Clinical Measures for the Cytokine Levels After and Before Hirudotherapy in Rheumatoid Arthritis Patients

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Abstract

Hirudotherapy is one of the oldest complementary medical methods using in treatment of disease like inflammatory disease, osteoarthritis, and after being surgery. The aim of this study was to identify what are roles of leech bioactive substances on the treating of rheumatoid arthritis disease. The study was carried out in Al-Najaf City/Center of leech therapy, during the period from October 2016 to October 2017, the study involved (140) RA patients who attended the hospital, the majority of the study were women (64.29%), and the most affected age groups were from 61-70 years old in percentage (42.86%) followed by the age group 71-80 years old (25%), also the results showed a highly significant increased mean serum level of hs-CRP (9.35 vs. 1.19 mg/ml), and cytokines including IL18 (59.87 vs.12.43 pg/ml), IL-6 (70.34 vs. 13.33 pg/ml), IL-8 (53.25 vs. 11.61 pg/ml), IL-10(44.58 vs. 10.50 pg/ml), IL-17(77.45 vs. 10.42 pg/ml), and TNF-α(68.30 vs. 11.81 pg/ml) for RA patients as compared to controls group, in addition the study showed that the patients after hirudotherapy have a significant decreased mean serum level of hs-CRP and cytokines (IL18, IL-6, IL-8, IL-10, IL-17, and TNF-α) as compared to the patients before hirudotherapy. It was concluded that leech therapy is a highly effective in treating RA patients by reducing the severity of disease. It was recommended to achieve more investigation for assessment the role of leech therapy and their bioactive substances in the treatment of RA disease.

Keywords: Hirudotherapy, Cytokines, Rheumatoid Arthritis patients.

Introduction

Hirudotherapy means leeches therapy, is the oldest therapy in medical treatment, its remedy attributed to the existence of chemical components found in the saliva of leeches [1] which are bioactive substances like hirudin, hyaluronidase, calinbdellins, eglins [2] enzymes like apyrase, collagenase, elastase and others [3], destabilize, trypptase inhibitor, acetyl choline and carboxypeptise A inhibitors [4].

Hirudotherapy is used in many countries like Germany, America, China, Russia, England, and Africa and in Ancient Egyptian from 1,500 BC [5]. Now hirudotherapy are widely used for large number of diseases such as skin disorders, cardiac thromboembolic conditions, nervous system abnormalities, reproductive system problems, inflammation, ulcerative lesions, eye and dental disorders, in addition, hirudotherapy has established itself in micro and plastic surgery as a protective method against venous congestion as well as for cosmetic purpose.

Leeches are considered as a new remedy for chronic and life-threatening diseases like cancer and metastasis. Mode of action of hirudotherapy depending on the injected of saliva in patient tissues through the blood withdrawal, a specific analgesic materials in saliva is pain relief [6], in addition, to many active ingredients with thrombolytic, anticoagulant, anti-inflammatory and lymph and blood circulation enhancing properties[7].

Rheumatoid arthritis RA, is a chronic, autoimmune and inflammatory disease [8], a prevalence of 0.5-1% of infection in industrialized world [9], have persisting synovial inflammation causing damage of joints and inability.

The diagnosis of auto antibodies like rheumatoid factor (RF) as well as the presence of anti citrullinated peptide antibody is the most important pretested investigation for rheumatoid arthritis [10], pathogenesis for this disease stays
inadequately concept in spite of most scientist studies reported that the synovial fibroblast as well as the immune cell was producing a large number of inflammatory cytokines that are played critical role in RA disease, earlier this disease was thought to be only Th1 cell mediated rheumatoid arthritis disease, recently, Th17 cell is considered important immune cell which plays effective role in RA disease [11] and a key cells in joint destruction [12].

Aims of the Study
The aim of this research is to identify what leech bioactive substances are, what to expect during the treatment with leeches and how they can help you to treat rheumatoid arthritis disease.

Materials and Methods
This prospective study was conducted from October 2016 to October 2017 at Al-Najaf City/ Center of leech therapy; involve (140) patients with active rheumatoid arthritis disease (the diagnosis made by the consultant medical staff at Al-Sader Medical City hospital based on clinical examination (>3 swollen and >3 tender joints), the diagnosis of rheumatoid factor (RF) autoantibody and other immunological laboratory investigations, in addition to (70) samples of healthy individuals as control group to comparison the results.

Selection of Leeches
Leech named Hirudomedicinalis is used for medical purpose because it is nonpoisonous leeches.

Duration of Treatment
The duration of treatment was fixed for 16 weeks with 3 leeches every time, the patient were taking the same drug internally or externally during the treatment with hirudotherapy.

Hs-CRP Test
Serum levels of HS-CRP were determined by immunoturbidimetric assay with the use of reagents and calibrators from Roche diagnostics.

Cytokine Tests
The serum level of cytokines including IL1β, IL-6, IL-8, IL-10, IL-17 and TNF-α were measured using Enzyme Linked Immune Sorbent Assay methods according to the kits from Bio-Source, Europe S.A. measured doing before and after the treatment with hirudotherapy for the same patient to evaluate the efficiency of treatment.

Calculation of the Results
The results of patient samples were calculated by interpolation from a standard curve using a curve fit equation for IL1β, IL-6, IL-8, IL-10, IL-17 and TNF-α

Statistical Analysis
The values of these parameters were presented as mean ± standard deviation (S.D.), and significant differences between means were assessed by ANOVA test, the least significant difference (LSD) or Duncan’s test by using the computer programmed social package for statistical analysis (SPSS) version 7.5 in which a probability (P) equals or less than 0.05 were considered significant.

Results
From the total, one hundred and forty patients have active rheumatoid arthritis disease were included at this study, female patients showed increases percentage frequencies when compared with male patients (64.29 vs.35.71%) (Table-1).

Table 1: Gender distribution of rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Controls</td>
<td>70</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>50</td>
<td>90</td>
</tr>
</tbody>
</table>

The distribution of patient samples according to age groups showed that the highly effected age group was between 61-70 years old in percentage (42.86%) followed by the age group 71-80 years old (25%)(Table-2).
Rheumatoid arthritis patients showed a highly significant increased serum level of hs-CRP as compared to controls (9.35 vs. 1.19mg/ ml)(Table 3-A). While after hirudotherapy, patients showed a significant decreased mean serum level as compared to the patients before hirudotherapy (5.49 vs. 9.35 mg/ ml) (Table 3-B).

### Table 3-A: Serum level of hs-CRP in rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (mg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70</td>
<td>1.19±0.81</td>
<td>N.S.</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>9.35±4.17</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Hs-CRP-high sensitive C reactive protein
Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

A significant (P ≤ 0.01) increased mean serum level of IL-1β in rheumatoid arthritis patient as compare to corresponding control (59.87 vs.12.43 pg/ml) was observed (Table 4-A), while patients after hirudotherapy showed a significant decreased mean serum level as compared to the patients before hirudotherapy (Table 4-B).

### Table 4-A: serum level of IL-1β in rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70</td>
<td>12.43±8.23</td>
<td>N.S.</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>59.87±28.34</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

The total patient showed highly significant (P ≤ 0.01) increase mean serum level of IL-6 when compared to controls (70.34 vs. 13.33pg/ml) (Table 5-A), while patients after hirudotherapy showed a significant decreased mean serum level as compared to the patients before hirudotherapy (Table 5-B).

### Table 5-A: serum level of IL-6 in rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70</td>
<td>13.33±10.14</td>
<td>N.S.</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>70.34±31.34</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).
Table 5-B: serum level of IL-6 in rheumatoid arthritis patients divided by hirudotherapy

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients before hirudotherapy</td>
<td>140</td>
<td>70.34±31.34</td>
<td>0.05</td>
</tr>
<tr>
<td>Patients after hirudotherapy</td>
<td>140</td>
<td>41.43±17.21</td>
<td></td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

Total patients were showed significantly increased mean serum level of IL-8 as compared to control subjects (53.25 vs. 11.61 pg/ml)/(Table 6-A). Patients after hirudotherapy showed a significant decreased mean serum level as compared to the patients before hirudotherapy (Table 6-B).

Table 6-A: serum level of IL-8 in rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70</td>
<td>11.61±7.10</td>
<td>N.S.</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>53.25±28.19</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

Table 6-B: serum level of IL-8 in rheumatoid arthritis patients divided by hirudotherapy

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients before hirudotherapy</td>
<td>140</td>
<td>53.25±28.19</td>
<td></td>
</tr>
<tr>
<td>Patients after hirudotherapy</td>
<td>140</td>
<td>26.49±13.16</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

A significant increase mean serum levels of IL-10 in rheumatoid arthritis patient as compared to control (44.58 vs. 10.50 pg/ml) was observed (Table 7-A), while patients after hirudotherapy showed a non-significant increased mean serum level as compared to the patients before hirudotherapy (Table 7-B).

Table 7-A: serum level of IL-10 in rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70</td>
<td>10.50±6.54</td>
<td>N.S.</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>44.58±23.10</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

Table 7-B: serum level of IL-10 in rheumatoid arthritis patients divided by hirudotherapy

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients before hirudotherapy</td>
<td>140</td>
<td>44.58±23.10</td>
<td></td>
</tr>
<tr>
<td>Patients after hirudotherapy</td>
<td>140</td>
<td>34.21±14.21</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

Total patient showed highly significant (P ≤ 0.01) increase mean serum level of IL-17 as compare to controls (77.45 vs. 10.42 pg/ml) (Table 8-A), while patients after hirudotherapy showed a significant decreased mean serum level as compared to the patients before hirudotherapy (Table 8-B).

Table 8-A: serum level of IL-17 in rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70</td>
<td>10.42±6.90</td>
<td>N.S.</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>77.45±33.10</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).
A highly significant increased mean serum level in TNF-α of rheumatoid arthritis patient as compare to controls (68.30 vs.11.81 pg/ml) was observed (Table 9-A). Patients after hirudotherapy showed a significant decreased mean serum level as compared to the patients before hirudotherapy (Table 9-B).

Table 8-B: serum level of IL-17 in rheumatoid arthritis patients divided by hirudotherapy

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients before hirudotherapy</td>
<td>140</td>
<td>77.45±33.10</td>
<td></td>
</tr>
<tr>
<td>Patients after hirudotherapy</td>
<td>140</td>
<td>40.23 ±19.17</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

Table 9-A: serum level of TNF-α in rheumatoid arthritis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70</td>
<td>11.81±7.56</td>
<td>N.S.</td>
</tr>
<tr>
<td>Patients</td>
<td>140</td>
<td>68.30±30.10</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

Table 9-B: serum level of TNF-α in rheumatoid arthritis patients divided by hirudotherapy

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Mean ± S.D. (pg/ml)</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients before hirudotherapy</td>
<td>140</td>
<td>68.30±30.10</td>
<td></td>
</tr>
<tr>
<td>Patients after hirudotherapy</td>
<td>140</td>
<td>33.63±18.11</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Highly significant difference (P value ≤ 0.01)
Significant difference (P value ≤ 0.05)
N.S.: not significant (P value > 0.05).

Discussion

Rheumatoid arthritis was most of commonly autoimmune arthritis disease involving approximately 690,000 cases in adults; women are three times more is affected with rheumatoid arthritis [13]. Up to our knowledge, this is the first study in Iraq which is trying to correlate between hirudotherapy and pro-inflammatory cytokines associated with RA disease as perfect biomarker to determine disease onset and progression.

The measurement of cytokine levels of Th1, Th2, and Th17 immune cells were investigated to be highest in rheumatoid patients, because these types of cytokines were playing a critical role in the pathogenesis of rheumatoid arthritis disease like IL-1β, IL-6, IL-8, IL-10, IL-17 and TNF-α by stimulating cartilage and bone inflammation and degradation and bone, attributed to an imbalance occurs in activities of pro- & anti-inflammatory cytokines leading to multisystem immune complications, there for serum level of cytokines may considered as indicator for the severity of disease, hence RA as example of autoimmune disease is consequence of a persistent an imbalance between these types of cytokines leading to chronic inflammation. We find highly significant increase mean serum levels of hs-CRP in patient as compared with control group, this results is in agreement with the results of Shrivastava and Pandey[14], who reported that the activity of RA disease correlated with hs-CRP, in addition to increased serum level of cytokines including IL-1β, IL-6, IL-8, IL-10, IL-17 and TNF-α rheumatoid patient as compared with control group, such finding may highlight the importance of these cytokines in the aetiopathogenic mechanism of the disease and such results are in agreement with the results of Scott [15], who demonstrated that IL-1β was increased in serum and synovial fluid of rheumatoid arthritis patient, it is associated with activity of rheumatoid arthritis disease as well as this result is supported by Rengel et al.[16], Who demonstrated that IL-6 and TNF-α are playing a key role in the cross talk between cytokines and IL-6 is the highly important pro-inflammatory mediator [8] can activate leukocyte and promote the release of TNF-α, that is acts as an indicator for inflammation.
severity [17] TNF-α is one of the most important pro-inflammatory cytokines activated monocytes and macrophages as well as activated NF-κB (nuclear factor-κB), through the PARs (Proteinase-activated receptors) /p38-MAPK (P38-mitogen-activated protein kinase) /NF-κB pathway. Elevated TNF-α levels can activate IKK (IkB kinase), which then degrades IkB and activates additional NF-κB, promoting further activation of cytokines [18].

TNF-α is the most important in regulating the pro-inflammatory cytokines formation, additionally to activation of synovial fibroblasts leading to the connective tissue growth factor production, leading to the osteoclasts hyper activation and joints destruction [19].

Additionally, it is well established that IL-10 as anti-inflammatory cytokine, has immune regulatory role by inhibiting cytokine synthesis and down regulate the function of antigen-presenting cell, the mechanism leading to increase this cytokine remain unclear may be explained by the increased production as compensatory phenomena to inhibit over production of pro-inflammatory cytokine and then inhibit sever inflammatory processes occurring in RA disease. In RA disease pro-inflammatory cytokine was appear to stimulate the synthesis of IL-10 leading to losing anti-inflammatory function and acquire pro-inflammatory role [20].

In agreement to the previous results, our studies demonstrate that there were elevated level of IL-17 in serum of patients, Th17 cells are responsible for the production of pro-inflammatory mediators such as chemokine CCL20, interleukin-22, and interleukin-26 [21]. IL-17 promotes the secretion of cytokine and chemokine which accelerates infiltration of neutrophils in the tissues leading to inflammation ending with injury, as well as, play active role in T-cell triggered inflammatory process [22].

Blockading of IL-17 was showed beneficial effects on murine arthritis; this blockade could be helpful in the treatment of rheumatoid arthritis in human [23]. Medical leech therapy is a type of conventional treatment for inflammatory disease throughout antiquity, and it is a kind of complementary medicine, recently in medical treatment has only focused on their mode of action. Hirudotherapy, an old technique, has been investigated by many researchers for possible effects on many diseases like inflammatory diseases, osteoarthritis, and after surgeries, Hirudomedicinalis is the widest type of leeches used in therapeutic purposes, leech secretes more than 100 bioactive substances like antistasin [24], hirudin, eglin [25], saratin, guamerin, bdellins, carboxypeptidase and complement inhibitors. They have anti-inflammatory, analgesic, platelet inhibitory thrombin and anticoagulant regulatory functions, in addition to antimicrobial and extracellular matrix degradative effects [26].

This technique is easy to apply, effective and cheap. In the present study, we found that there is a significant decrease in hs-CRP after hirudotherapy, as well as we found that the mean serum levels of IL-10, IL-6, IL-8, IL-10, IL-17 and TNF-α were significantly decreased in RA patients, especially if we consider that decreased synthesis of these cytokines will reduce the severity of disease, these results are explained by mechanisms of leech therapy which started when leeches bite, hyaluronidase and collagenase allow access to tissues and blood vessels; vasodilatation occurs through the action of histamine-like molecules, platelet function, kinin activity and the coagulation cascade are inhibited leading to suppressed inflammatory reactions, these studies supported by experiments working on mice which show a positive effect on repair of tissue wounds, also our results supported by researches reported that the anti-inflammatory functions of thrombin inhibitor hirudin in ischaemic flaps are mediated by mechanisms which involve the down-regulation of IL-6, TNF-α and ICAM-1 (Intercellular adhesion molecule -1) expression via the PARs/p38-MAPK/NF-κB pathways. The effect of hirudin on increasing flap survival may be related to enhanced angiogenesis and its antioxidation, anticoagulation, and antithrombus activities [27].

In addition to several evidence describing the regulation of leukocyte responses by thrombin involving hemotaxis and monocyte proliferation, hirudin is a protein binds to thrombin, causes depreciation of active thrombin leading to antithrombin activity [28], this substance is the most important one, because it has highest anticoagulant
activity with fewer adverse effects [29], hypolipidemia, anti-inflammatory effects and changes in viscosity [30]. Gelin is an eglin analog and effective thrombin inhibitor, gelin shows inhibitory effects on neutrophil elastase, chymotrypsin and cathepsin [31].

Factor Xa inhibitor breaks coagulation cascade and has anticoagulant effect, it has a crucial role in hirudotherapy for treating rheumatoid arthritis and osteoarthritis. In addition, eglin has possible anticoagulant effects [32] via direct or indirect inhibition of coagulation factors [33].

Destabilase is an enzyme with glycosidase activity [34] shows antibacterial and fibrinolytic actions [35], it has a major degradative action on stabilized fibrin and it has an anticoagulant action. In addition to hyaluronidase and collagenase enzymes support antimicrobial activity by facilitating spread of leeches bioactive substances [36].

Recently, anticoagulant peptide from different types of leeches have been identified [37] also such finding is supported by the results of Frolov and Litvinenko [38,39], who reported that Lymphocyte culture treated with hirudotherapy had decreased in pro-inflammatory cytokines production due to apoptotic process of lymphocytes influenced by leech antigens.

Conclusion
Although the cause of rheumatoid arthritis is not known we can conclude from this research that leech therapy is highly effective in treating RA patients by reducing the severity of disease and play a significant role in decrease pain, relieves joint inflammation as well as improves joint functions, therefore hirudotherapy is considered a universal safety treatment for centuries.

Leeches have a natural process that remove infected blood and release therapeutic components and enzymes from its saliva in the same time, leading to reduce inflammation and pain in the joints by affecting the level of pro-inflammatory cytokines which had active role in the process of inflammation of active RA disease.

Recommendation
Hirudotherapy must take a significant place as a natural therapeutic agent in the treatment of RA disease by decreasing the severity of disease, in sometimes is the best complementary medication, and surpasses pharmacological treatments because of its healing effects in the human body. This type of study needed more investigation to determine the exact role of hirudotherapy in the treatment of RA disease. Although modes of leeches’ action and their bioactive substances still await more exploration and remain among the biggest challenge to early complementary treatment of this disease.

References


