

DEUTERIUM DEPLETED WATER EFFECTS ON WALKER TUMOURS

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Abstract: The usual concentration of deuterium in water is about 144 ppm D/(D+H). It is known that the increase of deuterium in the body of living animals is a naturally bioaccumulation process and could be enhanced by adding in their diet heavy water – with obviously dramatic changes in health state. The aim of this study was to investigate the effect of DDW over experimental Walker tumours induced at Wistar rats and how far can influence the decrease of deuterium's value at treated animals. The result shows that DDW has a significant effect over the sub epidermal tumours and it has no side effects. The deuterium's analyses results show that DDW's rate of depletion is in correlation with a couple of factors (the concentration of deuterium depleting agent; the period of administration of deuterium depleting agent).

INTRODUCTION

The data from the literature emphasize the fact that the deuterium depleted water; which is less studied than the heavy water [1]; has remarkable biological effects; *in vitro* and also *in vivo*. Data from the literature emphasize the fact that the deuterium depleted water (DDW) presents biological qualities with favourable effects on animals' state of health [2]. Deuterium is the natural and stable; non-radioactive hydrogen's isotope; deuterium's concentration in natural water is about 144 ppm D/(D+H). Deuterium's concentration increase leads at the obtaining of heavy water which has noxious effects on living matter [3]; against the deuterium depleted water with remarkable biological effects *in vitro* but also *in vivo* [4].

LABORATORY TESTS

The research started from the data presented in the literature concerning the biological effects of deuterium depleted waters on the animals with neoplastic tumours and was oriented on the study of several deuterium depleted waters' (DDW) effects on Walker 256 tumour; using several experimental models. As followed objectives were: - the creation of experimental models on cancerized rats with Walker 256 tumour - solid form; - the testing of DDW on cancerized animals in different experimental conditions.

MATERIALS AND WORKING TECHNIQUES

As biological material for the achieving of the proposed objectives there were used: *Animals:* adult outbred rats from Wistar line (females and males) average weighting of 120 g raised and maintained in standard conditions. For the experimental research which were made; the maintaining of Walker 256 tumour – the solid and ascitical form; the prelevation of organs for the determination of

morphocytological and isotopical content; toxicity studies of DDW; preliminary studies to establish of the experimental studies; cancerisation; establish the diagram of testing the DDW on rat; for the achievement of the experimental protocol); there were needed approximately 400 animals; Wistar rats.

Tumour. It was used the Walker 256 tumour at the origin a carcinosarcoma spontaneously appeared in the region of the mammary gland at a gestant female rat. In present; the tumour's solid form is maintained through hypodermic successive transitions on Wistar rats. The tumoural connection vary with the age and the sex of the animals. Walker 256 tumour maintained through hypodermic grafts at Wistar rats metastasizes rarely ; and the invasion of regional ganglions takes place in terminal phase of the tumoural increase when the the canceration phenomena are increased. Walker tumour presents also the ascitic form which is maintained through intraperitoneal inoculations. Walker 256 is a standard tumour; used in the preclinical screening of antitumoural substances as in the ganglionic metastasation. To obtain solid hypodermic tumours at rats there were inoculated free ascitic tumoural cells which present a gripping index; constant and reproducible in 100%.

For hypodermic cancerisation of rats there were used ascitic cells ingathered from a inoculated donor with 14 days before. Tumoural cells which weren't ingathered on anticoagulant; were washed twice in medium and centrifuged at 1000 rotations per minute; for 10 minutes. The centrifugated cellular sediment it was resuspended in medium; and for the number of cells' establishment (9×10^6 cells in 0.5 ml per animal) the reckoning was made with Türk hemocytometer; and the cellular viability it was established through supravital coloration with methyl blue. As well; from the ingathered tumoural cells for the cancerisation there were made colored smears with MGG; for cytomorphological studies. The animals which were used for the experimental protocol were anesthetized with ether; weighted; marked; shaved and sterilized with sanitary alcohol where the inoculation with tumoural cells is made with unique use syringe and thin needle.

Deuterium Depleted Water (DDW) with a isotopical concentration of 30 ppm D/(D+H) and the GLF distilled water which were necessary for the obtaining of DDW 60 and DDW 100 variants were assured by ICIT Rm. Vâlcea. Deuterium depleted water was administered in animals' daily nourishment and as drinkable water instead of tap water; and for the hypodermic administration there were used assortments of deuterium depleted water; which were sterilized previous through autoclavation. DDW daily doses administered for each animal were of 2 ml and a quantity of 8-10 ml of DDW was taken from the nourishment and as drinkable water. The total quantity of DDW used in the experiment was 200 litres of DDW 30 ppm and GLF distilled water = 60 litres.

Other materials that were used:

- culture medium; serums; salt solutions; reactives;
- colorants-kits;
- sanitary materials;
- glassware; blades etc.

The experimental protocol was centred on the following objectives:

a) The effect of the different types of deuterium depleted water (ddw) on Walker 256 tumoural cells inoculated in pneumoderma at rats.

For the achievement of this experimental model it was injected air under the skin of the rat so that a cavity to be formed; a air bag; which was called by us *pneumoderma*

Pneumoderma's technique of execution: the rat which presents the laboratory animal frequently used in pneumoderma's execution; is shaved in the dorsal region after a easy anaesthesia with ether; afterwards the shaved area is disinfected with sanitary alcohol or iodine. After those operations; the rat is injected in the dorsal skin with 20 cm³ of air; with the means of a 20 ml syringe at which a thin needle is attached (no. 27). A unique air bag is formed; of regular shape; in which tumoural cells suspended in different solutions that we want to study are injected. In our conditions; tumoural cells are diluted in deuterium depleted water (DDW) and physiological serum for the control lot. In this experimental model; the *pneumoderma*; has the advantage that can be followed; in dynamics; the behaviour of the inoculated tumoural cells that are in direct contact with various assortments of deuterium depleted water. For the achievement of this experiment there were used normal Wistar rats which have received in their diet tap water (witness lot); and also normal rats which have received 30 days before cancerisation and follow-up after the cancerisation in the nourishment and drinkable water

deuterium depleted water (DDW lot) during all the period of the experiment. From among these animals which have been inoculated hypodermally with 9×10^6 Walker 256 ascitic tumoural cells.

There were formed the following lots:

LOT I - WITNESS - physiological serum. 20 normal Wistar rats (10 females and 10 males) which have received in their diet tap water; were inoculated in pneumoderma with 9×10^6 Walker 256 tumoural cells / animal; suspended in 5 ml of physiological serum. Before the inoculation in pneumoderma; the tumoural cells have been maintained in direct contact with physiological serum *in vitro* for ½ hour; and after that there were inoculated in pneumoderma. We mention that the physiological serum was administered in the moment of tumoural cells' inoculation; daily; 2 ml in pneumoderma; and when was the case air it was administered also to maintain the air bag.

LOT II - deuterium depleted water (DDW 100 ppm): 20 normal Wistar rats (10 females and 10 males) were inoculated in pneumoderma with 9×10^6 cells / animal; suspended in 5 ml of DDW 100 ppm. The tumoural cells; before the inoculation; were maintained for ½ hour in direct contact *in vitro* with DDW 100 ppm; and after that they were inoculated in pneumoderma. DDW 100 ppm was inoculated daily by 2 ml in pneumoderma starting from the moment of tumoural cells' inoculation to maintain the direct contact with the tumoural cells which were present in the pneumoderma.

LOT III - deuterium depleted water (DDW 60 ppm): 20 normal Wistar rats (10 females and 10 males) were inoculated in pneumoderma with 9×10^6 Walker 256 tumoural cells; suspended in 5 ml of DDW 60 ppm. Before the inoculation in pneumoderma; the tumoural cells have been maintained *in vitro*; for a hour; in direct contact with DDW 60 ppm. DDW 60 ppm was administered daily by 3 ml in pneumoderma for 10 days.

LOT IV - deuterium depleted water (DDW 30 ppm): 20 normal Wistar rats (10 females and 10 males) were inoculated for each animal 9×10^6 Walker 256 tumoural cells; suspended in 5 ml of DDW 30 ppm. Before the administration in pneumoderma; the tumoural cells have been maintained *in vitro* for 1/2 hour in direct contact with DDW 30 ppm.

Deuterium depleted water was administered daily by 2 ml beginning with the moment in which the tumoural cells have been inoculated in pneumoderma. It is mentioned that the deuterium depleted water assortments (DDW 100; 60 and 30) before the administration in pneumoderma were sterilised through autoclavation. In this experimental model the followed parameters were: tumoural growth; tumoural latency period; average time of survival for cancerised animals; anatomo-clinical aspects;

b) The verification of deuterium depleted water's (DDW) toxicity on normal Wistar rats. There were used 60 normal rats (30 females and 30 males); in average weight of 100 g; which received in nourishment and as drinkable water deuterium depleted water. During the DDW's period of administration there were supervised animals' state of health and the eventual secondary effects. At intervals of 30; 60; 90 days; by 10 animals (5 males and 5 females which have received DDW 30 in nourishment and drinkable water; have been sacrificed for the prelevation of blood; liver; muscular mass samples and conservation for the isotopical determinations; and also for the cytomorphological analysis.

c) Deuterium depleted water's effect on Walker 256 solid tumour's evolution transplanted hypodermally at rats. In this experiment there were used normal Wistar rats; which have received in their diet tap water; of both sexes; having an average weight of 120 g for the control lots and Wistar rats which for 30 days; before the cancerisation and further have received deuterium depleted water in nourishment and as drinkable water.

For the experimental model there have been made lots; as following:

LOT I - WITNESSES - hypodermally tumour: 20 rats which have received in their diet tap water; were inoculated hypodermally with 9×10^6 Walker 256 tumoural cells.

LOT II - WITNESSES – physiological serum: 20 rats which have received in their diet tap water; were inoculated hypodermally with 9×10^6 walker 256 tumoural cells. at 24 hours after the cancerisation are administered daily 2 ml of physiological serum hypodermally peritumoural.

LOT III - WITNESSES - physiological serum: 20 rats which have received in their diet tap water; were inoculated hypodermally with 9×10^6 walker 256 tumoural cells. At 7 days after the cancerisation are administered daily 2 ml of physiological serum hypodermally peritumoural.

LOT IV - DDW 30 ppm in diet: 20 rats which 30 days before the cancerisation and after have received DDW 30 ppm in their nourishment and as drinkable water. The rats are inoculated hypodermical with 9×10^6 Walker 256 tumoural cells.

LOT V – DDW 30 ppm in diet and hypodermical: 20 rats cancerised with 9×10^6 walker 256 tumoural cells. The rats have received ddw 30 ppm; for 30 days before the cancerisation and after in thier nourishment and in thier drinkable water; and after 24 hours after the cancerisation it was administered daily hypodermical peritumoural 2 ml of DDW 30 ppm.

LOT VI - DDW 30 ppm in diet and hypodermical: 20 rats cancerised with 9×10^6 Walker 256 tumoural cells. The rats have received in their nourishment and drinkable water DDW 30 ppm; for 30 days before the cancerisation; and after 7 days after the cancerisation it was administered daily hypodermical peritumoural 2 ml of DDW 30 ppm. Animals entered in the experiment after the cancerisation have been kept under daily observation to emphasize the apparition of tumoural nodules. The rats have been weighted and the tumoural incidence has been registered.

The followed parameters were: Latency period; Tumoural incidence; Average time of survival.

d) Microscopic exams. Microscopic exams which have been made had as purpose :

- Walker 256 tumoural cells viability determination through the colorant's exclusion method for the animals' cancerisation from the performed experiences;

- tumoural cells' reckoning was made with the Türk haemocytometer to establish the number of cells necessary to cancerise the animals;

- the realization; the coloration and the examination of the smears of tumoural cells colored with MGG; for the detection of eventual cellular modifications (form; dimension; nuclear outline; nucleolus; cytoplasm; etc);

- smears' execution from peripheral blood; hematogene marrow; ganglions; the coloration with MGG and the examination at microscope.

All those biological products resulted from normal or cancerised animals; and also from animals with Walker 256 tumours; which have received in their nourishment and as drinkable water deuterium depleted water.

RESULTS AND CONCLUSIONS

Results obtained from the testing of some assortments of deuterium depleted water (DDW) on Walker 256 tumoural cells inoculated at rats hypodermically in pneumoderma; are presented synthetic in Tab. 1-2 and Graph. 1-4.

Tab. 1.

The effect of some assortments of DDW on Walker 256 tumoural cells inoculated in pneumoderma

o crt.	LOTS	Animals' survival		
		30 days	40 days	60 days
	WITNESSES	0%	-	-
	DDW 100 ppm	100%	70%	30% without tumour
	DDW 60 ppm	100%	80%	40% without tumour
	DDW 30 ppm	100%	75%	35% without tumour

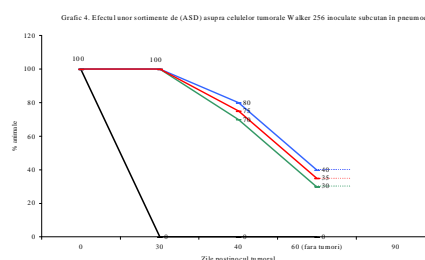
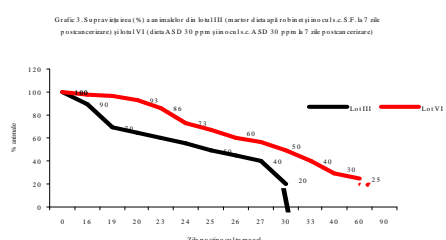
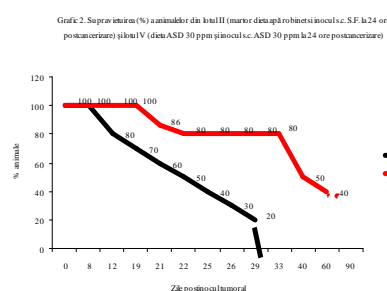
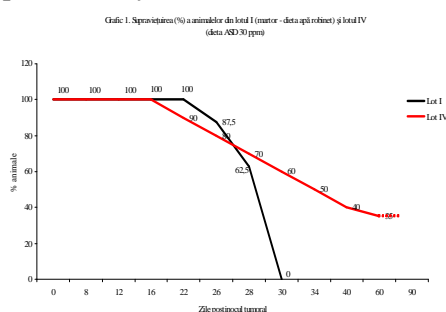
Tab. 2.

DDW's 30 ppm effect on Walker 256 tumoural cells inoculated hypodermically at rat.

o	LOTURI	Animals' survival %		
		3	4	60 days
	WITNESSES tumour	0	-	-
	DDW 30 ppm in diet before and after inoculation	6 5%	5 0%	35% without tumour
	WITNESS tumour physiological serum	0 %	-	-
	DDW 30 ppm in diet and s.c. 24	8	5	40% without
	DDW 30 ppm in diet and s.c. 7 days	5 0%	3 0%	25% without tumour

After the processing of the data obtained in the experiences made with deuterium depleted water (DDW) there were distinguished the following aspects:

- the tumoural incidence at hypodermically inoculated animals with 9×10^6 cells
- the latency period (tumour's apparition) for Walker tumour hypodermically inoculated was smaller at the witness lot (5-7 days) toward the lots where DDW was administered (10-14 days);
- average time of survival (ATS) was smaller at the witness lot (at 30 days 100% of animals were dead) toward the lots which have received deuterium depleted water (DDW) where the percent of living animals was at 30 days between 100% and 50%; and at 60 days after the cancerisation the survival percent vary between 25% and 40%



The antitumoural effects of deuterium depleted water (DDW) vary with the tumoural hystotype; with the anatomical area in which the tumour is present and the way in which the tumour develops (under the ascitic form or the solid form). The best results with DDW were obtained in our case with Walker tumour the solid form with hypodermically development; toward the intraperitoneal localisation; the ascitic form of Walker tumour; where the results with DDW were insignificant.

At the lots of animals at which DDW is administered appear variations in tumoural variations. Thus; we had animals with hypodermically tumours with rapid evolution as at the witness lot (percent that vary between 30 and 50% of animals) and animals with hypodermically tumours with slow evolution which lead to a increase of survival percent or a stopping of tumoural evolution and the complete regression of the tumour (percent that vary between 25 and 40% of animals).

Toward normal rats; the hypodermically cancerised rats with Walker 256 which have received in their diet tap water; as rats that for 30 days before and after the cancerisation have received in their

diet DDW and died because of the tumoural evolution; presents a haematological picture with normal values for leucocytes or leucopenia; a hyperneutrophilia with lymphopenia; and also low values of the elements of dendritic cells and NK/K. The rats that for 30 days before and after inoculation; with Walker tumour have received DDW during all the experiment; and in the end the ones which presented tumours with a big latency or a complete tumoural regression presents a haematological picture with hyperleukocytosis; partial blastised; with frequent dendritic cells and NK/K.

The red bone marrow and ganglions present an imunoblastic-plasmocitary increased proliferation. From the obtained results we can conclusion that:

- deuterium depleted water (DDW) administrated at rats in the nourishment and drinkable water in profilactical purpose and inoculated hypodermically peritumoural at cancerised rats with Walker 256 tumoural cells the solid form; had significant effects on the hypodermically tumours' evolution concretised in the survival's increase even in the evolution's stopping and the tumour's complete regression (the percent vary between 25 and 40% of animals);

- the preliminary haemathological exams explain the favourable effect of DDW through a significant increase of cellular immunity system's activity of modulation (CS);

- deuterium depleted water; regardless of concentration; of way and time of administration it was proved to be unotoxic for the rats.

DEUTERIUM'S CONCENTRATION DETERMINATION

PRIMARY DATA

Water's extraction from the biological samples and the mass spectrometry for the determination of deuterium's distribution in the organism have been made on biological samples resulted from: adult wistar rats (males and females) used in the laboratory experiments for ddw's toxicity testing.

Lots taken for study:

- Witness lot: nourished with standard nourishment and tap water (60 days)

- Lot I (experimental): nourished with standard nourishment and ddw 30 ppm; (60 days);

- Lot II (experimental): nourished with standard nourishment and ddw 60 ppm; (60 days);

Biological samples considered representatives and which were complied to the procedures necessary for deuterium's isotopical analysis through mass spectrometry (D) are of two categories: liquid samples (blood) and solid samples (haepathical tissue and muscular tissue).

The results for the isotopical analysis of biological samples resulted from the animals taken into study (M – males; F - females) are represented in Tab. 3 and Graph. 5.

Tab. 3

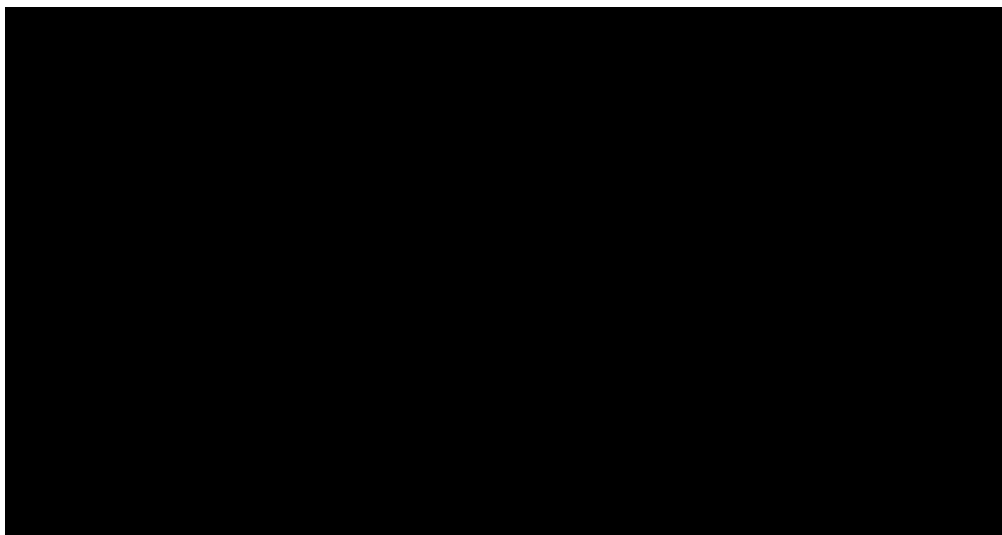
Results for the isotopical analysis (ppm D/[D+H]) from the biological samples resulted from the Wistar rats

Species / Individual	Nourishment's Deuterium concentration	Biological sample's Deuterium concentration
Rat Lot I – 1 (M) Blood	30	94.15
Rat Lot I – 2 (M) Blood	30	94.75
Rat Lot I – 3 (M) Blood	30	92.4
Rat Lot I – 1 (M) Liver	30	97.45
Rat Lot I – 2 (M) Liver	30	97.45
Rat Lot I – 3 (M) Liver	30	95.3
Rat Lot I – 1 (M) Muscle	30	94.65
Rat Lot I – 2 (M) Muscle	30	98.05
Rat Lot I – 3 (M) Muscle	30	96.15
Rat Lot II – 1 (M) Blood	60	112.05
Rat Lot II – 2 (M) Blood	60	110.85
Rat Lot II – 3 (M) Blood	60	108.05
Rat Lot II – 1 (M) Liver	60	112.75
Rat Lot II – 2 (M) Liver	60	110.3
Rat Lot II – 3 (M) Liver	60	108.9
Rat Witness – 01 (M) Blood	144	148.6

Rat Witness – 02 (M) Blood	144	148.65
Rat Witness – 03 (M) Blood	144	149.35
Rat Witness – 04 (F) Liver	144	146.8
Rat Witness – 05 (F) Liver	144	147.6
Rat Witness – 04 (F) Muscle	144	148.8
Rat Witness – 05 (F) Muscle	144	148.35

From the analysis made for tab. 3 and graph. 5 it can be observed that during lifetime (in normal conditions for development – 144 ppm) animal organisms have the tendency to accumulate the deuterium in the organism (the process of bioaccumulation). Concomitant; in comparison with average values of deuterium's distribution; bioaccumulation's grade of this isotope presents variations which appear in the presence of some depletant mediums of different concentrations.

DISCUSSIONS AND CONCLUSIONS



Graph. 5. Isotopic analysis result at Wistar rats – deuterium's variations

As well it can be observed that for the same concentration in deuterium from the nourishment and hydration medium appear only very little variations concerning the isotopic content in deuterium of the biological samples; maybe because of the existent ethological relations within the conspecific individuals from a group/lot and of each individual's appetite (hydric and nourishment necessary); dictated each specimen's phenotype and genotype. It also can be observed; because of the individual's pursuance in the presence of a much powerful depleting medium (30 ppm); comparatively with a less powerful one (60 ppm); a progressive decrease of deuterium content from individual's body which were raised in the mentioned conditions. In conclusion: the individual characteristics of a specimen (dictated by the genotype and the ethological component); but also the depletant's medium concentration (probably also the administration period) have a certain weight in the deuterium's bioaccumulation/elimination grade of determination in/from the body.

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