2009 - The effect of KAQUN-water on the immune parameters of healthy volunteers /NICS/

NATIONAL INSTITUTE OF CHEMICAL SAFETY

Report

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Budapest

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Antecedents

KAQUN HUNGÁRIA Ltd. (2144 Kerepes, Szabadság út 102), as Client has contracted the National Institute of Chemical Safety/NICS) (1096 Budapest, Nagyvárad tér 2.) as contractor in contract no. GOKBI-360/2009 to test the immune effects of KAQUN water = Q voda in healthy volunteers at the Department of Cytogenetics and Immunology of NICS. Kaqun is a special water with high oxygen content, suitable for use in the form of drinking water or bathing. In our study we examined the effect of 21 days of bathing and drinking on the immune parameters of healthy volunteers. The end points measured were: qualitative and quantitative blood counts, the ratio of lymphocyte populations, lymphocyte activation and the oxidative burst of neutrophil granulocytes. The measurements were carried out on the first day before the start of the treatment (0 point) and on the 8^{th}, 15^{th} and 21^{st} days.

The theoretical basis of immunology tests

Immune-toxicology examines the damaging/modifying effects caused by exposure at the workplace, environment or therapy on the immune system. Its task is to detect and assess the modifying factors affecting the immune system especially from the aspect of their effect on human health. An immune response may be elicited when the immune system is the passive target of a chemical agent or when the chemical, as an antigen, triggers a specific response. In consequence of the complexity of the immune system the chemical agents have a broad target of attack. They can affect the development, maturation, division, differentiation and function of cells, or modify the regulation of the immune system.

The immunology tests were carried out on peripheral blood samples. Blood cells consist of *red blood cells* (erythrocytes), *white blood cells* (leukocytes) and *platelets* (thrombocytes). The volume ratio of blood cells in the blood is characterized by the *hematocrit* value.

Erythrocytes are formed in the bone marrow, their development takes about 4 -5 days, while their nucleic acid content gradually degrades, and mature red blood cells do not have a nucleus. The blood of an adult contains an average of $4.5x10^{12}$ / l erythrocytes (for women the average is about $4,5x10^{12}$ / l, for men it is somewhat higher, $5x10^{12}$ / l). During maturation erythrocytes synthesize hemoglobin molecules, which are able to carry oxygen.

The average life-span of erythrocytes is 120 days, and they are degraded in the spleen and liver. More than 99% of the blood cells are erythrocytes.

White blood cells have an important role in the defence mechanisms of the body. Blood contains an average of $9x10^9$ / l white blood cells, but 4-10 x 10^9 / l is also within the normal range. There are 3 main types of leukocytes: *granulocytes, monocytes and lymphocytes*. 50-75 % of leukocytes of a healthy person are granulocytes, 20-45 % are lymphocytes and 2-9 % are monocytes.

The horseshoe shaped nucleus of immature granulocytes becomes lobed as they mature. Another characteristic feature is the presence of large quantities of granules in the cytoplasm – the biologically active material stored within them has a very important role in the development of inflammation and allergic reactions. The *neutrophil, basophil* and *eosinophil granulocytes* can be distinguished on the basis of their histological staining properties. Most of the granulocytes are *neutrophils* (3-6 x 10^9 / l). Since their half life in the circulation is short, (generally ~6 hours), they are produced in large quantities every day. They are the basis of cellular protection against infection, and can enter the tissues in large quantities. In the course of bacterial or fungal infection the neutrophil granulocytes phagocytose and destroy the pathogens. The intracellular killing of pathogens is achieved by oxygen-independent enzymes (lysosomal elastase, lysosime) and oxygen-dependent enzymatic systems (principally NADPH-oxidase). The activated phagocytic cells produce antimicrobial reactive radicals, so called reactive oxygen intermediates (ROI) in a reaction named oxidative burst.

Under normal conditions the number of *eosinophils* is far less in the circulation (1.5-3.0 x 10^8 /l); they are mostly found in the mucous membranes of the respiratory, urinary, and intestinal tract participating in the protection against parasites. The number of eosinophils circulating in the vascular system increases in the case of allergic reactions. The *basophils*, similarly to mast cells, contain heparin, histamine and other inflammatory mediators in their granules. Their number is low (<1 x 10^8 /l), they are important because they mediate immediate type hypersensitivity and anaphylactic reactions.

Normally the *lymphocyte* count is in the range of 1.5-3.5 x 10^9 /l, and their importance lies in mediating the adaptive immune response. They are relatively small cells, their round shaped nucleus fills the cytoplasm almost completely. Lymphocytes are classified into 3 main groups: *T lymphocytes* are responsible for the so called cellular immune response, while *B lymphocytes* are responsible for the humoral immune response, and the production of antibodies. The *NK cells* kill virus infected or cancerous cells.

Monocytes make up about 2-9 % of the white blood cells (1-8 x 10^8 /l), their nucleus is large, kidney or bean shaped. They originate from the bone marrow, they then enter the circulation where they spend about 72 hours, and then pass through the blood vessel wall and change into *tissue macrophages*. Their activation is initiated by lymphokines secreted by T lymphocytes, and as a result they become able to phagocytose foreign matter such as bacteria, and to release a number of inflammatory mediators (e.g. prostaglandin-E).

Platelets (*thrombocytes)* are cytoplasmic fragments of megakaryocytes surrounded by a cell-membrane; they do not have a nucleus. Their size is approximately 2-5 □m. When leaving the bloodstream or encountering damaged endothelial walls they are activated and play an

important role in blood coagulation. The average thrombocyte count is 3 x 10^{11} /l, but a value in the range of 1.5–4.0 x 10^{11} /l is normal.

The immune system has an evolutionarily old, non-specific arm which reacts immediately upon infection. Its most important elements are macrophages, granulocytes, NK cells and the complement system. Macrophages and *granulocytes* have an important role in the phagocytosis of pathogens and foreign particles, while *NK cells* destroy virus-infected and cancerous cells. The pathogen organisms that enter the body first meet this so-called innate immune system. Built on this, is the specific (antigen specific) adaptive immune system, which reacts slowly (in days) when first meeting the antigen, but has an immunologic memory; therefore it works fast and efficiently in the case of a second infection. T and B lymphocytes are the cells of the adaptive immune system. During the adaptive immune response *cytotoxic T (Tc)* cells are generated which are able to destroy the pathogens directly (cellular immune response), and *B lymphocytes*, which produce antibodies (humoral immune reaction). The presence of *helper T lymphocytes (Th)* is essential for the division and differentiation of the T and B cells. Cell-cell interactions and cytokines produced by leukocytes have an important role in the regulation of the immune response.

A number of molecules, "markers" appear on the surface of lymphocytes and with their help the lymphocyte populations can be distinguished from each other. These markers have been classified into groups, and each marker has been given a CD (Cluster of Differentiation) number. The basic lymphocyte populations (T, B, NK cells) can be defined with cell markers: *T lymphocytes* express CD3 (CD3+ cells), *helper T cells* also express CD4 (CD4+/CD3+ cells), *cytotoxic T cells* express CD8 besides CD3 (CD8+/CD3+ cells). Immature T cells express both the CD4, and the CD8 molecules (CD4+/CD8+ cells). *B lymphocytes* can be characterized by the CD19 cell surface antigen (CD19+cells). *NK cells* have CD56 surface molecules, but do not express CD3, therefore they are characterized as CD56+/CD3- cells. CD25 (IL-2 receptor) and CD71 (transferrin receptor) surface antigens cannot be detected on resting lymphocytes, they are expressed when the lymphocytes are activated (e.g. by an antigen). Therefore these surface molecules can be used to detect the activation of lymphocytes.

Immunotoxic materials can affect different immune parameters; therefore we have adjusted our measurements to characterize different functions. This is important, because the change in one parameter or another is not suitable to characterize the general condition of the immune system, conclusions can only be drawn from changes in the data pattern. We characterized the immune status of the studied subjects by measuring characteristics of white blood cells gained from peripheral blood. Qualitative and quantitative blood count was determined, and immune phenotyping was used to determine lymphocyte subpopulations and the CD25 (IL-2R) and CD71 (transferrin receptor) activation antigens expressed on lymphocytes with the aid of monoclonal antibodies produced against cell surface molecules.

Innate immunity was characterized with the help of a functional test: the killing capacity of white blood cells was determined by measuring the production of reactive oxygen intermediates (ROI) of granulocytes.

Test procedure

Selection of healthy volunteers

The selection of 30 healthy volunteers (15 women, 15 men) was carried out by KAQUN HUNGÁRIA Kft. Exclusion criteria in this study were: acute or chronic illness, infection, the use of any kind of drugs, and smoking, because these could affect immune parameters.
The participants were informed about the purpose and the course of the study, and they signed a *Declaration of Agreement* confirming that they had received information about the study and that their participation was voluntary.

Duration of the study and the procedure:

The examined persons participated in a 21 day bathing and water drinking treatment. The participants bathed once a day in the morning in individual bathtubs filled with 37 ^{o}C water containing stable oxygen, for a maximum of 50 minutes per occasion. The water drinking cure consisted of drinking 1.5 liter Kaqun drinking water every day in parallel with the baths. The bathing cure followed the standards established in the Kaqun Health Program Service.

The 21 days Kaqun bathing and the parallel water drinking treatment was divided into 4 groups, because only 7-8 persons could be examined in a single day. All four groups started on the first week, the first on Monday, the second on Tuesday, the third on Wednesday and the fourth on Thursday. The participants of the first group were always examined on Monday, the second on Tuesday and so on, see table below.

	1st week	2nd week	3^{d} week	4th week
Monday 1st group	day 1 blood sampling before treatment	day 8 blood sampling after treatment	day 15 blood sampling after treatment	day 21 blood sampling after treatment
Tuesday 2nd group	day 1 blood sampling before treatment	day 8 blood sampling after treatment	day 15 blood sampling after treatment	day 21 blood sampling after treatment
Wednesday 3^{d} group	day 1 blood sampling before treatment	day 8 blood sampling after treatment	day 15 blood sampling after treatment	day 21 blood sampling after treatment
Thursday 4th group	day 1 blood sampling before treatment	day 8 blood sampling after treatment	day 15 blood sampling after treatment	day 21 blood sampling after treatment

Methods:

Blood sampling:
Blood sampling at the site: day 1 before the bath, (0-point), then on days 8, 15, and 21 after the bath during the same part of the day. The blood samples were taken from the cubital vein of the examined persons in sitting position, under sterile conditions with venipunction. Standard 3 ml sterile vacuum blood sampling tubes containing anti-coagulant were used for blood sampling. One 3 ml tube with EDTA anti-coagulant for determining the qualitative and quantitative blood count, one 3 ml tube with heparin for the immunology tests. The blood samples were given unique identifiers marked on the blood sampling tubes.

The following tests were carried out on the blood samples:

1) Qualitative and quantitative blood count

The qualitative and quantitative blood count was carried out with an automated analyser in the blood sampling laboratory of OMFI (Bp. IX. Nagyvárad tér 2.).

Determined parameters:

- WBC leukocyte count,
- abs LY, abs MO, abs NEUTR, abs EO: the absolute number of lymphocytes, monocytes, neutrophil- and eosinophil granulocytes
- LY %, MO %, NEUTR %, EO %, BA %: percentile distribution of lymphocytes, monocytes, neutrophil- eosinophil- and basophil granulocytes
- RBC red blood cell count,
- Hb concentration of hemoglobin in the blood,
- HTK hematocrit,
- MCV mean cell volume,
- MCHC mean corpuscular hemoglobin concentration,
- RDW-CV red blood cell distribution width
- MCH mean cell hemoglobin,
- Thrombocyte count

2) Determination of immune parameters

Method:

The subpopulations and activation of circulating lymphocytes were determined by immune phenotyping, using flow cytometry. Heparinized whole blood was used for the measurement. The surface markers of peripheral lymphocytes were measured with fluorescent labelled monoclonal antibodies in a flow cytofluorimeter. The surface antigens

examined were: CD3 (T-cell receptor), CD4 and CD8 (T-cell co-receptors), CD19 (B-cell co-receptor), CD25 (interleukin-2 receptor), CD45 (protein-tirosine-phosphatase, pan leukocyte marker), CD56 (neural cell adhesion molecule, NK-cell marker), CD71 (transferrin receptor). Using 3 and 4 colour staining the following antibody combinations were used: (1) CD25-FITC / CD8-PE / CD3-PerCP / CD4-APC; (2) CD56-FITC / CD3-PerCP / CD45-APC; and (3) CD71-FITC / CD3-PerCP / CD19-APC. Standard forward and side scatter gating combined with CD45 was used to separate leukocyte populations and to set the lymphocyte gate. The lymphocyte subpopulations of the donors (T lymphocyte, helper T, cytotoxic T, B lymphocyte and NK-cell) were determined with the aid of cell markers. CD25 and CD71 surface antigens were used to determine the activation of lymphocytes.

Determined parameters:

- Ly, Mo, Neu, Eos: percentage of lymphocytes, monocytes, neutrophil- and eosinophil granulocytes

- Total T, T helper, T cytotoxic, Immature T, B cell, NK-cell: percentage of T lymphocytes, cytotoxic and helper T lymphocytes, immature T lymphocytes, B lymphocytes and NK-cells within lymphocytes

- Th/Tc: The ratio of helper and cytotoxic T lymphocytes

- Activated T: percentage of CD25 (IL-2 receptor) activation antigen carrying T cells within the T cells
  - Activated Th: percentage of CD25 activation antigen molecule carrying helper T cells within the helper T cells
  - Activated Tc: percentage of CD25 activation antigen expressing cytotoxic T lymphocytes within the cytotoxic T lymphocytes
  - CD71 positive T: percentage of CD71 (transferrin receptor) molecule carrying T cells within the T cells
  - CD71 positive B: percentage of CD71 (transferrin receptor) molecule carrying B cells within the B cells

3) Oxidative burst of neutrophil granulocytes

The production of reactive oxygen intermediates (ROI) which is directly proportional with the killing potential of white blood cells was measured with the aid of Bursttest (Phagoburst®) kit. Neutrophil granulocytes respond to activation by producing reactive oxygen intermediates, which oxidize the fluorogenic substrate. The quantity of oxidized substrate is proportional to the production of reactive oxygen radicals. Heparinized whole blood was used, and the measurement was carried out on a flow cytometer. We measured the quantity of oxidized substrate in the control and the stimulated samples, and determined the percentage of ROI producing cells. The activation stimuli: 1) fMLP chemotactic peptide (weak stimulus). 2) E. coli opsonized with antibody, which stimulates through the Fc receptors that recognize the constant part of the antibody (particulate stimulus) 3) PMA (phorbol-myristil-acetate), which transports signals through protein kinase C (strong stimulus)

Determined parameters:

Production of reactive oxygen intermediates (ROI)

Control, fMLP, E. coli, PMA: ROI production in unstimulated samples, and samples stimulated with fMLP, E. coli, and PMA

Percent of ROI producing cells
Control, fMLP, E. coli, PMA: Percent of ROI producing cells in unstimulated samples, and samples stimulated with fMLP, E. coli, and PMA

Statistical analysis:

Student's paired-t test was used for the group level statistical evaluation of the results, the level of significance was set at $p<0.05$.

Results and conclusions

1) Qualitative and quantitative blood count

The group results of qualitative and quantitative blood counts are shown in *table 1,* the individual results in *table 2.* No significant change was observed for the group average of white blood cell count in any of the groups. Individually both increased and decreased leukocyte counts could be observed during the three weeks of the study. No change was observed for the group average of lymphocyte counts. On the other hand a statistically significant decrease was observed in the group average of monocyte counts during the treatment in all three groups. In men the count decreased after the first and second week of treatment, while the change was not significant after the third week compared to the 0 point. At the individual level the monocyte count does not change or a slight decrease can be observed. In men the group average of neutrophil granulocyte count increases after the second and third week of treatment. At the individual level generally an increase can be detected, but in a few cases a reduction was observed during the three weeks of the study. The eosinophil count decreased for the whole group by the second and third week; in the case of men the reduction was present already after the first week. There was no significant change in the group average for women. Individually no change could be observed above the uncertainty of the measurement.

The percentage of white blood cells shows a similar change to that of the absolute numbers. The percent of monocytes decreased at the group level for all three groups already after the first week of treatment. Further change was not observed. The percent of neurophils increased for the whole group and for men after the second week of treatment, the percent of eosinophils decreased in the whole group and in men already after the first week of the treatment.

At the group level there were no changes in the red blood cell count and hemoglobin content. After the second week of the treatment a slight decrease in hemocrit was observed for the whole group. The average volume of erythrocytes (MCV) showed a very slight decrease by the second week, therefore the hemoglobin concentration for one erythrocyte (MCH) and the average hemoglobin concentration of the erythrocytes (MCHC) increased to a small extent.

A statistically significant increase in the group average of thrombocyte count was observed after two and three weeks of treatment both for the whole group and in men. Examining the individual results, the subjects usually did not show large changes in the thrombocyte count, and the thrombocyte count always remained within the normal range.

Biologically significant change was not observed in the qualitative and quantitative blood count either at group or individual level.

2) Determination of immune parameters

The measurements carried out with the flow cytometer produced very similar results to those carried out with the automated analyser regarding the percentile distribution of lymphocytes, monocytes, neutrophil- and eosinophil granulocytes. This can be considered as the internal control of the measurements.

The group averages of immune parameters are shown in *table 3,* the individual results in *table 4.* The percentage of monocytes decreases at group level for the whole group and for men by the second week of the treatment. At the same time the percentage of neutrophil granulocytes increases at group level for the whole group and for men by the second week of the treatment. The percent of eosinophils decreases in the whole group from the first week of the treatment, and in the case of men by the second week of the treatment the decrease is significant. In women the above parameters do not change significantly. In the course of the treatment the ratio of leukocytes changes statistically, which could be indicative, but the changes are so small that probably no physiological importance can be attached to them.

No significant changes were observed in the percentage of total T cells, helper T cells, immature T cells and B lymphocytes. The ratio of helper and cytotoxic cells did not change (Th/Tc) either. In the case of men the ratio of cytotoxic T cells showed a small, but significant reduction after the third week of the treatment. The percentage of NK-cells increased significantly after the second week of the treatment both for the whole group and for women. In men an increase was observed, but due to the large deviations in individual results, the change was not significant statistically. Individually, in general either there was no change or an increase was observed during the three weeks of the study. Although the ratio of cytotoxic T lymphocytes showed a significant decrease, at the individual level the changes were so small, that a physiological effect cannot be expected. Relatively bigger changes (increase) were observed in the ratio of NK-cells at the individual level, compared to the 0 point, which may have a functional impact: more NK cells are available to kill virally infected or cancerous cells.

The percentage of activated (CD25+) T lymphocytes increased by the second and third week in the whole group and in men. At the individual level there is either no change or an increase can be observed, but in a few cases the percentage of activated T cells decreased in the three weeks of the study. The percentage of activated (CD25+) helper T cells increased for the whole group by the second week of the treatment. In general individually there is either no change or an increase can be observed. The percentage of activated (CD25+) cytotoxic T cells increased significantly after the third week in the case of men. The increase in the expression of the CD25 cell surface molecule indicates the activation of T lymphocytes. These results indicate the intensification of the cellular immune response.

The percentage of transferrin receptor positive (CD71+) T lymphocytes did not change during the treatment. The percentage of B lymphocytes expressing transferrin receptors (CD71+) decreased significantly by the second week in the whole group, and by the third week this value returned to the original level. The individual data show such a large

distribution both individually and intra-individually that a biologically relevant conclusion cannot be drawn from these data.

Among the examined persons, there was a man whose percentage of B lymphocytes was well below the reference value. The reference range for B lymphocytes is 7.0-23%. The B cell percentage of the person indicated as Q3,Q33,Q63,Q93 (Gábor Rabb) was between 0.3-0.9% during the period of the study. The white blood cell count and the absolute lymphocyte count did not decrease, but the percentage lymphocytes was low measured with both test methods, and the percent of B lymphocytes was extremely low. The B cell count (data calculated with the aid of the absolute number of lymphocytes and the percentage of B cells) was at least one order of magnitude less than in the case of the other subjects. His data were not included in the statistical analysis of immune parameters, as in our opinion they would have falsified the data.
On the 15th of June 2009 the blood sample of the person coded Q35 (István Berei) deviated to such an extent from the values measured during the three other occasions regarding certain parameters (percentage of lymphocytes measured both with the automated instrument and flow cytometer, percentage of helper T and NK-cells) that his data measured on 15.06.2009 were omitted from the group level evaluation of the immune parameters.

3) Killing capacity of neutrophil granulocytes (production of reactive oxygen intermediates-oxidative burst)

The group averages for the production of reactive oxygen intermediates of neutrophil granulocytes are shown in *table 5,* the individual results in *table 6.* The reactive oxygen intermediate production (ROI) of neutrophil granulocytes increased significantly in all three groups from the first week of the treatment in the fMLP and PMA stimulated samples, and from the second week in the samples stimulated with E. coli. Individually, in general an increase was observed in ROI production, though in the samples stimulated with E. coli and PMA a decrease relative to the 0 point was observed for certain individuals after the first week.

The percentage of ROI producing cells increased significantly in all three groups from the first week and this is also true at the individual level.

The increase in ROI production, and the fact that more cells respond to stimulation, result in the increased killing potential (bactericidal effect) of neurophil granulocytes.

Summary

1. No biologically significant changes were observed in the qualitative and quantitative blood count either at group level or individual level during the 21 days of Kaqun treatment.

2. The percentage of NK-cells showed a statistically significant increase, and the individual changes (increase) relative to the 0 point were bigger, which may have a functional impact, namely that more NK cells are available to kill virus infected and cancerous cells.

3. A non-specific activation of T lymphocytes (indicated by the increase in the expression of the CD25 cell surface antigen) could be detected, presumably caused by the Kaqun treatment, indicating the increased activity of the cellular immune response.

4. Characteristically the value of several parameters changed significantly by the second week of treatment and during the third week the value of the parameter remained at the same level, or the change levelled to its original value (percent of neurophils, monocytes, activated (CD25+) T cells, activated (CD25+) helper T cells and CD71+ B cells). This suggests that two weeks treatment is the most effective for the change in immune parameters and after that the reaction of the body to the treatment decreases, that is, the effect cannot be boosted.

5. The increase of the production of reactive oxygen intermediates both at group level and at the level of the individuals results in the intensification of the killing potential of neurophil granulocytes.

28th of September 2009

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