RIBONUCLEASE-ENRICHED LACTOFERRIN

R-ELF

All Natural | Multi-Patented | Clinically Validated

Canine Bone & Joint Health Technology

OPTIMIZED BONE TURNOVER

REGULATED INFLAMMATION

ENHANCED FRACTURE HEALING

Nterminus

ABOUT THIS 'R-ELF' WHITE PAPER

Ribonuclease-enriched Lactoferrin (R-ELF) is a class of multi-functional, all natural, milk protein-complexes with several beneficial effects on bone physiology. The individual roles of lactoferrin (LF), a metal-binding protein, and ribonuclease (RNase), an angiogenic protein, have been well documented in medical literature over the past three decades. Recent advances in protein engineering and molecular modeling have made it possible to combine RNase and LF into R-ELF complexes. These bio-functional R-ELF complexes have demonstrated potent synergistic activity when compared to individual proteins. R-ELF complexes are known to regulate innate pathways of bone turnover, bone remodeling, joint inflammation, cartilage regulation, etc.

The purpose of this 'White Paper' is to provide clinical data and elucidate the recent advances in R-ELF research related to canine bone physiology, with emphasis on canine fracture healing after osteotomy procedures. Accordingly, this 'R-ELF White Paper' contains experimental/scientific data on:

i) Canine Case Studies: Since 2012, N-terminus Laboratory has collaborated with veterinary hospitals to measure the post-operative effects of R-ELF on the recovery and overall fracture healing of canine patients. Hundreds of dogs that underwent osteotomy procedures had their recovery rates monitored by veterinarians through radiographic images. This document cites THREE typical cases representing: i) CWTO, ii) TPLO for large dogs and iii) TPLO for small dogs. The full clinical data is currently being prepared for publication in a peer-reviewed veterinary journal.

ii) Human Clinical Trials: In 2008, two IRB-approved human clinical trials were conducted in collaboration with two major medical universities in Southern California. The purpose was to evaluate the effects of R-ELF on bone turnover and inflammatory responses. The results of these two studies were published in peer-reviewed medical journals: *Osteoporosis International* (in 2009) and *Inflammation Research* (in 2010).

iii) R-ELF Technology Grid: The Patents (issued) and Patent Applications (pending) on R-ELF spans three levels of intellectual property (IP). The foundational tier includes 2 patents that describe methods to isolate ultra-pure lactoferrin as the R-ELF base material. The second tier has 2 method patents on how to produce different types of R-ELF (Angiogenin or ANGex) Complexes. The final tier of technology transfer consists of 2 patents that describe the preparation of 'PORTIN' compounds for target-delivery of R-ELF *in vivo*.

iv) R-ELF Safety/Regulations: LF was granted GRAS (Generally Recognized as Safe) status by the US-FDA. LF is widely approved and used in the US, European Union, and Asia. A safety overview is included in this document.

AUTHOR

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Dr. Naidu is a medical microbiologist/immunologist and the Director of N-terminus Research Laboratory. He holds more than 35 years of scientific research and experience from academia, government and industry. Dr. Naidu has served as a professor on the faculties of California State University, Pomona, USA; University of North Carolina-Chapel Hill, USA; Lund University, Sweden; Pécs Medical University, Hungary; and the Institute of Preventive Medicine, India. He is an elected Fellow or Associate of 18 professional and scientific societies and consultant to public health agencies and major pharmaceutical enterprises.

Dr. Naidu is also a member of the advisory panels for several multinational corporations and an invited speaker at universities worldwide. He is the author/editor of 4 reference volumes, a prolific writer of 30 book chapters, and an investigative author of over 100 peer-reviewed research publications. Dr. Naidu's discoveries are the basis for the establishment of 5 start-up biotech companies in the United States. He holds multiple core patents in health-related applications.

Introduction

Dogs typically break their bones due to trauma (such as getting hit by a car), falling, or repetitive activity that places excessive physical force on the bones. Certain canine orthopedic procedures, such as Tibial Plateau Leveling Osteotomies (TPLO), also require surgically cutting and/or altering the bone structure. In dogs the majority of the traumatic bone fractures involve the hind legs; femur fractures can represent up to 45% of all fracture cases. The second most common long bone fracture in dogs is the tibia, followed by the radius and ulna. It is common for dogs to fracture both the radius and ulna in traumatic accidents such as car accidents or falls. Humeral fractures account for 10% of all limb fractures. Fractures disrupt circulation in the bone and lead to necrosis and hypoxia of adjacent bone tissue. Normal bone will respond to a fracture with undergoing an orderly regeneration of its tissue matrix. When the anatomical positions are also corrected, the bone will be able to regain all of its mechanical properties.

Bone is a highly vascularized tissue reliant on the close spatial and temporal connection between blood vessels and bone cells to maintain skeletal integrity. Skeletal development includes the coordination of multiple events such as migration, differentiation, and activation of multiple cell types and tissues. Reestablishment of circulation is an early event in fracture healing [1]. The development of a micro-vasculature and micro-circulation is critical for the homeostasis and regeneration of living bone, without which, the tissue would simply degenerate and die [2].

The strength of bone is defined both by its quality as well as density. Vascular tissue plays a major role in the formation of new bone and maintenance of skeletal strength [3]. It has also been shown that changes in blood supply to bone tissue lead to deterioration of bone health and can cause skeleto-muscular diseases [4]. Formation of new capillaries (or angiogenesis) within the bone tissue facilitates the supply of nutrients and removal of waste products. This process also promotes healing of fractures; and stimulates the remodeling and regeneration of bones [5].

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AUTHOR

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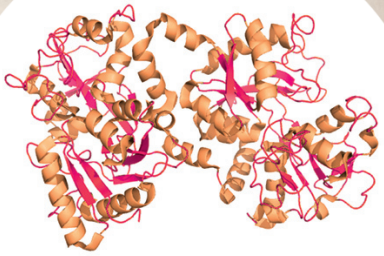
Dr. Borostyankoi is a veterinarian practicing in Southern California with special interest in small animal orthopedics, minimally invasive surgery and post-surgical rehabilitation. He is a Diplomate of the American College of Veterinary Surgeons. With a referral base of close to 3000 veterinarians, he performs hundreds of orthopedic surgeries for small animals every year.

Due to his vast experience, Dr. Borostyankoi has become a respected consultant in small animal orthopedics for the Southern California veterinary community. His research experience with surgical methods has contributed to the development and advancement of better orthopedic methods and technology. He has invented and improved several orthopedic implants. He greatly contributes to the continuous education of fellow veterinarians and lectures in the US and abroad.

Lactoferrin (LF) and Canine Bone Physiology



LF Ribbon Structure

LF regulates various cell types involved in skeleto-muscular regeneration. LF stimulates the proliferation of osteoblasts (bone forming cells) and chondrocytes (cartilage cells). LF inhibits osteoclastogenesis, reducing the number of cells that can actively resorb bone, thus producing a greater overall increase in bone volume.

Lactoferrin (LF) is an 80-kDa metal-binding glycoprotein found in exocrine secretions. The ability of LF to bind to large quantities of iron provides protection against pathogens and their metabolites by enhancing phagocytosis, cell adherence, and controlling the release of pro-inflammatory cytokines. LF is a primary constituent of immune homeostasis, functioning to reduce oxidative stress at the molecular level, and thus controlling excess inflammatory response [6]. LF levels in the blood are normally low (0.2–0.6 µg/mL), but at sites of inflammation, are significantly elevated (~200 µg/mL) due to its release from neutrophils. Elevated LF in the synovium is primarily due to anti-inflammatory response. LF regulates synovial iron load and has been shown to reduce inflammation in bone joints. Iron can suppress bone remodeling by reducing osteoblast formation and new bone synthesis [7].

LF has been shown to regulate various cell types involved in skeleto-muscular regeneration. It stimulates the proliferation and differentiation of osteoblasts, the bone forming cells [8]. LF also inhibits osteoclastogenesis and reduces the number of cells that actively resorb bone, thus producing an overall increase in bone volume [9]. LF is a positive regulator of bone with an important role in growth and healing. There is a growing interest in the potential use of LF for the improvement of bone health. In recent studies, dietary supplementation with LF demonstrated improved bone mineral density and bone strength. LF appears to be a promising candidate for the development of anabolic therapeutic agents for canine bone health [10].

LF - Documented Roles in Bone-Joint Physiology
Regulates synovial Fe(II) loading to reduce inflammation in bone joints
Binds to zinc and regulates MMPs from degrading bone cartilage
Modulates immune responses and prevents development of arthritis
Stimulates osteoblasts and regulates normal bone growth (formation)
Inhibits osteoclasts and regulates excessive bone resorption
Promotes strontium deposition in bone and as mineral transport protein
Evade infections at fracture site as a broad-spectrum antimicrobial agent

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Ribonuclease (RNase), Angiogenesis and Fracture Healing

Blood vessels play a pivotal role in skeletal homeostasis and bone repair; therefore, angiogenesis is important for successful bone remodeling [11,12]. Vascularization of the growth plate region is critical for two fundamental processes that determine the rate of bone growth – i) Chondrogenesis (cartilage production) and ii) Osteogenesis (bone formation). Precise coupling of these two processes is crucial during periods of rapid bone growth or fracture repair.

RNase, or angiogenin, is a 14-kDa, basic heparin-binding protein, which is a member of the pancreatic RNase superfamily. It is a key mediator in nearly all phases of angiogenesis, which makes it an important precursor in skeleto-muscular development [13]. Angiogenesis is a strictly regulated, multi-step process that occurs during normal bone remodeling and regeneration, pregnancy, and tissue repair conditions such as fractures and wound healing [14,15].

Bone tissue engineering in particular has benefited from the application of pro-angiogenic strategies. The need for an adequate vascular supply increases during healing,along with the challenges associated with vascularization of scaffolds implanted in vivo. The lack of a functional vascular supply can hamper the whole range of clinical applications of successful bone tissue engineering strategies, especially in canine bone grafts. These grafts depend on post-implant vascularization; accordingly, RNase could be promising in the establishment of a microcirculation in the engineered constructs.

RNase Ribbon Structure

Angiogenesis is a strictly regulated, multi-step process that occurs during normal bone remodeling and regeneration, pregnancy, and tissue repair conditions such as fractures and wound healing.

RNase - Multi-functional Role in Bone-Joint Physiology
Accelerates bone mineralization, bone regenerationand remodeling
Inhibits osteoclastic bone resorption
Scavenges free radicals as an antioxidant and protects bone integrity
Binds to endothelial cells and stimulates cell migration and invasion
Promotes cell proliferation/differentiation and mediates cell adhesion
Activates cell associated proteases and induces plasminogen activator
Prevents chrondrocyte apoptosis and preserves joint cartilage

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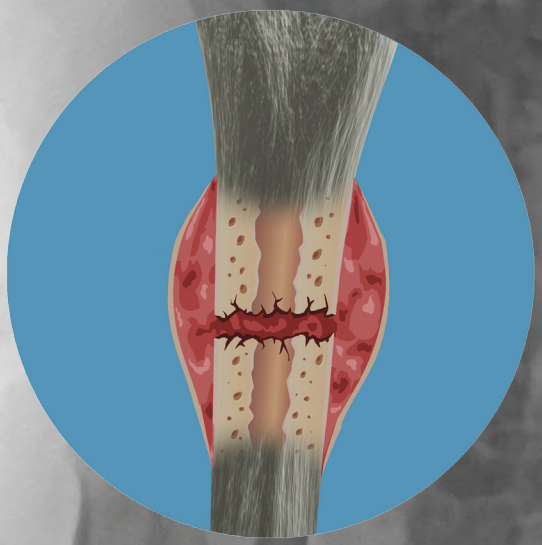
R-ELF AND CANINE FRACTURE HEALING

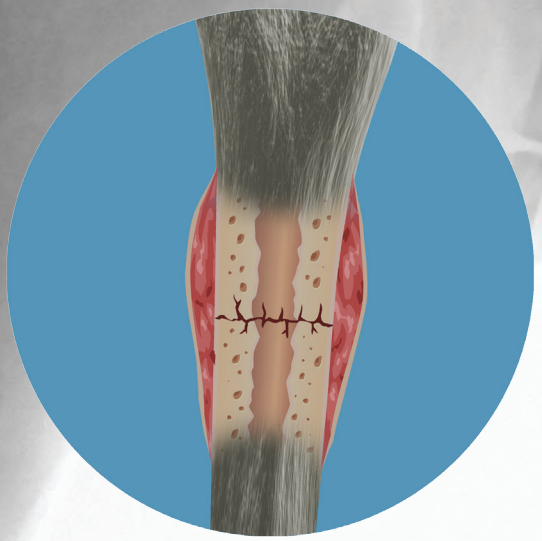
INFLAMMATION - I

Hematoma
Angiogenesis



INFLAMMATION - II

Immuno-modulation
Granular tissue



Ribonuclease-enriched Lactoferrin (R-ELF) is an all-natural, clinically validated, and multi-patented technology developed to improve canine bone and joint health. R-ELF is a precisely calibrated mixture of two bio-functional proteins that help regulate all phases of fracture healing. The ability of the LF protein in R-ELF complex to bind/transport metal co-factors (such as iron and zinc) could facilitate the elimination of harmful free radicals and regulate MMPs that hydrolyze and remove cellular debris from the fracture site. LF is a potent modulator of bone turnover that has been demonstrated to increase bone growth by 200% and down-regulate bone resorption by 50%. RNase, the immobilized enzyme in R-ELF complex, is critical for revascularization of regenerated tissue and in amplifying inflammatory cell mediators. The combination of LF and RNase in R-ELF complex elicits greater synergistic activity compared to the individual proteins. The following sections in this white paper (i) elucidate the effects of R-ELF on phases of fracture healing, (ii) review radiographic evaluations of canine osteotomy cases and (iii) cover bone turnover and inflammation profiles in human clinical trials.

Fracture healing involves a complex series of coordinated cellular and molecular processes leading to: removal of contaminating material, angiogenesis validating reinstatement of disrupted microcirculation, and restoration of bone continuity by activation, proliferation and chemotaxis of bone progenitors from surrounding periosteum and endosteum. Regulation of the various cells that orchestrate the fracture healing process depends on the biological effect evoked by angiogenin (RNase) and other growth factors/cytokines. R-ELF seems to be a potent regulator of canine bone turnover and fracture healing.

There are 3 distinct phases of fracture healing: i) Inflammation ii) Repair and iii) Remodeling.

i) Inflammatory Phase

The Inflammatory Phase begins immediately after the initial disruption of bone and surrounding soft tissues, and persists until the formation of cartilage or bone. This phase lasts for 3-4 days and potentially longer, depending on the degree of fracture. Hematoma sets the stage for the repair phase by releasing growth factors, which stimulate angiogenesis and bone formation. As an inflammatory mediator, R-ELF could stimulate angiogenesis and also signal early bone resorption by osteoclasts and proliferation of osteoprogenitor cells.

Mast cells containing vaso-active substances (such as R-ELF) help in the formation of new vessels. Within hours, a transient extra-osseous blood supply emerges from surrounding soft tissues, revascularizing the hypoxic fracture site. Mononuclear phagocytes delivered by these new vessels assist in the removal of necrotic bone and aid in callus formation.

ii) Repair Phase

During the Repair Phase, osteogenesis continues and a callus is formed to bridge the fracture site. At the end of this phase, bone union is achieved, but the structure of the fracture site differs from that of the original bone. The time required to achieve union varies based on fracture configuration and location, status of the adjacent soft tissues, as well as animal characteristics (species, age, health status, concurrent injuries/ diseases). Eventually, the injured bone regains enough strength and rigidity to allow low impact exercise.

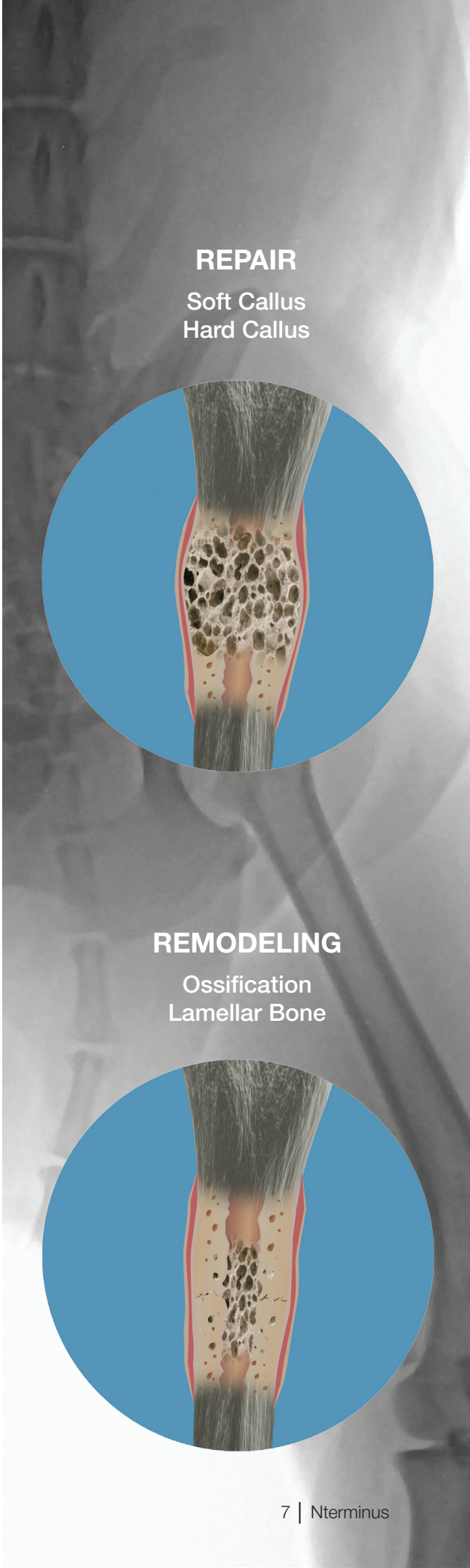
iii) Remodeling Phase

During the final Remodeling Phase, the large callus is reduced to the size of actual bone at the fracture site. The woven/ primary bone is replaced with secondary lamellar bone. This process may take months or even up to a year or more. The balanced action of osteoclastic resorption and osteoblastic deposition can be regulated by R-ELF. In spontaneous healing of a fracture, progression from soft to hard callus depends upon an adequate blood supply and a gradual increase in stability at the fracture site.

Most fractures, if left completely alone, would probably heal; however, due to any delayed union or malunion, the bone might lose its function. Successful healing of a fracture is not only determined by complete bony union on X-rays, but also by the functional use of the limb thereafter. R-ELF has been shown to promote functional fracture healing, while concurrently negating some of the problems of prolonged treatment such as decreased vascularization and poor bone turnover.

Bone grafts benefit from pro-angiogenic strategies, especially with adequate vascular supply from RNase during implantation and healing. R-ELF has been demonstrated to increase new bone formation and optimize bone resorption. Also, R-ELF is shown to improve anti-inflammatory response that could help fracture healing process.

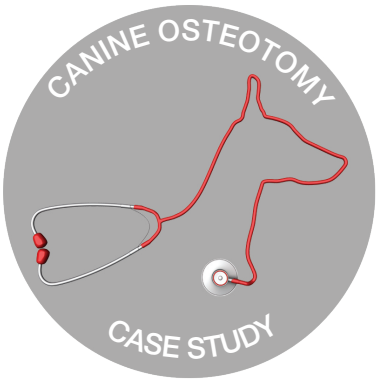
In summary, R-ELF is a multi-functional, all-natural, protein complex that promotes optimum bone turnover, modulates the inflammatory response, and regulates angiogenesis in canine physiology. These bio-activities are critical for canine bone tissue engineering. R-ELF could play a pivotal role in bringing out successful outcomes with various bone corrective procedures such as orthopedic surgeries, bone tissue grafts and implants. Furthermore, R-ELF could serve as a potent nutraceutical agent for a healing and/or aging canines that need bone and joint healthcare.



CANINE OSTEOTOMY CASE STUDY - I

R-ELF and Closing Wedge Tibial Osteotomy (CWTO)

Rupture of the cranial cruciate ligament can be partial or complete and the cartilages in the knee can be damaged because of the instability that results. Commonly used tibial bone cutting techniques include 'Closing Wedge Tibial Osteotomy (CWTO)' and 'Tibial Plateau Leveling Osteotomy (TPLO)' among other procedures. CWTO is often used in small dogs and in dogs with steep "tibial plateaus". While, TPLO is used in larger breed dogs if they are notably 'bouncy' or if they have small tibial crests (an anatomical feature of the tibia). The tibial plateau is the top of the tibia and forms the floor of the knee joint. This usually has a down-slope of about 22 degrees. In some breeds-like West Highland White Terriers- the slope can be 40 degrees or more. CWTO procedure reduces this down slope to something in the region of 5 degrees. This allows dogs to use the knee much more comfortably despite the ruptured cruciate ligament. In the CWTO procedure, two cuts are made in the tibia so that a wedge of bone can be removed. A wire is placed at the front of the bone between two bone tunnels and, by tightening it, the wedge shaped gap in the bone can be compressed. This reduces the slope of the tibial plateau. The bone is then stabilized with one or more bone plates and screws. Post-operative X-rays are taken to confirm satisfactory healing of the bone.



History/Diagnosis: The patient was evaluated for right hind leg lameness persisting for 4 weeks. The patient reportedly jumped out of owner's car and had been limping ever since. A family veterinary exam localized the lameness to a loose right stifle joint and sent the patient for orthopedic evaluation and surgery to a specialty clinic. The patient was ambulatory on the physical exam with a moderate lameness on the right rear leg. Palpation of the stifle joint revealed a mild joint effusion with a severe drawer motion in the stifle, consistent with a complete rupture of the cranial cruciate ligament. The referring veterinarian's radiographs revealed a non-displaced proximal fibula fracture. Patient also had a likely pre-existent 'Medial Patella Luxation (MPL)', a common developmental disorder in small dogs.

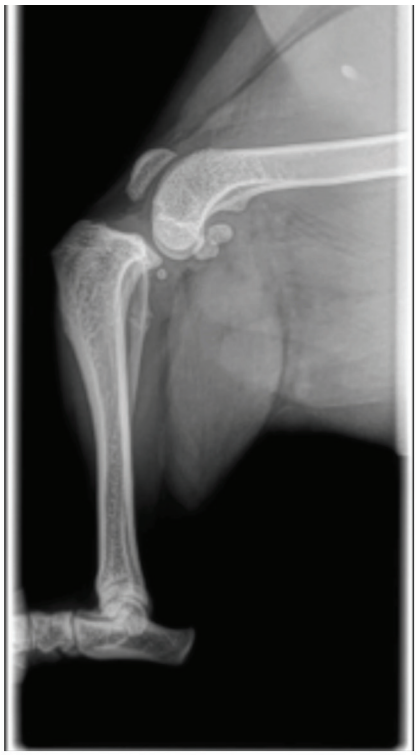
Surgical Procedure: A 'Closing Wedge Tibial Osteotomy (CWTO)' was performed on the right rear leg to correct the cranial cruciate ligament-deficient stifle. The procedure also included a MPL repair with a femoral trochlear resection and tibial crest transposition. The tibial crest was affixed in its new position with two K-wires. The routine surgical procedure is designed to reduce the tibial plateau slope by removing a wedge shaped piece of bone from the proximal tibia and to fix the MPL condition at the same time. The created wedge shaped bone defect then is collapsed and held together with an orthopedic wire and bone plate. The size of the wedge is determined by the 'Tibial Plateau Angle (TPA)'. The surgery resulted in a significant cortical alignment deficiency with aligning the cranial cortex.

Post-Operative Care / Effects of R-ELF: Post-surgical recommendations included antibiotics, NSAIDs, and activity restriction. Additionally, R-ELF (160 mg/day) was orally administered as bone and joint supplement. The veterinary reported that expected bone healing time for a clinically normal dog, such as this patient, is normally 8 weeks with continued activity restriction. However, an expedited 7-week bone healing was observed from radiographic evidence with the R-ELF intake; accordingly, the patient was released with no restrictions at the 7-week check up. The radiographs also showed complete bone remodeling of the previously created caudal cortical misalignment – this improvement is usually not observed until 12+ weeks.

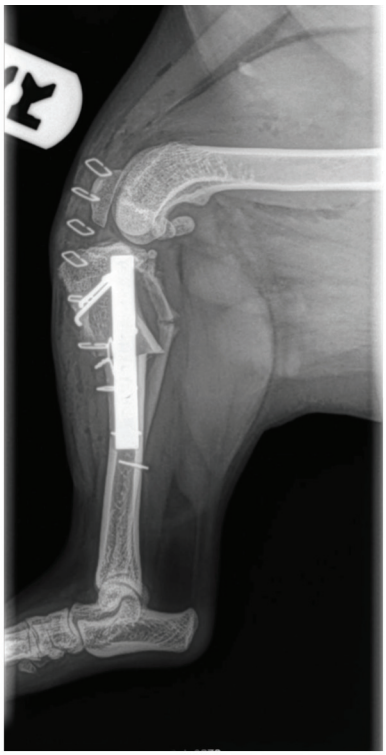
R-ELF demonstrated canine fracture healing/bone reunion ~30% faster.

R-ELF shortened post-osteotomy recovery time in dogs by 3-4 weeks.

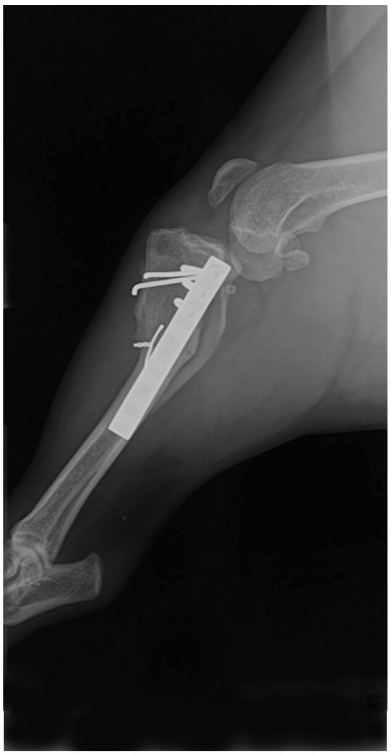
Patient: Cairn Terrier (Spayed Female)
Age/Body Weight: 2-year / 21 lb
Clinical Condition: Rupture of Cranial Cruciate Ligament + Medial Patella Luxation
Surgical Procedure: Closing Wedge Tibial Osteotomy (CWTO)



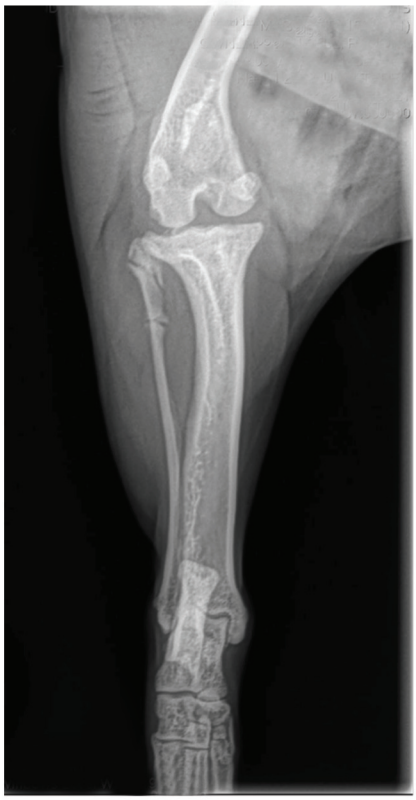
PRE-PROCEDURE

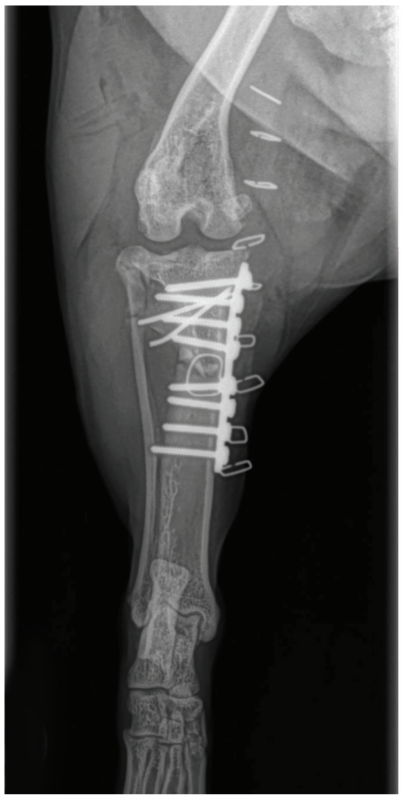


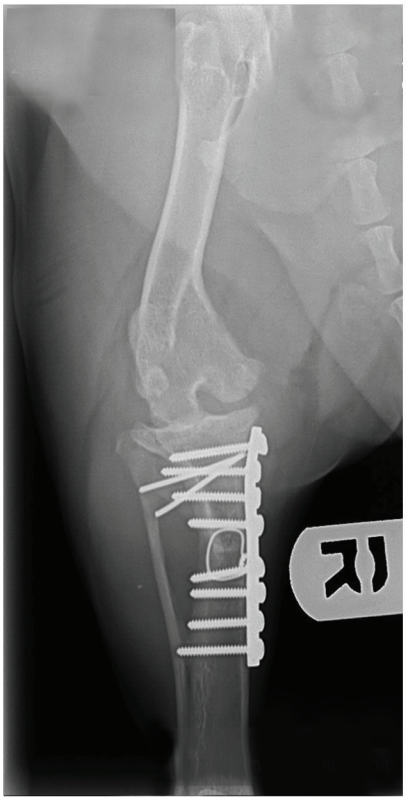
DAY 0



WEEK 7







CANINE OSTEOTOMY CASE STUDY - II

R-ELF and Tibial Plateau Leveling Osteotomy (TPLO) - Large Dogs

A ruptured cranial cruciate ligament is one of the most common orthopedic injuries in dogs. It is estimated that 1 million dogs suffer from this injury every year in the USA alone. TPLO has become the standard procedure to fix such injuries due to its estimated 90% successful outcome. The procedure entails cutting the tibia bone under the knee joint and changing the position of the top segment to other ligaments to take over for the ruptured ligament. The ligament is stabilized with a special bone plate and screws. Part of the success of this procedure depends on a proper bone healing. If the bone is not healed there is a risk that the surgically created position may fall out of alignment; therefore, dogs need to be limited in their activity during the healing period. This is achieved initially with cage confinement and later with indoor confinement and controlled leash walks.

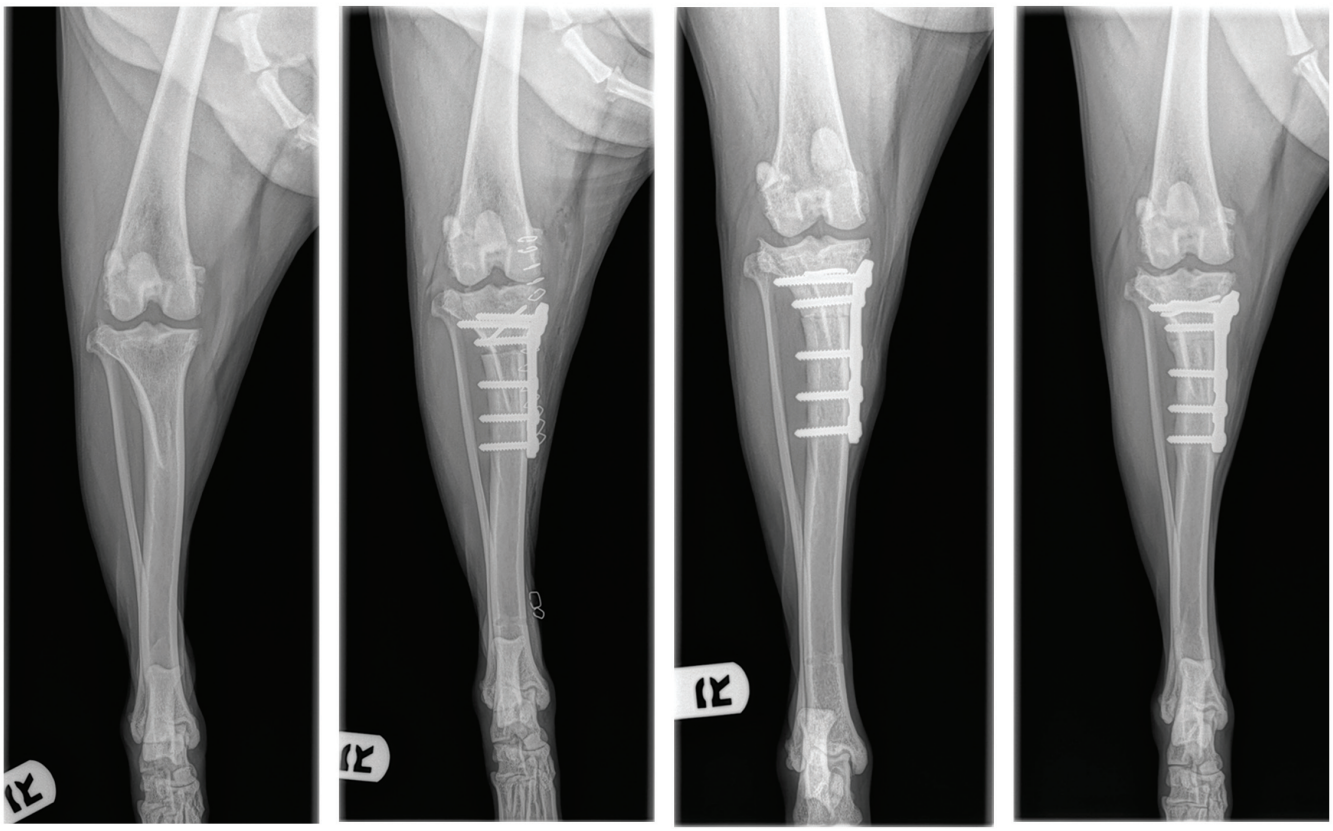
History/Diagnosis: The patient had a history of limping on right rear leg for 4 weeks. During the physical examination, the patient walked with a narrow gait on the rear legs with moderate right rear leg lameness. A palpable joint effusion was observed in the right stifle joint and flexing of the knee seemed painful. The stifle was slightly unstable on palpation with a small degree of drawer motion in the joint. Both hips had decreased range of motion with minimal discomfort on extension. Based on the radiographs, bilateral hip dysplasia and right anterior cruciate ligament trauma (partial tear) was diagnosed.

Surgical Procedure: A Tibial Plateau Leveling Osteotomy (TPLO) was performed on the patient. The stifle was opened up with a mini arthrotomy to remove the damaged portion of the ligament and to release the medial meniscus. The proximal tibia was cut with a 21-mm TPLO blade to mobilize the proximal tibia. The tibial plateau was rotated 7-mm to eliminate the tibial thrust. The segment was stabilized with a 3.5-mm Universal TPLO plate, 6 x 3.5 mm cortical screws and a 0.062 inch Kirschner wire.

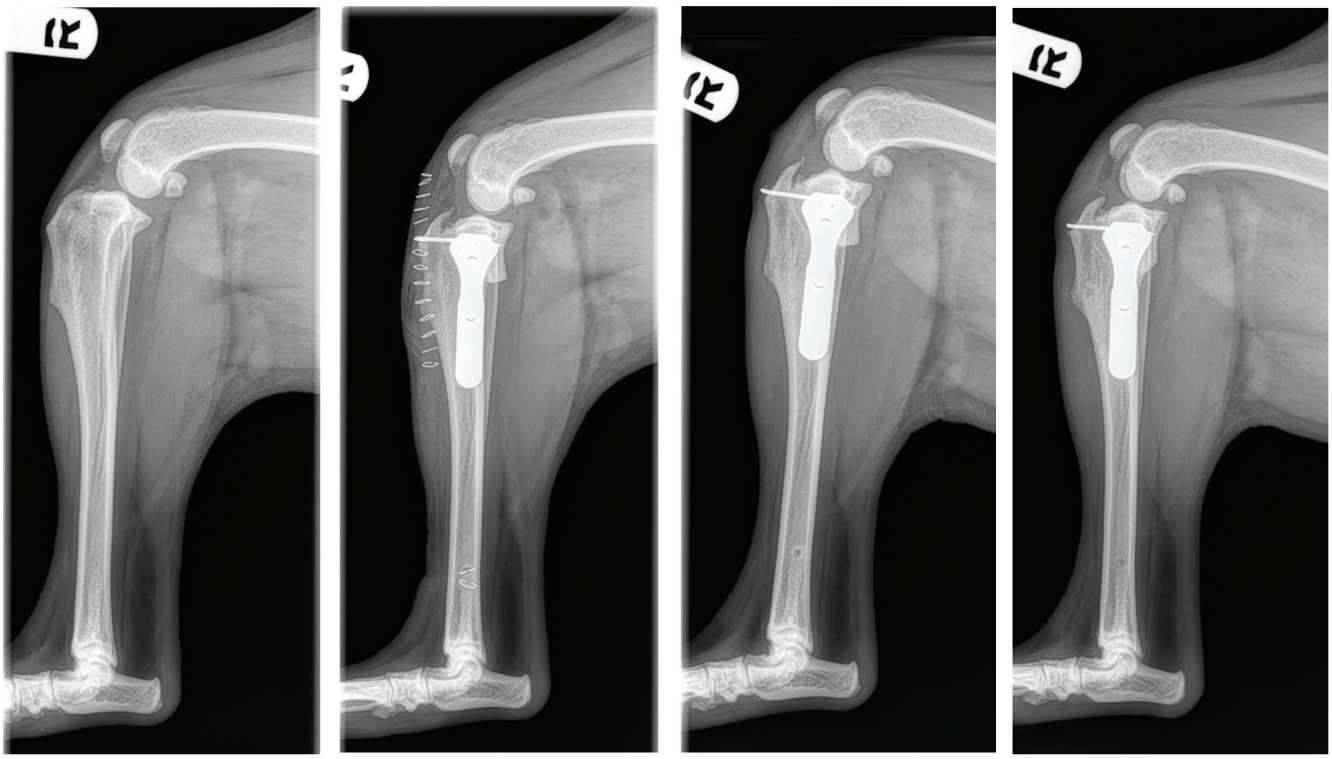
Post-Operative Care / Effects of R-ELF: Post-surgical care included the usual 4 weeks of cage confinement and an additional 4 weeks of indoor confinement with controlled leash walks. In addition, the patient was orally administered a daily dose (240 mg) of R-ELF. Follow up radiography (Day-0, Week-4, and Week-9) showed rapid bone healing. The patient was released from the confinement and restricted activity to full activity at the 9-week check up. At the 9-week evaluation, the patient was fully active on her operated leg with no sign of lameness. The veterinary surgeon reported that the operated leg could be not differentiated from the intact leg just by observation (without checking the chart or touching the legs). The radiographic images showed complete bone healing and remodeling.

In over 100 vet-supervised clinical cases, post-surgical R-ELF supplementation seemed to help canines achieve satisfactory bone healing/turnover within 7-8 weeks instead of the previously recorded 12+ week recovery. In several veterinary clinics, dogs were released out of confinement, taken off exercise restrictions, and returned to full function within 8 weeks. This significantly decreased the post-operative recovery time of the patient. *Note: In this case study, the client has missed the 8-week recheck appointment and the evaluation was postponed to 9th week.*

Patient: German Shepherd (Spayed Female)
Age/Body Weight: 4-year / 59 lb
Clinical Condition: Bilateral Hip Dysplasia + Right Anterior Cruciate Ligament Trauma
Surgical Procedure: Tibial Plateau Leveling Osteotomy (TPLO)



PRE-PROCEDURE DAY 0 WEEK 4 WEEK 9



CANINE OSTEOTOMY CASE STUDY - III

R-ELF and Tibial Plateau Leveling Osteotomy (TPLO) - Small Dogs

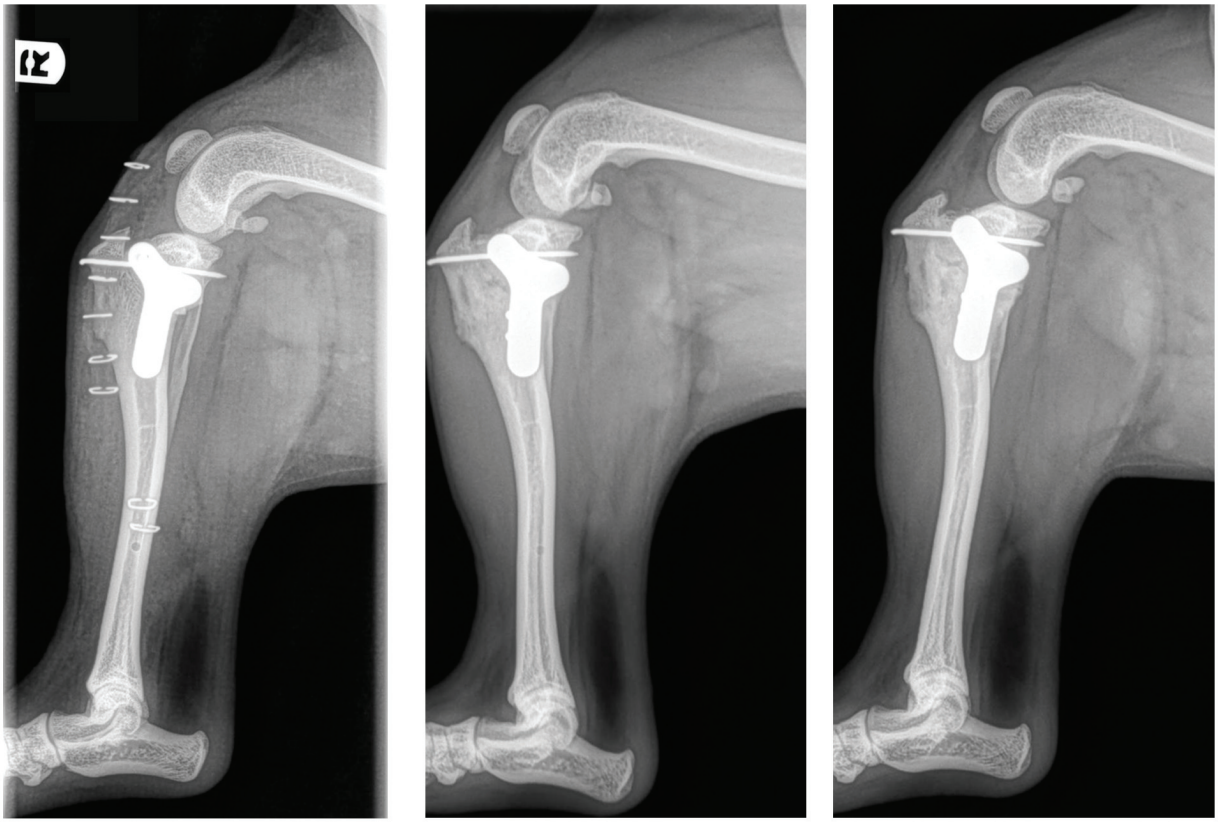
The TPLO procedure was initially developed for knee repair of larger dogs to stabilize the stifle joint during weight-bearing and compensate for the loss of the cranial cruciate ligament. The outcomes of TPLO were so successful on large dogs that this procedure eventually extended to most cruciate ligament repair on all size and breeds dogs, such as this Case Study-3 patient – a small-sized dog with 9.5 kg bodyweight.

History/Diagnosis: Patient had a history of non-weight bearing lameness of the right rear leg. During the physical exam the dog was walking with moderate right rear leg lameness. There was a palpable joint effusion present in the right stifle joint and flexing the knee was painful. The stifle was partly unstable on palpation with a drawer motion in the joint. Veterinary diagnosis indicated a rupture in the right anterior cruciate ligament and an unstable knee joint.

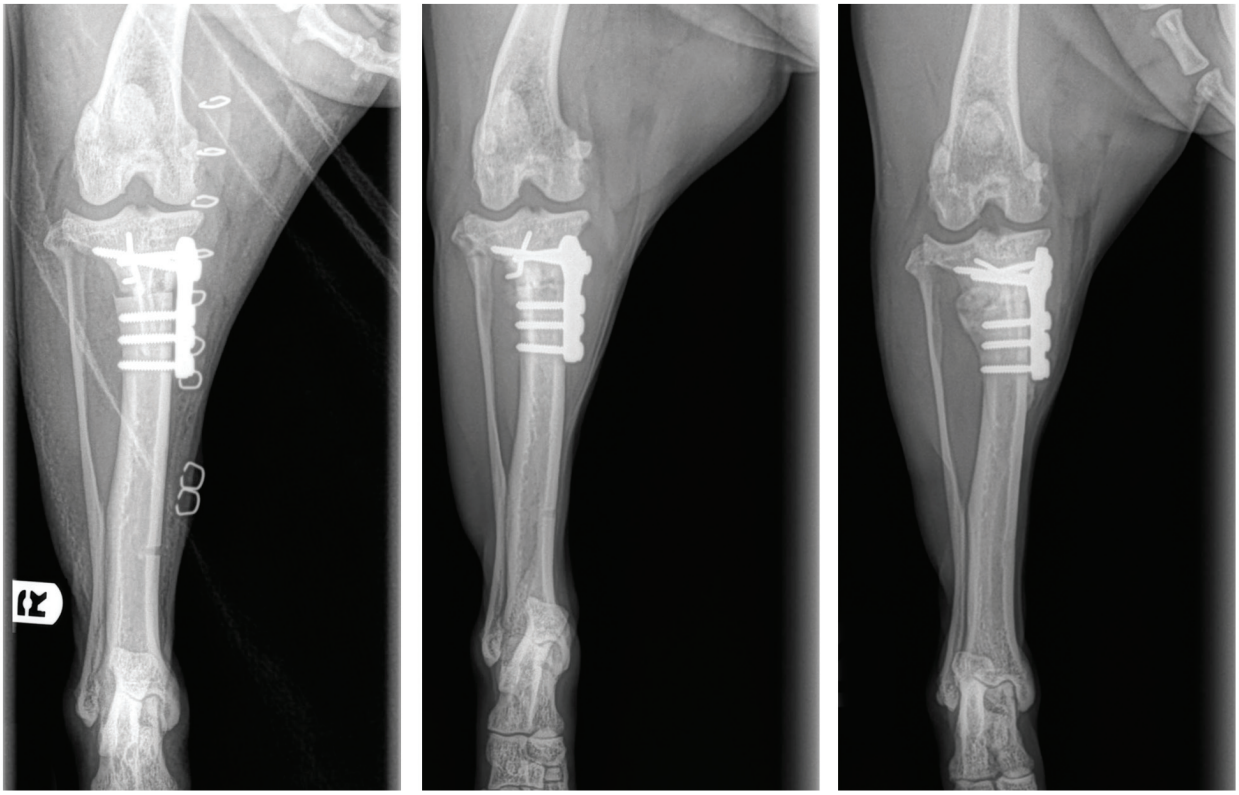
Surgical Procedure: A right Tibial Plateau Leveling Osteotomy (TPLO) was performed by opening of the stifle with a mini arthrotomy to remove the remnants of the damaged ligament and to release the medial meniscus. The proximal tibia was cut with a 12-mm TPLO blade to mobilize the joint surface – the tibial plateau. The proximal segment was rotated 4.5-mm to eliminate the tibial thrust. The segment was stabilized with a 2.0 mm Cadmus stile TPLO plate, 6 x 2.0 mm cortical screws and a 0.045 inch Kirschner wire.

Post-Operative Care / Effects of R-ELF: During post surgical care (on top of the usual 4 weeks of cage confinement and an additional 8 weeks of indoor confinement with leash walks) the patient was orally administered R-ELF (160 mg) on a daily basis. The routine X-ray checkups (Day-0, Week-4, and Week-8) indicated expedited bone healing. The patient was released from confinement and restricted activity to full activity 4 weeks sooner than expected in clinically similar cases where dogs wer. By the 8-week recheck the bone was completely healed and the dog regained full activity.

Patient: Bichon Frise (Neutered Male)
Age/Body Weight: 7-year / 21 lb
Clinical Condition: Rupture of the Right Anterior Cruciate Ligament + Unstable Knee Joint
Surgical Procedure: Tibial Plateau Leveling Osteotomy (TPLO)

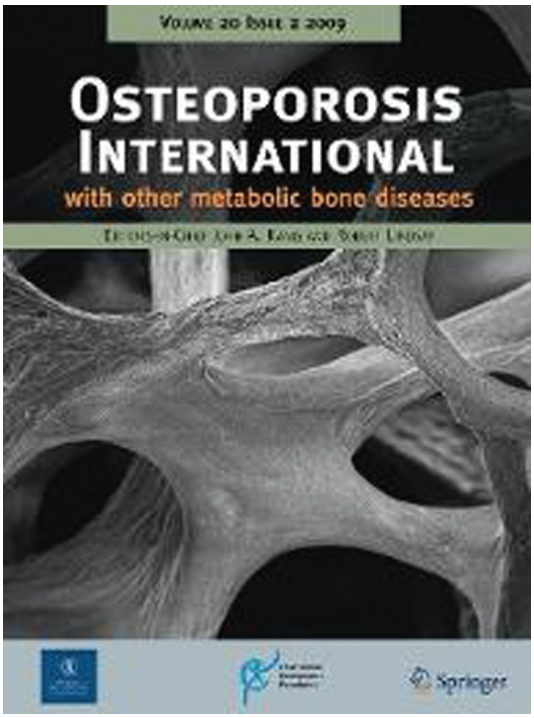


DAY 0 WEEK 4 WEEK 8



HUMAN CLINICAL TRIAL - I

R-ELF and Bone Turnover in Post-Menopausal Women



AUTHORS: Bharadwaj S, Naidu AGT Betagiri G, Prasadarao NV, Naidu AS
ARTICLE: Milk ribonuclease-enriched lactoferrin induces positive effects on bone turnover markers in postmenopausal women.
JOURNAL: *Osteroporos Int* 20:1603-1611(2009)

SUMMARY

Osteoporosis, a major health issue among post-menopausal women, causes increased bone resorption and reduced bone formation. A reduction in angiogenesis could also contribute to this imbalance. Current treatments such as hormone replacement therapy and bisphosphonates have drawbacks of severe side effects. Milk ribonuclease (RNase) is known to promote angiogenesis, and lactoferrin (LF) to stimulate bone formation by osteoblasts. The effect of RNase-enriched lactoferrin (R-ELF) supplement on the bone health of post-menopausal women was examined.

Methods: A total of 35 healthy, post-menopausal women, aged 45 to 60 years, were randomized into placebo or R-ELF supplement groups. The bone health status was monitored by assessing bone resorption markers, serum N-telopeptides (sNTx) and urine deoxypyridinoline (uDpd) cross-links; and serum bone formation markers, bone-specific alkaline phosphatase (sBAP) and osteocalcin (sOC).

Results: R-ELF supplementation demonstrated a decrease in urine Dpd levels by 14% (19% increase for placebo) and serum NTx maintained at 24% of the baseline (41% for placebo), while serum BAP and OC levels showed a 45% and 16% elevation (25% and 5% for placebo).

Conclusions: R-ELF supplementation showed a statistically significant reduction in bone resorption and increase in osteoblastic bone formation, to restore the balance of bone turnover within a short period.

Background: Bones undergo a continuous remodeling process through repeated cycles of destruction and rebuilding. In healthy young adults, the amount of new bone formation approximately balances the amount of bone resorption. As the age increases, however, the balance shifts to favor bone resorption. Current efforts to treat bone diseases have primarily concentrated on the development of drugs to block bone resorption, which decrease the formation or activity of osteoclasts. Present treatment options for postmenopausal osteoporosis include hormone replacement therapy (HRT) and bisphosphonates. HRT is known to reduce osteoclast activity, but is prone to adverse effects, such as increased risk of breast cancer [16,17]. Bisphosphonates have a risk of development of esophageal ulcers [18]. Therefore, it is imperative to explore and develop strategies for enhancing the bone formation and simultaneously prevent bone loss without side effects. Angiogenesis or formation of new capillaries within bone tissue could facilitate supply of nutrients and removal of waste metabolites and facilitates healing of fractures, remodeling and regeneration [19,20].

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17. Collins JA, et al (2005) Breast cancer risk with postmenopausal hormonal treatment. *Hum Reprod Update* 11:545–560.
18. Makins R, Ballinger A (2003) Gastrointestinal side effects of drugs. *Expert Opin Drug Safety* 2:421-429.
19. Glowacki J (1998) Angiogenesis in fracture repair. *Clin Orthop* 355S:S82–S89.
20. Carano RAD, Filvaroff E (2003) Angiogenesis and bone repair. *Drug Discov Today* 8:980–989.

Study Design

Women (n=35) included into the study were assigned to placebo group or R-ELF group, randomly. Subjects (n=15) in the placebo group and were supplemented with 100% RDA (Recommended Daily Allowance) of calcium, in tablet form. Subjects of the R-ELF group (n=20) were administered with two R-ELF capsules of 125 mg each, along with 100% RDA of calcium administered orally from Day-1 to Day-180. Venous Blood (by standard venipuncture) and urine samples were collected from each subject on Day-0 (baseline before supplementation), Day-15, Day-30, Day-60, Day-90 and Day-180 of the study. Standard systolic/diastolic blood pressures as well as the body weight were also monitored at baseline and the aforementioned days.

R-ELF and Bone Resorption Markers

The median sNTx and uDpd changed gradually to a peak level for both placebo as well as R-ELF groups, as shown in *Figure 1 (AB)*. The resorption markers for each observational day were also normally distributed (P: 0.34 – 0.99). sNTx for the placebo group increased from 15.8 ± 2.6 nM BCE (Bone Collagen Equivalents) on Day-0 to 22.1 ± 1.7 nM BCE on Day-180, while R-ELF group displayed a relatively smaller rise (12.9 ± 3.3 to 16.1 ± 2.0 nM BCE).

The change in bone turnover markers, calculated as a percent of the corresponding baseline levels are represented in *Figure 2 (AB)*. Median sNTx for the placebo group showed an increase of 40.1% in 180 days, reflecting significant bone resorption while the R-ELF group showed a relatively smaller rise of 24.5% during the same period (P<0.001, 95% CI). The duration to achieve 80% of the peak change in sNTx is about 25 days for R-ELF group compared to 45 days for the placebo group, indicating that R-ELF supplementation induces its effects within a short time. The calculated parameters for the bone markers and the results of statistical tests are summarized in *Table 1*.

FIGURE-1

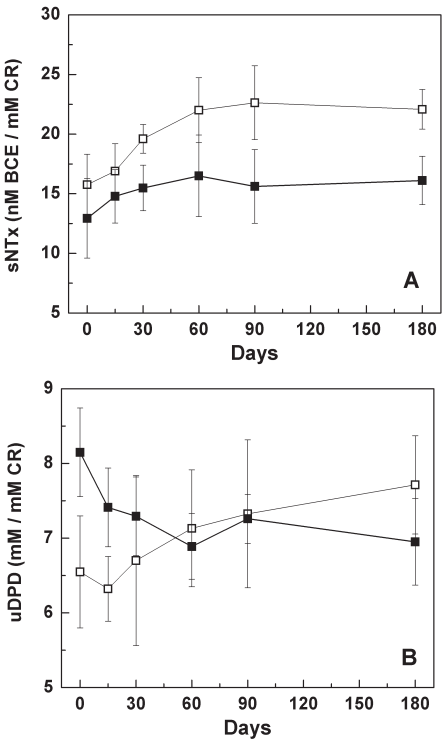


FIGURE-2

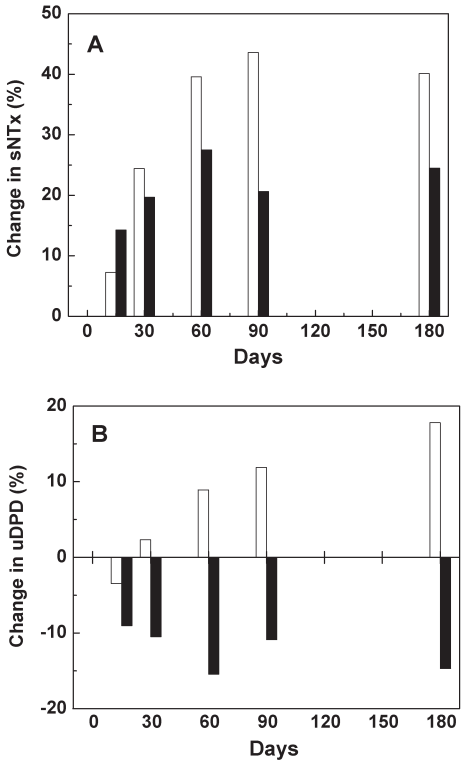


TABLE-1: *Progress of Biochemical Bone Resorption Markers*

Marker	Change from Baseline (%)		Peak level (in Days)	*t*-test [d]
	Median [a]	Peak [b]		
Serum NTx				
Placebo	32.0 ± 7.6	41.6	45	15.18
R-ELF	20.2 ± 4.0	24.1	25	(< 0.001)
Urine Dpd				
Placebo	5.6 ± 3.3	19.2	95	4.11
R-ELF	-10.7 ± 2.2	-14.0	31	(< 0.01)

[a] Median ± SEM
[b] Peak change in the marker obtained from a non-linear least squares fit to logistics curve
[c] Not determined as there was no significant change in serum OC for the control group
[d] Value of the t statistic. Significance P (95% CI) is given in parenthesis

FIGURE-3

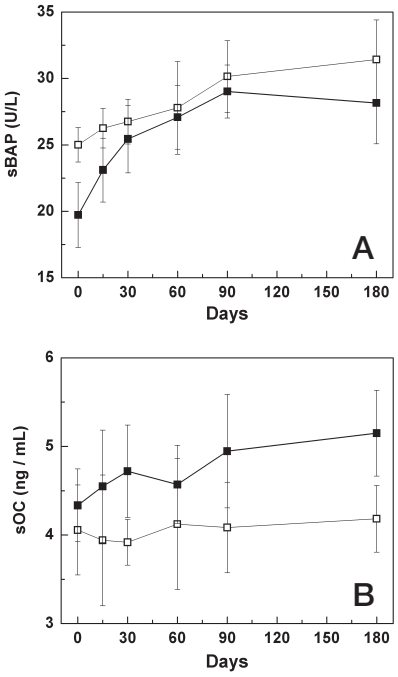
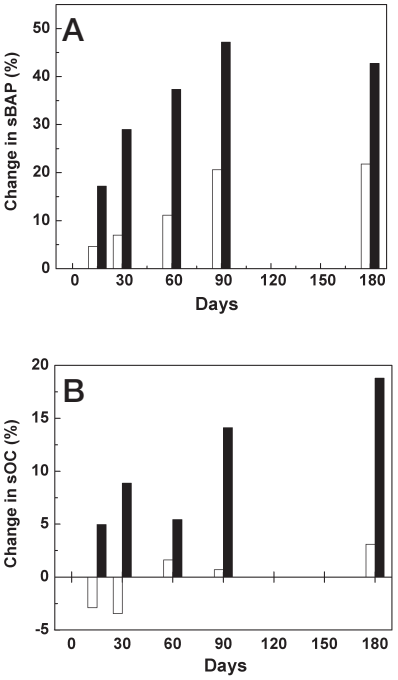


FIGURE-4



An interesting reversal of trend was observed with median uDpd levels although the placebo group showed an increase from 6.6 ± 0.8 to 7.7 ± 0.7 by Day-180, the R-ELF group decreased from 8.2 ± 0.6 to 6.9 ± 0.6 nM Dpd/mM Creatinine. The overall rise of 17.8% indicated a significant level of bone resorption in the placebo group. In contrast, Dpd levels of the R-ELF group showed a reduction in resorption by ~10% within 30 days and continued to fall to 14.6% by the end of the study (P = 0.0021, 95% CI). The R-ELF group achieved 80% of peak change in Dpd levels in about 30 days, compared to 95 days taken by the placebo group.

R-ELF and Bone Formation Markers

The two markers of bone formation, sBAP and sOC were determined at the same pre-defined intervals during the study. *Figure 3 (AB)* shows the measured variation of median sBAP and sOC levels for both groups. As with the bone resorption markers, the sBAP and sOC data sets for each observational day were normally distributed (P: 0.39 – 0.98).

The variation of sBAP and sOC from their respective baseline levels is depicted in *Figure 4 (AB)*. Median sBAP gradually increased to a peak level for both the groups (25.0 ± 1.3 to 31.4 ± 3.0 U/L for placebo and 19.7 ± 2.4 to 28.1 ± 3.1 U/L for the R-ELF group). Although median levels for R-ELF group are lower than those for the placebo group, the % change from baseline was better for the R-ELF group. The increase in 42.7% for R-ELF group compared to 25.7% for the placebo was also statistically significant (P<0.001, 95% CI). The peak level attained was about twice that of the placebo group, and 80% of peak change was achieved within ~45 days of R-ELF supplementation. In the case of sOC, mean marker levels maintained within ± 3% of baseline for placebo (4.1 ± 0.5 to 4.2 ± 0.4 ng/mL), while those for the R-ELF group increased linearly by 18.8% from 4.3 ± 0.4 to 5.2 ± 0.5 ng/mL. The results summarized in *Table 2* indicate that change in sOC with supplementation was statistically significant (P<0.001, 95% CI).

TABLE-2: *Progress of Biochemical Bone Formation Markers*

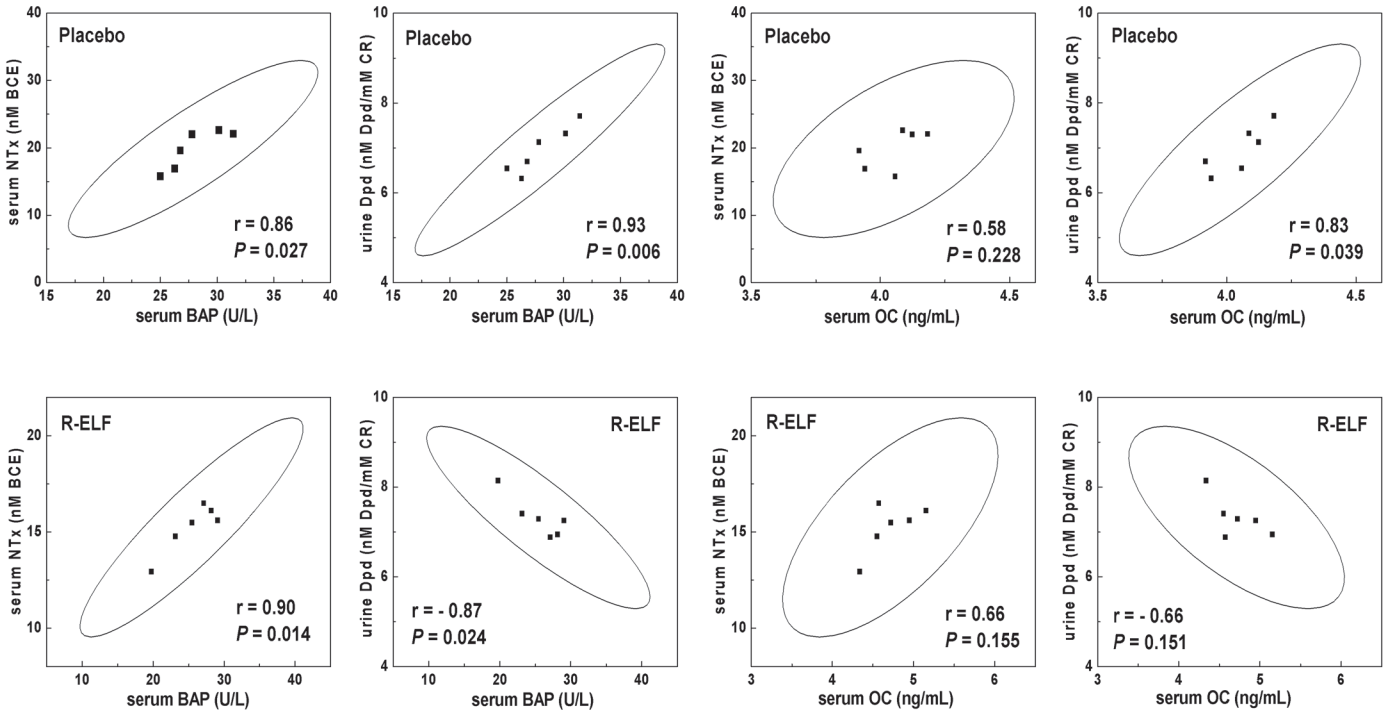
Marker	Change from Baseline (%)		Peak level (in Days)	*t*-test [d]
	Median [a]	Peak [b]		
Serum BAP				
Placebo	9.1 ± 3.9	25.4	115	4.10
R-ELF	33.2 ± 7.2	44.9	45	(< 0.001)
Serum OC				
Placebo	0.4 ± 1.0	5.1	- [c]	-20.3
R-ELF	7.2 ± 2.8	16.4	-	(< 0.001)

[a] Median ± SEM
[b] Peak change in the marker obtained from a non-linear least squares fit to logistics curve
[c] Not determined as there was no significant change in serum OC for the control group
[d] Value of the t statistic. Significance P (95% CI) is given in parenthesis

Bone formation markers were observed to be highly correlated with bone resorption markers for both groups, with Pearson correlation coefficient (r) values in the range 0.58 – 0.93 for the placebo and 0.66 – 0.90 for the R-ELF group, respectively. The correlation plots along with the statistical parameters are shown in *Figure 5*. The positive correlation observed for placebo group is generally improved with R-ELF supplementation, as seen from the higher r and smaller P values for sNTx with sBAP as well as sOC. In case of uDpd, the positive correlation seen with the placebo group is changed to negative for the R-ELF group – r changing from 0.93 to -0.87 for sBAP and from 0.83 to -0.66 for sOC, respectively. This change reflects the effect of R-ELF supplementation to reduce resorption markers while increasing the formation markers to restore balance of bone turnover.

FIGURE-5

Correlation plots of bone formation markers vs. bone resorption markers. Top row corresponds to the placebo group and the bottom row to the R-ELF group. The Pearson correlation coefficient (r) and significance (P) are shown for each plot.



Bone formation (osteoblastic activity) increased by ~200% (almost doubled) with R-ELF intake compared to the placebo.

A significant reduction (>50%) in bone resorption (osteoclastic activity) was measured in the R-ELF group.

R-ELF restored the innate balance of bone turnover within 2 to 4 weeks of supplementation.

HUMAN CLINICAL TRIAL - II

R-ELF and Improvement of Inflammatory Responses

AUTHORS: Bharadwaj S, Naidu AGT Betagiri G, Prasadarao NV, Naidu AS
ARTICLE: Inflammatory responses improve with milk ribonuclease-enriched lactoferrin supplementation in postmenopausal women.
JOURNAL: *J Inflamm Res* 59(11):971-978 (2010)

OBJECTIVE AND DESIGN

A six-month, randomized clinical study was conducted to evaluate the effect of a ribonuclease-enriched lactoferrin (R-ELF) supplement on the circulating cytokine levels and bone health of post-menopausal women.

Subjects: Healthy postmenopausal women (n=35), aged 45-60 years, were randomized into placebo and R-ELF groups.

Treatment: R-ELF group was supplemented with R-ELF (2 x 125 mg/day) and calcium (100% RDA), while the placebo group received only the calcium supplement. Serum levels of receptor activator for NF-κB ligand (RANKL), C-reactive protein (CRP) and various pro- and anti-inflammatory cytokines were determined by ELISA.

Results: Pro-inflammatory cytokines IL-6 and TNF-α decreased significantly (-44% and -10% respectively) while anti-inflammatory IL-10 increased (140%) with R-ELF supplementation. RANKL and CRP were modestly reduced (-50%) relative to the placebo, while RANKL elevated initially.

Conclusions: R-ELF supplementation showed beneficial effects towards improvement of inflammatory status.

Background: The immune and the skeletal systems share several regulatory factors, such as cytokines, transcription factors, and receptors. The immune cells and osteoclasts are derived from the same hematopoietic precursor cells, which originate in bone marrow and interact with bone cells [21,22]. Inflammation is the primary defense mechanism of the body characterized by a 'high alert' state of the immune system, triggered by the release of several pro-inflammatory cytokines. It also affects the processes of bone resorption and formation, due to the presence of pathways that are common to both bone cell maturation and inflammation. Pro-inflammatory cytokine levels are known to rise during aging and stress [23]. Elevated levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 andIL-1ß receptors suggest subtle changes in the immune system [24]. Thus, the body is in a state of mild, continuous inflammation, characterized by a rise in the levels of acute phase proteins such as C-Reactive Protein (CRP), a well-known bio-marker for inflammation. In healthy, elderly individuals higher serum CRP levels were associated with high bone turnover rate, resulting in low bone mineral density [25].

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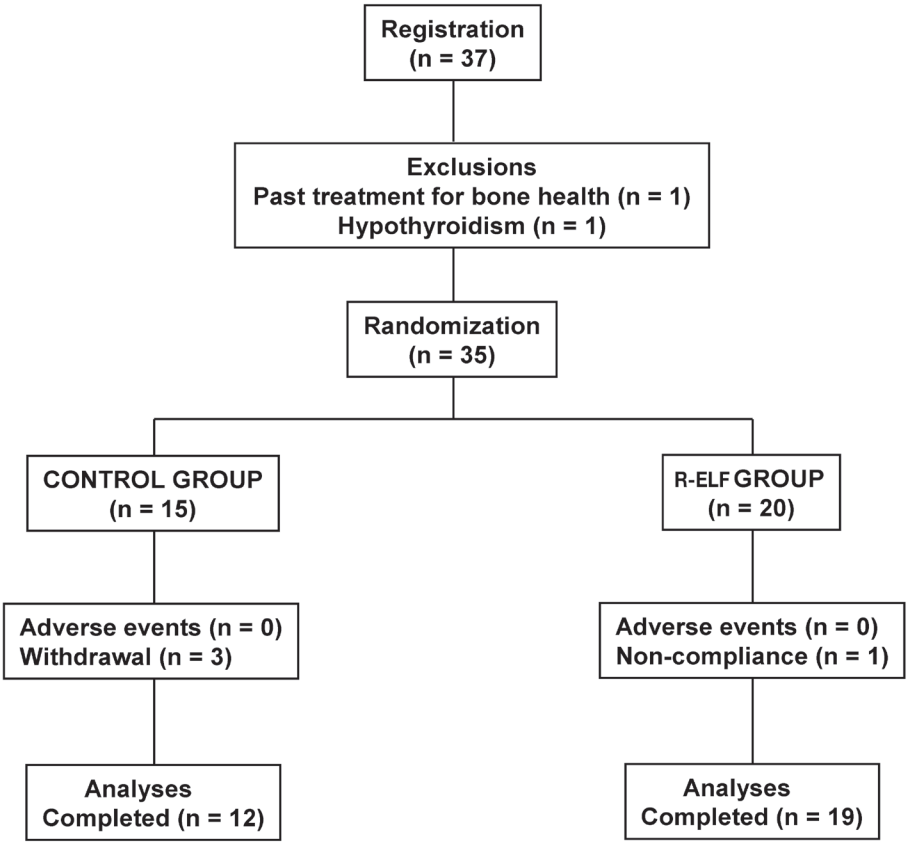
23. De Martinis M, et al (2005). Inflammation-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. *FEBS Lett* 579: 2035-9.
24. Bruunsgaard H (2006) The clinical impact of systemic low-level inflammation in elderly populations. With special reference to cardiovascular disease, dementia and mortality. *Dan Med Bull* 53: 285-309.
25. Koh JM, et al (2005) Higher circulating hsCRP levels are associated with lower bone mineral density in healthy pre- and postmenopausal women: evidence for a link between systemic inflammation and osteoporosis. *Osteoporos Int* 89: 735-42.

Study Design



R-ELF is a ribonuclease (angiogenin)-enriched LF either co-isolated from bovine milk (50:50 ratio wt/wt) or both proteins admixed to obtain required ratios [US Patents: 7601689 & 8003603]. Thirty-five women included in the study were randomly assigned to one of the two groups: placebo group or R-ELF group. Fifteen subjects assigned to the placebo group were supplemented with 100% RDA (Recommended Daily Allowance) of calcium, in a tablet form. Whereas 20 subjects of the R-ELF group were given with two R-ELF capsules of 125 mg each, along with 100% RDA of calcium administered orally from Day-1 to Day-180. Venous blood samples were collected, by standard venipuncture technique from each subject on Day-0 (baseline before starting the supplement), Day-30, Day-90 and Day-180 of the study.

Inflammatory cytokines and markers: The levels of inflammatory cytokines – IFN-γ, IL-6, IL-12+p40, TNF-α, IL-1ß, IL-10 and TGF-ß were determined by enzyme-linked immunosorbent assay (ELISA) using respective monoclonal antibodies. High purity C-Reactive Protein (CRP) and anti-CRP rabbit polyclonal antibody were obtained from Calbiochem/EMD (San Diego, CA) and anti-RANKL rabbit polyclonal antibody from Abcam Inc. (Cambridge, MA). CRP assay had a sensitivity of 8 pg/mL and detection limit of 1 ng/mL.

Statistical analysis: Cytokine data from each day was analyzed for measures of central tendency, deviation and distribution of data. Data were considered outliers, if they were >1.5 times the inter-quartile range (IQR) above the third quartile or below the first quartile. Outliers were discarded from datasets for statistical tests of significance. In view of the small size of placebo and R-ELF groups, median and standard error of the mean were used as the preferred measures of central tendency for this modest set of data. The Kolmogorov-Smirnov test (KS test) was used as a test for normal distribution of data within each set. Student's unpaired two-sample t-test was used for comparison of the mean observed change in markers for placebo and R-ELF data sets to establish the effect of R-ELF supplementation. OriginPro Ver. 8 (OriginLab, MA USA) software was used for data analysis.

Pro-Inflammatory Cytokine Levels Improved with R-ELF Intake

The median IFN-γ ranged from 14.4 ± 1.7 to 18.3 ± 2.9 pg/mL for the placebo group while the values increased from 28.6 ± 13.7 to 32.4 ± 12.8 pg/mL for the R-ELF group and the difference between them was statistically significant (P = 0.0007 for median IFN-γ, placebo vs. R-ELF). IFN-γ levels for the R-ELF group decreased (-6.6%) within thirty days but increased considerably (+42%) for the placebo. By the end of study, although IFN-γ levels for the R-ELF group increased (+13.5%), the increment was smaller compared to the placebo (+27%) *(Figure 6A)*. Median IL-1ß levels ranged from 10.2 ± 3.8 to 15.0 ± 10.2 pg/mL for the placebo and from 22.6 ± 10.9 pg/mL to 23.3 ± 19.9 pg/mL for the R-ELF group (P = 0.0131 for median IL-1ß, placebo vs. R-ELF).

A large decrease in IL-1ß levels was observed initially but the trend is reversed by Day-90 and turned out significantly positive (+46.5%) for the placebo, while it remained close to the baseline (+2.8%) for the R-ELF group *(Figure 6B*).

TNF-α levels ranged from 239.3 ± 15.8 on Day-0 to 238.3 ± 69.5 pg/mL for placebo, while a decrease from 279.7 ± 31.0 to 251.4 ± 43.2 pg/mL (P = 0.0341 for median TNF-α, placebo vs. R-ELF) was observed with R-ELF supplementation. A large initial decline in TNF-α levels (-27.4% for R-ELF vs. -15.5% for placebo) was sustained until Day-180 for the R-ELF group (-10.1%), but not with the placebo (-0.4%) *(Figure 6C)*.

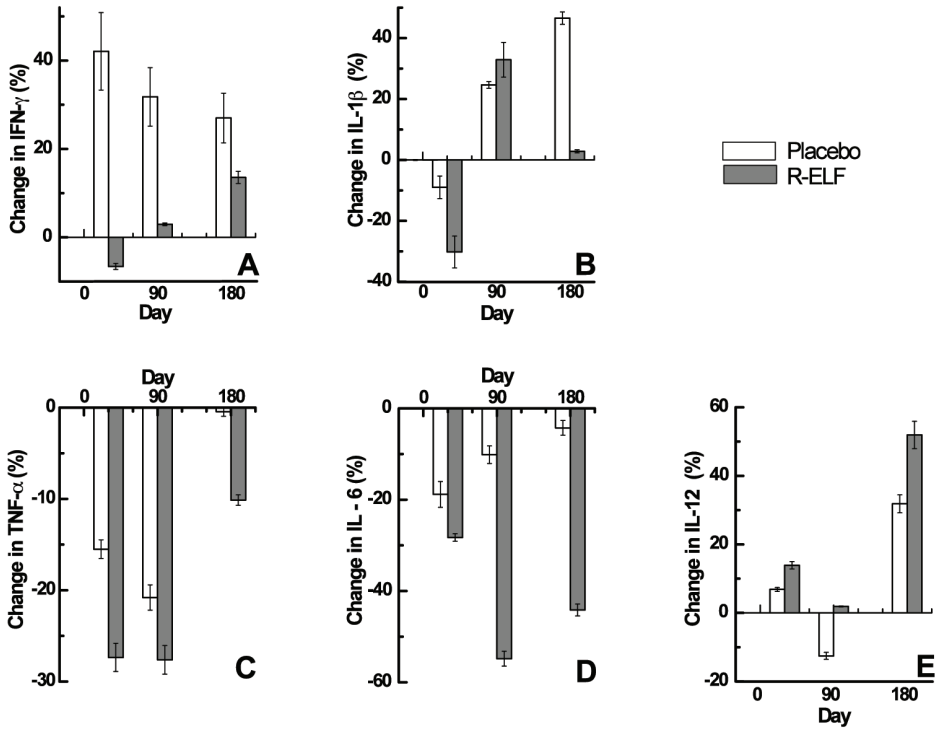
Furthermore, median IL-6 levels slightly decreased for the placebo from 2.1 ± 1.5 on Day-0 to 2.0 ± 2.1 pg/mL by the end of study, while IL-6 decreased from 5.5 ± 3.9 to 3.0 ± 4.1 pg/mL for the R-ELF group. As shown in *Figure 6D*, by the end of study, the decrease in IL-6 was significant (P = 0.0338 for median IL-6, placebo vs. R-ELF) for the R-ELF group (-44.1%) compared to the placebo (-4.3%).

Levels of IL-12+p40 (also referred to as IL-12) were similar for both groups; placebo ranging from 68.5 ± 1.6 to 90.2 ± 1.6 pg/mL and R-ELF group from 73.1 ± 4.0 to a slightly higher 111.1 ± 5.6 pg/mL. R-ELF supplementation significantly raised IL-12 levels by 51.9% by Day-180 compared to placebo, with an increase of 31.8% from their respective baseline levels (P < 0.0005 for mean IL-12, placebo vs. R-ELF) *(Figure 6E)*.

These results demonstrate that supplementation of R-ELF induced down-regulation of pro-inflammatory TNF-α and IL-6, and a moderate rise in IL-1ß and IFN-γ levels within 6 months.

FIGURE-6

Effect of R-ELF on Pro-Inflammatory Cytokines.
Median change in the serum levels of pro-inflammatory cytokines – IFN-γ (panel A), IL-1ß (panel B), TNFα (panel C), IL-6 (panel D) and IL-12 (panel E) – in subjects from placebo (open bars) and R-ELF (filled bars) supplemented groups.

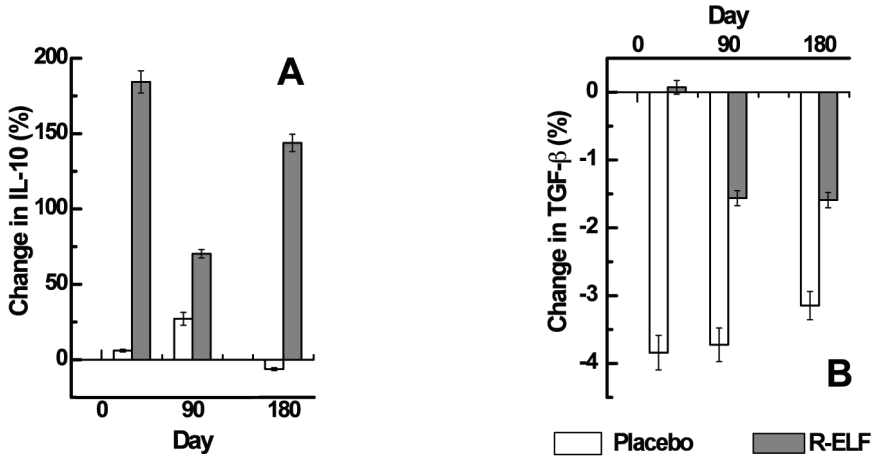


R-ELF: Effects on Anti-Inflammatory Cytokines

Levels of IL-10 for the placebo were close to the baseline median value of 17.4 ± 2.7, until Day-180 (16.3 ± 3.6 pg/mL), while the levels increased from 7.7 ± 9.2 to 18.8 ± 4.2 pg/mL for the R-ELF group. A major positive change was observed in IL-10 with R-ELF supplementation (+150%) compared to placebo (-6.2%) (P = 0.0235 for change in median IL-10, placebo vs. R-ELF) by the end of study *(Figure 7A)*.

Median TGF-ß levels slightly decreased for both the groups, 786.3 ± 73.1 to 761.5 ± 85.1 pg/mL for the placebo, compared to 739.4 ± 58.9 to 727.6 ± 51.9 pg/mL for the R-ELF (P = 0.0072 for median TGF-ß, placebo vs. R-ELF). This decline was smaller for the R-ELF group (-2%) than the placebo (-3%) *(Figure 7B)*.

FIGURE-7: ***Variations in Serum Levels of Anti-Inflammatory Cytokines with R-ELF supplementation.*** *Median change in the serum levels of anti-inflammatory cytokines – IL-10 (panel A) and TGF-ß1 (panel B) in subjects from placebo (open bars) and R-ELF (filled bars) supplemented groups.*



These results indicate a relatively positive change in anti-inflammatory cytokines with R-ELF supplementation, compared to calcium supplementation alone. The correlation between pro- and anti-inflammatory cytokines was analyzed and the results are presented in *Table 3*.

TABLE-3: *Correlation between Pro-inflammatory and Anti-inflammatory Cytokines*

Cytokine	Day-0		Day-180	
	IL-10	TGF-ß	IL-10	TGF-ß
PLACEBO Group				
IFN-γ	0.89 (<0.005)	nc*	0.97 (<0.0005)	nc
IL-6	0.79 (0.05)	nc	0.97 (<0.0005)	nc
TNF-α	nc	nc	0.86 (0.05)	nc
IL-1ß	0.67[a] (0.066)	nc	0.98 (<0.0005)	nc
IL-12	nc	nc	nc	nc
R-ELF Group				
IFN-γ	nc	nc	0.95 (<0.005)	nc
IL-6	0.82[a] (0.086)	-0.41[b] (0.271)	0.92 (<0.05)	-0.50 [b] (0.167)
TNF-α	nc	nc	nc	nc
IL-1ß	nc	nc	nc	nc
IL-12	nc	nc	nc	nc

* nc – No correlation observed
[a] 90% CI,
[b] Not statistically significant, all other correlations are significant to 95% CI

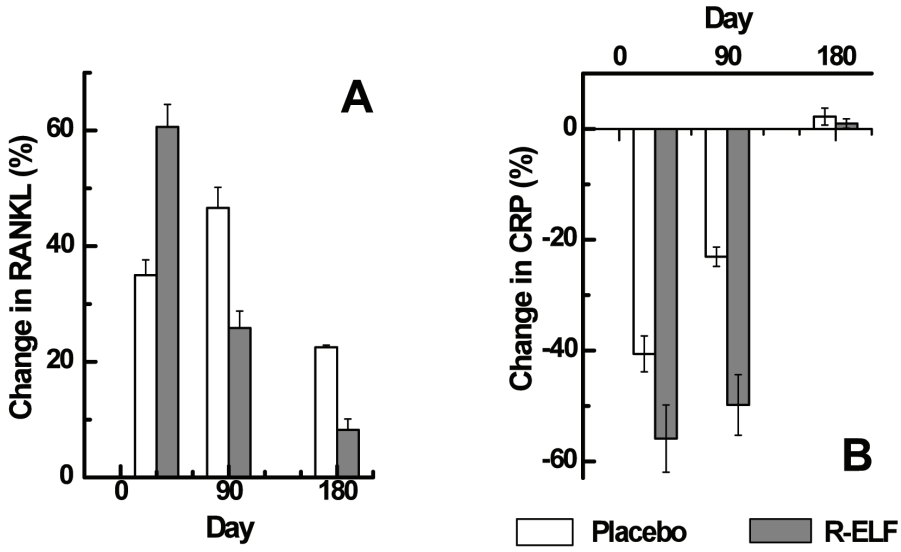
The correlation matrix for placebo and R-ELF groups are generally maintained from Day-0 to Day-180 and improved with R-ELF supplementation. Most cytokine correlations also remained statistically significant at 95% confidence interval. The correlation of IL-6 versus IL-10 improved with R-ELF supplementation. In general, TNF-α, IL-12+p40 and TGF-ß did not show any correlation, neither with the progress of study nor with supplementation. IL-10 was highly correlated with IFN-γ and IL-6 at the beginning of the study for both groups, which improved by the end of the study. Interestingly, IFN-γ was not correlated with IL-10 at baseline but demonstrated a high degree of correlation after R-ELF supplementation. These positive correlations suggested a moderate elevation of anti-inflammatory cytokines that could reduce pro-inflammatory response. Inverse correlation is a favorable outcome of reduction in pro-inflammatory and elevation of anti-inflammatory cytokines, in which could diminish inflammation. An improved negative correlation was observed between IL-6 and TGF-ß with R-ELF supplementation, although not statistically significant

R-ELF Reduced the Levels of CRP and RANKL

RANKL is a well established marker for the onset of osteoporosis in postmenopausal women. RANKL, a mediator of osteoclast formation, indicated a sharp rise by Day-30 followed by a steady decline for both placebo and R-ELF groups (*Figure 8A*). However, in response to R-ELF supplementation there was a larger and rapid decline by -34.8% (60.6% on Day-30 to 25.8 on Day-90) compared to 11.6% increase (35.0% on Day-30 to 46.6 on Day-90) with calcium supplementation alone. By the end of the study, RANKL levels were further decreased to 8.2% for R-ELF group and remained at 22.5% for placebo (P = 0.0202 for median change in RANKL, placebo vs. R-ELF).

CRP levels showed a sharp decrease for both the groups by Day-30, but the trend reversed and CRP levels increased to baseline by the end of the study (*Figure 8B*). A relatively large reduction in CRP levels was observed for R-ELF group (-55.9% by Day-30), which sustained for a long time (-49.8% by Day-90) (P = 0.0286 for mean change in CRP, placebo vs. R-ELF). On the other hand, placebo group regained CRP levels rapidly, -40.6 to -23.1% of its baseline CRP level in sixty days. These results suggest an improvement in the inflammatory status and reduced bone resorption in postmenopausal women with R-ELF supplementation.

FIGURE-8: ***Changes in Serum Levels of RANKL and CRP with R-ELF Supplementation.*** *Median change in the serum levels of RANKL, a marker of osteoclast formation (panel A) and CRP, an inflammatory marker (panel B) in subjects from placebo (open bars) and R-ELF (filled bars) groups.*



R-ELF was shown to regulate both pro-inflammatory and anti-inflammatory responses – critical for fracture healing and optimal joint function.

R-ELF TECHNOLOGY GRID

Patents and Intellectual Properties (IP)

The R-ELF Technology Grid is landscaped on THREE levels of Intellectual Property (IP). Since, R-ELF is based on lactoferrin (LF) and ribonuclease (RNase), two glycoprotein fractions from milk, their purity and structure/function-related attributes define the potential bio-activity of the final R-ELF complex.

Level-1: *Ultra-Pure Lactoferrin (LF-TCR) Technology:* Due to microbial and endotoxin contaminants, most commercial LF preparations are not suitable for making protein complexes. It is also important to ensure that LF protein is not denatured and the bio-burden or other chemical impurities do not interfere in the LF-RNase interaction to form stable complexes. To circumvent these issues, LF-(TCR) was developed using a novel decontamination technology consisting of food-grade surfactants, antioxidants and polyphenols. The compounds utilized in the multi-tier TCR process are also known to provide additional nutraceutical benefits. The multi-functional in vitro performance of LF-(TCR) is summarized in 2 US patents (7125963 &7326775). In conclusion, LF-(TCR) is a functionally enhanced ultra-pure lactose-free milk protein produced by an innovative protein engineering technology.

Level-2: *R-ELF (or ANGex) Technology:* Canine bone physiology, especially fracture healing, requires an amplified inflammatory response and an enhanced bone turnover for callus formation as well as bone reunion. The LF protein is shown to support these functions. Formation of new capillaries and revascularization of the bone matrix is essential for regaining total bone function; since, bone is the central site for synthesis and transport of blood cells. RNase-mediated angiogenesis could help reach this functional target. R-ELF technology was developed [US Patents 7601689 & 8003603] to immobilize RNase on milk lactoferrin substrate via non-covalent interactions and conjugation methods including biotin-avidin affinity binding and disulfide bonding techniques.

Level-3: *Neo-PORTIN Technology:* Bone fracture healing is an energy consuming process with a high metabolic demand. The activation of immune cells via inflammatory response and elevated bone turnover needs ATP input. Furthermore, target delivery of minerals and cellular building blocks requires transport mechanisms and target delivery systems. A combination of CoQ10 (ubiquinone for ATP synthesis) and LF (the innate metal-binding transport protein with special cellular receptors) complexed with RNase may help these cellular energy-dependant functions. A technology to trigger and release bio-energy (US Patent 7956031) and an R-ELF/CoQ10 composition for target transport (US Patent 8021659) has been developed to promote canine bone health.

R-ELF TECHNOLOGY GRID

Ultra-pure Lactoferrin (LF-TCR) Technology

US Patent: 7125963
Issued: Oct 24, 2006


The Director of the United States Patent and Trademark Office

Has received an application for a patent for a new and useful invention. The title and description of the invention are enclosed. The requirements of law have been complied with, and it has been determined that a patent on the invention shall be granted under the law.

Therefore, this

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US Patent: 7326775
Issued: Feb 05, 2008


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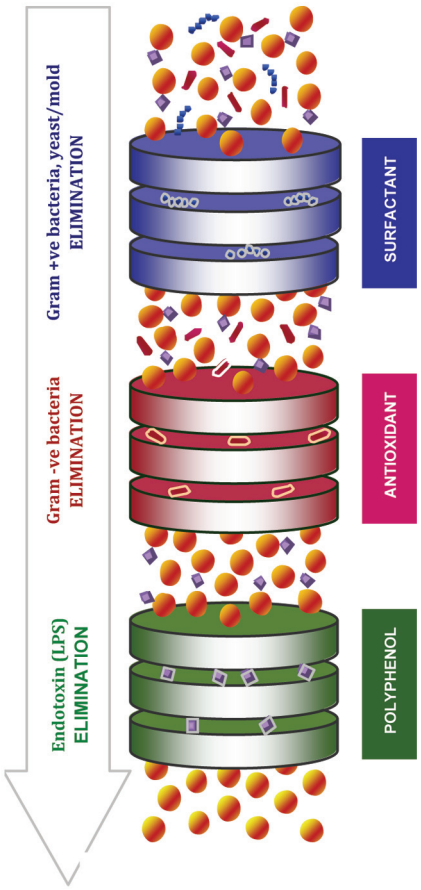
Treatments for contaminant reduction in lactoferrin preparations and lactoferrin containing compositions

A method of preparing an ultra-cleansed lactoferrin preparation, termed treatment for contaminant reduction (TCR) is provided which includes the steps of treating commercial lactoferrin preparations with at least one each of surfactants, antioxidants and polyphenols to form purified lactoferrin (LF-TCR) and drying the LF-TCR. Additionally a therapeutic lactoferrin composition is provided which contains LF-TCR and optionally surfactants, antioxidants, polyphenols, tissue/membrane diffusion facilitating agents and anionic compounds. The therapeutic lactoferrin composition can additionally contain bioactive agents, dietary supplements, nutraceuticals/functional foods, prophylactic agents, therapeutic agents and probiotic lactic acid bacteria.

Several lactoferrin (LF)-based dietary supplements are currently available in health food markets worldwide. A majority of such products are derived from partially isolated (enriched) LF fractions from colostrum or whey concentrates. Bulk isolation of LF directly from milk is limited and relatively an expensive process. High purity LF preparations commercially exist; however, products from such protein materials are cost-prohibitive and fall short of consumer acceptance without valid functional assurance. The nutraceutical benefits of LF as a dietary supplement for human or animal health application requires an innovative technology compatible with large-scale manufacturing practices. Such technology transfer should ensure the highest standards of product safety, quality assurance and delivery of an optimal dosage for an effective functional outcome. Furthermore, the microbiological and toxicological quality issues compromise the in vivo performance standards of LF as a potent dietary supplement. The following QA/QC issues are critical for the development of LF as a canine bone health supplement.

LF-(TCR) is an ultra-purified, activated milk glycoprotein, which is functionally superior than most commercial LF (from milk, whey or other sources). It takes four liters of bovine milk to produce a single 80 mg dose of ultra-pure LF.

FIGURE-9



Lactoferrin-(TCR)

Based on the types and levels of contaminants, as well as the microbiological quality assurances implemented with cGMPs in the commercial-scale manufacturing of LF, a multi-tier TCR process has been developed using natural substrates as decontaminant agents (*Figure-9*). This patented process extends the scope of TCR process in combination with synergistic compositions, to enhance multifunctionality of LF. LF-(TCR) is a powerful physiological system to complex or transport other bioactive compounds. The TCR process could be used as a stand-alone technology or integrated with different lab-scale, pilot-scale or commercial-scale technologies practiced in the isolation and purification of LF.

The TCR process includes the creation of a surfactant tier analogous to the physiological gastric detergents, which selectively disrupt the cell membranes of contaminant microbes. Natural and/or food-grade surfactants for use in the present invention include plant-derived saponins, food-grade polysorbates and bile salts. This tier also utilizes carbonate or bicarbonate anions at specific ratios in combination with natural antioxidants such as vitamins A, C or E to enhance the anion-dependent LF bioactivity. For the purpose of restoring the anion-dependent bioactivity of commercial LF, methods related to generating carbonated aqueous systems or anaerobic encapsulations are also suitable. Finally, the ultra-cleansing TCR process uses effective and permissible amounts of food-grade polyphenols to neutralize endotoxin contaminants in commercial LF preparations. Polyphenols of particular use for this purpose include oleoresins, aquaresins, oleuropeins, terpenes, flavonoids and biliproteins.

TABLE-4: *Enhanced Multi-functional Performance of LF-(TCR)*

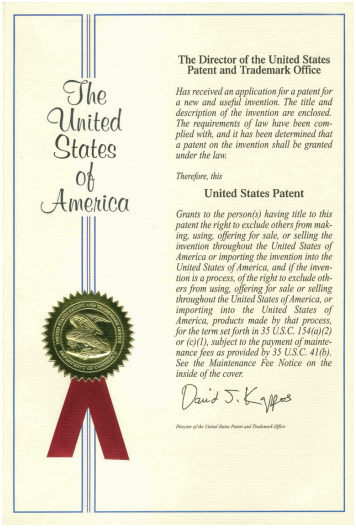
FUNCTIONAL ACTIVITY	LF (Whey)	LF (Milk)	*LF-(TCR)*
ANTIOXIDANT ACTIVITY			
Total Antioxidant Status	<1.0 µ.mol/mg	5.3 µ.mol/mg	**72.9 µM/mg**
FRAP Value (at 24-h)	NT	583 mM	**994 mM**
BACTERIOSTASIS ACTIVITY			
Escherichia coli	15.4-h	26.2-h	**>48-h**
Salmonella Typhimurium	12.5-h	22.9-h	**>48-h**
PREBIOTIC ACTIVITY			
Lactobacillus spp. (n=13)	96%	147%	**200%**
Bifidobacterium spp. (n=3)	110%	146%	**213%**
Lactococcus lactis	105%	120%	**186%**
Streptococcus thermophilus	92%	116%	**192%**

The multi-functional in vitro performance of LF-(TCR) is summarized in Table-4. In conclusion, LF-(TCR) is a functionally enhanced bio-active molecule produced by an innovative patented protein engineering technology [US Patents 7125963 & 7326775].

R-ELF TECHNOLOGY GRID

R-ELF (or ANGexTechnology

US Patent: 7601689
Issued: Oct 13, 2009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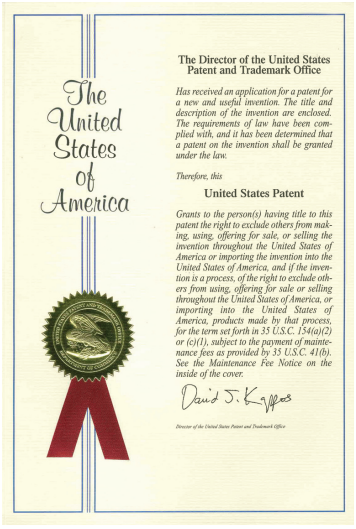
US Patent: 8003603
Issued: Aug 23, 2011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Angiogenin complexes (ANGex) and uses thereof.

Stabilized angiogenin compositions and methods of preparing a stabilized ANG compositions by non-covalent immobilization on a naturally occurring substrate, such as a protein, lipid, nucleic acid or nucleotide substrate, are disclosed.

R-ELF complexes are formed with RNase (Angiogenin or ANG) and a second protein, such as lactoferrin (LF), based on functional association or a synergy that may enhance the biological function of the complex. R-ELF protein complexes can be formed by physical, charge and/or chemical interactions. RNase (ANG) and a protein substrate (such as lactoferrin, LF) may be complexed together directly or they may be complexed by means of an appropriate bi-functional reagent. A non-covalent complex may be formed by means of electrostatic interactions which may be enhanced by inclusion of appropriate buffers and/or salts.

The formation of a R-ELF complex may be confirmed by using co-immuno-precipitation techniques. In preferred embodiments, the protein substrate is from the group including, but not limited to transport proteins, subepithelial matrix proteins and antimicrobial proteins. Transport proteins include but are not limited to lactoferrin, transferrin, ovo-transferrin (conalbumin), ceruloplasmin, metallo-thionein and transfer factors. Subepithelial matrix proteins include but are not limited to fibronectin, fibrinogen, laminin, vitronectin, osteopontin, native collagens and denatured collagen (gelatin). Antimicrobial proteins include but are not limited to peroxidases (lacto, myelo and salivary forms) and lysozyme.

Both LF and RNase co-exist in milk and other biological fluids as key regulatory molecules with specific functions in various physiological pathways. From a bone metabolism standpoint, both proteins elicit potent effects individually as anabolic and anti-resorptive agents during aging process, disease and repair conditions. For the first time, a synergistic effect of these two bio-active molecules mixed together as an R-ELF complex to advance bone health was discovered and patented.

R-ELF complexes (or ANGex) have higher protein stability and have been shown to play a multi-functional role in bone regeneration, joint lubrication, bone turnover and fracture repair.

FIGURE-10

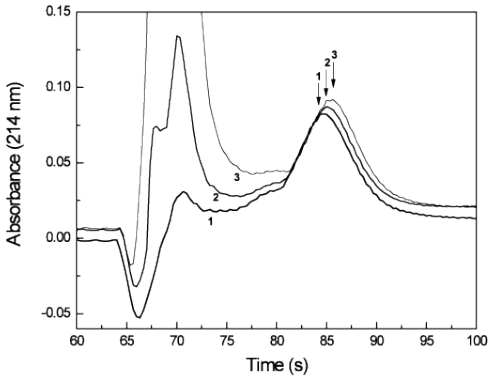


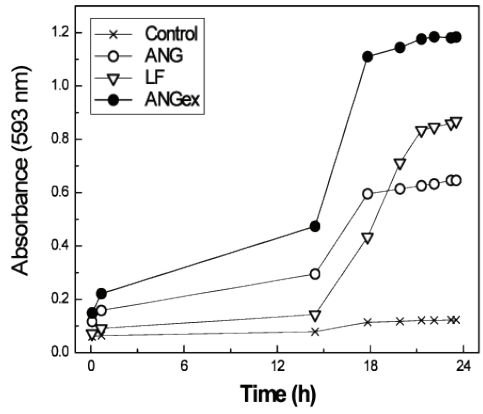
Figure-10: ***ANGex Detection by Chromatography:*** *Cation-exchange chromatogram is shown for the separation of ANGex from free ANG, using NaCl gradient of 20-100% B in 20 min. Free, native LF (1 mg/mL) elutes as a single peak at retention time of 84.6 min (Curve 1). The retention time of this peak increases upon ANGex formation with 1 mg/mL (Curve 2) and 4 mg/mL (Curve 3) of ANG and LF (1 mg/mL) in solution. Elution of ANGex was monitored by the absorbance at 214 nm using a UV-vis detector.*

The structure-function enhancement of R-ELF complex was initially evaluated for antioxidant activity by kinetic ferric reducing/antioxidant power (FRAP) assay [26]. The antioxidant efficiency (AE) was measured as change in absorbance with the concentration of R-ELF formed at different RNase:LF ratios. When individually tested, free RNase showed an AE value of 0.6 Abs/mM. However, when mixed with 25 μM of LF, the AE value increased to 0.7, indicating a synergistic potentiation in the antioxidant power. After 24-h, the maximum AE value attained individually for RNase and LF were 0.64 and 0.87, respectively; however, after the two proteins were mixed, the resulting R-ELF complex exhibited an AE value of 1.18, which is a 35% and 84% increase from that of LF and RNase, respectively.

Specific ratios and molecular stoichiometry between LF and RNase are critical in the functional design of the R-ELF complex [US Patents 7601689 & 8003603]. The synergistic potentiation in antioxidant capacity of the R-ELF complex indicated a re-arrangement of molecular structure-function of the two regulatory proteins. Further in vitro studies also revealed a synergistic enhancement in antimicrobial and enzymic activities of R-ELF complex. The molecular and functional design (interplay) of these two key regulatory proteins in the R-ELF complex has been established in randomized placebo-controlled human clinical trials.

26. Benzie IFF, Strain JJ (1999) Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymol (Oxidants and Antioxidants Part A)* 299:15-27.

FIGURE-11



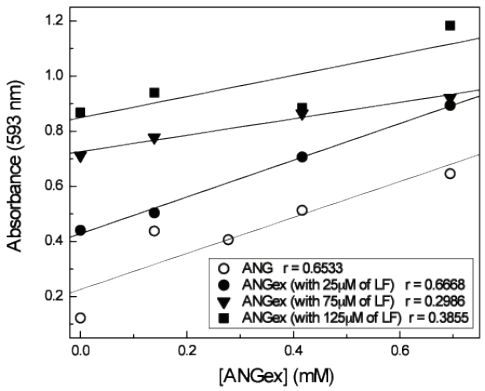
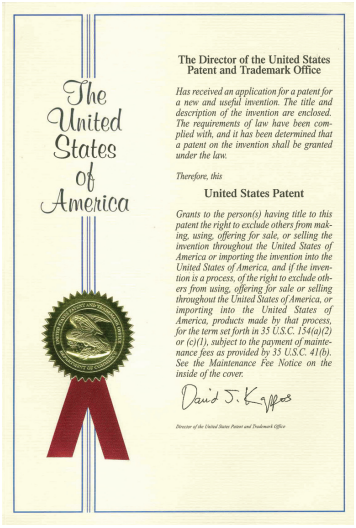


Figure-11: ***Antioxidant Activity of ANG, LF and ANGex:*** *Antioxidant activity was determined by FRAP assay with Vitamin C, TROLOX and FeSO4 as standards, by following the increase in absorbance at 593 nm with time. Top panel shows the FRAP reaction kinetics data for LF, ANG and ANGex. . The concentrations of the three standards, LF, ANG and ANGex were 10 mg/ml (125 μM for LF, 694 μM for ANG). Bottom panel compares the antioxidant efficiency (r) as measured by the change in absorbance with the concentration of ANGex. Lines represent ANGex formed at varying concentrations of ANG with 0 (○), 2 (●), 6 (▼) and 10 mg/mL (■) of LF. ANGex formed with 2 mg/mL of LF exhibits the highest antioxidant efficiency with r = 0.046.*

R-ELF TECHNOLOGY GRID

R-ELF and Neo-PORTIN Technology

US Patent: 7956031
Issued: Jun 07, 2011


The United States of America

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David J. Kappos

Director of the United States Patent and Trademark Office

Metallo-lactoferrin-coenzyme compositions for trigger and release of bioenergy

Formulations are provided for the trigger and release of bioenergy. The formulations generally include a trigger complex, an elemental complex and a coenzyme-vitamin B complex. The trigger complex is high in fiber and includes at least one metal-binding protein in an alkaline buffer system. The elemental complex includes one or more trace element as a suitable salt. The coenzyme-vitamin B complex includes one or more coenzyme, coenzyme precursor and/or B-vitamin. The compositions can be administered orally in a variety of forms.

This invention describes methods to prepare specific combinations of LF-(TCR)-CoQ10 mixtures to trigger the release of bio-energy (bio-E) in the form of adenosine triphosphate (ATP). Additionally the invention discloses compositions of functional delivery systems to recreate physiological proton gradients for rapid activation and release of cellular and extracellular ATP.

Coenzyme Q10 (CoQ-10) is a fundamental molecule for production of cellular energy in most living organisms. Although found in all human cells, CoQ-10 occurs at relatively elevated concentrations in bone cells with high energy requirements such as osteoblasts, osteoclasts and chondrocytes. The total body content of CoQ-10 has been estimated at 0.5-1.5 g. Normal blood levels range from 0.7-1.0 µg/mL. [Folkers 1996].

Fracture healing is an intensive metabolic process with high energy (ATP) consumption. This CoQ10/Lactoferrin based technology supports canine fracture healing by providing bone cells and inflammatory cell mediators with direct ATP release.

A vital role in the production of cellular energy combined with its potent antioxidant activity makes CoQ-10 an essential bone health supplement. Furthermore, its multi-functional properties including vitamin-like adjuvant activity, protection against age-related degeneration, support of homeostasis, prophylactic and therapeutic effects against several diseases, makes CoQ-10 an important nutraceutical agent.

Embodiments of the invention provide formulations that provide effective assimilation of CoQ-10 while supporting angiogenesis and vascular generation, particularly in bone fracture healing.

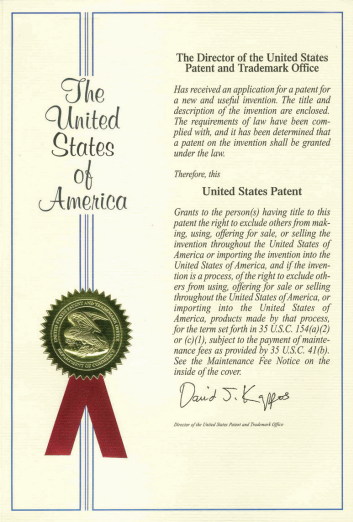
27. Folkers K (1996) Relevance of the biosynthesis of coenzyme Q10 and the four bases of DNA as a rationale for the molecular causes of cancer and a therapy. *Biochem Biophys Res Commun* 224:358-61.

Neo-PORTIN with CoQ10 helps support the metabolic energy demand of bone cells during bone turnover and fracture healing.

Coenzyme Q10, lactoferrin and angiogenin compositions and uses thereof

Methods of enhancing the bio-availability of coenzyme Q10, and methods of supporting the cardiovascular system to accommodate the increase in cellular energy synthesis as a result of the bio-availability of coenzyme Q10 are described. Compositions which include coenzyme Q10, lactoferrin and/or angiogenin are described for use in the related methods, for multi-functional health applications

US Patent: 8021659
Issued: Sep 20, 2011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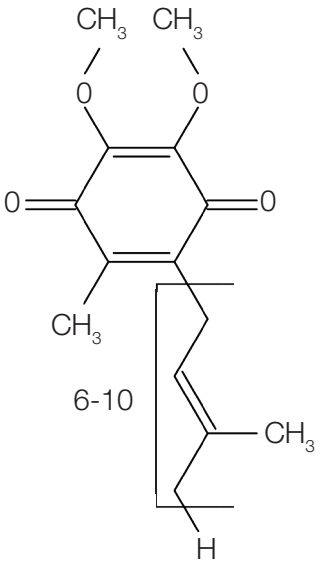
Bio-availability of bone minerals; receptor-mediated endocytic release of cations, anions and GAG-molecules such as chondroitin, glucosamine and hyaluronic acid require energy (ATP)-dependant active transport in vivo. This physiological function could be achieved with R-ELF complex (with calibrated LF:RNase wt/wt ratios from 50:1 to 9:1) admixed with CoQ-10.

R-ELF could perform an efficient target delivery function with specific transport mechanism(s) in vivo. There are three forms of LF proteins based on iron saturation status: apo-LF (iron-free), mono-ferric form (one ferric ion), and holo-LF (binds two Fe^{3+} ions). The ability to bind ferric iron with high affinity (KD approximately 10-20 M) and retain it at low pH gives LF its antioxidant and antimicrobial properties. The ability to keep iron bound at low pH has in vivo significance, especially at sites of inflammation where the pH drops below 4.5 due to neutrophil degranulation.

Besides iron, LF is capable of binding a large amount of other compounds and substances such as glycosaminoglycans (GAGs; chondroitin, glucosamine, hyaluronic acid), DNA, or other metal ions including manganese (Mn^{3+}), cobalt (Co^{3+}), copper (Cu^{2+}), zinc (Zn^{2+}) etc. Apart from CO_3^{2-}, LF can also bind a variety of other anions such as oxalates, carboxylates, and others. Accordingly, LF could affect bone metabolism with transport and distribution of various minerals, anionic and cationic substances into the extra-cellular matrix (ECM). R-ELF, with its mineral transport activity, may help promote calcium deposition in the bone. Osteoblast-mediated Ca(II) deposition in the ECM is a critical step in bone tissue generation.



Co-enzyme Q10

Neo-PORTIN with metal-transport protein – LF-(TCR), provides an efficient target delivery system for minerals and nutrients that are essential for bone physiology.

R-ELF COMPLIANCE AND SAFETY

R-ELF: Regulatory Compliance, QA/QC and Safety

Lactoferrin (LF) is a bio-active milk protein – therefore, the source of the dairy plays a critical role in the large-scale production of LF for nutraceutical applications. The LF used in the R-ELF and Neo-PORTIN complexes is isolated from cow's milk certified by the New Zealand Food Safety Authority (NZFSA). The operation of farm dairies, manufacture, storage and transport of dairy products is approved by the New Zealand Ministry of Agriculture and Forestry (MAF), following a stringent 'Bio-Security Monitoring Program'.

The dairy milk is processed in an ISO 9001:2000 facility that meets the requirements set out in the internationally recognized standard. LF is isolated from fresh milk through patented serial fractionation and chromatographic techniques, to remove milk sugars (i.e. lactose), milk fats and other milk proteins. Most commercially available LF is obtained through high-temperature spray-drying process, which can denature heat-sensitive LF protein. In contrast, LF-(TCR) utilizes a patented freeze-drying technique that preserves LF protein structure and function.

Purified milk LF is subjected to a series of QA/QC tests to ensure the quality of the final protein product. These tests include Chemical Analysis for protein content, levels of heavy metals (if any), water activity, etc.; Microbiological Analysis for total bio-burden, coliform counts, etc. The LF protein is also subjected to functional tests such as iron-binding activity prior to QA/QC release. LF base material is further tested for functional properties such as antioxidant, antimicrobial and prebiotic activities (see LF-TCR patents) before integrating into R-ELF (ANGex) or Neo-PORTIN complexes.

MAF BIOSECURITY NEW ZEALAND

Kosher Certified
GMO-Certified
Lactose-Free
Hormone-Free
Antibiotic-Free

In the **United States**, milk derived LF has been granted GRAS (Generally Recognized As Safe) status by the U.S. Food and Drug Administration (US-FDA) under 21-CFR.179.36(f).

In the **European Union**, milk-derived LF is permitted by the European Food Safety Authority (EFSA) under EU Council Directive 83/417/EEC. The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) endorsed LF with no safety issues in a range of matrices including food supplements, infant formulas, dietetic food for special medical purposes, and sports nutrition. After evaluation of animal and in vitro data, the NDA found no safety issues at proposed intakes ranging from 667 mg/100 g of baby foods and foods intended for children aged 1-3 years to 4000 mg/100 g for energy bars for sportsmen and women. In reaching this decision, the NDA Panel considered that LF up to the highest dose (2000-mg/kg bw per day) tested in the sub-chronic rat study did not show adverse effects.

In **Japan**, LF is specified in the 'List of Existing Food Additives', which is a list of the permitted natural additives set by the Japanese Ministry of Health, Labor and Welfare (JMHLW). In **South Korea**, LF concentrates are listed as 'Authorized Natural Additives. In **Taiwan**, LF may be used in special nutritional foods under the following condition – only for supplementing foods with an insufficient nutritional content and may be used in appropriate amounts according to actual requirements.

LF is considered safe and has been approved worldwide for various human and animal health applications.

ABOUT US

N-terminus (a division of en-N-tech, Inc) is a pioneer in the research of bio-active molecules and a global leader in successful technology transfers that impact areas of dietary supplements, food safety, veterinary and human medicine.

N-terminus is internationally recognized for its cutting-edge research and intellectual properties on protective and therapeutic aspects of 'natural' bioactive molecules. N-terminus applies its scientific expertise in the discovery and development of novel technologies in veterinary and human health.

N-terminus is committed to establish and maintain the reputation of highest quality and reliability with health technologies that it offers and to advance the field applications of this emerging multifunctional molecule through education, research and publication.

N-terminus was started in the year 2000 and employs a team of professors and medical scientists from the fields of microbiology, immunology, biochemistry, and physiology. Since its formation, this researc facility has been playing an active role in educating the world about the research and health implications of natural bio-active molecules. Based in Southern California, N-terminus collaborates with several academic and corporate research institutions worldwide.

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