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A method to induce Interleukin-1 Receptor Antagonist Protein from autologous whole blood

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ABSTRACT

Objective: Current orthopedic therapies, aimed solely at symptomatic control, are unable to restore the cytokine imbalance that produces the hallmark clinical profile of osteoarthritis. While a myriad of chemical factors in the cytokine network stimulate local joint inflammation and pain, Interleukin 1 (IL-1) is widely recognized as a key offender and a potential therapeutic target. The purpose of this article is to describe a novel, on-site, point of service process (Arthrokinex™) to induce Interleukin 1 Receptor Antagonist Protein (IL-1-Ra or IRAP) from whole blood aimed at inhibiting the destructive intra-articular effects of IL-1.

Methods: 53 patient charts were included in this retrospective chart review study. Venous blood from the selected participants had been harvested and centrifuged to isolate Platelet Rich Plasma and Platelet Poor Plasma. These layers were extracted and incubated for 30 min in a specialized syringe containing medical grade concentrator beads. After centrifuge filtration, the supernatant containing IL-1-Ra was extracted. Anti-inflammatory (IL-1-Ra, IL-10) and pro-inflammatory (TNF α, IL-1 β) cytokines of baseline whole blood were compared to the conditioned serum following quantification using ELISA.

Results: On average, a 32-fold increase (baseline, 550 pg/mL; post conditioning 17,537 pg/mL) in IL-1-Ra concentration was observed after the brief interaction of blood with the concentrator bead surface. IL-1-Ra, if present in concentrations that are 10–100 times higher than IL-1β, will block the interaction of IL-1β with cell surface receptors. At these increased concentrations, Arthrokinex™ induced IL-1-Ra joint injections produce an IL-1-Ra to IL-1β ratio of 999:1. Post conditioning levels of IL-1β and TNF α were not clinically significant.

Conclusion: The Arthrokinex™ blood conditioning process has the ability to rapidly induce IL-1-Ra without increasing the pro-inflammatory cytokine profile.

1. Introduction

The poorly understood, multi-factorial pathogenesis of osteoarthritis (OA) provides a significant challenge to treat the estimated 27 million people in the US [1] affected by the progressively debilitating disease. Articular cartilage destruction, subchondral bone remodeling and synovitis are the chief causes of the clinical manifestation of OA, which include pain, swelling, and stiffness of the affected joint. Axiomatically, these symptoms can pose a dramatic hindrance on daily activities depending on severity. Analgesic drugs, nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular (IA) corticosteroid injections are the currently recommended pharmacologic interventions for knee OA [2]. Given the limited scope and effectiveness of these treatment options aimed solely at symptomatic control of the disease, many patients are forced to undergo surgery. Regenerative therapies including platelet rich plasma and mesenchymal stem cells, are on the rise despite conflicting evidence of supportive data. Early data indicated the potential musculoskeletal benefits and cost effectiveness of platelet rich plasma (PRP) injections. The majority of trials have failed to provide evidence for the increased use of PRP therapy [3]; however it is difficult to pinpoint if this is due to the actual treatment regimen or the lack of standardized protocols, platelet separation techniques and outcome measures. Interestingly, a recent systemic review and meta-analysis reported PRP IA injections are significantly superior to placebo and hyaluronic acid for the treatment of knee OA (all other outcomes were excluded) [4]. Despite inconsistent results, the market value of

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PRP is estimated to reach $126 million by 2016 [5]. A number of recent studies are beginning to emerge that reveal the potential for autologous adipose tissue derived mesenchymal stem cells, albeit to a much smaller degree than PRP, for the treatment of OA [6]. The need for an out-patient, on-site, point of service, low cost symptom relieving and possibly chondroprotective drug is evident given the already high prevalence of arthritis in the US, which is expected to increase to nearly 67 million people by the year 2030 [7], the significant financial burden to the patient ($703/year) and insurer ($3080/year) [8] and the lack of effective non-surgical options.

A recently improved knowledge of the molecular mechanisms underlying this disease has led to the exploration of biotherapeutic strategies. One approach that holds promise is the inhibition of interleukin-1β (IL-1 β), a major cytokine promoting the catabolic activity associated with OA affected joints [9]. Attur et al. [10] reported the presence of biologically active IL-1β in OA-damaged cartilage providing the rationale to explore blockade of this molecule as a target to facilitate cartilage repair and potentially reverse degradation. Different methods to specifically inhibit Interleukin 1 (IL-1) have been tested. Briefly, those include the application of soluble IL-1 receptors, monoclonal antibodies against IL-1 or IL-1 receptor 1, blocking the formation of active IL-1β, gene therapy, and the application of IL-1 receptor antagonist protein (IL-1-Ra) [9], which serves as the focus of this investigation. It is unclear which method is most effective; however, the success of three commercially available IL-1-Ra products (recombinant Anakinra™, autologous Orthokine™ and Arthrex™) led to the development of our novel IL-1-Ra formulation process. The primary purpose of this investigation was to test our hypothesis that our on-site, point of service, minimal manipulation processing of whole blood would induce sufficient IL-1-Ra levels and IL-1-Ra:IL-1β block ratios. Secondary outcomes were twofold: (1) to ensure the Arthrokinex™ process did not increase the concentration of anti-inflammatory cytokines (TNF-α and IL-1β) and (2) to evaluate cytokines levels in the serum samples after being stored at −20 °C for at least one year.

2. Materials and methods

2.1. Study design

This was a retrospective chart review/proof of concept investigation aimed to quantify the ability of the Arthrokinex™ process to enhance IL-1-Ra in whole blood. All aspects of the study protocol were extensively reviewed and approved by IntegReview Institutional Review Board (IRB) as being considered exempt from requiring IRB approval as it met all requirements outlined in 45 CFR 46.101(b)(4), specifically (1) the research involves only the collection or study of pre-existing data, documents, records, pathologic specimens or diagnostic specimens and (2) the information will be recorded in such a manner that the subjects cannot be identified, directly or through identifiers linked to the subjects.

2.2. Participants

Existing charts were reviewed and a total of fifty three (53) patient charts met all inclusion criteria for this analysis: age >21 years, chronic OA for at least 3 months, patients were diagnosed with OA according to the American College of Rheumatology (ACR) criteria, radiographic evidence of OA and ⩾4 pain grade (on a numeric scale of 1–10). Exclusion criteria included: patient charts of those in generally poor health, pregnant or breast feeding, drug dependent (chronic opioid use, alcohol, etc.), undergone surgery or treatment of the affected joint within the last 3 months, lacked the mental ability to understand the treatment plan, systemic disease of the musculoskeletal system, bone cancer, metastasis or tumor-like lesions in the immediate proximity to the treated joint, fracture in the last 3 months, acute bacterial infection, blood clotting disorders, major psychiatric disease requiring therapy, and continuous corticoid or NSAID therapy due to other diseases. Informed consent was obtained from each participant and all work was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.3. Processing of whole blood (Arthrokinex™)

Using aseptic techniques, 60 mL whole blood from the median cubital vein of fifty three (53) participants was harvested into a sterile 60 mL syringe containing 3 mL of anticoagulant citrate dextrose (ACD) solution and centrifuged (3200 rpm, 15 min). The resultant Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP) were then extracted and the remaining layers, containing buffy coat and erythrocytes, were discarded. Both the PRP and PPP were transferred to a specialized, closed-system, centrifuge tube containing medical grade concentrator beads, mixed and allowed to incubate for 30 min at ambient temperature. After the short incubation period, centrifuge filtration (2000 rpm, 3 1/2 min) through a sterile 0.45 μm filter was completed and the resulting sterile filtrate was slowly drawn into 1 mL syringes. The 1 mL syringes could be used immediately for intra-articular injection or stored at −20 °C for future use.

2.4. Biomarker assays

The primary outcome of measuring IL-1-Ra (pre- and post-conditioning) was achieved by using the highly sensitive, commercially available quantitative sandwich enzyme-linked immunoassay technique (R&D Systems, Quantikine ELISA; Minneapolis, MN, USA. The manufacturer reports this kit, when run in accordance with standard Quantikine protocols, to be extremely sensitive (minimum detectable dose ranged from 2.2 to 18.3 pg/mL), specific (no significant cross-reactivity or interference was observed), precise (intra- and inter-assay CVs were 3.7% and 6.7%) and linear (all diluted samples fell with the dynamic range of the assay). Since sample concentrations were expected to fall outside the range of provided standards, serum was diluted 100 fold by adding 5 μl of sample to 495 μl of calibrator diluent. Resulting concentrations were calculated by subtracting the average zero standard optical density and log transforming IL-1-Ra concentrations versus the log of the optic density on a linear scale, and the best fit line determined by regression analysis. IL-1-Ra concentrations were only accepted if the standard curve correlation coefficient (r) reached 0.99 and the CV of each sample was under 20%.

As a secondary outcome, serum levels (pre- and post-conditioning) of pro-inflammatory (TNFα, IL-1β) cytokines and another anti-inflammatory cytokine (IL-10) were measured separately using ELISA. All kits reported comparable sensitivity, specificity, precision and linearity as described above. Similar to IL-1-Ra, all kits were run in accordance with standard Quantikine protocols.

2.5. Statistical analysis

SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Wilcoxon signed rank test were performed to analyze the statistical difference between baseline and post-processing cytokine levels. All results shown are the mean ± SEM of two or more experiments.

3. Results

3.1. Participant characteristics

Of the 53 participants, 25 men and 28 women were included. The mean age was 59.8, ranging from 25 to 85 years. According to the World Health Organization guidelines, 16 of the 53 (30%) participants were classified as overweight (BMI ⩾ 25 kg/m^2) and 30 participants (57%) are classified as obese (BMI ⩾ 30 kg/m^2). Of the 30 obese patients, 21 fall into the category of class I obesity (BMI 30–34.99 kg/m^2), 6 were classified as class II obese (BMI 35–39.99 kg/m^2) and 3 were characterized as class III obese (BMI ⩾ 40 kg/m^2) (Table 1).

3.2. Primary outcome: Arthrokinex™ process induced IL-1-Ra levels

Autologous conditioned serum resulted in a markedly increased induction of Arthrokinex™-derived IL-1-Ra (17,537 ± 1234 pg/mL) (Table 2). These values are comparable to IL-1-Ra levels produced by Orthokine™ [11] and Arthrex™ [12]. On average, after the short 30 min incubation period, a 32 fold increase in IL-1-Ra was observed between baseline and post-Artrhokinex™ serum. IL-1-Ra levels increased by at least a factor of 10 in 50 (out of 53) serum samples. Furthermore, 5 samples obtained IL-1-Ra levels that increased at least 20 times from baseline, 14 samples that increased at least 30 times from baseline, 11 samples that increased at least 40 times from baseline and 13 samples that increased at least 50 times from baseline. One sample increased by a factor 120, representing the highest increase of IL-1-Ra observed. This robust and rapid increase in IL-1-Ra synthesis resulted in a mean serum IL-1-Ra to IL-1β ratio of 999.0 (Fig. 1).

3.3. Secondary outcomes: IL-10, TNF-α, IL-1β

In addition to the 32 fold increase in IL-1-Ra, a statistically significant increase in the anti-inflammatory Interleukin 10 cytokine ($p < 0.001$) was observed. Despite this statistical significance, the clinical significance of this increase is negligible. A statistical increase was also observed in the pre and post-Arthrokinex™ levels of IL-1β and TNF α (Table 2); however, similar to IL-10, these increases are not clinically significant.

3.4. Storage of autologous conditioned serum

Following our novel conditioning process, approximately 6–12 mL of concentrated IL-1-Ra rich serum is extracted. In order to test the storage capacity of Arthrokinex™, a small, separate subset of patients' (n = 21) conditioned sera that had been stored at −20 °C for at least one year was analyzed using ELISA. Mean IL-1-Ra levels remained markedly elevated (16,167 ± 109 pg/mL) and were similar to mean levels of IL-1-Ra observed soon after processing. A similar trend was observed in each of the additional cytokine levels measured in this investigation (IL-10, 31.9 ± 4.1 pg/mL; IL-1β, 42.5 ± 3.7 pg/mL, TNFα, 13.1 ± 0.7 pg/mL).

Table 1
Patient demographics and clinical characteristics.

N	53
Male/female	25/28
Mean age (range)	59.8 (25–85)
BMI classification	
Overweight (BMI ⩾ 25 kg/m^2)	16/53 (30%)
Obese Class I (BMI 30–34.99 kg/m^2)	21/53 (40%)
Obese Class II (BMI 35–39.99 kg/m^2)	6/53 (11%)
Obese Class III (BMI ⩾ 40 kg/m^2)	3/53 (6%)

Table 2
Cytokine induction following Arthrokinex™ procedure.

	Baseline (pg/mL)	Post-Arthrokinex™ (pg/mL)	Fold increase	P value
IL-1-Ra	549.6 ± 52.6	17,537 ± 1234	31.9	<0.0001
IL-1 β	7.3 ± 0.8	17.5 ± 2.0	2.4	<0.0001
IL-10	24.9 ± 2.7	31.7 ± 4.8	1.3	<0.001
TNFα	9.8 ± 1.9	24.9 ± 2.7	2.5	<0.0001

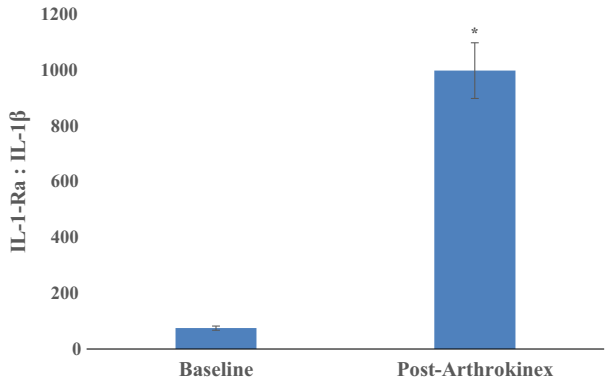

Fig. 1. Autologous conditioned serum (Arthrokinex™) produces a favorable cytokine profile by shifting the anti-inflammatory to pro-inflammatory ratio. Enhanced concentration of IL-1-Ra has the potential to block the destructive effects of IL-1β.

4. Discussion

The primary aim of this investigation was to determine the capacity of the Arthrokinex™ conditioning process to induce synthesis of IL-1-Ra from a patient's whole blood. The desired therapeutic effect of Arthrokinex™ is facilitated by the ability of IL-1-Ra to limit the destructive inflammatory intra-articular actions of IL-1β. Supranormal levels of IL-1β and TNFα, mainly produced by activated synoviocytes, mononuclear cells and articular cartilage, drive the catabolic response and augment the pathogenesis of OA by stimulating release of other cytokines (IL-8, IL-6 and nitric oxide) and prostaglandin E_2. Additionally, IL-1β and TNFα can increase their own production through autocrine signaling which could further shift the equilibrium between IL-1β and IL-1-Ra. All of these cytokines diffuse into the synovial fluid and promote cartilage matrix degradation [13].

Consistent with previous studies designed to physico-chemically induce anti-inflammatory cytokines [11,14,15], our novel process provides sufficient levels of IL-1-Ra to competitively inhibit IL-1β [15]. IL-1-Ra, if present in concentrations that are 10–100 times higher than IL-1β, will block the interaction of IL-1β with cell surface receptors as well as soluble IL-1R type 2 [16]. The volume of the synovial fluid in an OA-affected knee increases to approximately 13.6 mL [17] and contains 34 pg (2.5 pg/mL) of IL-1β [18]. All serum levels in this trial exceeded the 100:1 threshold so it is therefore reasonable to conclude that Arthrokinex™ can consistently produce IL-1-Ra levels that will inhibit IL-1β.

The capability of IL-1-Ra to treat knee OA symptoms is confirmed in some [14,15], but not all [19] clinical trials. The largest trial (n = 376) conducted by Baltzer et al. [14], was designed to compare the clinical effectiveness of intra-articularly administered IL-1-Ra (Orthokine™) to hyaluronan (HA) and saline. Patients in each group had two appointments with a physician per week for three consecutive weeks. Patients in the saline and HA group received a total of three injections and the patients in the IL-1-Ra

group received a total of six injections. Orthokine™-treated patients showed significant improvements in all outcome measures compared to HA and saline-treated patients. Certainly, the slightly different treatment regimens among groups should be considered; however, this preliminary study provides evidence for the efficacy of intra-articular administration of IL-1-Ra as a treatment strategy to combat the immobilizing effects of knee OA. Previously, Yang et al. [15] reported a superior biological response elicited by Orthokine™ compared to physiological saline in the treatment of knee OA. Despite the comparable improvement on the WOMAC in patients treated with Orthokine™ and placebo, KOOS (Knee Injury and Osteoarthritis Outcome Score) symptoms and sport parameters were significantly improved in the treatment group. Additionally, other clinical observations in Orthokine™-treated patients were consistently improved but were not statistically significant. Ultimately, the authors concluded the use of Orthokine™ cannot yet be recommended for the treatment of knee OA since the primary outcome of the investigation, 30% improvement in WOMAC between groups, was not met.

Surprisingly, several recent review articles [20–22] have ignored the success of Orthokine™, but instead focused solely on the inability of Anakinra™ to provide symptomatic relief of KOA that was significantly superior to placebo. In an open label randomized, multicenter, double-blind, placebo-controlled trial (n = 160), patients were divided into 3 groups and received a single intra-articular dose of Anakinra™ (150 mg or 50 mg) or placebo [20]. After 4 weeks, WOMAC global scores for all group improved with a non-significant difference between placebo and Anakinra™ 50 mg and between placebo and Anakinra™ 150 mg. A statistically significant improvement ($p = 0.051$) was approached between the placebo group and the Anakinra™ 150 mg group in the WOMAC pain sub-score on day 4. However, it is difficult to directly compare this trial to previous findings since only a single injection was administered. Additional trials are needed to determine whether repeated injections deliver therapeutic levels of IL-1-Ra to reduce pain.

Several clinical human trials have reported improved clinical outcomes of patients with muscle [23] and ligament injury [24–28] as well as spinal disorders [29] following IL-1-Ra treatment. Of particular interest, Darabos et al. [26] compared IL-1β levels in the synovial fluid (SF) of 10 patients treated with IL-1-Ra to 10 patients treated with placebo (physiological saline) following ACL reconstruction surgery. Surgery caused an immediate elevation of synovial fluid concentration of IL-1β levels in almost all patients (19 of 20). After 10 days, patients treated with IL-1-Ra had concentrations that were equal to or below the concentration in a normal knee and statistically lower than placebo. The authors concluded that the dramatic decrease in IL-1β facilitated by IL-1-Ra application could augment the ACL healing process. The same authors confirmed these results in a larger trial (n = 62) and observed several postoperative outcomes associated with IL-1β concentrations [27]. The most important finding was the significant reduction in bone tunnel widening in autologous conditioned serum (ACS) treated patients at 6- and 12 months. Treated patients had significantly fewer joint effusions and performed better on functional tests at 6 months and had significantly greater range of motion at 12 months. Patient-administered outcomes (WOMAC and IKDC) were significantly improved in ACS-treated and placebo-treated groups; however, patients treated with ACS reported consistently lower pain scores and significantly improved WOMAC stiffness scores ($p = 0.047$) compared to placebo.

Treatment of OA continues to be a challenge for clinicians and investigators. As the population continues to age, a greater percentage of the population will likely develop OA and require surgical intervention unless a disease modifying drug is developed. To date, the Osteoarthritis Society International (OARSI) [30] and European League against Rheumatism [31] recommend acetaminophen as the first choice of oral analgesics to treat mild-moderate knee OA, and if successful, should be used as the preferred long-term analgesic due to its efficacy, relative safety and low cost. However, the evidence suggests that NSAIDs are superior to acetaminophen for improving knee pain in people with OA [32] and should be administered to patients who do not respond to acetaminophen [30,33–35] or as an initial therapy option for patients with moderate to severe pain [2,36]. Despite the superior clinical response, NSAIDs should be used with caution due to the well documented serious gastro-intestinal, renal and cardiovascular toxicities [37] including the FDA warnings about stroke and myocardial infarction. Another recommended treatment option includes the short-term use of IA corticosteroid injections [35]; however, similar to NSAIDs treatment, serious potential side effects of long term administration abound [38]. Since knee OA is a chronic disease, these short term treatment options highlight the significant unmet need for a disease-modifying OA drug that does not have any major side effects.

5. Conclusions

Our novel process to induce extremely high levels of the potent receptor antagonist for IL-1 requires a short incubation time to allow same day, point of care service to patients, utilizing a closed loop system to reduce the risk of contamination, and does not introduce any additional chemicals to the biotherapeutic product. Extremely high levels of IL-1-Ra are consistently achieved without augmenting key pro-inflammatory cytokines. Additionally, it is relatively inexpensive and can be safely stored without degrading IL-1-Ra or compromising the IL-1-Ra:IL-1β block. A large, well-designed, randomized clinical trial is needed to assess the symptom relief and chondroprotective effects of IL-1-Ra. Meanwhile, the Arthrokinex™ conditioning process offers an alternative, point of service molecular approach to rapidly induce IL-1-Ra which has the potential to provide therapeutic benefit in the treatment of mild to moderate OA.

Disclosure statement

The research being reported in this publication was supported by Memorial Clinical Research, a division of Barreto Healthcare Clinic. Angelique Barreto has equity ownership in, serves as an advisor for, serves on the board of directors of Arthrokinex Joint Health LLC., which is developing a process related to the research being reported. The terms of this arrangement have been reported to the IRB. Timothy Braun is not affiliated with Memorial Clinical Research and reports no conflicts of interest.

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