Actido™

Salient Characteristics definition and evidence-based key qualities outlook and substantiation

1. Executive Summary

Actido™ is a pure botanical extract produced from fresh cucumbers with anti-inflammatory actions. The indicated use of Actido™ is the prevention and the symptomatic treatment of inflammatory states of the skin, the joints and the skeletal musculature.

Actido™ is based on Natexin™, the commercial designation of the mix of different active compounds obtained by Naturalea through its patented proprietary manufacturing process which includes the full control over the entire supply chain, from the cultivars to the final product.

The unique and highly efficient extraction process ensures that all different active compounds composing Natexin™ express their synergic action to the fullest potential.

Cytotoxicity, genotoxicity and acute oral toxicity of the extract have been tested according to the reference standards, showing that Actido™ is safe at the tested dosage.

Actido™ acts at the earliest stages of the inflammatory cascade, by suppressing the levels of TNF- α, a key regulator of the inflammatory immune response. This activity has been documented by a full data set including in vitro and in vivo evidence.

2. Introduction

2.1 Inflammation

Inflammation is the body's natural response to harmful stimuli and is characterised by the movement of plasma and leukocytes from the blood into injured tissues. This particular type of immune response is important for the body to ward off harmful pathogens as well as to trigger tissue repair processes and helps to restore homeostasis at infected or damaged sites.

At a molecular level, inflammation is the result of a proinflammatory cascade of mediators including cytokines, such as tumor necrosis factor (TNF)-α, interleukins (IL)-1β and IL-6, chemokines, and inducible enzymes such as cyclooxygenase (COX)-2, all of which play critical roles in controlling the inflammatory process. Vascular endothelial cells respond to the activation of the inflammatory cascade by undergoing a number of pro-inflammatory changes, which increase leukocyte adhesion, transendothelial migration, and the recruitment of different blood cell types, including monocytes that locally differentiate into macrophages. Overall, these cellular and molecular events results in the onset of the four classic hallmarks of local inflammation: redness, heat, swelling, and pain.

In normal circumstances, the immune system has several mechanisms to resolve the inflammatory responses. The resolution of inflammation requires the termination of the pro-inflammatory signalling pathways, the clearance of inflammatory cells, and the restoration of normal tissue function. A failure of these mechanisms may lead to chronic inflammation and, in turn, underline the pathogenesis of diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD), COPD (chronic obstructive pulmonary disease) and other chronic inflammatory disorders.

2. Introduction

2.2 TNF-α

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine produced predominantly by activated macrophages and T lymphocytes, but it can also be secreted by a broad variety of other cells types. In healthy individuals TNF-α is generally not detectable. However, specific noxious events (i.e. ultraviolet light, trauma or toxins) can trigger TNF-α expression, thereby elevated serum and tissue levels are found in inflammatory and infectious conditions, with serum levels correlating with their severity [1].

TNF-α is a key mediator in the early steps of acute inflammation. Indeed, TNF-α act synergistically with IL-1, by initiating the proinflammatory cytokine cascade. Most of the local proinflammatory effects of TNF-α depends on its actions on vascular endothelium and endothelial-leukocyte interactions [2]:

- In response to TNF-α, endothelial cells display adhesion molecules for leukocytes, including E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). This response leads to recruitment of leukocytes at the site of injury.

- TNF-α induces the expression of the enzyme cyclo-oxygenase 2, which promotes the synthesis of prostacyclin (PGI2), a potent vasodilator, resulting in increased local blood flow.

- TNF-α increases vascular permeability, allowing the trans-endothelial passage of fluid and macromolecules and the formation of oedema.

- TNF-α induces the expression of pro-coagulant proteins, such as tissue factor, and down-regulate anticoagulant protein, such as thrombomodulin TNF. The prothrombotic TNF-α effect is a mechanism to locally contain the infection.

In addition to its local effects, TNF-α is directly linked to the induction of cachexia, is associated with chronic diseases, and affects thermoregulation, lipid metabolism, blood flow , coagulation and insulin resistance.

In view of the central role of TNF-α in the innate host inflammatory response, blocking the production or the action of this cytokine is an effective treatment option for a variety of conditions associated with excessive or poorly controlled inflammation. Various strategies have been used for neutralizing TNF-α so far, including antibodies, soluble receptors, recombinant TNF binding proteins, and non-specific agents (e.g. pentoxifylline, thalidomide and metalloproteinase inhibitors)[3].

2.3 Cucumis Sativus: historical notes and pharmacological activities

Cucumber (Cucumis sativus L.) is a member of the Cucurbitaceae family like melon, squash and pumpkins. It is native to India, found wild in the Himalayas but it is commercially cultivated worldwide as a seasonal vegetable crop. Fruits are widely consumed fresh, fermented (pickles) or as cooked vegetables. They contain water (96.4%), protein (0.4%), fat (0.1%), carbohydrate (2.8%), mineral (0.3%), calcium (0.01%), phosphorus (0.03%), iron (1.5 mg/100 g) and vitamin B (30 IU/100 g). Additionally, a number of enzymes have been described in these fruits together with a high concentration of ascorbic acid, lactic acid and essential oils [4,5]. Due to the high water content, and very low calories cucumber is traditionally used in rural and urban areas to remove general debility and as a cooling agent.

Throughout history, different parts of the plant such as leaves, fruits and seeds have been explored for their therapeutic benefits. In the Indian traditional medicine, particularly in Ayurveda, cucumber is widely used for various skin problems including swelling under the eyes and sunburn for its refreshing, cooling, healing, soothing, emollient and anti-itching effect to irritated skin [6,7]. In Chinese folk medicine the leaves, stems and roots are generally used as anti-diarrheal, detoxicant and anti-gonorrhoeal agents [8].

More recently, several pharmacological activities have been reported with this plant. Volatile oil of cucumber showed antifungal activity against human pathogen fungi and antibacterial activity against Gram-positive and Gram-negative bacteria[5,9]. Fruits and peel extracts have shown to have some antidiabetic and hypolippidemic activity when administrated to experimental animals and in 2009 Gill et al., suggested it might have an ulcer protective potential based on experiments in rats [10-13].

Cucumber fruits and seeds are also recommended for the treatment of various skin problems. The pulp is primarily composed of water with ascorbic acid (vitamin C) and caffeic acid, which gives a soothing effect on skin and reduces swelling. Being naturally rich in lactic acid, it is also widely used for the chemical peeling of the skin to reduce the thickness of the stratum corneum in dry skin, icthyosis, follicular hyperkeratosis, seborrheic keratosis, actinic and keratosis [14,15].

2. Introduction

Cucumber sativus fruit has been referred as a potential antiwrinkle agent in cosmetic products given its significant free radical scavenging activity observed *in vitro* [16–18]; moreover, it was found to have anti-hyaluronidase and anti-elastase actions, confirming the potential role of this fruit on skin care [19]. The topical application of extracts of cucumber was observed to decrease skin moisture and melanin content and to increase trans-epidermal water loss (TEWL), thus suggesting a possible use also as a whitening and anti-acne agent[20].

3. Actido™

3.1 Overview

Actido™ is a pure botanical extract with anti-inflammatory actions produced from fresh cucumbers and containing Natexin™, the commercial designation of the mix of different active compounds obtained by Naturalea through its patented proprietary manufacturing process. Cucumber is naturally rich of phyto-constituents with pharmacological activities, Actido™ anti-inflammatory effect is due to the synergistic activity among several molecules. The proprietary manufacturing process provides a highly efficient extraction of these compounds, thus ensuring the maintenance of the pharmacological synergism among the active ingredients.

Actido™ acts in the early stages of inflammation, by suppressing the activity of TNF-α, a key regulator of the inflammatory immune response. **(Figure 1)**

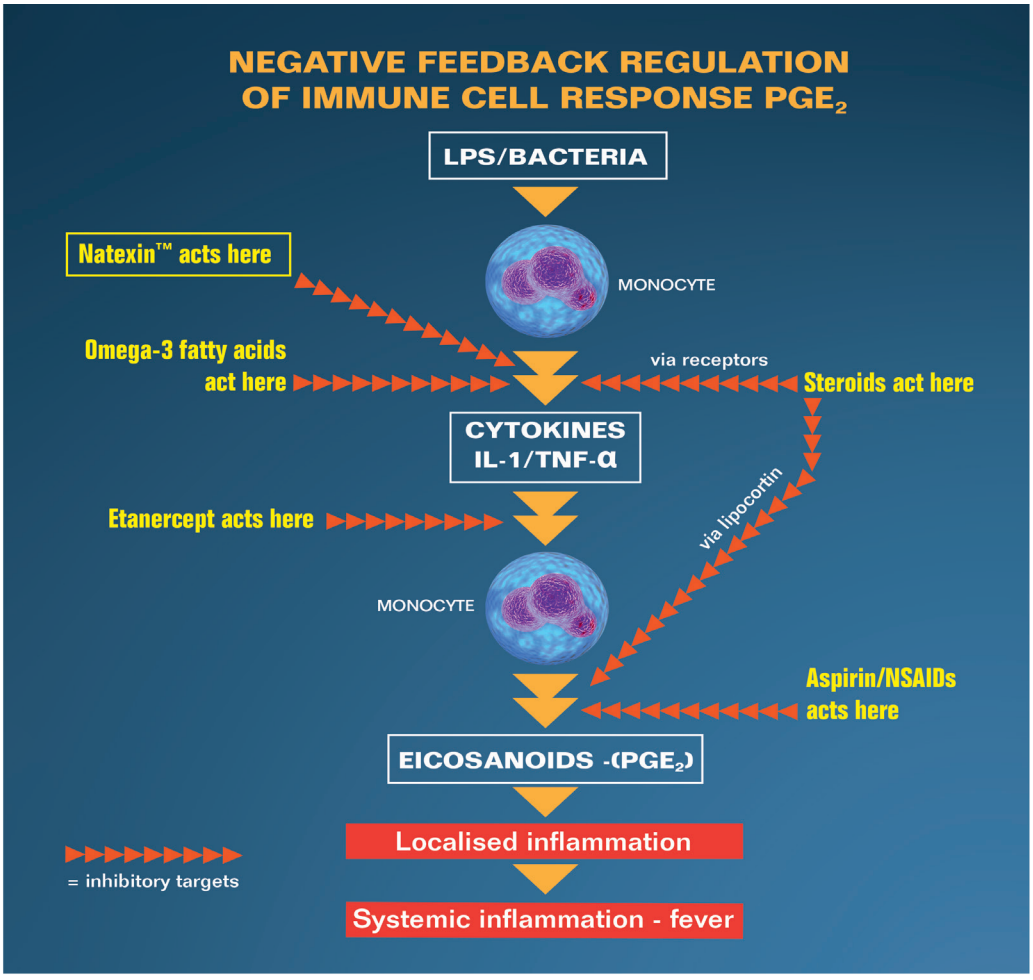

Figure 1 - The mechanism of action.

Actido™ acts in the early stages of inflammation by suppressing the activity of TNF-α.

3. Actido™

Inflammation can result from intense physical activity, and is involved in a number of disease states. Moreover, a mild pro-inflammatory state due to increased peripheral levels of inflammatory cytokines is often associated with aging and with the major degenerative diseases of the elderly [21].

A botanical extract with strong anti-inflammatory activity is a true innovation to the market, with possible applications in the prevention and in the symptomatic treatment of inflammatory states affecting different body districts, including the skin, the joints and the skeletal musculature. Actido™ is particularly indicated for athletes and sportive amateurs, elderly people and in skin care.

3.2 The quality

Actido™ is the result of a long process of research and development aimed at producing a botanical extract with the highest standards of safety and quality. Actido™ is obtained via a proprietary process from selected cultivar. Naturalea controls all the supply chain from the field to the final consumer to assure continuity of supply and constant levels of quality both in the raw materials and in the final product.

Actido™ is a whole fruits extract obtained by using polar organic solvents, mainly water/ethanol blends, and under mild conditions, for preserving its content of Natexin™, the unique mix of natural components,to the greatest possible extent. High-tech, mechanical purification stages have been developed for maximizing the effectiveness of the extraction process and isolate Actido™ from the fruits. In its final form, the extract is a soluble and highly purified powder obtained by a freeze-drying process.

The extract, the manufacturing process and its applications are patent protected.

3.3 The safety

Actido™ safety profile was assessed through several *in vitro* and *in vivo* studies.

3.3.1 *In vitro* genotoxicological study

Two *in vitro* genotoxicological tests were carried out in compliance with the OECD 471:1997 guidelines. Bacterial reverse mutation assay were performed on *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102, and *Escherichia coli* strain WP2uvrA. Genotoxic potential of Actido™ at doses up to 5 mg/plate was measured in the presence and absence of an exogenous source of metabolic activation.

According to the reference standards, Actido™ proved to be not mutagenic for all the test strains, in either the presence or absence of metabolic activation.

3.3.2 Evaluation of cytotoxicity *in vitro*

The *in vitro* cytotoxicity test for Actido™ was done according to the ISO 10993-5:2009 protocol using mouse fibroblast 3T3 cell line. MTT assay was performed to calculate the percentage of viable cell in the presence of serial dilutions of extract compared to the sample without extract (treatment medium) and to a positive control (0.5 mg/ml SDS solution in treatment medium). Based on the results obtained, Actido™ was considered not toxic with IC_{50} value of 14.003 mg/ml.

3.3.3 Evaluation of acute oral toxicity *in vivo*

A toxicology study was performed according to Organization for Economic Cooperation and Development (OECD) guideline 420 adopted on December 17th, 2001.

Actido™ acute oral toxicity was assessed in a 14 days *in vivo* rat study, following administration of a single oral dose (2000 mg/Kg). The assay involved the daily evaluation of the following data: mortality, body organ functions, tegumentary apparatus conditions, mucosae conditions, somatomotor activity and sensorium conditions. Body weight was weekly noted. After the observation period of 14 days, all surviving rats were sacrificed and subjected to complete necropsy. The treated rats did not show any case of mortality or adverse clinical signs during the study. During the first week treated rats showed lower body weight increase compared to the standards of species and rats. During the second week, the body weight gain was found to be lower in three treated animals. On necropsy, no major gross

pathological changes were observed.
Based on these findings, Actido™ has a LD50 > 2000 mg/Kg and is included in class 5/NC of GHS classification according to the OECD N° 420.

3.4 The efficacy

Actido™ exerts an anti-inflammatory action by suppressing the expression of TNF-α, an early proinflammatory cytokine. This action has been investigated in a variety of experimental settings in collaboration with leading Universities and Medical Research Centers:

- European Oncology Hospital, Cell Biology and Immunotherapy Unit
- Bologna University, Laboratory of Cellular Biotechnology
- Torino University di, Department of Applied Botanic

3.4.1 *In vitro* evidence

Actido™ inhibits TNF-α expression in animal and human *in vitro* cell models in a dose-dependent manner

The first line evidence has been collected in *in vitro* cell models under lipopolysaccharide (LPS)-induced inflammation. Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria and has long been recognized as a key factor in septic shock and, more generally, in inducing a strong immune response in normal mammalian cells [22,23].
Activation by such stimulus results in the production of inflammatory signaling mediators that include nitric oxide, prostaglandin E2 (PGE2) and pleiotropic cytokines such as IL-6 and TNF-α.

In the first instance, Actido™'s activity was assessed on D1 cells. D1 cells are a dendritic cell line derived from mouse spleen. These cells belong to the innate immune system and are recruited to sites of infection and inflammation for antigen uptaking and processing. LPS-stimulated D1 cells were treated with Actido™ at various concentrations and the protein secretion of IL-6,IL-12, IFN γ and TNF-α was measured in the supernatant. Stimulation with LPS led to the release of all the tested proinflammatory cytokines.

Treatment with Actido™ suppressed this inflammatory response by downregulating cytokine secretion in a dose-dependent way. Similar results were obtained also in human monocyte-derived dendritic cells generated *in vitro* from peripheral blood mononuclear cells (PBMCs) of healthy donors.

Actido™ modulates upstream mediators and downstream effectors of inflammation in porcine Aortic Endothelial Cells

A different set of experiments was performed using an *in vitro* model of inflammation with primary culture of porcine Aortic Endothelial Cells (pAECs) stimulated with LPS. These cells were treated with Actido™ at different concentrations; then cell viability, and the expression of genes involved in the onset or maintenance of inflammation were evaluated: TNF-α, HO-1, VCAM-1 and ZO-1.

VCAM-1 is an immunoglobulin-like adhesion molecule expressed on activated endothelial cells. Upon leukocyte binding, VCAM-1 activate molecular signals that alter endothelial cell shape and allow the opening of passageways for leukocytes. ZO-1 is one of the protein forming the thigh junctions, the structures that mediate intercellular adhesion in epithelial and endothelial cells. Downregulation of tigh junction proteins is one of the mechanisms mediating the increase in endothelial permeability occurring during acute inflammation. Heme oxygenase-1 (HO-1) is an enzyme involved in modulating oxidative injury and maintaining cellular homeostasis. Its expression is a protective cellular response against various stressors such as hyperoxia, hypoxia, heavy metals and endotoxins.

Stimulation with LPS induced cellular detachment and cytotoxicity. Moreover, LPS upregulated the inflammatory mediators TNF-α, and VCAM-1, and decreased the expression levels of ZO-1 and HO-1. Treatment with Actido™ suppressed the inflammatory response in LPS-stimulated pAECs. Indeed, it inhibited the LPS-induced cell detachment in a dose-dependent manner. After treatment with Actido™, the expression of both TNF-α and VCAM-1 were significantly reduced; conversely, an upregulation of the protective enzyme HO-1 and a sustained expression of ZO-1 was observed.

These results show that Actido™ can repress the expression of TNF-α and is effective also in modulating downstream effectors of inflammation, such as VCAM-1 and ZO-1.

Actido™ inhibits TNF-α expression in whole blood stimulated with LPS without affecting IL-6 levels

To study the effect of Actido™ in whole blood, post-weaned pigs were used as models. Heparinized blood samples were collected from 3 pig and used for whole blood LPS stimulation. LPS and/or Actido™ were added to blood and incubated. Aliquots of each blood sample were collected at different time points for RNA extraction and plasma was isolated for cytokines assay.

LPS added to whole blood caused a significant increase in the levels of TNF-α a mRNA and protein levels after 18h of incubation. On the contrary, whole blood added with Actido™ showed a significant decreased response to LPS stimulation. Indeed, the amount of TNF-α at both RNA and protein level was almost undetectable at the same time point. **(fig. 2)**

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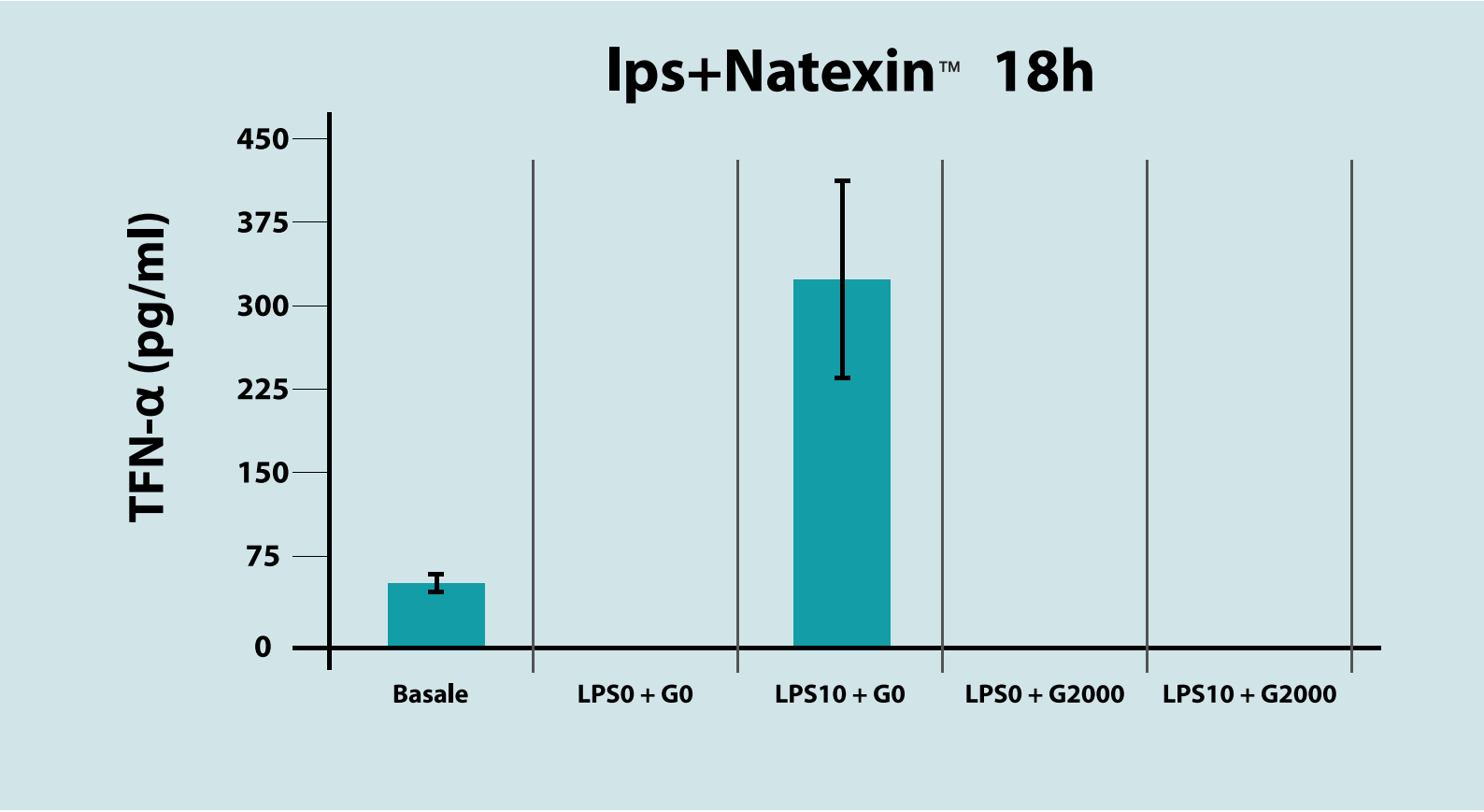

Figure 2. Effect of Actido™ extract on TNF-alpha concentration in blood.
The inflammatory response is activated using LPS, a know and most used inflammation factor. The addition of LPS cause an inflammation state, as can be seen by the increase in the amount of the concentration of TNF-alfa in the blood sample. The addition of Actido™ Extract reduce the inflammation state to zero.

Regular exercise reduces the risk of chronic metabolic and cardiorespiratory diseases, in part by exerting anti-inflammatory effects. One of the mechanisms underlying this action is the induction of an anti-inflammatory environment with each bout of exercise via muscle contraction-induced factors, the so-called "myokines"[24]. Although mostly regarded as a pro-inflammatory mediator, interleukin 6 plays a central role in the cytokine response to exercise and exerts many anti-inflammatory actions[25]. Indeed, the plasma IL-6 concentration increases exponentially during physical activity, with mechanisms regulated

primarily by muscle glycogen content. Increased plasma levels of IL-6 are related to exercise intensity, duration, the mass of muscle recruited, and one's endurance capacity and are, in turn, responsible for a subsequent rise in circulating levels of the IL-1 receptor antagonist (IL-1RA) and IL-10, a potent promoter of an anti-inflammatory state[26,27]. IL-6 has therefore a central role in mediating the anti-inflammatory effects of exercise, particularly when the exercise is prolonged and glycogen-depleting.

For this reason, also the levels of IL-6 were evaluated in whole blood experiments. Interestingly, the LPS-induced production of IL-6 was not downregulated by the Actido™ treatment **(fig. 3)**.

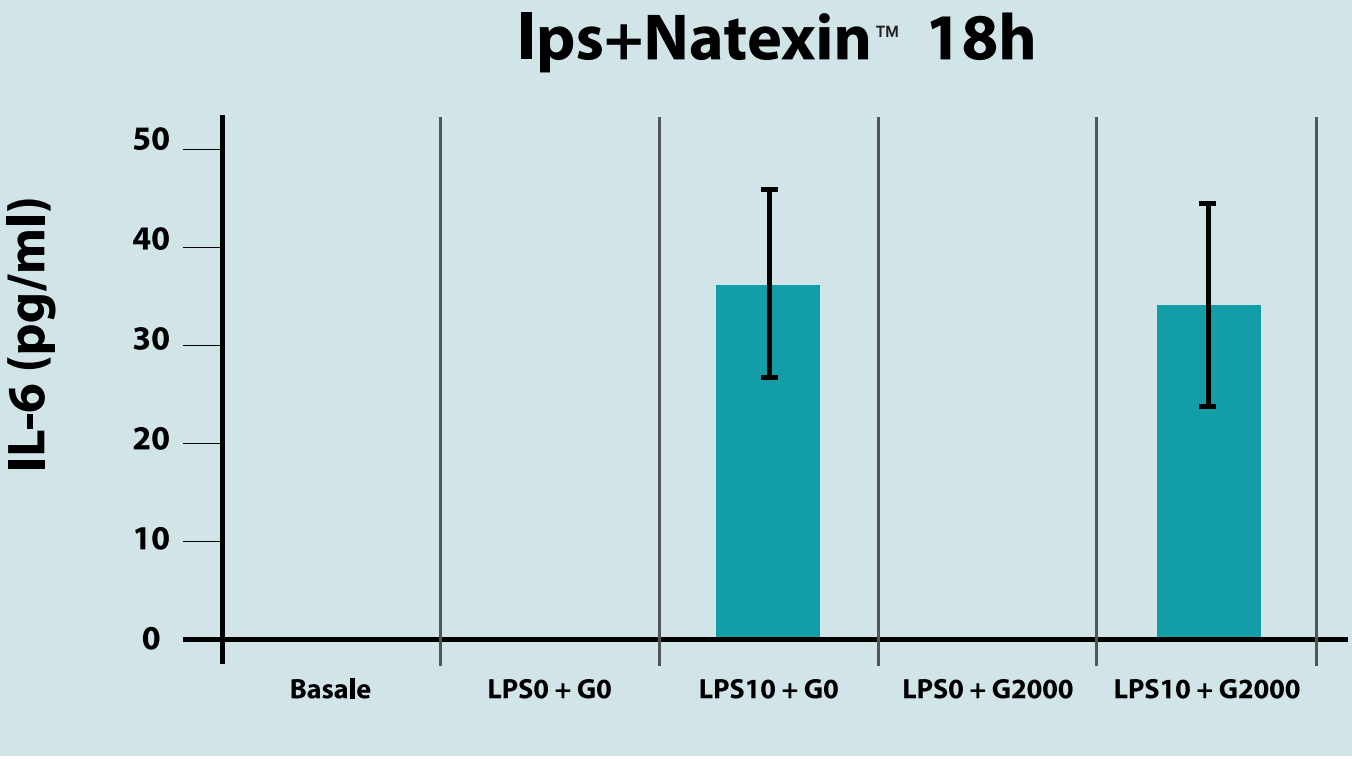

Figure 3. Effect of Actido™ extract on IL-6.
As shown on the picture above our extract has almost no activity towards this special cytokine.

This results suggest that Actido™ extract exerts its anti-inflammatory action without affecting the secretion of IL-6, thus maintaining the protective, anti-inflammatory effect mediated by this cytokine during and immediately after exercise. To the best of our knowledge, this is a very unique feature among botanical extracts and make Actido™ particularly suitable for the use by amateur and professional sportspeople.

3.4.2 *In vivo* efficacy studies

To assess the anti-inflammatory and alleviating properties of Actido™ *in vivo*, two human observational studies were performed, involving respectively a selected cohort of professional athletes and the general population.

Observational study in professional athletes

The first study was carried out in collaboration with the Italian Carabinieri Corp. Data from twenty Olympic athletes from various track and field specialties were retrieved during a one year observational study. All of them were assuming two to four capsules a day of Actido™, a dietary supplement based on Actido™ extract, to treat persistent pain affecting muscles, joints or tendons and compromising their daily workout. The treatment period varied from 2 to 4 weeks. Pain assessment before and after the treatment was performed using a self-completed 0-10 numeric Visual Analog Scale for Pain with 0 representing “no pain” and 10 representing the “worst possible pain” **(Fig.4)**.

3. Actido™

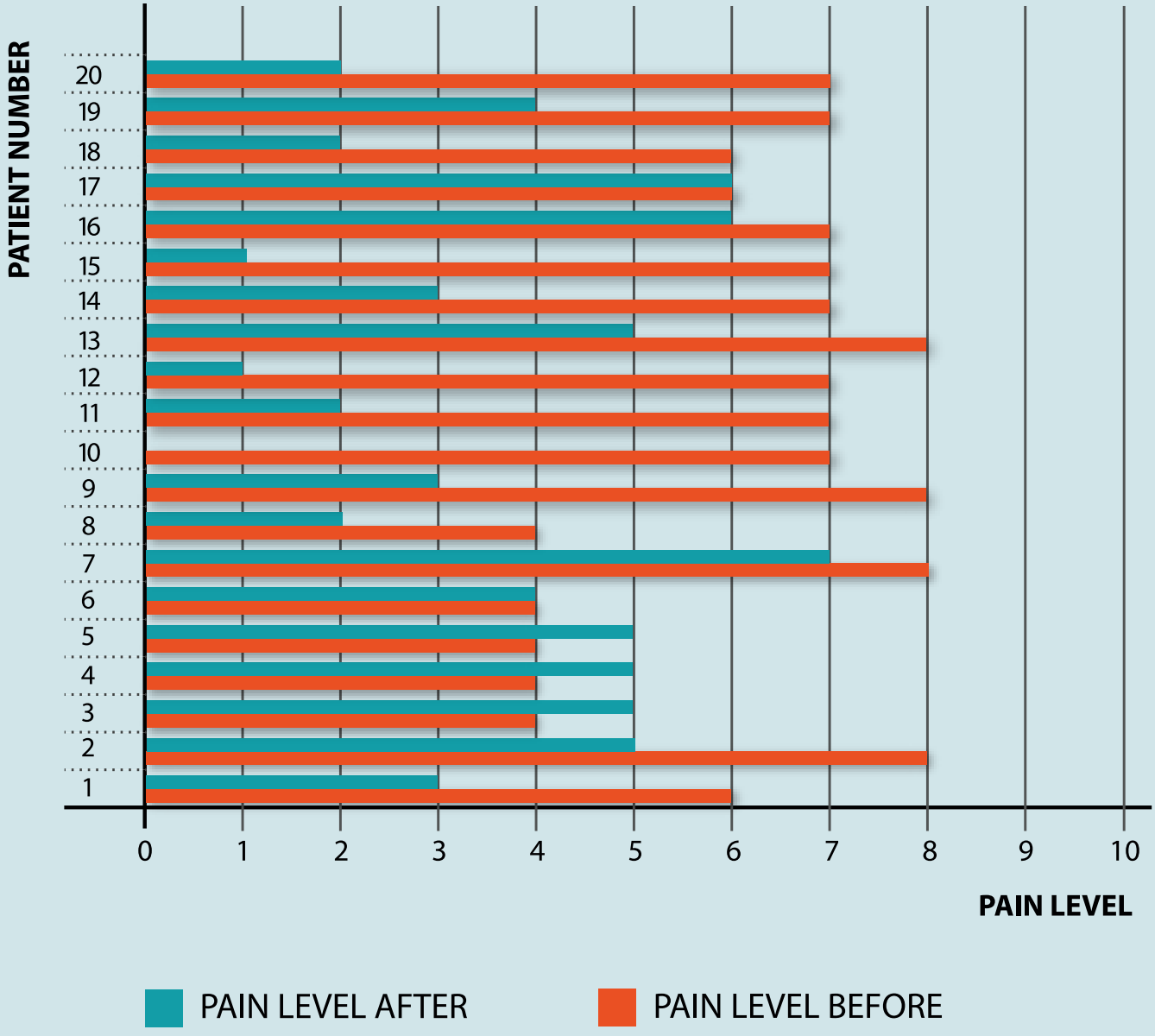

Figure 4 **Pain level in the treated subjects.**
Pain assessment before (red columns) and after (blu columns) the treatment with Actido™.

3. Actido™

The efficacy of the treatment in pain-relief was calculated as the difference between the estimated levels of pain before and after the treatment and evaluated in qualitative terms as “strongly positive”, “positive”, “slightly positive” or “negligible” according to the value obtained.

None of the subjects involved in the study reported side effects. The large majority of the athletes (65%) had “positive” or “strongly positive” results in terms of pain relief **(Fig.5)**;

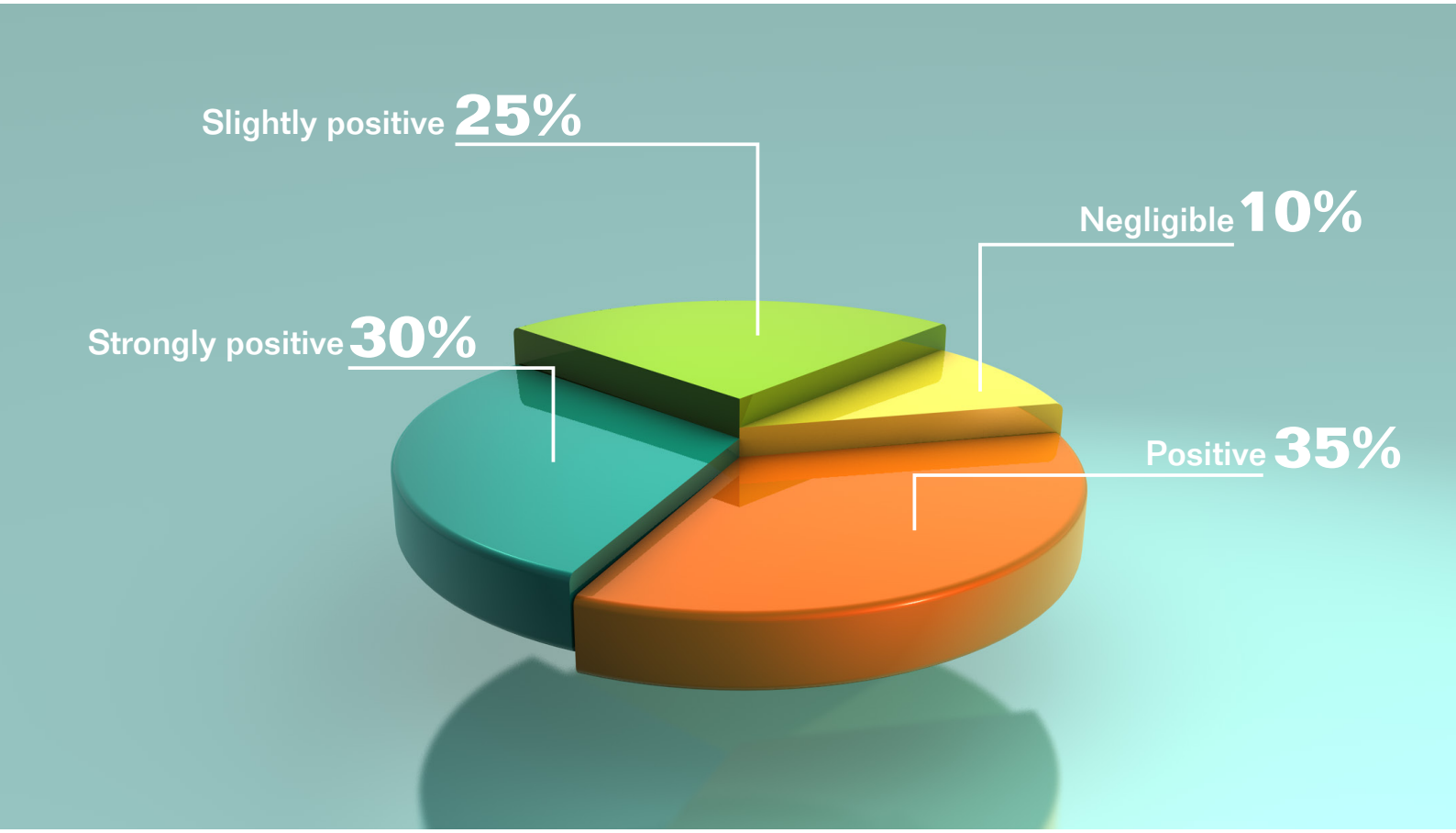

Figure 5. The pain-relieving efficacy in the tested population.
The pain-relieving efficacy was calculated as the difference between the level of pain before and after the treatment. According to the obtained results, a qualitative evaluation was assigned: “0: Negligible”, “1-2: Slightly Positive”, “3-4: Positive”, “≥ 5: Strongly Positive”.

All of them were able to get back to their training program after the treatment. These results provide evidence that the administration of Actido™ extract could be a new and useful strategy in the treatment of muscular, tendon and joint pain. Actido™ extract does not to contain any doping substance and is therefore suitable for athletes both before and during competitions.

Observational study in the general population

The second observational study, involved the retrospective collection of data coming from 10 specialist physicians who had prescribed Actido™ to patients affected by various acute inflammatory conditions associated with pain. The treatment period varied from 10 to 30 days for a total of 50 patients taking a daily dose of two capsules twice a day.

A weekly monitoring report was prepared for each patient to provide a qualitative summary of the development of the symtoms. The score was assigned on a scale of 0 to 10, where 0 represented “no effect after treatment”, 4 a “negative result”, 5 a “negligible result” and 10 a “complete remission”. The evaluation criteria for the assignment of the scores included both the resolution of the pain symptoms and the functional recovery of patients.

3. Actido™

The assigned score for each patient is shown in **figure 6**.

Figure 6. Efficacy scores collected from the 50 patients.
The scores were assigned considering equal to 0 the condition of "no effect after treatment", 5 a "negligible result", 10 a "complete remission". The red line represents the threshold dividing negligible (Efficacy score < 5) from non-negligible results (Efficacy score > 5).

Overall, in 80% of the treated patients, a “positive” or “strongly positive” outcome was assessed **(Fig.7)**;

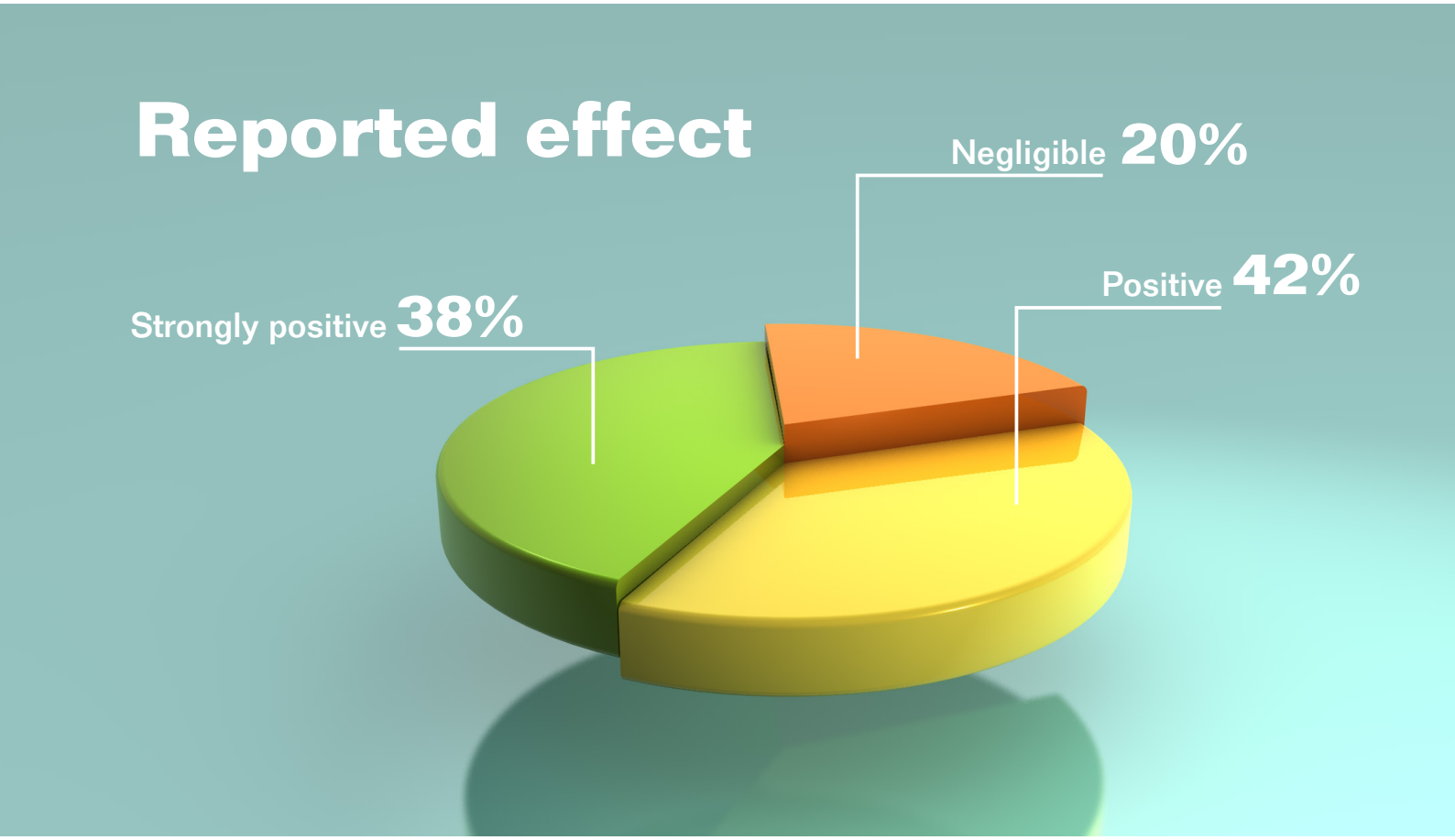

Figure 7. Distribution of the efficacy scores in the tested population.
According to the assigned scores, a qualitative evaluation was adopted: “< 5: Negligible Result”, “?: Positive”, “?: Strongly Positive”.

no adverse event had been reported even during the longest treatment periods. This second observational study confirmed that Actido™ extract is safe and effective in treating a inflammatory-related pain symptoms. Additionally, as the patients presented a wide range of inflammatory conditions, the study suggested that Actido™ may be a promising treatment option for a broad spectrum of painful conditions affecting different body districts.

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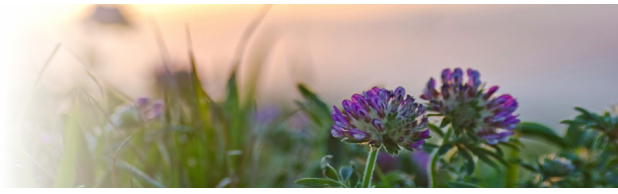

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