Title: DEUTERIUM DEPLETED WATER (DDW) USING AS ADJUVANT IN CANCER THERAPY FOR CYTOSTATICS TOXICITY REDUCING

Abstract: The hereby invention addresses to a new application of Deuterium Depleted Water (DDW), namely, its administering, on a concentration of 60 ppm, as daily diet, to adult healthy outbred Wistar rats produces a distinguished lowering of toxic effects produced by cytostatics (Cyclophosphamide, 5-Flourouracil, 5-Fluorouracil, and Vinblastine) when used in these animals mono-chemotherapy. Also, the 60 ppm DDW administering under similar conditions generates a significant cytostatics toxicity (Cyclophosphamide, 5-Flourouracil, and Vinblastine) used in polychemotherapy on pet dogs having different types of cancer. These relevant findings confirm the idea of 60 ppm DDW as a new and efficient adjuvant in cancer chemotherapy on pets and humans.

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DEUTERIUM DEPLETED WATER (DDW) USING AS ADJUVANT IN CANCER THERAPY FOR CYTOSTATICS TOXICITY REDUCING

The patent refers to DDW using as reducer of cytostatics toxicity. It is well known that when treating the cancer, the chemotherapy is used both on human and animals. Besides the favourable effects obtained when treating the tumours, chemotherapy has toxic immune-suppression effects on patients, or under certain conditions, it can even trigger secondary cancers. This is why the researchers focused on finding that medical means or other nature means to reduce cytostatics toxicity, and consequently to improve therapy factor and to implicitly ameliorate the cancer patients’ life condition.

The positive results of procaine as adjuvant to colorectal cancer chemotherapy is well known (Europ. Journal of Cancer, 1995, 31A, p. 1283 –1287). Sergio Caffagi, Mauro Exposito et al. have realized the synthesis of a new cytostatics – CIS-diamino-platinum – (II) that contains procaine chloral hydrate, which is a kind of cytostatics with anti-tumoral effect both in vivo and in vitro (Anticancer research, 1992, 12, p.2285-2292).

In vitro and in vivo, this new cytostatic has a renal toxicity much lower than classic cis-platinum cytostatic, and this is the reason why it is used in oncological hospitals.

A method to in vivo establish the efficient Deuterium Depleted Water concentration for cancer therapy on lab animals is known, method (Patent Application: a 2003/00685) that could be applied in experimental oncology. It is ascertained, by this method, that continuously administering of Deuterium Depleted Water (DDW), with a concentration of 60 ppm, over a period of 60 days prior tumor grafting, and then the administration of this water over a period extremely long (700 days) inhibits some experimental malign tumor development and growth at Wistar outbred rats, finally resulting in cancer significant percentage ingrowths, as well as in significant
prolongation of survival time of the animals having tumors (due to a very slow tumoral growth).

The problem solved by this invention is finding a therapeutic means to avoid the toxic side effects of cancer chemotherapy. The hereby invention finds out a new application of DDW, and this is that the administering of 60 ppm Deuterium concentration DDW, as daily diet, leads to a visible decrease of some cytostatics toxic effect on hepatic, renal and hematopoietic area. These effects recommends 60 ppm concentration Deuterium Depleted Water use as adjuvant with benefic effects on chemotherapy.

The advantages of Deuterium Depleted Water administering during mono-chemotherapy are the followings:

- It reduces the toxic effects within cyto-hematological status, both on peripheral blood and on lympho-nodal area
- It has a hepato-protective effect
- It reduces the intensity of degenerative nephritis modifications generated by cytostatics on kidneys level
- It reduces P450 enzyme activity involved in cytostatic metabolism
- Determines the minimization of glycolysis and implicitly of serum glycoproteins concentration, which demonstrates that the cytostatics toxicity is reduced;
- Determines the level lowering of glutation-S-transferazes (GST) implied in cytostatics metabolism – a lower level of these enzymes reached after DDW administering shows the positive role this kind of water has in influencing the toxicity minimization of cytostatic products.
- It has a protective effect against oxidizing process, with a reduction of oxidizing stress factor.
Herein below, there is an exemplification of DDW use as adjuvant in cytostatic toxicity minimization, related to the figures that illustrate:

- figure 1: $^3$H-thymidine distribution in thighbone marrow of adult Wistar rats, after 5 days from the last cytostatic administering;
- figure 2: $^3$H-thymidine distribution in thighbone marrow of adult Wistar rats, after 10 days from the last cytostatic administering;
- figure 3: $^3$H-thymidine distribution in lymphnodes of adult Wistar rats, after 5 days from the last cytostatic administering;
- figure 4: $^3$H-thymidine distribution in lymphnodes of adult Wistar rats, after 10 days from the last cytostatic administering;

60 ppm concentration Deuterium Depleted Water was used for healthy adult outbred Wistar rats. Mono-chemotherapy was applied on these animals using the following cytostatics: Vinblastine (VBL); Cyclophosphamide (CFS); 5-Fluorouracil (5-Fu); Farmarubicine (FARM). The administered doses were:

- VBL – 0.1 mg/kg body weight;
- CFS – 5 mg/kg body weight;
- 5-Fu - 10 mg/kg body weight;
- FARM – 1 mg/kg body weight;

The cytostatics were intra-peritoneally administered (i.p.) during 5 days consecutively; the doses were established depending on lethal dose of 50% (LD50).

The animals were distributed into two groups, as:

1) animals that had received tap water, as a daily diet, before the beginning of the cytostatic therapy, during cytostatic therapy and after the last cytostatic dose (TW$^1$-control group);

$^1$TW – Tap Water
2) animals that had received 60 ppm Deuterium Depleted Water, as a daily diet, before the beginning of the cytostatic therapy, during cytostatic therapy and after the last cytostatic dose (DDW\textsuperscript{2}-control group);

Animals sacrifice occurred over 5, respectively 10 days from the last cytostatic dose. After animals’ sacrifice, some tests were performed: bio-chemical, cyto-histological& morphological tests (on liver, spleen, kidneys), and test for 3HTdR incorporation into lymphoid organs (bone marrow and lympho-nodules).

The four cytostatics have as a side effect myelinic suppression, and this is why the cyto-hematologic test has been performed on smears of peripheral blood from hemato-genering marrow and from lymphatic ganglions.

The heart, the liver, the kidneys and the intestines of the animals were also morphologically examined. The slides have been May-Grünvald-Giemsa stained.

Biochemical exams comprised sialic acid pouring into the serum extracted from the whole blood; lipid peroxidizing (MDA); oxidative stress factor determination (ISO).

Also, the enzymes implied in cytostatics metabolism (P450-cytocromes, triggering enzymes, GST-glutation S-transferaize) were examined, too.

Proteins; glycoproteins; the serum-proteins degree of glycolysis and the electrophoresis of these serum-proteins in agarose gel were examinated.

\textsuperscript{3}HTdR incorporation was identified in tighbone marrow and in the lymphonodules of healthy adult Wistar rats.

All these examinations were performed both on DDW-control group and on TW-control group.

\textsuperscript{2} DDW – Deuterium Depleted Water
A. Health development at the animals in the two groups: DDW and TW-control

The protective effect of the 60 ppm Deuterium Depleted Water against the toxicity generated by cytostatics has been studied using lots of 88 Wistar rats, each lot, males and females, with an average weight of 172 g. The animal lots were established as per GLP on pharmacological studies on laboratory-tested animals. The possibility of acute cytostatic toxicity reducing by DDW administering as animals' daily diet was noted.

Taking into account the seriousness degree, the depressive effect on hematopoiesis of immunological area of the body is the most important side effect, myelin suppression being the limitative factor for cytostatic dose, as the 4 kinds of cytostatic have as main side effect the myelin suppression. The lowering of this toxic phenomenon intensity at the rats from DDW-control group was observed, comparing to the intensity of this toxic phenomenon at the animals in the TW-control group.

The life quality condition of the rats was observed, respectively the improvement of this factor at the DDW group, through diminuation or elimination of the other side phenomena accompanying the cytostatics administering, as comparing to TW-control group.

Thus, it is known that the most serious adverse reactions accompanying the cytostatics administering occur to:

a) digestive system: the 4 types of cytostatics are generating nausea, vomit, inflammations of mucous membranes (from stomatitis to ulceration, depending on the administered dose), anorexia, diarrhea, or constipation;

b) urinary system: cyclophosphamide can produce micro and macroscopic hematurias

c) cardiovascular and pulmonary system: cyclophosphamide can produce pneumonia and late pulmonary fibrosis; cyclophosphamide, 5-fluorouracil
and farmarubicine can produce cardiac insufficiency depending on the administered dose;

d) skin: all the cytostatics can produce alopecia, nail and skin pigmentation;

e) peripheral and central nervous system: 5-fluorouracil and vinblastine can produce ataxia, paresthesia, peripheral neuritis.

All this manifestations have been parallel observed at the two groups of animals.

Other observed parameters were:

- spontaneous mortality rate
- body weight loss
- digestive, nasal hemorrhage
- apathy
- anorexia

The above parameters observation has led to the following findings:

- at the DDW group animals receiving cyclophosphamide, 5-fluorouracil or farmarubicin, there were no records of any spontaneous dead animals. Within this group of animals there were no any signs of toxicity during the 5 days of therapy, or during post-therapy period. At the TW-control group being similarly treated, the spontaneous mortality occurred and it was necessary to sacrifice the animals showing obvious toxicity signs

- at the DDW group animals receiving vinblastine (in this case, the most toxic cytostatic) low intensity toxic effects were recorded and respectively, a low percentage of mortality (25% versus 66% at TW-control group).

- body weight increase demonstrated some differences between the two groups of animals. So, at the TW-control group, an easy body weight slow down was recorded, until the 5th day of the post-therapy period. At the DDW group receiving vinblastine, the animals body weight increase was far more evident comparing to TW-control;
clinical condition of the animals demonstrated that in case of DDW group no any cytostatic typical toxicity phenomena occurred (except for the group receiving vinblastine, when some low intensity phenomena occurred), compared to TW-witnesses group that demonstrated obvious toxicity phenomena.

B. Biochemical examinations

a) Sialic acid dosing

Taking into consideration the importance of sialic acid (N-acetil-neuraminic acid) in manifesting the cell biological properties, the quantitative changes of this acid have been analyzed at the DDW animals group receiving, as a daily diet, Deuterium Depleted Water before the beginning of cytostatic therapy, during this therapy and also after the last cytostatic dose, comparing to the animals in TW-control group.

The study was performed on a number of 32 Wistar outbred rats.

For each sampled serum quantity, sialic acid has been dosed after the serum hydrolysis with 0.1N sulphuric acid, by Kattermann micro-method. The free or bonded sialic acid dosage is based on oxidation of sialic acid with periodic acid, and on the mono-aldehydes generation that combined with 2-sulphobarbituric 2-thiobarbituric acid produced a stain compound that was spectrophotometrically measured.

The sialic acid level was examined in Wistar rats serum. The findings are shown in Table 1
Table 1

<table>
<thead>
<tr>
<th>Lot No.</th>
<th>Cytostatic</th>
<th>Number of rats</th>
<th>TW–control Group Sialic acid mmol/l (average values)</th>
<th>DDW Group (daily diet with Deuterium Depleted Water) Sialic acid mmol/l (average values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cyclophosphamide</td>
<td>8</td>
<td>2.77</td>
<td>2.68</td>
</tr>
<tr>
<td>II</td>
<td>5-fluorouracil</td>
<td>8</td>
<td>3.04</td>
<td>2.43</td>
</tr>
<tr>
<td>III</td>
<td>farmarubicine</td>
<td>8</td>
<td>2.88</td>
<td>2.62</td>
</tr>
<tr>
<td>IV</td>
<td>vinblastine</td>
<td>8</td>
<td>3.00</td>
<td>2.82</td>
</tr>
</tbody>
</table>

The findings regarding average values of sialic acid levels showed in the above Table 1 demonstrate that for all the administered cytostatics the sialic acid levels are lower at DDW – group.

b) Lipids peroxidizing

Amongst the biological effects produced by free radicals, the high degree of lipid peroxidising can be explained also by a higher probability for the reactive species of oxygen to encounter lipid compounds, elements of cell membranes or cell organelle. The lipid peroxidising is a typical example for reaction having a chained radicals mechanism. One single peroxil radical entered into reaction with fat poly-unsaturated acid could not only result in altering the structure of the acid, but it could generate also a series of fat acid peroxil radicals. These ones would, then, interact with other lipids, keeping active the transfer reaction of one electron, and the oxidative destroying of the substratum. The toxic aldehydes resulted from lipids in cell
membranes peroxidizing have their own biological effects. Being unsaturated α-β, they are electrophile species with a high reactivity, and are easily interacting with SH-groups of the proteins or with the thiols having small molecular mass (glutathione), making inactive the proteins and enzymes with essentials –SH groups. Thus, a series of cyto-pathological consequences of the lipid peroxidizing in membranes are explainable.

The measurements within present invention approach the oxidative stress parameter modifications induced to the two groups of animals - TW-control and DDW - due to the cytostatics administering.

On this purpose, measurements of the reactions of lipidic peroxidizing, thiol-oxidizing were performed and also, murine ceruloplasmin measurement was done. These measurements were esteemed to be important for oxidative stress to be established, as follow:

- *the reaction between 2thiobarbituric (TBA) and malondialdehyde (MDA) as index of lipid peroxidizing.*

TBA reacting materials generation is frequently used to esteem what are the organs possible to show lipid peroxidizing. The lipid peroxidizing of the biological samples might be esteemed by measurement of MDA formed as a result of endoperoxides breaking down during the last stage of poly-unsaturated fat acids oxidizing. This method is the most simple one and presently, the most used. Malondialdehyde (MDA) represents the final product of lipid peroxidizing and the by-product of prostaglandins and thromboxanes biosynthesis.

- *Ceruloplasmine dosing*

Ceruloplasmine was dosed into extra-cell fluids by Ravin method. This dosing method was chosen to illustrate the hereto invention because ceruloplasmine is able to catalyze the Fe²⁺ oxidizing to Fe³⁺ after its releasing from transferrin. This
activity represents a mechanism by which the lipid peroxidizing and free radicals generation promoted by iron ions could be prevented. It is known that lipid peroxidizing can be started by the transit metals ions, especially by the iron and copper ions existing in all the biological systems. Any increase of iron and copper salts concentration will result in tissular attacks and would enhance the oxygen toxic effects. Ceruloplasmine stops the redox cycles necessary for toxic effects initiation.

To esteem the oxidative stress, as per the scope of this invention, a new parameter has been introduced, namely the oxidative stress factor, which is defined as being the ratio between oxidative reactions and the anti-oxidant ones. Both these reaction types are measurable by means of ceruloplasmine and lipid peroxides values referred to thiol-oxidation basis. Thus, the oxidative factor could be calculated using the formulae:

\[
\text{Oxidative Stress Factor (OSF)} = \frac{\text{lipid peroxides \times ceruloplasmine}}{\text{thiol-albuminous groups}}
\]

The obtained results are shown in Table 2

<table>
<thead>
<tr>
<th>LOT</th>
<th>Peroxides</th>
<th>Ceruloplasmine</th>
<th>Thiol-albuminous</th>
<th>Oxidative Stress Factor (OSF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/100 ml serum</td>
<td>(U.I.)</td>
<td>nmol/l</td>
<td></td>
</tr>
<tr>
<td>DDW + CFS</td>
<td>5.65</td>
<td>64</td>
<td>247</td>
<td>1.46</td>
</tr>
<tr>
<td>DDW + 5-FU</td>
<td>5.32</td>
<td>81</td>
<td>285</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>69</td>
<td>397</td>
<td>1.08</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td>DDW+FARM</td>
<td>5.74</td>
<td>71</td>
<td>178</td>
<td>1.28</td>
</tr>
<tr>
<td>DDW+VBL</td>
<td>6.54</td>
<td>91</td>
<td>349</td>
<td>1.70</td>
</tr>
<tr>
<td>TW+CFS</td>
<td>6.47</td>
<td>63</td>
<td>267</td>
<td>1.52</td>
</tr>
<tr>
<td>TW+5-FU</td>
<td>6.14</td>
<td>110</td>
<td>204</td>
<td>3.31</td>
</tr>
<tr>
<td>TW+FARM</td>
<td>7.48</td>
<td>80</td>
<td>358</td>
<td>2.98</td>
</tr>
</tbody>
</table>

It is found that the Oxidative Stress Factor values are smaller at the group of animals treated with cytostatics and Deuterium Depleted Water as daily diet, and this fact demonstrates that, within the ratio oxidants/anti-oxidants, the anti-oxidant branch is stronger, having effect on activating the protection system against oxidative stress accompanying the cytostatic metabolization.

c) The study of enzymes implied in cytostatic metabolization

The enzymes implied in cytostatics metabolization have been classified as activation enzymes (or phase I enzymes) such as P450 cyto-chroms and detoxifying enzymes (or phase II enzymes) such as glutathione S-transferases (GST). The activation enzymes (MFO) role is to transform the hydrophobic substances into hydrophilic substances. The homo-oxygenating substances catalyze the insertion of one single oxygen atom from the oxygen molecule on an organic substratum, the other oxygen atom being reduced to water. This process occurs due to the reactions of substratum oxidizing as below:

\[ \text{AH} + \text{O}_2 + 2\text{NADPH} \rightarrow \text{AOH} + 2\text{NADP}^* + \text{H}_2\text{O} \]

This enzymatic system is located to the level of the endoplasmic reticulum membrane of the mitochondrion and of the nucleus’ envelop. To study it, the cell organelles are to be separated through repeated centrifuging with a final separation of microsomes of
105,000 xg by ultra-centrifuging. At the microsomes level, the P450 cytochroms (a kind of iso-enzymes family) activity and concentration is measured, which are the most important MFO representatives.

The P450 concentration is spectrophotometrically determined after CO and Natriumdithionit reduction of microsomal P450 (Omura and Sato method). As P450 is a isoenzyme family it intervenes both in activation and in detoxification of xenobiotics and so, also in cytostatics’ activation and detoxifications. The enzymatic activity has been determined for a single substratum, namely for p-nitroanisol, which is a de-metilation reaction.

Among the enzymes implied in cytostatic detoxification there are also the glutathion S-transferases (GST). These are a group of enzymes that catalyze the conjugation of some electrophile products (of endogen or exogen origin) with glutathiones (GSH) according to the reaction:

RX +GSH → GS-R + HX

The GST activity determination has been done using the Habrig method.

One of the important properties of this enzymatic system is the fact that, similar to P450 cyto-chromes, it shows a reduced substratum specificity, which is a property needed for xenobiotics detoxification, and it also shows a high specificity of their metabolism.

Together with GST, the GSH (the main sulph-hydrdric non-protein compound from alive bodies) intervene within the detoxification processes working as a electrophile “scavenger”, which is also a reaction possible under the condition of GST absence:

R⁺ + GSH → R-GS + H⁺

In mammals' cell, the GSH has a double functionality oscillating between the reduced form and the oxidized one. It takes part to H₂O₂ reducing, which is a reaction
catalyzed by glutathione peroxidase, being, therefore, implied in some normal radicalic or pathologic processes control. 

GSH was determined through the aloxane method.

The measurements results are shown in Table 3

Table 3

<table>
<thead>
<tr>
<th></th>
<th>P450 Activity nmoles/mg proteins/min</th>
<th>GSH Snmoles/mg proteins</th>
<th>GST nmoles/mg proteins/minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFS+TW</td>
<td>0.7414</td>
<td>0.598</td>
<td>2.580</td>
</tr>
<tr>
<td>CFS+DDW</td>
<td>0.3999</td>
<td>0.368</td>
<td>1.096</td>
</tr>
<tr>
<td>5-FU+TW</td>
<td>0.7341</td>
<td>0.598</td>
<td>1.47</td>
</tr>
<tr>
<td>5-FU+DDW</td>
<td>0.4557</td>
<td>0.478</td>
<td>1.37</td>
</tr>
<tr>
<td>FARM+TW</td>
<td>0.6031</td>
<td>0.581</td>
<td>1.73</td>
</tr>
<tr>
<td>FARM+DDW</td>
<td>0.4739</td>
<td>0.419</td>
<td>1.313</td>
</tr>
</tbody>
</table>

From Table 3, the following conclusions can be drawn:

- due to Deuterium Depleted Water utilization as daily diet, some changes in activity of the enzymes in phase I and phase II are noted comparing to TW-control group;
- in case of cyclophosphamide, which is a nonspecific form without alkylation activity, its activation occurs at the liver level under the action of P450. The cyclophosphamide administering causes a decrease of approximately one time of the phase I and II enzymes at the group TW-CFS;
- Deuterium Depleted Water utilization together with the cyclophosphamide causes a visible diminuation enzymes’ activity;
- 5-fluorouracil induces the phase I enzymes, enhancing the P450 activity to approximately twice times (double) at the control, comparing to the ones at the group of animals treated with Deuterium depleted Water;
- farmarubicine is metabolized into liver through reduction and hydrolysis. In case of farmarubicine administering together with Tap Water, this highly induces the P 450 activity and concentration of P450, and in case of Deuterium Depleted Water administering, the P450 activity is much more reduced;
- at the all lots of animals treated with cytostatics and DDW, both GSH concentration and GST activity have been much lowered comparing to the ones recorded for those lots of TW-control animals treated with cytostatics and tap water.

d)  **Protein, glycoprotein and serum protein glycolysis degree examination**

The methods used for these measurements are the well known ones (the measuring burette and the (glucidic) glucose compound dosing through the reaction with orto-toluidine).

The findings of this examination are shown into the Tables 4 and 5.
Table 4

<table>
<thead>
<tr>
<th></th>
<th>Total protein g/dL</th>
<th>Glucose (glucides) compound mg/dL</th>
<th>Glycolysis degree mg glucides/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFS+TW</td>
<td>6.9</td>
<td>45.49</td>
<td>6.59</td>
</tr>
<tr>
<td>CFS+DDW</td>
<td>5.5</td>
<td>30.1</td>
<td>5.47</td>
</tr>
<tr>
<td>5-FU+TW</td>
<td>6.6</td>
<td>45.49</td>
<td>6.89</td>
</tr>
<tr>
<td>5-FU+DDW</td>
<td>6.2</td>
<td>26.27</td>
<td>4.2</td>
</tr>
<tr>
<td>FARM+TW</td>
<td>5.08</td>
<td>49.41</td>
<td>9.72</td>
</tr>
<tr>
<td>FARM+DDW</td>
<td>6.1</td>
<td>38.8</td>
<td>6.3</td>
</tr>
<tr>
<td>VBL+TW</td>
<td>5.15</td>
<td>41.56</td>
<td>8.06</td>
</tr>
<tr>
<td>VBL+DDW</td>
<td>4.5</td>
<td>34.0</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Tabelul 5

<table>
<thead>
<tr>
<th></th>
<th>Glycolysis Degree with Tap Water mg glucides/g proteins</th>
<th>Degree of glycolysis with Deuterium Depleted Water mg glucides/g proteins</th>
<th>Percentage of protein glycolysis degree lowering at the animals receiving DDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclophosphamide</td>
<td>6.59</td>
<td>5.47</td>
<td>17%</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>6.89</td>
<td>4.2</td>
<td>39%</td>
</tr>
<tr>
<td>farmarubicine</td>
<td>9.72</td>
<td>6.3</td>
<td>35%</td>
</tr>
<tr>
<td>vinblastine</td>
<td>8.06</td>
<td>7.5</td>
<td>7%</td>
</tr>
</tbody>
</table>
From Tables 4 and 5 the following conclusions can be drawn:
- the 60 ppm concentration Deuterium Depleted Water including in the daily diet of the animals has caused the lowering of the glycolysis degree and implicitly the serum glycoproteins concentration lowering;
- a different effect in case of each cytostatic is noted;

e) Measurement of serum proteins gel electrophoresis in agarose

The used method is the application of a double diffusion in agarose gel using two lectins from wheat seed embryo and from Raphanus Sativus.
The fact that the serum sampled from cytostatics & DDW –treated animals group has not interacted with lectin confirms the idea that DDW has a favorable effect both on glycolysis degree and on serum glycoproteins distribution.

f) Measurements on \(^3\text{HTdR (\(^{3}H\) Tritiated Thymidine)}\)

The \(^3\text{HTdR}\) incorporation was measured in thighbone marrow and in lympho-nodules of adult Wistar rats from both groups (DDW and TW-control).
The incorporation capacity determination was performed by intra-peritoneally injecting of a quantity of 37kBq/g body weight (1μCi/g) of 3H-thymidine with a specific activity of 925 GBq/mmol (25Ci/mmol), and the radiochemical purity was higher than 95%. The biological samples were sampled after 5 hours from \(^3\text{HTdR}\) injecting, then they were dissolved in Soluene-350, re-suspended in liquid scintillator (Hyonic fluor). The activity measurement was performed using a Tri-Carb, Packard Model device.
After 5 days from the last cytostatic dose, a significant percentage increase of \(^3\text{HTdR}\) (7% and 15%) distribution was registered both in marrow and in lympho-nodules, at the animals receiving 60 ppm concentration Deuterium Depleted Water as a daily diet, comparing to TW-control (Fig. 1 and 3).
After 10 days from the last cytostatics dose, the $^3\text{HTdR}$ distribution in bone marrow and in lympho-nodules of DDW group of animal was more significant comparing to TW-control, (14% - 25%) as shown in Fig. 2 and 4.

The findings demonstrate a real improvement of AND synthesis. This significant increase of AND synthesis is also a result of cytostatic-produced toxicity reducing through daily administering of 60 ppm Deuterium Depleted Water, which makes possible the DDW application as adjuvant for cytostatics’ toxicity reducing.

C) Cyto-hematological examinations

These examinations were intended for some cyto-morphological aspects revealing, to demonstrate the modifications occurring within the hematopoietic area, as consequences of 60 ppm Deuterium Depleted Water administering as a daily diet, comparing to TW-control group that received tap water as a daily diet. The examinations were done using samples from peripheral blood, hematogenous marrow and from lymphatic ganglions. The prepared samples were classically stained with May-Grünwald-Giemsa. The examinations led to the following findings:

- the toxicity of all cytostatics used in the present invention had very high values within hematopoietic territory of the TW-control group, to which 150 ppm Deuterium tap water was been administered as daily diet:
  - anemia and granulopenia in the peripheral blood of the cyclophosphamide-treated animals
  - leukocytosis in peripheral blood of the 5-fluorouracil-treated animals;
  - leukopenia, anemia and granulopenia in the peripheral blood of the farmorubicin-treated animals
anemia, thrombocytopenia, leukopenia and granulopenia in the peripheral blood of the vinblastine-treated animals;
- hyporegenerative anemia in medulogram;
- the 60 ppm Deuterium Depleted Water administering as the daily diet, significantly reduced, to almost zero, the toxic effect from hematopoietic zona;
- cyto-hematological status revealing the differences between TW-control group and DDW group is manifested both in peripheral blood and in lympho-nodal area, but not in the medullar zona of hematopoiesis.

**D) Histological examinations**

The tissue samples taken from Wistar rats used on the present invention purposes were fixed into a solution of 10% formaldehyde. To demonstrate the histological modifications, the Masson method using paraffin insertion and tri-chrome staining technique was applied.

The microscopically aspects of histological examinations revealed the following findings:

a) **liver**

- at all the animals treated with 60 ppm Deuterium Depleted Water as daily diet (the DDW group) tissular territories with morphologically non-modified hepatocytes and extension-limited, hepatocyte degeneration zones were recorded. The degenerative modifications were manifested through a hepatocytarian tumefaction, granular dystrophy or granulo-vacuolar dystrophy and through cytoplasm oxiphiliation. All the lesions of this kind are considered to have reversible potential;
- at the DDW and vinblastine treated animals, the hepatocytary regeneration is clearly manifested;
- at the animals in the TW-control group hepato-steatosis has been noted, which is considered to be an aggravation of the degenerative lesions.

b) kidneys

- the presence of the mono-nuclear infiltration at the most of the animals in the TW-control group as well as its absence under the condition of Deuterium Depleted Water administering suggests the protective effect of the DDW on the renal interstitium;
- granulo-vacuolar nephrosis was more reduced in terms of extension and intensity at the DDW group;

c) intestine

- in the most of the cases from the both groups of animals no any modifications have been noted in the structure of intestinal walls.

d) myocardium

- no any significant modifications have been noted on the myocardium morphology

From histological examination the following conclusions can be drawn:

- the 60 ppm concentration Deuterium Depleted Water administering as daily diet has an hepato-protective effect, with liver morphology preservation, comparing to hepato-steatosis installed to the animals that have been administered tap water in their daily diet
- the hepatocytary regenerative phenomena are far more obvious in case of vinblastine administering together with Deuterium Depleted Water
- the 60 ppm concentration Deuterium Depleted Water administering as daily diet has a protective effect on renal interstitium;
- the 60 ppm concentration Deuterium Depleted Water administering as daily diet has an impact in reducing the intensity of nephrocitary degenerative modifications observed in case of normal water administering.
The relevant results of all the examinations demonstrate that 60 ppm Deuterium Depleted Water administering as a daily diet, at healthy adult female and male outbred Wistar rats before, during and after cytostatics administering (mono-chemotherapy) determines mainly a hepato-protective effect, an obvious reducing of toxic effect of cytostatics within the hematopoietic area, the reducing of nephrocytary degenerative modifications intensity and the lowering of glycolysis degree of the serum protein. All these results demonstrate that Deuterium Depleted Water of 60 ppm concentration reduces the toxicity of administered cytostatics and constitute a pro-argument for the idea that this kind of water using as a benefic adjuvant in cancer chemotherapy.

The results obtained with 60 ppm DDW as benefic supplement in various types of cancer poly-chemotherapy on pet dogs are a serious evidence supporting this idea. Thus, 26 dogs of different outbred, sex and age, with different cancer types clinically and para-clinically diagnosed through cyto-morphological and hematological examinations or through lympho-nodal piercing, have been divided into two groups:

**Group A:** dogs treated with cytostatics (poly-chemotherapy as successive treatment sessions, with different periods of breaks depending on the anatomo-clinical type of cancer) and to what 60 ppm DDW had been administered as daily diet, with 14 days before the poly-chemotherapy begun, during the therapy period and after therapy completion.

**Group B (control):** dogs that had been treated with the same kind of cytostatics and in the same way and to what 150 ppm Deuterium concentration tap water (TW) had been administered.

Poly-chemotherapy was applied using the following cytostatics:

1. *Cyclophosphamide:* 80-100 mg/m²; a treatment session at 14 days;
2. *5-Fluorouracil or Metrotrexat:* 50-75 mg/m²; a treatment session at 7 days;
3. *Vinblastin:* 2-3 mg/m²; a treatment session at 7 days;
4. *Vincristin:* 1 mg/m²; treatment session at 3 days;
All these cytostatics, as it is well known, demonstrate toxicity elements, from what we might mention the following:

- cyclophosphamide that firstly affects the tissues having a fast turn-over, causes the urinary bladder fibrosis, hyper-uremia hepatotoxic modifications, myelo-suppressive effect, leukopenia and trombocytopenia, hemorrhaging-necrotic cystitis, alopecia, etc.

- 5-Fluorouracil causes: medullar aplasia; nausea; vomiting; diarrhea; digestive hemorrhages; ulcers; lacrimal channel stenosis; conjunctivitis; etc.

- Vinblastin causes myelin-suppression, urinary retention, neuro-muscular and pulmonary disorders;

- Vincristin causes leukopenia; trombocytopenia; alopecia; rash; oedema; paresthesia; deep tendonal reflexes lose; myalgia; paraplegic ileus; polyuria or disuria; urinary retention, etc;

The 60 ppm DDW & cytostatic treated dogs manifested an almost normal appetite comparing to the dogs receiving exclusively cytostatics.

As regarding locomotion disorders (that are specific to advanced cancers), the 60 ppm DDW & cytostatic treated dogs had a muscular tonus clearly superior to the ones treated only with cytostatics. One of the explanations is that a better toxins elimination occurred through urine.

The dogs having malign lymphoma treated with 60 ppm DDW & cytostatic showed an obvious micro-poly-adenopathy comparing to the macro-poly-adenophaty noted at the dogs treated only with cytostatics.

These aspects are explained also by the cytopathic effect that DDW has on malign lymphocytes.

At the group of dogs treated only with cytostatics, a series of disorders appeared, such as:

- renal: glomerulonephritis; proteinuria and nephritic syndrome; Bence-Jones protein hematuria in case of B plasmacytom;
- splenomegaly clinically and echografically evidenced;
- mucosas might show a hemorrhagicary syndrome; micro-hemorrhages at the tongue bridle level, at the soft palate level, etc.
- tachycardia and tachypnea – signs of respiratory and systemic cardio-circulation disorders;
- gastro-intestinal manifestations: constipation or diarrhea; progressive weakness (weight lose) of the animals, gastro-intestinal ulcers with melena and hematemesis etc.
- skin paraneoplastic manifestations: alopecia; dermato-fibrosis; erythema; urticaria; rash; skin and mouth ulcers;
- hematological manifestations: erytrocytosis in case of renal, liver, pancreatic tumors; anemia in case of pancreatic tumors and melanoma; granulocytosis in melanoma and lymphoma; trombocytosis in leukemia and carcinoma.

All these manifestations showed in toxic status description, as a result of cytostatics used by us in poly-chemotherapy schemes applied to pet dogs having different types of cancers are very much reduced when 60 ppm DDW had been administered as a daily diet for these animals.

The mixed treatment consisting in cytostatics and 60 ppm DDW administering, caused also a lowered level of the urea and serum creatinine both during and after the therapy.

For example: at an 11 years, male German Dog, with cellular lymphoma B diagnosis, before the cytostatic & 60 DDW therapy, the serum urea values were of 58.8 mg/dL, during the therapy, they were of 37.9 mg/dL and post-therapy the values were of 26.3 mg/dL. At the same animal, the serum creatinine values were of 1.50 mg/dL before the therapy, of 1.27 mg/dL during the therapy and of 0.9 mg/dL after therapy.
At another dog, 5 years, female Rotwtweiller breed, diagnosed with cytemic centroblastic lymphoma B, that was treated with 60 ppm DDW & cytostatic, the following biochemical findings were recorded:

- **creatinine** 1.48 mg/dL before the therapy, 1.8 mg/dL during therapy and 1.38 mg/dL after therapy;
- **trombocytes** 135 thousands/mm³ before therapy, 209 thousands/mm³ during therapy and 201 thousands/mm³ after therapy
- **leukocytes** 9.45 thousands/mm³ before therapy, 12.69 thousands/mm³ during therapy and 8.70 thousands/mm³ after therapy;

While at a 2.8 years, female Pekinese dog, diagnosed with Waldenström malady that was treated only with cytostatics, the following biochemical results were recorded:

- **urea**: 30.6 mg/dL before the therapy, 43.24 mg/dL during therapy and 52.6 mg/dL after therapy;
- **alkali phosphatase**: 32 U/L before the therapy, 360U/L during therapy;
- **leucocytes**: 95.07 thousands/mm³ before therapy, 43.3 thousands/mm³ during therapy and 146.05 thousands/mm³ after therapy;

Another interesting aspect is related to the 60 ppm DDW effect on immune cellular system of dogs having different cancer types and treated with cytostatics. Thus, 60 ppm DDW acts both on cell clones responsible for cellular and humoral mediated immunity. The 60 ppm DDW initiates an apoptosis process that is cyto-morphologically translated by reducing the proliferative cells number, and consequently, tumor volume decrease, preventing in the same time the immediate recurrence and, thus, resulting in long-lasting remissions with uttermost positive consequences for cancer-diagnosed animals life condition.

The 60 ppm DDW is the cause of cell basic compound regeneration for immune answer (B lymphocyte in all its different types, dendritic cell and NK-K cell complex).
All these facts result in post-therapeutically improvement immune status of these animals receiving 60 DDW and cytostatics, and, in this way, the therapeutical remission is extended. All these are benefic actions causing a better protection against cytostatic toxicity. Thus, a better therapeutic index has been obtained, which means both a prolongation of pets' live and a remarkable improvement of the confort of these animals having different types of cancers.

**Conclusion**

The results obtained on dogs confirm the results obtained in case of rats, and relieve the fact that 60 ppm DDW has certain properties for cancer organism detoxification, organism that is to be subject to toxic stress generated by cytostatic (side effect) used in anti-cancer polychimiotherapy. These are the reasons we assert the idea of using the 60 DDW as adjuvant, in reducing the toxicity of the cytostatics used in cancer polychimiotherapy applied on pets and humans.
CLAIMS

1. The use of Deuterium Depleted Water of 60 ppm concentration as adjuvant in reducing the toxicity of cytostatics used in mono-chemotherapy with: cyclophosphamide – CFS; 5-fluorouracil – 5-FU; farmarubicine – FARM and vinblastine – VBL, by administering this kind of water as a daily diet for adult and healthy Wistar outbred rats, before, during and after cytostatic treatment.

2. The 60 ppm DDW application as adjuvant for reducing the toxicity of cytostatics used in mono-chemotherapy with: cyclophosphamide – CFS; 5-fluorouracil – 5-FU; farmarubicine – FARM and vinblastine – VBL, by administering this kind of water as a daily diet for pet dogs having different types of cancer, before, during and after polychemiotherapy.
Figure 1

$^3$H-thymidine distribution in thighbone of adult Wistar rats, after 5 days from the last administration of cytostatics

![Graph showing activity distribution in TW control and DDW control for thighbone (TW) and thighbone (DDW).]

Organ

Figure 2

$^3$H-thymidine distribution in thighbone of adult Wistar rats, after 10 days from the last administration of cytostatics

![Graph showing activity distribution in TW control and DDW control for thighbone (TW) and thighbone (DDW).]

Organ
Figure 3

$^{3}$H-thymidine distribution in lymphonodes of adult Wistar rats, after 5 days from the last administration of cytostatics

![Graph showing $^{3}$H-thymidine distribution in lymphonodes of adult Wistar rats.](image)

Organ

Figure 4

$^{3}$H-thymidine distribution in lymphonodes of adult Wistar rats, after 5 days from the last administration of cytostatics

![Graph showing $^{3}$H-thymidine distribution in lymphonodes of adult Wistar rats.](image)

Organ