Healing Process of Autogenous Bone Graft in Spontaneously Hypertensive Rats Treated With Losartan: An Immunohistochemical and Histomorphometric Study

Walter Cristiano Gealh, DDS, MSc,* Cassiano Costa Silva Pereira, DDS, MSc, PhD,† Eloa Rodrigues Luvizuto, DDS, MSc, PbD,‡ Idelmo Rangel Garcia-Júnior, DDS, MSc, PbD,§ Cristina Antoniali, PbarmD, MSc, PbD,‖ and Roberta Okamoto, DDS, MSc, PbD¶

Purpose: Hypertension is a major risk factor for cardiovascular diseases, which has been related to such changes as gradual bone loss and a decrease in bone mass index. The cellular and molecular mechanisms that involve hypertension and osteoporosis are not fully understood. Many patients have high blood pressure, controlled or uncontrolled, and may use at least 1 antihypertensive drug, and an understanding of the interference of hypertension with bone healing is very important when considering oral rehabilitation with implants and bone grafts. This study investigated the interference of hypertension in bone metabolism during the repair process of autogenous bone grafting and analyzed the influence of losartan, an antihypertensive drug and angiotensin II receptor antagonist, through histometric and immunohistochemical analyses by examining the protein expressions of osteocalcin, osteoprotegerin, receptor activator of nuclear factor-κB, receptor activator of nuclear factor-κB ligand, tartrate-resistant acid phosphatase, vascular endothelial growth factor, and platelet endothelial cell adhesion molecule.

Materials and Methods: The groups studied include 24 normotensive Wistar rats and 24 spontaneously hypertensive rats divided into groups treated and not treated with losartan. Rats were subjected to block bone graft surgery in the mandible and were sacrificed at 7, 14, and 28 days.

Results: Histometric analysis was performed to evaluate the amount of bone tissue formed at the interface of the recipient bed and bone graft. The total area of formed bone tissue was outlined and calculated. Immunohistochemical analysis was semi-quantitative and the significance of the differences between groups regarding the percentage of newly formed bone tissue interface and protein expression were determined by ANOVA analysis of variance and Kruskall-Wallis followed by Tukey test or Holm Sidak to detect differences between groups. The results were considered statistically significant when $P < .05$.

Conclusion: The untreated hypertensive rats showed a delay in the repair of autogenous bone block grafts compared with untreated Wistar rats. Furthermore, the use of losartan for lowering blood pressure in these animals was shown to improve the healing process, despite not showing important statistical differences.

© 2014 American Association of Oral and Maxillofacial Surgeons
Arterial hypertension is defined as increased blood pressure (systolic and diastolic) beyond the limits currently set by the Seventh Report of the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. It affects close to 1 billion people worldwide and approximately 65 million in the United States, making it the leading cause of death in industrialized countries.

Changes in skeletal structure, such as gradual bone loss in the femur and a decrease in body bone mass index, have been strongly linked to hypertension and death from infarction, and the cellular and molecular mechanisms that involve hypertension and osteoporosis are not fully understood.

The renin-angiotensin system plays an important role in blood pressure regulation and remodeling of various tissues owing to the ability of angiotensin II (Ang II) to interfere with bone metabolism through receptors located in osteoblasts and osteoclasts or through capillary blood flow from bone marrow. There are 2 major subtypes of angiotensin receptors, AT1 and AT2, and most actions are mediated by AT1 receptors.

Losartan, a drug belonging to the group of angiotensin receptor antagonists (ARBs), has an antihypertensive action that selectively blocks the AT1 receptors of Ang II in vascular smooth muscle and the adrenal gland, thereby blocking its vasoconstrictor effects and secretion of aldosterone. Currently, this drug is of interest because it is not only effective, but also has the capacity to protect target organs, with a low incidence of adverse effects.

Considering that bone tissue presents a balance between osteoblastic and osteoclastic activity, 2 proteins (osteoprotegerin [OPG] and receptor activator of nuclear factor-κB ligand [RANKL]) can be used as markers of cellular activity. RANKL appears as a protein released by osteoblasts that binds to the receptor activator of nuclear factor-κB (RANK), a receptor located on the surface of osteoclast precursors. Once the binding between RANK and RANKL occurs, the differentiation of osteoclast precursors in active osteoclasts takes place, thus beginning the process of bone resorption. OPG moderates osteoclastogenesis by acting as a decoy receptor by binding to RANKL and preventing its binding to RANK. The balance in expression between the osteoclastogenesis stimulator (RANKL) and inhibitor (OPG) influences the amount of bone resorbed.

Tartrate-resistant acid phosphatase (TRAP) is an enzyme found in cells of osteoclastic origin and erythrocytes that is released during bone resorption and after osteoclast removal from its site of action. It is considered an important marker of osteoclast activity.

Revascularization is essential in bone grafting, in which angiogenesis and collateral blood vessel growth characterize, in part, repair and tissue remodeling. It has been shown that the amount of progenitor cells and the angiogenic potential are decreased in cases of hypertension. In spontaneously hypertensive rats (SHRs) and in patients with essential hypertension, endothelial functions are impaired and could compromise the regeneration of blood vessels. Thus, proper treatment of hypertension is critical, because the decrease in blood pressure is the primary mechanism that triggers the activation of vasculogenesis and the growth of blood vessels.

Although there are reports showing that Ang II receptors are expressed in osteoblasts and osteoclasts, the effects of this peptide on bone metabolism are controversial. Therefore, this study assessed how the proteins osteocalcin, OPG, RANK, RANKL, and TRAP are expressed, denoting the activity of bone cells and vascular proliferation by the labeling of vascular endothelial growth factor (VEGF) and platelet endothelial cell adhesion molecule (PECAM) during the repair process of autogenous bone grafts in normotensive rats and SHRs treated and not treated with losartan.

Materials and Methods

This research project was submitted to and approved by the ethics committee on animal experiments (protocol number 2008-005159).

Twenty-four adult male Wistar-Kyoto rats (Rattus norvegicus Albinus) and 24 SHRs (body weight, 250 to 300 g) from Physiology Lab were used. Throughout the experiment, the animals were kept in individual cages in an environment with a stable temperature (22 ± 2°C) with a controlled light cycle (12 hours light, 12 hours dark) and with balanced feed (Ração Mogiana Alimentos SA, Campinas, Brazil) and controlled amounts of water, except 12 hours before the surgical intervention, when the animals were kept on a fasting diet.

The animals were divided into 4 groups: in group 1 (Wistar; control), 12 normotensive rats were not treated with losartan; in group 2 (Wistar-L; second control), 12 normotensive rats were treated with losartan; in group 3 (SHR), 12 hypertensive rats were not treated with losartan; and in group 4 (SHR-L), 12 hypertensive rats were treated with losartan. The animals were sacrificed at 3 different periods (7, 14, and 28 days), with 4 mice in each period.

Method and Means of Administering Losartan

Losartan was administered orally (30 mg/kg body weight per day) after dilution in 50 mL of water. This volume of liquid was offered to each animal daily, starting 7 days before the surgical procedure and extending...
until the day of euthanasia. Animals in the untreated groups had only water in the same quantity (50 mL). Every 7 days, the animals were weighed and the drug dose was recalculated and adjusted according to the current weight.

Every day the vials were checked to ensure that the entire medication had been taken and, before surgery, the blood pressure of each animal was measured to verify the effectiveness of the drug.

Excluded were animals with a weight outside the accepted range, animals that did not consume all the water (and, hence, the drug) because this was the means of controlling drug ingestion, and animals that did not exhibit a lower blood pressure after receiving losartan.

SURGICAL TECHNIQUE

The methodology used to perform autogenous bone grafting was based on the description of Jardini et al., with modification in the method of graft fixation proposed by Gealh et al. in which self-drilling miniscrews, maintaining the medial muscle insertion in the mandible, and obtaining compression between the graft and the recipient bed were used, in accord with the technique used in clinical practice.

In all surgical procedures, the animals received general intramuscular anesthesia with xylazine hydrochloride at a dose of 0.03 mL/100 g of body weight and ketamine hydrochloride at a dose of 0.07 mL/100 g of body weight.

After the anesthesia took effect, trichotomy was performed, antisepsis was performed with topical polyvinylpyrrolidone-iodine, and the operative field was isolated. The right parietal region was chosen as the donor area. An incision was made in the shape of a half-moon in the coat lining of the skull to expose the cortical bone and remove the entire thickness of the diploe of the right parietal bone using a surgical trephine 3.8 mm in diameter and a constant speed of 800 rpm under continuous irrigation with saline solution (Fig 1).

A linear incision was performed in the skin of the right mandibular angle, cutaneous and subcutaneous, allowing visualization and dissection of the masseter muscle. Another incision was made at the lower edge of the jaw, detaching the periosteum and exposing only the lateral face. The bone block removed from the parietal region was fixed to the lateral face of the right mandible using a compression technique (Fig 2). Then, 5-0 nylon sutures were used in the deep muscle tissue and skin surface.

All animals received a single antibiotic dose as immediate postoperative medication (benzathine administered intramuscularly at the dose of 0.2 mL per animal). At 7, 14, and 28 days after completion of grafting, the animals were sacrificed by an anesthetic overdose. After sacrifice, removal of the right hemimandible was performed, as was dissection and removal of the surrounding muscle tissue (Fig 3).

HISTOLOGIC PROCESSING

The pieces obtained were fixed in 10% formalin solution for 24 hours and decalcified in a solution of 4.13% ethylenediaminetetraacetic acid (pH 7.0; Merck, Darmstadt, Germany) for 3 months, with the solution changed every 15 days. After decalcification, these pieces were dehydrated, cleared, and embedded in paraffin to obtain longitudinal serial sections 6 μm thick.

Histometric evaluation was performed by staining with hematoxylin and eosin. Immunohistochemical processing was performed by inhibiting endogenous peroxidase activity with hydrogen peroxide. Antigen retrieval was performed with citrate buffer and blocking of nonspecific reactions was performed with skim milk and bovine albumin during the incubation of antibodies. Primary antibodies were used for anti-OPG, anti-RANK, anti-RANKL, anti-OC, anti-TRAP, anti-VEGF, and anti-PECAM (all from Santa Cruz Biotechnology, Santa Cruz, CA). Biotinylated anti-goat antibody produced in rabbits was used as the secondary antibody (Pierce Biotechnology, Rockford, IL). The immunohistochemical reaction signal was amplified using a streptavidin system (DAKO Kit, Dako Co, Carpinteria, CA) and visualized by dianinobenzidine as chromogen (Dako Co). Counterstaining was obtained using Harris hematoxylin (Merck).

An optical microscope with objective ×25 amplification (Leica Aristoplan Microsystems, Leitz, Bensheim, Germany) coupled to an image-capturing camera (Leica DFC 300FX, Leica Microsystems, Heerbrugg, Switzerland) connected to a microcomputer.
(Pentium III) with digitalized image analyzing software (Leica Camera Software Box, Leica Imaging Manager IM50 Demo Software) was used.

**Results**

Histometric analysis was performed using a light microscope to evaluate the amount of bone tissue formed at the interface between the recipient bed and the bone graft. Imagelab 2000 (Diracon Bioinformatics Ltda, Vargem Grande do Sul, SP, Brazil) was used for quantitative analysis at 7, 14, and 28 days. The total area of formed bone tissue was outlined and calculated using Imagelab (Table 1). Statistical analysis of data was performed using SigmaStat 3.1 (Systat Software, San Jose, CA).

Immunohistochemical analysis was semi-quantitatively performed with the aid of an optical microscope to evaluate the immunostaining against OPG, RANK, RANKL, OC, PECAM, and VEGF. Because immunohistochemical analysis consists of a qualitative examination, it was stratified with a value scale.
than .05. Results were followed by the Tukey test or the Holm-Sidak test to by analysis of variance and the Kruskal-Wallis fol-

RANKL, OC, TRAP, VEGF, and PECAM was determined for the percentage of newly formed bone tissue at

3.1. The significance of differences among groups ranging from 0 to 3 (0, no protein expression; 1, protein with little expression; 2, intermediate protein expression; 3, high protein expression) and subsequently subjected to statistical analysis.

Statistical analysis was performed with SigmaStat 3.1. The significance of differences among groups for the percentage of newly formed bone tissue at interface and the protein expression of OPG, RANK, RANKL, OC, TRAP, VEGF; and PECAM was determined by analysis of variance and the Kruskal-Wallis followed by the Tukey test or the Holm-Sidak test to detect differences among groups. Results were considered statistically significant at a P value less than .05.

ANALYSIS OF RESULTS

7 Days

For histometric analysis of the amount of bone formed at the interface between the graft and the recipient bed, no statistical difference between groups was seen (P < .05; Fig 4).

Semiquantitative analysis indicated a statistically significant difference in the expression of OPG (P < .05) between the Wistar and SHR groups; however, there was no significance between the other groups (Figs 5, 6). Likewise, there was a statistically significant difference (P < .05) in the expression of RANK predominantly in the SHR group (Figs 5, 7). For RANKL, there was a statistically significant difference (P < .05) between the Wistar and Wistar-L groups, with a predominance of protein in the treated group and no difference among the other groups (Figs 5, 8). The protein expression of VEGF and PECAM showed statistically significant differences (P < .05), with increased expression of VEGF in the SHR and SHR-L groups and increased expression of PECAM in the SHR group (Figs 5, 9, 10). TRAP and OC showed no statistically significant differences (P < .05; Figs 5, 11, 12).

14 Days

Statistical analysis for the quantity of bone formation at the interface was significantly different (P ≤ .01). In the analysis among groups, the Wistar group predominated over the others. There also was a statistically significant difference (P ≤ .01) between the SHR and SHR-L groups, with greater bone formation in the SHR-L group. There was no statistically significant difference between the Wistar-L and SHR groups (P = .05; Fig 4, Table 1).

The expression of OPG showed no statistical difference among groups (Figs 5, 6). The expression of RANK was statistically higher in the SHR and SHR-L groups compared with the Wistar group (α = 5%; Figs 5, 7). There was a statistical difference in the expression of RANKL, which was most abundant in the SHR-L and Wistar-L groups compared with the Wistar group (α = 0.05; Figs 5, 8). The expression of TRAP was statistically higher in the Wistar-L than in the SHR-L group (P < .05), whereas the expression of OC was statistically higher in the Wistar and Wistar-L groups than in the SHR group (P < .05; Figs 5, 11, 12). The proteins VEGF and PECAM showed no statistical difference among groups (α = 5%; Figs 5, 9, 10).

28 Days

Despite the Wistar and Wistar-L groups exhibiting a larger amount of newly formed bone, histometric statistical analysis showed no significant difference between groups for the amount of bone at the end of this period (P < .05; Fig 4).

Labeling of OPG showed no statistical difference among groups (Figs 5, 6). RANK expression was statistically different between the Wistar and Wistar-L groups, with the protein predominating in the Wistar-L group (Figs 5, 7). There was a predominance of RANKL in the SHR and SHR-L groups, but with no statistical difference between the other groups (Figs 5, 8). Animals in the Wistar group showed higher expression of TRAP and OC protein, with a statistically significant difference (P < .05) compared with the SHR and SHR-L groups, respectively (Figs 5, 11, 12). The labeling of VEGF and PECAM protein showed no statistically significant difference among groups (P < .05; Figs 5, 9, 10).

Discussion

Many studies have suggested an association between hypertension and changes in calcium metabolism, leading to increased bone loss, secondary activation of the parathyroid gland, and the consequent increase in the removal of calcium from bones.26,27 Thus, research studies have been conducted to clarify the relation between hypertension and bone metabolism and the interference that antihypertensive drugs can cause.
Ang II, the largest and most powerful peptide effector of the renin-angiotensin system and essential in long-term control of arterial hypertension, can control the growth of bone cells alone or in combination with other regulatory factors through the stimulation of AT1 receptors, thus increasing DNA synthesis in osteoblast cultures. According to Lamparter et al, Ang II stimulates DNA synthesis, cell proliferation, and collagen synthesis in osteoblast precursor cells. However, in mature osteoblasts, this effect is not observed, and paracrine effects may be responsible for metabolic changes in these cells, as suggested by Lamparter et al.28

Hagiwara et al reported that Ang II is a potent suppressor of the differentiation and mineralization of osteoblast cells. Such a phenomenon may explain why the process of bone repair in animals with hypertension is delayed, as seen in this experiment, in which histometric analysis showed that the Wistar group presented a larger amount of newly formed bone in the interface between the receiver bed and the graft compared with the SHR group (Table 1). Another aspect to be considered is that, although there was no statistically meaningful difference, immunoblot levels for OC in the SHR group were always lower than in animals in the normotensive groups (Figs 5, 12). This is noteworthy because this protein is an important marker for the process of bone mineralization. In other words, in hypertensive animals, the mineralization process is slowed down compared with that in normotensive animals.

According to Shimizu et al, Ang II indirectly promotes the differentiation and activation of osteoclasts through the stimulation and regulation of RANKL by osteoblasts. In the differentiation process, osteoclasts do not receive the direct action of Ang II. It directly induces the expression of RANKL in osteoblasts by the activation of AT1 receptors, which in turn leads to indirect activation of osteoclasts. In SHRs, an increased production of Ang II is expected, because this is one of the causes of hypertension, with a consequent increase in the production of RANKL by osteoblasts. This explains the fact that, in this work, an increased expression of RANKL was observed in SHRs compared with control animals (Figs 5, 8). It is interesting to note that this increased concentration of RANKL did not reflect a higher concentration in the immunostaining for TRAP (Figs 5, 11), a protein responsible for osteoclastic activity. Performing a general analysis of the OPG, RANK, and RANKL system showed that, in a global manner, bone turnover was slower in the SHR and SHR-L groups because, despite the high concentrations of RANK and RANKL in these groups, there were high levels of OPG (Figs 5, 6), with subsequent

**FIGURE 4.** Histometric analysis of the amount of bone formed at the interface between the graft and the recipient bed (magnification, ×25). At 28 days, there is less bone formation in the SHR group than in the Wistar and SHR-L groups. SHR, spontaneously hypertensive rats not treated with losartan; SHR-L, spontaneously hypertensive rats treated with losartan; Wistar, normotensive rats not treated with losartan [control]; Wistar-L, normotensive rats treated with losartan [control].

neutralization of high levels of RANK and RANKL, resulting in lower expression of TRAP (Figs 5, 11).

Therefore, one would suspect that the class of drugs with a mechanism of action related to Ang II would be antihypertensive angiotensin-converting enzyme inhibitors (ACEIs) or ARBs and might interfere somehow with bone metabolism. The use of ARBs can increase the tendency for the development of postmenopausal osteoporosis through the decrease of transforming growth factor-β1 (TGF-β1), which has been related to the maintenance of bone mass.31,32

Nishiya and Sugimoto33 examined the effects of antihypertensive drugs on the functions of osteoblasts and found that calcium channel blocker drugs stimulate osteoblast differentiation, whereas ACEI or ARB drugs do not appear to affect the functions of osteoblasts. According to Broulik et al.,34 enalapril (an ACEI) and losartan showed no effect on bone metabolism in normotensive rats and the administration of losartan in dosages similar to those recommended for the treatment of hypertension caused no significant changes in bone density, mineral content, or femur morphometry in healthy animals. Moreover, rats that had undergone ovariectomy in addition to long-term use of ARB drugs showed a decrease in serum levels of TGF-β1, whereas no such change was observed at the bone level and no metabolic acceleration relative to bone mass loss was detected.35
FIGURE 6. Immunohistochemical analysis of osteoprotegerin on days 7, 14, and 28 (magnification, ×25). SHR, spontaneously hypertensive rats not treated with losartan; SHR-L, spontaneously hypertensive rats treated with losartan; Wistar, normotensive rats not treated with losartan (control); Wistar-L, normotensive rats treated with losartan (control).


FIGURE 7. Immunohistochemical analysis of receptor activator of nuclear factor-κB on days 7, 14, and 28 (magnification, ×25). SHR, spontaneously hypertensive rats not treated with losartan; SHR-L, spontaneously hypertensive rats treated with losartan; Wistar, normotensive rats not treated with losartan (control); Wistar-L, normotensive rats treated with losartan (control).

FIGURE 8. Immunohistochemical analysis of receptor activator of nuclear factor-κB ligand on days 7, 14, and 28 (magnification, ×25). SHR, spontaneously hypertensive rats not treated with losartan; SHR-L, spontaneously hypertensive rats treated with losartan; Wistar, normotensive rats not treated with losartan (control); Wistar-L, normotensive rats treated with losartan (control).


FIGURE 9. Immunohistochemical analysis of vascular endothelial growth factor on days 7, 14, and 28 (magnification, ×25). SHR, spontaneously hypertensive rats not treated with losartan; SHR-L, spontaneously hypertensive rats treated with losartan; Wistar, normotensive rats not treated with losartan (control); Wistar-L, normotensive rats treated with losartan (control).

FIGURE 10. Immunohistochemical analysis of platelet endothelial cell adhesion molecule on days 7, 14, and 28 (magnification, ×25). SHR, spontaneously hypertensive rats not treated with losartan; SHR-L, spontaneously hypertensive rats treated with losartan; Wistar, normotensive rats not treated with losartan (control); Wistar-L, normotensive rats treated with losartan (control).


FIGURE 11. Immunohistochemical analysis of tartrate-resistant acid phosphatase on days 7, 14, and 28 (magnification, ×25). SHR, spontaneously hypertensive rats not treated with losartan; SHR-L, spontaneously hypertensive rats treated with losartan; Wistar, normotensive rats not treated with losartan (control); Wistar-L, normotensive rats treated with losartan (control).

However, in a study by Ma et al., bone loss induced in ovariectomized SHRs was clearly decreased by the use of telmisartan, an ARB drug.

Histometric analysis in the present study showed that although there was no statistical difference, the Wistar-L and SHR-L groups showed greater bone formation compared with the Wistar and SHR groups, respectively (Fig 4, Table 1). The authors believe that, as reported by Shimizu et al., there was a decrease in osteoclast differentiation mediated by the action of losartan on AT1 receptors, but one can disagree with the findings by Nishiya and Sugimoto, Broulik et al., Li et al., because such differences were not meaningful, indicating that losartan does not affect bone metabolism from a clinical viewpoint.

Few studies have addressed factors related to capillary density in spontaneously hypertensive animals, namely those related to the development of hypertension or to the possible prevention of capillary rarefaction from the use of antihypertensive drugs. Ang II, which also can be locally produced by endothelial cells, may have an important role in the coordination of capillary cell growth and bone formation during tissue remodeling. It also enhances the formation and proliferation of endothelial cells, and this induction enhances biological cellular activity, which may affect the potential for vascular remodeling. According to Emanuelli et al., hypertension causes a negative influence on vascular growth, and this effect is probably mediated by the decrease in the level of proangiogenic growth factors such as VEGF. The accuracy of this assertion can be confirmed in the present study from the lowest expression of VEGF and PECAM in the SHR groups compared with the Wistar groups (Figs 5, 9, 10).

Rizzoni et al. suggested that normalization in capillary density in the skeletal muscles of SHRs can be obtained with antihypertensive therapy, specifically with losartan and enalapril. Blockers of the renin-angiotensin system, such as losartan, seem to prevent or reverse the microvascular rarefaction in the heart and brain of rats. You et al. reported that antihypertensive therapy combined with diuretics and ACEI drugs provided the quantity and potential “restoration” of progenitor cells, restoring the decreased angiogenic potential in SHRs.

An important fact is that antihypertensive treatment can normalize the processes involved in cell differentiation and proliferation mediated by several growth factors. ACEIs and ARBs have shown improved vascular function in SHRs, suggesting that restoration of endothelial function may contribute, at least in part, to the beneficial effects of these treatments. Niida et al. reported that Ang II increased VEGF expression, which in turn stimulated
osteoclastogenesis through the expression of receptors on hematopoietic cells.

Data from this study agree with those reported by You et al., Rizzoni et al., Munzenmaier and Greene, Riveiro et al., Potenza et al., and Niida et al. in which the use of antihypertensive medications contributed to the improvement in standards of angiogenesis in hypertension settings. However, in the present study, the use of losartan in normotensive animals caused a decrease in the immunoblots of VEGF and PECAM, probably owing to the decrease in normal blood pressure patterns, thus compromising the revascularization process (Figs 5, 9, 10).

Bone metabolism is regulated by a range of mediators, many of which are still to be discovered. Thus, the authors agree with Asaba et al. that more studies and research are needed to better understand bone dynamics, especially when systemic changes such as hypertension are associated. It can be concluded, despite the limitations of the present study, that SHRs presented a delay in the repair of autogenous bone block grafts compared with Wistar rats, and that the use of losartan for lowering blood pressure in these animals showed improvement in the scarring process, despite not showing statistical differences. The authors suggest that controlled high blood pressure with losartan produces better conditions for bone healing than uncontrolled hypertension. Other antihypertensive drugs must be studied and clinical research must be performed.

References