgery, each animal was injected intramuscularly with antibiotics (Baytril ®, Bayer Korea, Seoul Korea) at a dose of 0.2 ml/kg

and analgesics (Nobin®, Bayer Korea, Seoul Korea) at a dose of 0.44 mg/kg once daily for 1 week.

4. Histologic evaluation

All animals were sacrificed by heart perfusion, and block biopsy specimens including the defect and surrounding tissue were taken. The specimens were fixed with the mixture of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). After demineralization with 10% ethylene diamine tetraacetic acid (EDTA), the specimens were dehydrated with a graded series of ethanol, embedded in paraffin, sectioned at 5 μm with microtome, and stained with Masson's trichrome.

5. Histomorphometric analysis

Histologic observation and histomorphometric analysis were carried out using a light microscope with image analysis system (i-Solution® , iMTechnology, Inc. Korea). The histomorphometirc measurements included an evaluation of the area of newly-formed bone tissue expressed as a percentage of the total defect area.

III. Results

1. Membrane charge analysis

The negatively charged membranes were initially charged to average surface voltage of -1000 V. All membranes underwent a charge decay during the first 24 h after which they were stabilized at about 40% of the initial charge. After the initial charge decay, the charged membranes maintained the residual polarization unaltered for three months (Figure 2).

2. Clinical observation

Unfortunately, one rabbit was excluded from evaluation because of death, but the other animals remained healthy during the observation period, and were histologically processed for analysis.

3. Histologic observation

1) Control groups
At 2 weeks, approximately 0.66 mm extension of new bone formation was observed at the end of the defect. The remaining parts of defect filled with
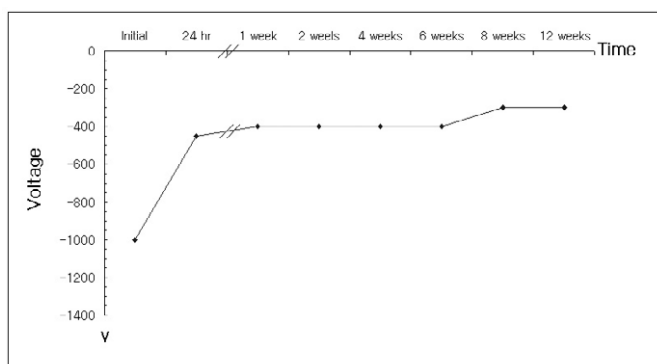

Figure 2. Charge retention of negatively charged membranes. After the initial charge decay, the negatively charged membranes maintained a fairly constant residual charge.
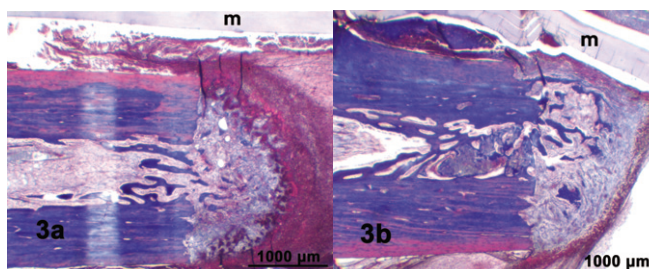
Figure 3. Histologic finding of control & test groups treated with membrane (m) at 2 weeks after surgery ; Photomicrograph shows initiation of new bone formation at the end of the defects. Also, the new bone formation was seen at the bone marrow cavity of the bone fragments. (Masson's trichrome, × 20) (a:control, b:test)
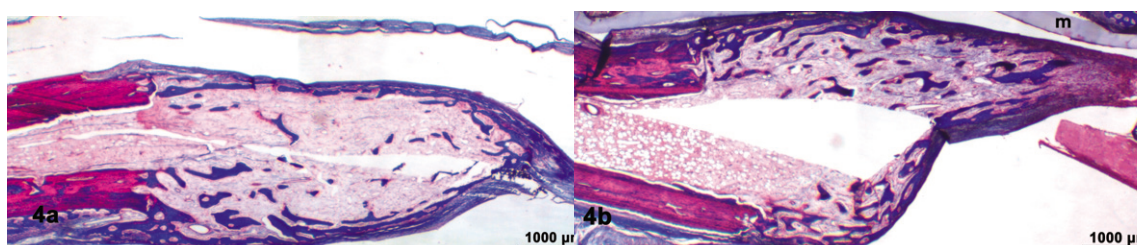

Figure 4. Histologic finding of control & test groups treated with membrane (m) at 4 weeks after surgery ; Photomicrograph shows that the new bone was growing at the end of the bone fragment. Newly formed bone in test group was more abundant than in control group. (Masson's trichrome, ×20) (a:control, b:test)
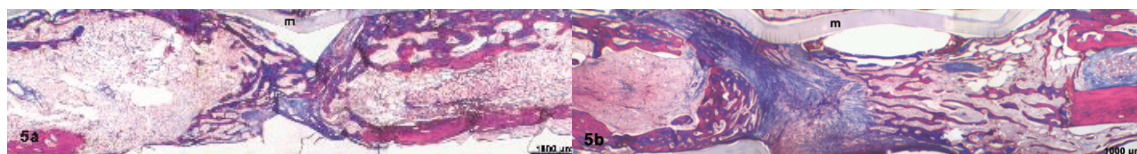

Figure 5. Histologic finding of control & test groups treated with membrane (m) at 6 weeks after surgery ; Photomicrograph shows that the bone formation was more organized and the gap was almost bridged. Newly formed bone in test group was similar with that in control group. (Masson's trichrome, ×20) (a:control, b:test)

blood clot, rich in cells and blood vessels. Moreover, the new bone formation was seen at the bone marrow cavity of the bone fragment (Figure 3a).

At 4 weeks, the new bone was growing at the end of the bone fragment. Centrally, the defect was filled with large amounts of connective tissue with clear signs of mineralization (Figure 4a).

At 6 weeks, the bone formation was more organized and the gap was almost bridged. It was found that a thin bone at the internal surfaces of the membrane, with a component of fatty marrow within the former defect. The central part of the defect was filled with new bone, collagen fiber (Figure 5a).

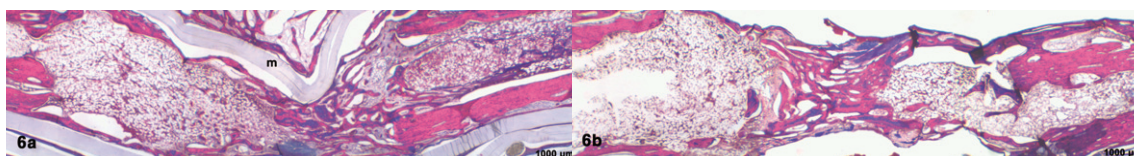At 8 weeks, Although the membrane was folded,

Figure 6. Histologic finding of control & test groups treated with membrane (m) at 8 weeks after surgery ; Photomicrograph shows that the complete bony unio was achieved in defect area and more maturated mineralized bone was observed in the inner surface of membrane. Newly formed bone in test group was similar with that in control group. (Masson's trichrome, ×20) (a:control, b:test)

Table 1. The area of newly formed bone to total defect (%)

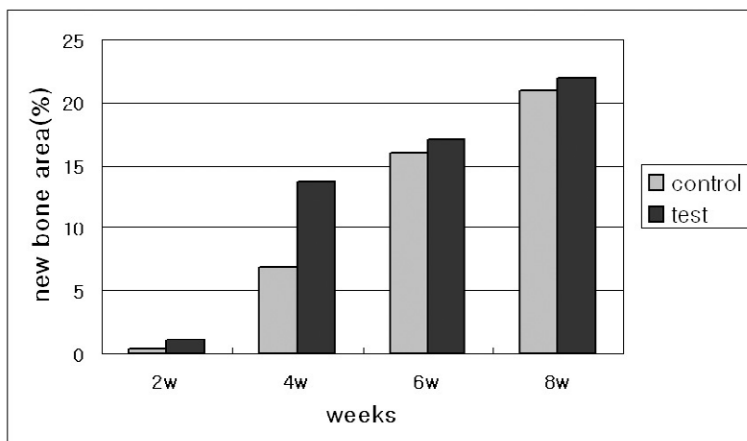| Groups	Time(weeks)			
	2	4	6	8
control	0.32	6.86	15.94	20.92
test	1.10	13.75	17.15	21.93

Figure 7. The area of newly formed bone to total defect. At 2 and 4 weeks, the amount of the newly formed bone in test group was markedly more than that in control group.

defect had a thin bony bridge at the internal surfaces of the membrane. A larger component of fatty marrow within the former defect was also present. A small amount of collagen fiber is seen at the central part (Figure 6a).

2) test groups

At 2 weeks, approximately 0.80 mm extension of new bone formation was observed at the end of the defect, and strong new bone formation was seen at the marrow cavity of the bone fragment (Figure 3b).

At 4 weeks, the new bone was growing at the end of the bone fragment. Centrally, the defect was filled with large amounts of connective tissue with clear signs of mineralization (Figure 4b).

At 6 weeks, along with the inner surface of membrane, mineralized bone was observed with a progression of remodeling. Coarse collagen bundle

occupied the inner side of the defect with about 2 mm of remaining bony-union in the center of defect (Figure 5b).

At 8 weeks, complete bony bridging in defect area was observed with a larger component of fatty marrow. The progression of remodeling in the central portion was observed (Figure 6b).

4. Histomorphometric analysis (Table 1, Figure 7)

At 2 weeks, the amount of the newly formed bone in test group was more than that in control group. The proportion of new bone formation to total defect area was 0.32% in control group, and 1.10% in test group.

At 4 weeks, also, newly formed bone in test group was more abundant than in control group. The proportion of new bone formation to total defect area was 6.86% in control group, and 13.75% in test group.

At 6 and 8 weeks, no obvious difference was found between the two groups but newly formed bone in test groups were slightly more than that in control groups.

IV. Discussion

In previous studies, placement of membranes alone does not significantly promote healing of long bone segmental defects. Bluhm and Laskin[36] covered 8 mm rat fibular defects with e-PTFE membranes and allowed healing for 12 weeks. They showed that only one of 10 defects were partially bridged on radiographs. Nyman et al.[37] using 10 mm rabbit radius defects, reported narrow bony bridging on the internal membrane surface after 27 weeks of healing, whereas the main part of the defects was filled with soft tissue. Thus, this study

combined placement of membranes with negatively electric fields. That is, PTFE membranes were used as a carrier of negatively electric fields.

Interest in the medicinal use of electricity was accelerated in 1953 when Yasuda induced osteogenesis by electrical stimulation[12]. Various studies have been conducted in order to evaluate the effect of electrical stimulation for osteogenesis and demonstrated that negative electrical stimulation may have contributed to the acceleration of osteogenesis[10,34]. Although the exact action mechanism of electrical stimulation for osteogenesis is currently unclear, various possible modes have been reported[16-24]. Of electric stimulation systems, direct current is partially invasive, and has the potential of tissue hazards and the risk of infection. Thus, Bassett[27] was the first to use a pair of Helmholtz coils to produce a magnetic field(and induce an electric field) across a fracture site and increase osteogenesis. Brighton[38] developed a capacitively coupled electromagnetic field composed of metal plates placed on the skin overlying the nonunion fracture site and induced fracture repair. However, these also are inconvenient, complex, and require additional devices. As compared to the methods described above, in the present study, negatively charged PTFE membranes were used as carrier of electric fields. It is more non-invasive, simpler than those methods, and does not require additive devices for electrical stimulation in vivo.

In this study, the negatively charged membranes were charged to a surface voltage of -1000 V, because the initial charge represents the polarization that yielded the best results in experiments on bone cells differentiation and attachment[39]. However, membranes voltage varied between -300 and -500 V after one day because of the initial charge decay. Although follow-up measurements of the membrane polarization continued in vitro throughout the

experimental period, it is difficult to apply these results to the membranes placed in the rabbits. This is due to the local presence of biological products and mediators potentially disturbing for the membranes electrical charge. At the end of this study, the charge measurements of the membranes placed in the rabbits were not performed because the specimens contained membrane. Therefore, it was questionable that the negatively charged membranes maintained the residual polarization unaltered throughout the experimental period. However, several studies indicated that after a few weeks of electrical stimulation, continued stimulation was not productive for additional bone formation[40,41]. Marino et al.[41] commented that the osteogenic responses to acute and chronic stimulation are quite different, and that chronic stimulation is counterproductive because it adversely affects the repair process.

The most significant finding in this study was the accelerated early new bone formation observed with the negatively charged membranes. At two weeks, newly formed bone tissue was markedly more in test group than in control group. The proportion of new bone formation to total defect area was 0.32% in control group, and 1.10% in test group. At 4 weeks, the proportion of new bone formation to total defect area was 6.86% in control, and 13.75% in test. This observation was similar to the finding of the previous study[33]. In rabbit calvarial defect model, Chierico et al.[33] demonstrated that a mean total area of 27.95% of newly-formed bone was observed in the negatively charged sites at 10 days while the neutral and positively charged domes yielded insignificant amount of osteogenesis at the same evaluation interval. They reported more new bone formation at an early healing period than in this study. It is suspected that this difference may be due to differences in the size of defect and in the healing pattern of radius and calvaria of rabbits.

In this study, newly formed bone in test groups were slightly more than that in control groups at 6 and 8 weeks, yet no obvious difference was found between the two groups . It could be explained that the effects of the negative charge have been overshadowed by the regenerative potential of the membrane itself.

In conclusion, although the number of samples was small, this study showed that the negatively electrical stimulation accelerated early bone healing. Therefore, the combination of negatively electrical stimulation and PTFE membrane is noninvasive, useful, and valuable method in long bone healing. However, definitive conclusions cannot be made as to the validity of this new approach due to the limitations of the study. Further experimentation is needed using larger study sample size and research protocols allowing testing various types and degrees of polarization.

V. Summary

The purpose of this study was to evaluate the effects of negatively electric field on bone healing in rabbit segmental long bone defects using negatively charged PTFE membrane.

Ten millimeter segmental defects in the rabbit radius were used as the experimental model. After membranes were then charge injected using a corona-charging apparatus, the left defects were covered with non charged PTFE membranes as control groups, whereas the right defect was covered with negatively charged PTFE membranes as test group. The animals were divided into 4 groups of 2 rabbits each, and sacrificed at 2, 4, 6, and 8 weeks.

Histomorphometric analysis showed a more newly formed bone in negatively charged membrane at early healing period. At 2 weeks, the proportion of new bone formation to total defect area

was 0.32% in control group, 1.10% in experimental group. At 4 weeks, the proportion of new bone formation to total defect area was 6.86% in control, and 13.75% in experimental. At 6 and 8 weeks, no obvious difference was found between the two groups but newly formed bone in test groups were slightly more than that in control groups.

In conclusion, negatively charged membranes showed more newly bone tissue than noncharged membranes at an early healing period. Although the number of samples was small, this study showed that the combination of negatively electrical stimulation and PTFE membrane may be of value in long bone healing.

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Charged membrane에 의한 negative electric field가
토끼 장골의 골 치유에 미치는 영향

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골재생을 위한 술식은 자가골, 합성골 등의 이식술, 골견인술, 골유도 재생술등이 있으며, 더 나은 결과를 위해서 성장 인자나 cytokine의 적용, 전기적 자극 등이 이용될 수 있다. 이 중 골재생을 위한 전기적 자극을 이용한 골재생 방법에서 비교적 양호한 결과가 보고되어지고 있으며, 전기적 자극은 크게 direct current, inductive coupling, capacitive coupling으로 나뉘어 사용, 연구되어지고 있다. 하지만, 위의 전기적 자극들은 비교적 침습적이고, 환자들에게 불편감을 줄 수 있으며, 부가적인 장치가 필요한 단점이 있다. 따라서, 본 실험에서는 골재생을 촉진하기 위한 비침습적인 전기자극의 방법으로, negatively charged membrane을 이용하여, 토끼 요골의 골절성 결손부에서 negative electric field가 골재생에 미치는 영향을 연구하고자 하였다.

8마리 토끼의 양 요골에 10mm의 골절성 결손부를 형성한 후, 코로나 방전 장치로 -1000V로 대전시킨 polytetrafluoroethylene membrane을 사용하여, 실험군에는 negatively charged membrane을, 대조군에는 noncharged membrane을 적용시킨 후, 2, 4, 6, 8주째 2마리씩 희생하여 조직학적, 조직형태학적 분석을 실시하였다.

2주째, 대조군에서 골결손부에 대한 신생골의 비율은 0.32%, 실험군에서는 1.10%로 나타났으며, 4주째 대조군에서 골결손부에 대한 신생골의 비율은 6.86%, 실험군에서는 13.75%로, 대조군에 비해 실험군에서 더 많은 양의 신생골이 관찰되었다. 6주와 8주째도 대조군에 비해 실험군에서 더 많은 신생골이 관찰되었으나, 그 차이는 크지 않았다.

결론적으로, 토끼 요골의 골절성 결손부의 골치유에서 negatively charged membrane을 이용한 전기적 자극은 초기 골치유를 촉진시키며, 따라서, 이러한 방법의 전기 자극은 장골의 치유에 있어 비침습적이며, 유용한 수단이라고 사료된다.