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The effects of Bio-normalizer against chronic virus and acute toxic hepatitis

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TRIAL PROTOCOL

(confidential)

Pilot Clinical Trial (Phase II)

Title: THE EFFECTS OF BIO-NORMALIZER AGAINST CHRONIC VIRUS AND ACUTE TOXIC HEPATITIS

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INTRODUCTION

Over the last several years, much progress has been made in the treatment of adult patients with chronic hepatitis B and hepatitis C in the absence of significant other illnesses. Usually, adults included in clinical treatment trials suffered from stable active viral infection with intact immune systems, elevated alanine aminotransferase (ALT) levels with serologic or histologic evidence of hepatitis, and clinically compensated liver disease [ ]. Special patient populations such as children with lymphoproliferative diseases subjected to multiple transfusions and anticancer immunosuppressive therapy fall outside these categories. Treatment of these patients remains experimental.

Inadequate needle sterilization has contributed to the sp[read of HBV among these children in the vast majority of cases. The consequences of chronic HBV infection in children can be devastating. Not only chronic liver failure occurs, but the children are at risk for hepatocellular carcinoma development [ ]. It was shown recently [ ] that hepatitis B is related to the development of liver cancer in children in high endemicity areas of Asia, where hepatocarcinoma is the most common tumor in children. Results using α_2-interferon therapy for combating chronic HBV in children are similar to those seen in adults [ ].

Since the discovery of HCV in 1989, enormous progress has been made in the ability to detect, diagnose, and treat this infection [ ]. Chronic HCV evolves in 50-70% of patients who acquire acute infection [ ]. Only approximately 50% of the patients with HCV have symptomes or signs of liver disease [ ]. The serum ALT level does not correlate with the histological activity of the disease [ ]. It may be normal for a long time in the face of viremia and active liver disease on biopsy. In contrast to HBV, spontaneous remission is either extremely rare or does not occur in patients with chronic HCV [ ]. It is generally estimated that liver-related mortality from chronic HCV is 5-10% and hepatic failure develops in approximately 25% of patients who have progressed to cirrhosis [ ]. Therefore, chronic HCV is the major indication for liver transplantation [ ]. Common knowledge of chronic hepatitis C in children, as well as in other populations, is evolving as markers of the disease are developed. The recent progress of serologic tests for the detection of antibodies to HCV (anti-HCV) permits a reappraisal of the diagnosis of HCV infection in patients. However, serologic screening appears to identify not more than 70% of patients with ongoing HCV infection, genuine infection may be present even in the absence of a detectable humoral immune response [ ]. Furthermore, patients receiving immunosuppressive therapy may develop anti-HCV reactivity only after treatment withdrawal. Therefore, evidence of HCV infection may be provided by up-to-date highly sensitive methods such as HCV-RNA detection by PCR or anti-HCV detection by EIA 3 or 4 immunoassays [ ].

Hepatitis C occurs primaraly in children who have received multiple blood transfusions [ ], and in children on hemodialysis [ ]. Hepatitis C virus (HCV) infection also plays an important role in the etiology of liver disease in patients treated for childhood leukemia (ALL and AML) [ ].

An optimum therapy for HCV does not exist, however, interferon therapy is under evaluation. Thus, in one clinical trial, 36% of children who have been treated with α_2-interferon for 6 month had normal ALT activity [ ].

Management of chronic hepatitis.

Actually, α interferon therapy has been shown to be effective for adult patients with compensated liver disease due to hepatitis B and hepatitis C in the absence of significant other medical problems. At present, α_2-interferon is the most widely used for treatment HBV and HCV and is considered to be relatively non-toxic. However, α_2-interferon is inactive when taken orally, therefore, it needs to be injected. Furthermore, interferon treatment of hepatitis B and C virus infections has been hampered by overall initial response rates of less than 50%, a relapse rate that is more than 50% for patients with HCV, and rare responses in individuals with chronic HBV who are immunosuppressed (transplant recipients, dialysis patiemts, AIDS patients, children treated for acute lymphoblastic leukemia). Some of patients failed to response to interferon therapy due to the presence of interferon antibodies. These facts and the high cost of the drug prompted researchers and physicians to develop other medicine such as antiviral agents and immunomodulators. Immunomodulatory agents used in the treatment of chronic HBV are corticosteroids, colony stimulating and transfer factors, interleukin 2, and thymosin. Some of them, being tested in clinical trials, showed negligible activity in comparison with interferons [ ]. Most currently availuble antiviral agents (acyclovir, nucleoside analogues, AZT, interferons) inhibit viral DNA synthesis by inhibition either reverse transcriptase or DNA polymerase [ ]. Unfortunately, drugs of this class may also inhibit the host cellular and mitochondrial DNA synthesis, interfering with critical cellular functions, which lead to their high toxicity. It was suggested that in future the optimal treatment for chronic HBV infection may involve administration of different combinations of antiviral and immunomodulatory agents.

Overall, fewer agents have been tested for activity against HCV than with HBV. The lack of experimental models for chronic HCV infection and the recent discovery of the virus are the major reasons for this discrepancy. Until now, optimal management of HCV with α_2 interferon, which is the only licensed preparation, remains unclear, because neither a longer duration of therapy nor a higher interferon dose can cause the improvement of response rates [ ]. Antiviral agent ribavirin has been tested in clinical trials and showed a slight efficacy in the improvements in ALT levels, despite persistence of HCV RNA [ ]. Inhibitors of hepatic fibrosis and the protease inhibitors appear to be extremely promising new classes of antiviral substances to combat HCV infection [ ].

Bio-Normalizer (BN), a health Japanese food supplementation, produced by the yeast fermentation of *Carica papaya* and some other tropical herbs, is known for a long time as a very effective agent capable of improving digestive processes and liver functions. A mechanism of such kind a therapeutic activity remains obscure. Intensive studies over last 8 years have shown that BN exhibited free radical scavenging [ ], antioxidant [ ], and immunomodulating [ ] properties. It was found in the *ex vivo* experiments that BN significantly suppressed lipid peroxidation in the rat brain subjected to ischemia-reperfusion [ ], expressed antiinflammatory and antifibrotic activity [ ], improved the macrophage functions [ ]. BN has been recently discovered as a potent stimulator of endogenous interferon production in mice [ ]. Taking into account all these experimental facts, knowing that BN consists of protease/antiprotease system of papain, and it is practically non-toxic natural substance, we assumed that BN may protect liver cells against lesion induced by the virus infection and due to that may be efficient agent for a long-term treatment of patients with chronic viral HCV and HBV, and non-viral

toxic hepatitis. In present study to assess the HCV infection among children, who have been treating with chemotherapy and multiple blood transfusions, EIA 4, forth generation recombinant immunoblot assay (Organon, USA) and reverse-transcription polymerase chain reaction (PCR) were used.

PATIENTS AND METHODS

Patients.

30 patients, 17 (55%) males and 13 (45%) females aged between 2 and 15 years (median age 8.86 years), who have been treating at Russian Institute of Pediatric Hematology since 1991, were studied.

24 of them (80%) were suffered from acute lympholeukemia (ALL), 3 (10%) -were with acute myeloleukemia (AML), and 3 (10%) - were with aquired aplastic anemia. Details on antileukemic and antianemic treatment administered in this period are reported elsewhere in literature [1-3] and presented in patients' Case Reports. All the patients had received blood derivatives from volunteer donors as part of their treatment. The exposure to blood transfusions was expressed in blood units (one blood unit corresponds to one unit of packed erythrocytes, platelets, or frozen plasma derived from one donor). The mean number of units transfused was 8.9 per patient (range, 1 to 20 units).

Clinical trial design. This was a Phase II, open-label, controlled, randomized study. A main inclusion criteria was an elevated level of alanine aminotransferase (ALT) activity in the blood serum. Children fulfilling all the eligibility criteria were randomized according to the blind numbering to two groups:

Group A (15 children, odd numbers) - control group, patients were treated with standard chemotherapy or maintaining therapy. General characteristics of the control group patients are presented in Table 1.

Group B (15 children, even numbers) - experimental group, patients were additionally given 9 g BN a day for 28 days. Entire daily amount of BN was divided into three equal doses and given orally 3 times: 1) early in the morning before breakfast, 2) 1 hour after lunch, 3) just before sleeping. General characteristics of the experimental group patients are summarized in Table 2.

The total study period for each subject was 58 days: a 2-day baseline evaluation period followed by a 28-day testing period and a 28-day after BN treatment period.

This pilot clinical trial was performed in accordance with protocol approved by the Ethic Committee of the Institute of Pediatric Hematology. Informed consent of patient or his/her parents was obtained before the study.

The entire study was conducted between January 1995 and April 1995.

Methods.

Hepatitis diagnosis. Infection with hepatitis B virus (HBV) was assessed by serologic assays that allowed to determine the presence of HBs and HBe antigens, as well as

antibodies to HBe, HBcor, and HBs parts of a viral genome. Special diagnostic KITs for HBV determination were used (, USA).
A confirmation of HCV diagnosis was much more complicated, so far highly sensitive tests were applied.
Anti-HCV detection. Patient serum samples were tested by an EIA 4 (Organon HCV, 4th generation, Organon Diagnostic Systems, USA) that detects antibobies to core antigen, a fusion of the c100-3 with c33-core antigens of HCV, and NS5 protein, the putative HCV replicase, according to the manufacturer's instructions.
HCV-RNA detection. Freshly-collected serum samples stored frozen at -70°C were used for HCV-RNA extraction. Primer used for retrotranscription and nested PCR (polymerase chain reation) amplification were localized in concerved sequences of the 5'-noncoding region of the viral genome. Sensitivity of the method was 1 to 10 copies of template. To avoid false-positive results, all recommended precautions were strictly followed. An adequate number of controls was included in each set of experiments.
Non-viral toxic hepatitis associated with intensive anticancer chemotherapy was confirmed clinically, biochemically by the presence of elevated transaminase activities, and serologically by the absence of HBV and HCV viremia or antibodies to hepatitis viruses.

Interferon status. The entire content of interferons in the blood plasma was determined by ELISA assay (IFNg ELISA Kit, Endogen; Boston, Massachusetts). The α-interferon and γ-interferon production by phytohemagglutinin-stimulated peripheral blood mononuclear cells were assessed semi-quantitatively by means of the targeting virus-transformed cells. (Details of the latter assay will be provided a bit later!). Results were expressed as a dilution of the blood leukocyte supernatant induced a definite suppression in a targeting cell viability.

Cell immunology. To assess T-lymphocyte subpopulations, CD4+, CD8+, and their ratio were analyzed using monoclonal antibodies. Flow cytometry of peripheral blood cells was done using standard methods and a FACS microflow cytometer (Coulter, France). Details of such an analysis reported elsewhere [ ].

Biochemical assays. ALT, AST, and lactate dehydrogenase (LDG) activities, bilirubin and γ-GTP content were determined in order to find out evidence of liver chronic or acute failure. A standard Beckman Laboratory System analyzer CX40 was used.

Free radical status. Oxygen radical production by the blood neutrophils and monocytes was measured by luminol- and lucigenin-dependent chemiluminescence [ ]. Intensity of spontaneous lipid peroxidation in plasma was determined using TBA method [ ].

Statistical analysis. Non-parametric procedures were used throughout. Paired comparisons were made with the Wilcoxon signed ranks test. Statistical significance was assumed at $p < 0.05$. Results are expressed as mean ± SEM.

RESULTS

Patients.

Fifteen of the BN group patients, 8 males and 7 females aged between 2 and 15 years were suffered from ALL (15 subjects, 100%). A single dropout from the BN group occured 1 week into the study due to the child death of the relapse of underlined disease. During the clinical trial five (36%) patients were subjected to intensive chemotherapy, six (43%) - were on the maintaining therapy, and the others - were under observation without any anticancer treatment (Table 1). Nine (64%) of the 15 patients studied had evidence of exposure to HCV as shown by the presence of circulating anti-HCV and/or HCV-RNA (Table 3). The HCV viremia was found for three patients. Ten (71%) of the 15 patients were infected with HBV that followed from the data of serologic analyses (Table 5). Seven (50%) patients of this group were infected by a combination of HBV and HCV. Three (21%) patients exhibited some clinical features of toxic hepatitis induced by anticancer drugs. The diagnosis of acute toxic hepatitis was confirmed biochemically.

Fifteen of the control group patients, 8 males and 7 females aged from 4 to 13 years were mostly with ALL (10 subjects, 66%), AML (3 subjects, 20%), and acquired aplastic anemia (2 subjects, 14%) (Table 2). All patients were subjected either to intensive course of chemotherapy (10 subjects, 66%) or to maintaining therapy. Nine (69%) of them were exposed to HCV as was shown by serologic or PCR analyses; eleven (85%) children had serologic evidence of HBV viremia, and eight (62%) patients studied were infected both HBV and HCV (Tables 4 and 6). Antibodies to HCV were found in the 8 patients, while HCV viremia was confirmed by PCR assay for the 4 patients only. Toxic hepatitis was defined clinically and by the elevated level of transaminases in four (13%) children.

CONCLUSIONS

1. A short-term administration of BN showed good and satisfactory tolerability in 100% of patients studied. There were no drop-outs from clinical trial depending on BN toxicity and its side adverse effects.
2. A one month BN administration led to significant improvment of ALT levels in % of patients. This efficacy is higher than those found earlier for α_2 interferon treatment.
3. After 1 month BN administration, % of patients possessed significantly suppressed clinical symptomes of chronic hepatitis.
4. An efficacy of BN therapy was equal in HBV and HCV infected patients.
5. BN administration inhibited lipid peroxidation in plasma. It suppressed oxygen radical overproduction (% of patients) by neutrophils and monocytes, but increased an inhibited level of oxygen radicals in some patients (%).
6. A one month BN administration did not affect HCV and HBV viremia, but did increase antiHCV and antiHBV antibodies in % of patients.
7. A Phase III clinical trial with prolonged period of BN administration (up to 6 month) could be recommended for children and adults with chronic hepatitis treated for a variety of tumours.