



2010 - Report on the examination of KAQUN oxygen-rich water's role in reactive oxygen species generation in in vitro system /HAS/

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Report on the examination of KAQUN oxygen-rich water's role in reactive oxygen species generation in in vitro system

KAQUN Hungária Kft. and the Department of Surface Chemistry and Catalysis HAS Isotope Research Institute concluded a research contract for the examination of KAQUN oxygen-rich water in order to assess whether the clinically tested beneficial effect of the water stated for the immune system that could be supported by assessing the immunological parameters of volunteers may be evidenced at any basic level of the mechanisms or not. For this purpose we examined KAQUN oxygen-rich water's effect exercised on peroxide production and reactive oxygen species generation in a purposefully selected in vitro system, which may be important in vivo for influencing apoptotic systems. We also examined how the different effects exercised on water – heat effect, nitrogen and carbon dioxide rinse – influence KAQUN water's effect on peroxide production.

The performed examinations, their results as well as the conclusions deduced from them will be presented below, and also a recommendation for further reasonable examinations will be given.

The applied examination method

Horse radish peroxidase – peroxide – benzidine system

The principle of the method

Horse radish peroxidase produces reactive oxygen species from peroxide. This converts benzidine into a colourful product with kinoid structure. The change of colour concentration may be photometrically measured at 620 nm. Thus the system – being a highly sensitive procedure - is suitable for detecting reactive oxygen species. By the use of this method it can be assessed whether oxygen-rich water increases the quantity of reactive oxygen species in the system or not. Peroxidase first reduces molecular oxygen solved in the oxygen-rich water to peroxide, then thus produces a greater quantity of reactive oxygen species from the increased peroxide in the system.

This method may also be used for measuring antioxidant capacity. The presence of an antioxidant, e.g. ascorbic acid, poliphenol, or uric acid inhibits the formation of the colour.

Description of the process

- 1) Peroxidase + H₂O₂ + benzidine → reactive oxygen species + kinoidic benzidine
- 2) O₂ + peroxidase → H₂O₂ + benzidine → reactive oxygen species + kinoidic benzidine

Reagents

- 1) Horse radish peroxidase 9000 U/l
Benzidine HCl 233 µmol/l
NaCl 155 mmol/l
- 2) Carbamide peroxide, stabilized 2.5 mmol/l

For the examinations we prepared liophilized reagent 1) which was solved in 10 cm³ oxygen-free or –poor, ion-exchanged water. The solution is stable at a temperature between + 2 and + 8 °C for 2 weeks, and between + 15 and + 25 °C for 2 days. Reagent 2) is stabilized carbamide peroxide which was solved in 100 cm³ oxygen-free or –poor water. The solution is stable at a temperature between + 2 and + 8 °C for 1 week.

- Measured samples:
- 200 µl KAQUN water
 - 200 µl KAQUN water boiled for 10 minutes
 - 200 µl KAQUN water rinsed with nitrogen
 - 200 µl KAQUN water rinsed with carbon dioxide

200 µl oxygen-free, or –poor, ion-exchanged water control

Measuring equipment: LKB UV-Vis spectrophotometer

1 cm³ narrow cuvette

Temperature: 25 °C

We carried out the measurements in the method that 1 cm³ of reagent 1) was added to 200 µl sample, the sample was homogenized, then the reaction was started by adding 200 µl of reagent 2). Absorbance and its change were immediately measured for 3 minutes. Absorbance intensity was proportional to the quantity of the produced reactive oxygen species. Intensity measured in control water was considered 100%, the intensity measured in KAQUN water samples was compared to this.

Results and their evaluation

Examination results are represented by the attached Tables 1 and 2, and Figures 1 and 2. Based on the results it can be clearly concluded that in the applied in vitro system, in KAQUN oxygen-rich water a reactive species concentration showing the maximum may be reached in 10 seconds, whereas in the control water this process is slow, showing significantly lower maximum. The produced reactive oxygen has short life. The increase measured in comparison to the control is resulted by the fact that the oxygen-rich water allows an increase in peroxide quantity according to the reaction outlined above. We also examined whether KAQUN water's effect increasing peroxide quantity changes or not in open bottle, or to the effect of rinsing with nitrogen, carbon dioxide or boiling. From the data in Table 2 the following decreases can be assessed: 6.4% in a bottle open for 5 days, 4.7% for rinsing with nitrogen, 6.6% for rinsing with carbon dioxide, and 49.9% for boiling for 10 minutes. Boiling caused the greatest decrease of efficiency, which is naturally no surprise as the oxygen content of water increases at cooling, and decreases at heating. Whereas at a temperature of 0 °C maximum 14.5 mg oxygen can be solved in 1dm³ water, at 25 °C only 8.5 mg. KAQUN water contains 18-20 mg oxygen per dm³, which is 6 to 8 times higher than average oxygen content.

The reaction applied in in vitro system also happens the same way in the cell system as both peroxide generation from molecular oxygen and substrate oxidation take place in the cell wall while reactive oxygen is produced. Here NADH also participates in the reaction. In perfect systems there is a balance in these processes. The lack of reactive oxygen species means a problem similar to their permanent overproduction causing oxidative stress state. The extremely quick reactive oxygen increase measured in in vitro system allows the hypothesis that adding the appropriate quantity of oxygen-rich water in in vitro conditions might lead to a quick production of greater quantity of OH species in the Fenton (Haber-Weiss) reaction. It is known that several publications deal with the topic that the intracellular oxidative state, reactive oxygen species (ROS) might play an important role in apoptosis.

Programmed cell death is of high importance in the development of multi-cell living organisms and in the operation of the immune system. A great part of physiological cell death takes place by means of apoptosis, and it is a basic part of the differentiation of both animal and plant tissues. During experiments it became clear that in the development of high order organisms cell death leads to the formation of different organs, organ systems

and parts of the body, besides it plays a role in eliminating different structures used in certain development phases that are not needed any longer. Apoptosis is indisputably important in the formation of the immune system. The development of T and B lymphocytes is a complex process. During the generation of the ever renewing lymphocyte stock there will always be clones that are unable to work or are autoaggressive. These need to be removed from the operating lymphocytes. This removal is of high importance so that they can function efficiently. Clones will be destroyed by means of the apoptosis mechanism. By this method the organism prevents autoimmune reactions acting against own cells. The disorders occurring in the control of the apoptotic system may lead to the generation of several diseases, mentioning just a few: autoimmune diseases, immune deficiency syndrome, rheumatoid arthritis, etc. The normal function of apoptosis is essential for wound healing as well.

Apoptosis is started and controlled by cell signals. The discussion of the complicated apoptotic cascade may not be the subject of the present report. As reactive oxygen species play an important role in this mechanism, in the sense of the above outlined processes KAQUN oxygen-rich water – if it can provide a higher concentration of molecular oxygen at cell level – may have an effect on the starting of the non-operating apoptosis, or on the increasing of the reduced apoptosis. All this can have a beneficial effect on certain diseases. It is evidenced that for example increased apoptosis have a favourable effect on rheumatoid arthritis. The clinical effects evidenced so far by KAQUN water can probably be explained by the stimulation exercised on the apoptotic process as well. The results of the examinations performed in different cell lines by using the water can be interpreted similarly.

Recommendations for further examinations

The experiment results presented in our report prove that KAQUN oxygen-rich water is able to increase the quantity of peroxide and reactive oxygen species in in vitro peroxidase – peroxide system. These experiments should be repeated in cell lines where apoptotic cascade only works weakly or it does not work at all. This means first of all the grade of apoptosis must be measured in the cell line. Several cell populations are suitable for these examinations. It is obvious to use tumour cell lines as in these the catalase linked to the membrane protects

the tumour cells from apoptosis induced by intracellular ROS. This takes place in a way that catalase decomposes peroxide very efficiently thus preventing the Fenton (Haber-Weiss) reaction in which the OH species giving apoptotic signal are generated. In the lack of peroxide, HOCl synthesis will also be inhibited, which is also an apoptotic signal, which means the tumour cell is in catalase protection. The inhibition of catalase leads to the intracellular signalisation of ROS. Thus the quantity of the produced peroxide is accumulated and Fenton (Haber-Weiss) reaction and the generation of HOCl take place, which produces reactive oxygen species.

The examination of KAQUN oxygen-rich water in cell system will probably result the fact that the quantity of the peroxide produced in the method as presented in this report – which may be reached by dosing GOX (glucose oxydase), HRP (horse radish peroxidase), and MPO (mieloperoxidase) – exceeds the effect of catalase and starts the apoptotic cascade through

the Fenton (Haber-Weiss) and HOCl. This can be evidenced by measuring the extent of apoptosis.

In *in vivo* conditions the question is what molecular oxygen concentration may be provided constantly from the oxygen-rich water at cell level.

Literature

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