Caloric Restriction Diet Induces Specific Epigenotypes Associated with Life Span Extension

A.I. Bozhkov* and Yu.V. Nikitchenko

Biology Research Institute V.N. Karazin Kharkov National University, 4 Pl. Svobody, 61022 Kharkov, Ukraine

Abstract: The lifespan of Wistar rats on caloric restriction diet (CRD) is correlated with the changes in prooxidant-antioxidant balance, in the contents of triiodothyronine, and thyroxin (epigenotype characteristics).

It has been shown that in a month after the moment of one-month-old rats began to receive CRD the part (up to 15 %) of experimental animals died without any apparent cause, irrespective of the degree of calorie restriction (40 %, 57 % and 60 % weight-loss).

The rest of animals with 40 %, 57 % and 60 % weight-loss had longer life span in comparison with control group.

The CRD-induced life-span prolongation in animals was accompanied by the induction of specific epigenotypes featured by acceleration of the electron transfer rate in electron transport chain and subsequent reduced production of reactive oxygen species and increased antioxidant activity. The activity of glutathione reductase, glutathione transferase, NADH-cytochrome C reductase, isocitrate dehydrogenase was elevated. The activity of Se-dependent glutathione peroxidase was higher more than 30 times as compared with control. Likewise, the epigenotypes of animals with 40 %, 57 % and 60 % weight-loss CR, were characterized by 37, 43 and 56 % decrease in triiodothyronine and 50, 25, 39 % decrease in tyrosine, respectively. The observed induction of specific epigenotypes is associated with higher life-span and is related to the multivariant metabolic strategies of adaptation to CRD.

Keywords: Caloric restriction diet, aging, prooxidant-antioxidant system, epigenotype.

1. INTRODUCTION

The first study of life-span gain possibility in experiments by caloric restriction diet (CRD) was published more than 70 years ago by C.M. McCay [1]. In 60-th of the last century in Biology research institute of the Kharkov National University in laboratory of professor V.N. Nikitin the complex research of CRD influence on the life-span of Wistar rats was conducted [2, 3]. The conversion of one-month-old animals to CRD was shown to be accompanied by decrease of metabolism rate [4], rate of physiological systems becoming [5] and extension of average and maximal life-span [6].

For the last years there the numerous studies on CRD influence on average and maximal life-span of different objects from yeast cells [7] to monkeys [8] are conducted. And now one may say about formed conception of direct relation of metabolism rate and life-span. The free-radical theory of aging conformed to this conception [9, 10] It was shown that calorie-restricted animals have decreased rate of reactive oxygen species generation and as a consequence number of products of free-radical damages of macromolecules in organism [11, 12]. As known in case of “excess” of free-radical reactions products their cytotoxic effects reveal and pathologies development occurs [13, 14].

It would seem there is a logic direct relation between CRD and free-radical hypothesis. However along with destructive processes reactive oxygen species induce the synthesis of antioxidants able to neutralize their cytotoxic action [15-17].

Despite of vast numbers of researches on CDR model some specialists prejudiced verity of life-span extension and supposed that it could be only effect of animal selection [18, 19].

One may assume that CRD executes some interrelated functions: selection of genotypes with potentially high life-span, induction of antioxidant system that can provide resistance of organism both to reactive oxygen species, and to others stress-factors.

Earlier we assumed that conversion of animals to CRD induced the formation of specific epigenotypes providing extension of average and even maximal life-span [18]. For epigenotypes providing potentially high life-span the cooperative changes in set of metabolic systems and first of all in prooxidant-antioxidant, hormonal and also nucleic-protein and lipid metabolism can be typical [20-22].

On this basis, the aim of this work was to research mitochondrion functional activity, content of some products of free radical processes, activity of

*Address correspondence to this author at the Biology Research Institute V.N. Karazin Kharkov National University, 4 Pl. Svobody, 61022 Kharkov, Ukraine; E-mail: bozhkov@univer.kharkov.ua

E-ISSN: 1929-5634/13

glutathione-dependent and $NADP^+$-dependent enzymes of mitochondrion of rat liver and also content of thyroid hormones in blood serum of rats maintained during 2 months (from one month age) on CRD, providing different degree of animal growth inhibition.

2. MATERIALS AND METHODS

The research was conducted on males Wistar rats maintained at standard vivarium conditions in accordance with the guidelines of the European Convention for the Protection of the Vertebrata using for the experimental and scientific aims (Strasburg, 1986). The animals of control groups were fed *ad libitum*. The rats of experimental groups at one-month age were conversed from standard diet to one of calorie restricted diets resulted in growth inhibition of different degree: diet by McCay-Nikitin [2], resulted in growth inhibition by 63 % («severe»), and two diets resulted in less growth inhibition, — by 57 % («moderate») and 40 % («mild»). At 3- and 24-months age the part of rats was decapitated at ether narcosis; the blood serum, and mitochondrion and post-mitochondrion fractions from liver were collected. In each group for assessment of life span were used 30-40 animals [23]. The thyroxin (T_4) and triiodothyronine (T_3) contents in blood serum were estimated using the standard radioimmunological kits ("Total T4 RIA" and "Total T3 RIA", IMMUNOTECH, Czech Republik). The rate of respiration and oxidative phosphorylation at FAD^+-(succinate) and NAD^+- (glutamate+malate, β-oxibutirate)-dependent substrate oxidation in mitochondrion was estimated polarographically: the rate of oxygen consumption was registered in non-phosphorylating (V_2), phosphorylating (V_3) and disengaged (V_{3p}) states; the efficiency of phosphorylation was estimated (ADP/O; the proportion of ADP quantity transformed in ATP, to oxygen quantity consumed by mitochondrion in phosphorylating state 3) [24]. The rate of rotenone-resistant NADH-cytochrome c-reductase and NADH-oxidase activity of mitochondrion was determined by NADH oxidation fluorimetrically in hypotonic medium with cytochrome c [25].

The ICDH (C1.1.1.42) activity was determined in liver mitochondria spectrophotometrically by the rate of $NADP^+$ reducing in a medium containing 34 mM tris-HCl buffer, pH 7,4, 0,34 mM EDTA, 1,5 mM $NaCl_2$, 0,1 mM $NADP^+$ 1,5 mM isocitrate, 0,2% Triton X-100 at 37°C. The ICDH activity is expressed in nmol of NADPH/min per mg of protein [26],

Determination of glutathioneperoxidase activity (GP EC 1.11.1.9).

GP activity was determined in the cytosol and mitochondria of liver spectrophotometrically at 340 nm using the method [27] with changes in medium containing 50 mM K^+, Na^+-phosphate buffer (pH 7.4), 1 mM EDTA, 0,15 mM NADPH, 1 unit of yeast glutathione, 0,2% Triton X-100 and 3 mM of Na azide to inhibit catalase. The samples were incubated at 37 °C. The activity is expressed in nmol of NADPH/min per mg of protein with the molar extinction coefficient $6{,}22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

Determination of glutathione-S-transferase activity (GT EC 2.5.1.18).

GT activity was measured in the cytosol and mitochondria of tissue spectrophotometrically at 340 nm [28] in a medium containing 0,1 M K^+-phosphate buffer, pH 6,5, 1 mM 1-chloro-2,4-dinitrilbenzol, 5 mM of reduced glutathione 0,2% Triton X-100 at 37°C. The activity was calculated with the molar extinction coefficient $9{,}6 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

Determination glutathione-reductase activity (GR EC 1.6.4.2).

GR activity in the cytosol and mitochondria of tissue was measured spectrophotometrically by loss of NADPN [29] in medium containing 50 mM K^+-phosphate buffer, pH 7,4, 1 mM EDTA, 0,16 mM NADPN, 1 mM GSSG, 0,2% Triton X- 100, at 37°C. The activity is expressed in nmol of NADPH/min per mg of protein with the molar extinction coefficient $6{,}22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

The rate of ascorbate-induced lipid peroxidation of mitochondrion was determined in medium with 100 мМ tris-HCl, pH 7.4, 0.5 мМ ascorbate, 12 мкМ Fe^{2+} by uptake of malonic dialdehyde with thiobarbituric acid [30], with using of molar extinction coefficient $1.56 \cdot 10^5\ M^{-1} \cdot sm^{-1}$. The Schiff bases content in mitochondrion of liver was estimated fluorimetrically after their extraction by chloroform-methanol (1:2) from suspension of organelles [31]. The results were expressed in relative units take the intensity of fluorescence of 1 μg of quinine sulfate in 1 ml of 0.1 N H_2SO_4 as 1950 units. The protein content was determined by Lowry assay in Miller modification [32]. In each group for assessment of biochemical parameters were used 7 animals.

The data obtained were analyzed statistically by non-parametric (Wilcoxon-Mann-Whitney test) and

parametric (t-test) methods using “Statistika V.6”. The compliance of feature distribution to normal one was analyzed by Shapiro-Wilk test. The correlation analysis was conducted by Pearson. The animal survival rate was estimated by Kaplan-Meier method and comparison of curves of survival rate was drawn by Gehan with Yates correction. Differences between treatment groups were considered significant if P was less than 0.05 ($p<0.05$).

In the study were used the reagents produced by Applichem.

3. RESULTS

3.1. The Life-Span of Experimental Animals Maintained on Calorie Restricted Diets

One-month rats were divided on 4 experimental groups: the 1st group – control – remained on standard diet; the 2nd group was restricted in food quantity by such way that after 2 months this group animals weight was 40 % less than control group – conditionally “mild” diet; the 3rd group was restricted by 57 % at “moderate” diet and the 4th group was restricted by 63 % at “severe” diet (Figure **1**).

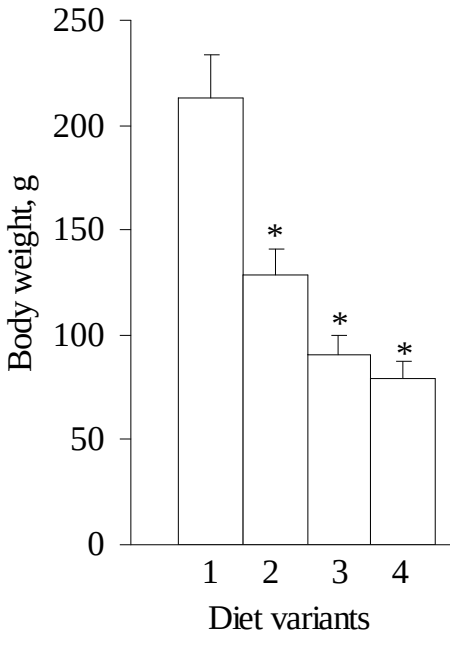

Figure 1: Weight of 3-month old animals (g).

(1) - tacking the standard diet.

(2) - tacking the “mild” calorie-restricted diet since the second month of their life.

(3) - tacking the “moderate” calorie-restricted diet since the second month of their life.

(4) - tacking the “severe” calorie-restricted diet since the second month of their life.

* $p<0.05$ as compare to control.

Thus, in the control group, fed by standard diet, individual animals died after 10 months of life and mass death began with 18 months. Last animal in this cohort died at the age of 33 months. It should be noted that the curve of the control group mortality was not linear, but S-shaped (Figure **2**).

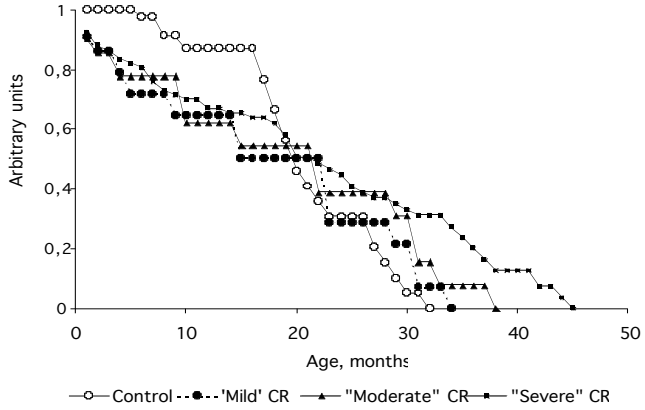

Figure 2: The survival in three groups of animals with age.

(−○−) – animals in “control” group.

(··●··) – animals tacking the “mild” calorie-restricted diet.

(−▲−) – animals tacking the “moderate” calorie-restricted diet.

(−▪−) – animals tacking the “severe” calorie-restricted diet.

The same non-linear mortality characterized the 2nd and 3rd experimental groups. At the same time, the for 4th group of animals, which lagged in body weight by 63%, almost linear curve of mortality was observed (Figure **2**).

Importantly that immediately after the transfer of experimental animals on the CRD some part of animals (10-15%) perished with no pathological manifestations.

The marked difference in the survival of animals on different CRD was shown in later stages of ontogeny (Figure **2**).

It should be paid attention on the time of the death of the last animal in each experimental group. So, in the control group the last animal died at the age of 33 months, in the 2nd - 35 months, in the 3rd group - 39 months and in the 4th group at 46 months (Figure **2**).

We have suggested, that transfer of animals on CRD performs a dual function: 1. provides the selection of animals already having a specific pattern of metabolic rate - epigenotype 2. "selects" animals with epigenotypes able to induce those metabolic chains that can enhance resistance to a limited diet and as a result, gain life-span. To check this suggestion the

pattern of metabolic values was determined - epigenotype after 2 months of the transfer of animals on different diets. Unlike epigenome characterizing the pattern of expressing genes under epigenotype we understand already implemented metabolic pattern, i.e. implemented epigenome. It should be noted that epigenotype gives more information to understand the biological functions than the epigenome does. In epigenotype characteristic we included the content of thyroid hormone (thyroxine, triiodothyronine) and values of prooxidant-antioxidant systems of experimental animals.

3.2. The Content of Thyroid Hormones (Thyroxine and Triiodothyronine) in Animals Maintained for Two Months on Different Calorie Restricted Diets

The triiodothyronine content in animals maintained for 2 months on different CRD was significantly reduced compared to control animals (Figure **3A**). There was correlation between the weight loss caused by the "mild", "moderate" and "severe" CRD and reduction of serum triiodothyronine in animals (r=0,983). Thus, if the body weight was reduced by 40, 57 and 63%, the content of triiodothyronine was decreased by 37, 43 and 56%, respectively (Figure **3A**).

The content of thyroxine in the blood serum of animals maintained on the CRD was significantly lower than in control animals (Figure **3B**). However, this reduction was not dependent on the type of CRD. Thus, in the case of "mild" CRD thyroxine content was 50% lower compared to the control and at a "moderate" and "severe" CRD only for 25 and 39%, respectively (Figure **3B**).

The tiriyodtironin and thyroxine are known to perform similar metabolic functions, however, the activity of triiodothyronine is 4-5 times higher than that of thyroxine. Perhaps this explains the absence of a direct correlation between the decrease in the amount of thyroxine in the blood and weight loss of the animals on the CRD.

Consequently, the content of thyroid hormones is reduced in the animals on CRD. This result suggests that after 1 month on CRD animals survived such only those who have low thyroid function.

The triiodothyronine and thyroxine are known to be involved in the regulation of a large number of metabolic processes, and in particular, the oxidative breakdown of fats, carbohydrates, protein synthesis, the synthesis of sex hormones, mineral metabolism, which results in the regulation of growth and development.

Such a wide range of metabolic activity of thyroid hormones is accompanied by many cooperative

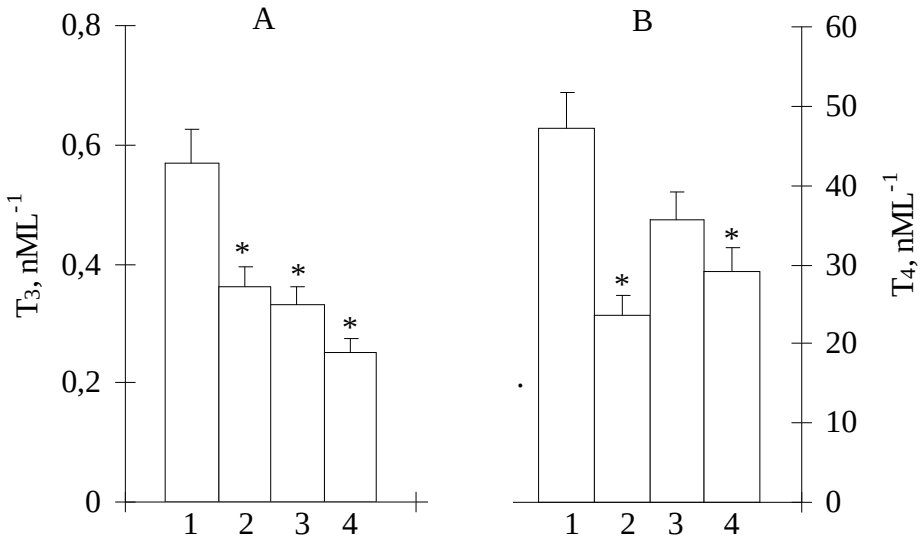

Figure 3: The concentration of thyroxine (T3) (A) and trriiodothyronine (T_4) (B) in rat blood serum in.
(1) – animals in “control” group.
(2) – animals tacking the “mild” calorie restricted diet.
(3) – animals tacking the “moderate” calorie restricted diet.
(4) – animals tacking the “severe” calorie restricted diet.
* p <0.05 as compare to control.

Table 1: The Effect of Different Hypocaloric Diets on Respiration and Oxidative Phosphorylation in Mitochondria of Rat Liver in the Process Substrate Oxidation of Succinate (V2, V3, V3p – Oxigen Uptake (n Atoms) / min • mg Protein; n = 7 - 9)

Parameter	Control	Diet		
		mild	moderate	severe
Substrate oxidation - succinate				
V_2	27.03 ± 1.91	23.06 ± 1.23	22.33 ± 0.77*	22.06 ± 1.04*
V_3	136.3 ± 10,2	108.2 ± 8,1*	97.44 ± 6.0*	95.67 ± 6.9*
V_{3p}	139.9 ± 9,6	122.2 ± 8.7	112.9 ± 5.5*	117.2 ± 4.7*
Respiratory Control	5.064 ± 0,194	4.776 ± 0.075	4.347 ± 0.177*,**	4.306 ± 0.141*,**
ADP/O	1.724 ± 0.044	1.650 ± 0.058	1.724 ± 0.041	1.773 ± 0.076

changes of metabolic chains in the body. Such co-operative changes can lead to the induction of specific epigenotype, which provides a potentially high life-span of animals with altered epigenotype.

We hypothesized that epigenotype of potentially high life-span may be associated with changes in the activity of mitochondrial oxidative phosphorylation, the activity of pro-and antioxidant systems.

3.3. Values of Oxidative Phosphorylation of Rat Liver Mitochondria Treated with 2 Months Different CRD

To determine the efficiency of oxidative phosphorylation in mitochondria of liver 3 different substrates were used: succinate-FAD-dependent, glutamate + malate - NAD-dependent and β-hydroxybutyrate - NAD +-dependent substrates.

The maintenance of animals on CRD for 2 months was accompanied by a reduction of oxidative phosphorylation in all three states: V2 (non-phosphorylating), V3 (phosphorylating) and V3r (fragmented) in Chansu (Table **1**).

However, the greatest reduction was shown for V3, and it depended on the degree of diet restriction. So for the "mild" CRD it was 21 % and for the "moderate" and "severe" – 29 and 30 % respectively. Reduction in the intensity of phosphorylation for V2 and V3 did not depend on the degree of calorie restriction and was manifested only for "moderate" and "severe" diets (Table **1**).

Respiratory control was reduced insignificantly only in the case of "moderate" and "severe" CRD compared with the respiratory control of animals on the standard diet. However, the efficacy of phosphorylation (ratio of ADRs / O) remained the same for all the groups of animals (Table **1**).

Therefore, the maintenance of experimental animals on CRD with different degrees of caloric restriction and varying degrees of growth retardation for 2 months was accompanied by a slight decrease in the oxygen consumption rate and the degree of coupling of oxidative phosphorylation in the mitochondria of the liver when used glutamate + malate and β-hydroxybutyrate as oxidation substrates.

Thus, V2 did not change compared to control animals (Table **2**, **3**). Phosphorylating oxidation speed (V3) decreased only in the case of "moderate" and "severe" CRD by 21 and 22 % respectively when using glutamate + malate, and by 21 and 32 % respectively when using β-hydroxybutyrate (Table **2**, **3**).

In the case of the glutamate + malate oxidation respiratory control decreased in animals maintained on a "mild", "moderate" and "severe" diets, and in the case of β-hydroxybutyrate the decrease is shown only for animals on "moderate" and "severe" CRD (Table **2**, **3**). However, the efficiency of phosphorylation (ADP / 0) remained unchanged in all groups of experimental animals (Table **2**, **3**).

These results allow us to conclude that the CRD reduces the rate of oxidative phosphorylation in liver mitochondria. The degree of reduction of phosphorylation depends on the caloric restriction. It was shown in most for the "severe" CRD that provides the greatest effect of increasing life span. At that the rate of phosphorylating oxidation (V3) and the degree of coupling of oxidative phosphorylation (respiratorycontrol) decreased mostly. This indicates a certain decrease in the flux density of electrons in electric transport chain of the mitochondria of liver cells

Table 2: The Effect of Different Hypocaloric Diets on Respiration and Oxidative Phosphorylation in Mitochondria of Rat Liver in the Process Substrate Oxidation of Glutamate + Malate (V2, V3, V3p - Oxigen Uptake (n Atoms) / min • mg Protein; n = 7 - 9)

Parameter	Control	Diet		
		mild	moderate	severe
Substrate oxidation glutamate + malate				
V_2	12.77 ± 0.48	13.31 ± 0.62	13.56 ± 0.54	13.64 ± 0.71
V_3	108.9 ± 3.9	94.99 ± 5.5	85.88 ± 5.5*	86.3 ± 4.7*
V_{3p}	145.5 ± 4.4	131.0 ± 9.6	109.1 ± 3.4*	119.4 ± 5.1*
Respiratory Control	8.616 ± 0.176	7.116 ± 0.126*	6.301 ± 0.193*,**	6.341± 0.218*,**
ADP/O	2.754 ± 0.043	2.753 ± 0,051	2.575 ± 0.071	2.786 ± 0.064

Table 3: The Effect of Different Hypocaloric Diet on Respiration and Oxidative Phosphorylation of Mitochondria of Rat Liver in the Process of Substrate Oxidation of β-Oxybutyrate (V2, V3, - Oxigen Uptake (n Atoms) / min • mg Protein; n = 7 - 9)

Parameter	Control	Diet		
		mild	moderate	severe
Substrate oxidation				
V_2	9.93 ± 0.41	9.68 ± 0.55	10.05 ± 0.37	10.52 ± 0.39
V_3	45.85 ± 2.40	41.62 ± 2.02	36.46 ± 1.45*	31.41± 2.01*,**
Respiratory Control	4.646 ± 0.197	4.325 ± 0.134	3.630 ± 0.083*	2.985± 0.153*,**
ADP/O	2.538 ± 0.088	2.670 ± 0.064	2.507 ± 0.105	2.547 ± 0.077

of animals primarily on "severe" CRD and, consequently, a decrease in the rate of generation of free radicals and reduce cross-processes in the cells of these animals.

3.4. Some Values of Lipid Peroxidation in Liver of Rats Maintained for 2 Months on "Severe" CRD

The activity of free radical processes in the liver of animals was assessed by the rate of accumulation of TBA-active products and the number of Schiff bases - the end products of peroxidation.

It was found that in the animals maintained for 2 months on "severe" CRD the content of TBA-active products was lower by almost 30% than in the liver of control animals (Figure **4**).

Therefore, maintenance of the animals on the "severe" CRD was accompanied by reduction of products of free radical reactions, which correlated well with a reduction of oxidative phosphorylation and a decrease in the density of electron transport in electric transport chain of the mitochondria.

However, the reduction products of free radical processes in animals maintained on the CRD can be also connected with the high activity of their antioxidant enzymes.

3.5. Activity of Antioxidant Enzymes in Liver of Rats Maintained for 2 Months on a Different CRD

To clarify the role of antioxidant enzymes in life-span increase of rats the activity of glutathionetransferase (GT), isocitratedehydrogenase (ICDH), Se-dependent glutathioneperoxidase (Se-GP) and glutathionreductase (GR) was determined.

The activity of all the enzymes varied differently against different CRD. If the activity of GR remained unchanged, the GT activity slightly (11%) increased compared to control, and only on the "severe" CRD (Table **4**).

At the same time the activity of ICDH and the Se-GP increased dramatically. Thus, the activity of ICDH increased 1.7-fold, 1.9 and 1.8 times compared with

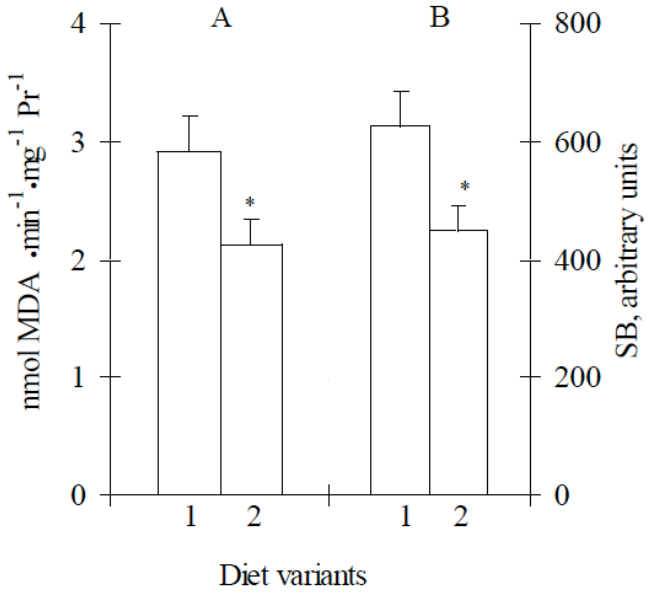

Figure 4: The intensity ascorbat-induced peroxidative oxidation of lipids (nmol MDA min^{-1}mg Pr $^{-1}$ and content of shiff bases in rat liver mitochondria.

(1) – animals in “control” group.

(2) – animals tacking the “severe” calorie restricted diet.

Table 4: Activity of Glutathione -Dependent Antioxidant Enzymes (Se-Dependent Glutathione Peroxidase, Glutathione Transferase and Glutathione Reductase) and $NADP^+$-Dependent Isocitrate Dehydrogenase in Liver Mitochondria of Rats of the Control Group, a Group of Animals, which was on a Standard Diet and the Groups of Rats were in the Standard Diet (Control) and Various Hypocaloric Diets

Parameter	Control	Diet		
		mild	moderate	severe
Se-dependent glutathione peroxidase, nmol NADPH/ min • mg protein	13.9±1.5	262.8±34.1*	448.9±31.2*, **	509.9±19.6*, **
Glutathione transferase, nmol1-clorine-2.4- dinitro-benzene/min • mg protein	120.2±6.3	128.6±10.2	135.6±13.5	137.5±6.1#
Glutathione reductase, nmol NADPH/ min • mg protein	25.7±1.5	26.7±2.2	29.0±1.6	29.1±09
Isocitrate dehydrogenase , nmol NADPH/ min • mg protein	177.9±27.6	306.6±29.4*	341.6±18.2*	322.4±10.4*

Notes: * - p <0.05 compared with control, ** - p <0.05 compared with "mild" diet, # - 0.05 <p <0.1 compared with the control.

the control, respectively, for the "mild", "moderate" and "severe" diets (Table **4**). The activity of Se-GP increased even more. Thus, it was higher 19, 32 and 37 times in the animals maintained for 2 months on the "mild", "moderate" and "severe" CRD respectively compared to the control (Table **4**).

Consequently, the response of the studied antioxidant enzymes on CRD varied. The activity of Se-GP increased the worst (very strongly) compared to the control level. The degree of increase in the activity depended on the degree and extent of calorie restriction and growth retardation (r=-0,991). It should be noted that high correlation was revealed between the changes of the Se-GP activity of and the content of T3 in serum of animals maintained for two months at various CRD (r = -0,967).

It is known that the outer mitochondrial membrane is rotenone-sensitive chain NADH oxidation, which is involved in the reduction of dehydroascorbic acid, and thus may be involved in improving the reliability of the antioxidant system of the cell. The activity of the redox-chain was judged by the change of the activity of NADH-oxidase and NADH – cytochrome c reductase in animals maintained for 2 months on different CRD.

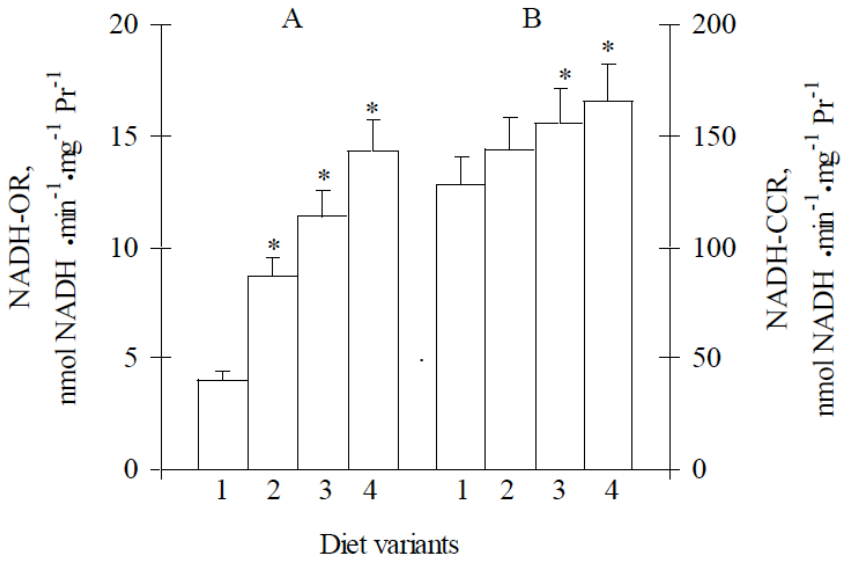

Figure 5: The activity of NADH-oxidase (A) and NADH cytochrome-C reductase (B) in.
(1) – animals in “control” group.
(2) – animals tacking the “mild” calorie restricted diet.
(3) – animals tacking the “moderate” calorie restricted diet.
(4) – animals tacking the “severe” calorie restricted diet.
* p <0.05 as compare to control.

It was found that the maintenance of experimental animals on different CRD was accompanied by significant increase of NADH-oxidase activity and the degree activity increase is strongly correlated with the level of calorie restriction. Since in the animals maintained on a "mild", "moderate" and "severe" diets NADH-oxidase activity increased by 2, 2.9, and 3.5 times, respectively, compared to the control (Figure **5**).

Activity of NADH-cytochrome-c-reductase also increased, depending on the degree of calorie restriction, but much less compared to the NADH-oxidase (Figure **5**).

It should be noted that there is a pronounced correlation (r=0,972 and r=0,970) between the increase of activity of NADH-oxidase and NADH-cytochrome-c-reductase and growth retardation of the animals maintained on the CRD.

4. DISCUSSION

The increase of average and maximum life-span of rats maintained on CRD is well known [2, 6, 7] and our results confirm this. In addition, we noticed that the transfer of 1 month rats on CRD was followed by the death of 10 to 15% of the animals for a month and it didn’t depend on the degree of caloric restriction. The superiority of the experimental animals maintained on CRD in life-span compared with the control group was clearly manifested in the later stages of ontogeny (Figure **2**).

The results on the dynamics of mortality and life span leads to the following assumptions:

1. Initial (selected for the experiment) cohort of animals is highly heterogeneous in their ability to respond to caloric restriction.

Thus, 10 - 15% of the animals are not able to survive in such conditions and die. In some animals, about 15-20%, a significant increase of life-span compared to control animals is observed (Figure **2**).

2. There is no direct correlation between the degree of caloric restriction and life-span increase.

Thus, the difference in life-span between the groups maintained on the "mild" and "moderate" diets were little, while on the "severe" CRD there was significant increase of life-span compared to others groups of animals (Figure **2**).

These results can be explained on the basis of the concept of epigenotype [33]. The essence of the concept is that the metabolic pattern is interoperable sequence of biochemical cycles, which provides a coordinated response to various stimuli (factors). Since epigenotype consists of a large number of on the one

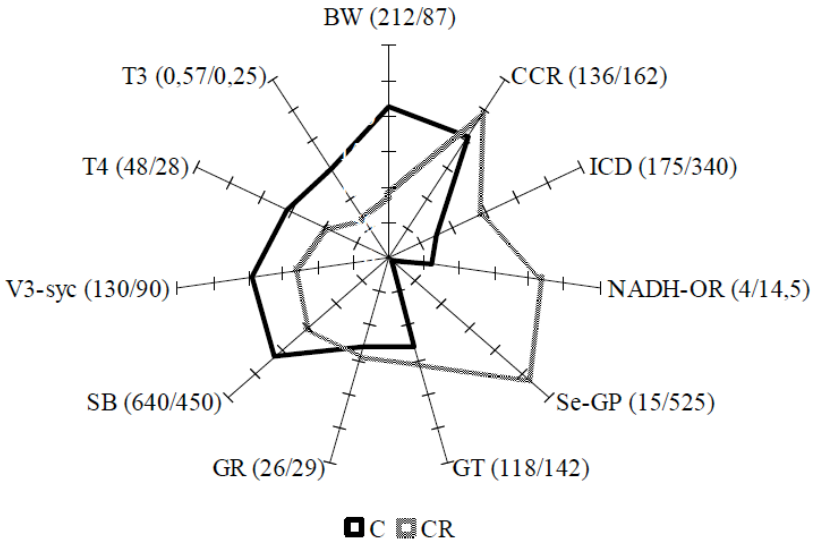

Figure 6: Epigenotype of the "control" group of animals (C) and of the "experimental" group (CR) tacking the "severe" calorie restricted diet since the second month of their life.

BW − body mass; CCR − cytochrom-c-reductase; ICD − isocitrate dehydrogenase; NADH-OR − NADH-oxidoreductase ; Se-GP − Se-dependent glutathione peroxidase; GT – glutathione S-transferase; GR − glutathione reductase; SB – Schiff bases; V3-syc – phosphorylating states; T4 – thyroxin; T3 – triiodothyronine.

hand related, and on the other relatively autonomous elements (biogeochemical cycles), then the cell can generate an infinite number of combinations - epigenotype. Epigenotype diversity can be repeatedly extended by the presence of alternative metabolic pathways in biological systems.

These results suggest that the transition from one to another epigenotype occurs in salutatory manner.

Thus, life-span varies quite dramatically on CRD providing 60% of body weight restriction, while at the restriction by 40 and 50% there were almost no differences in life-span.

We believe that the transfer of 1 month animals on CRD, which induces metabolic shift from anabolic to catabolic type, results in the death of the animals whose epigenotype for unclear reasons can not "do" a saltatory transition to a epigenotype providing catabolic pathway. Therefore on the first stage CRD selects epigenotype.

Later in the "selected" epigenotype cooperative changes of all the elements of the metabolic system occur. But since there is an infinite variety of metabolic variants of epigenotypes, in the population there will be individuals with different types of epigenotype among which there will epigenotypes providing high life-span. Consequently, on the conditional second stage CRD performs the function of specific induction of epigenotype.

Increase of life-span of individuals with specific epigenotypes is realized as hormesis effect [33].

The data obtained in this study suggested the epigenotype concept. Thus, the transfer of animals on CRD is characterized not mere by saltatory increase of the Se-dependent glutathione peroxidase (30 times) or isocitrate dehydrogenase activity, but also by changes in relation of the various antioxidant enzymes, hormonal status, and other values of metabolism. Presenting the epigenotype measures as the spatial portrait, the differences of epigenotypes of control animals and animals maintained for 2 months on "severe" CRD is rather obvious and compelling (Figure **6**).

These results allow us to hypothesize that the CRD performs a dual function: 1 – "selection" of animals with specific epigenotype; 2 – induction of hormesis response on the life-span. It is important that forming of specific epigenotypes will influence not only on life-span, but also on the response of the organism to other factors, susceptibility to infectious agents or pharmaceuticals.

REFERENCES

[1] Mc Cay CM, Crowell MF, Maynard LA. The effect of retarded growth upon length of life span and span and upon ultimate body size. J Nutr 1935; 10: 63-79.

[2] Nikitin VN, Stavitskaya LI. Retarding the growth of food and its effect on age-related changes of the body. I. The change of tissue phosphorus fractions and structural proteins of the

liver in young rats under the influence of long-retarding growth and subsequent fattening food. Proceedings of the Institute of Biology and Biology. Faculty of KSU. Kharkov: Univ Kharkov Press 1960; 29: 111-23.

[3] Nikitin VN. Experimental approaches to life extension. Problems Ageing Longevity 1991; 1(1): 5-10.

[4] Lints FA. The rate of living theory revisited. Gerontology (Schweiz) 1989; 35(1): 36-57. http://dx.doi.org/10.1159/000212998

[5] Knight WD, Witte MM, Parsons AD, Gierach M, Overton JM. Long-term caloric restriction reduces metabolic rate and heart rate under cool and thermoneutral conditions in FBNF1 rats. Mech Ageing Dev 2011; 132: 220-29. http://dx.doi.org/10.1016/j.mad.2011.04.001

[6] Masoro EJ. Caloric restriction and aging: an update. Exp Gerontol 2000; 35: 299-305. http://dx.doi.org/10.1016/S0531-5565(00)00084-X

[7] Lin S-J, Kaeberlein M, Andalis AA, Stutrz LA, Defossez P-A, Culotta VC, *et al*. Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. Nature 2002; 418(18): 344-8. http://dx.doi.org/10.1038/nature00829

[8] Mattison JA, Lane MA, Roth GS, Ingram DK. Calorie restriction in rhesus monkeys. Exp Gerontol 2003; 38: 35-46. http://dx.doi.org/10.1016/S0531-5565(02)00146-8

[9] Skulachev VP. A biochemical approach to the problem of aging: "Megaproject" on membrane-penetrating ions. The first results and prospects. Biochemistry (Moscow) 2007; 702(12): 1385-96. http://dx.doi.org/10.1134/S0006297907120139

[10] Page MM, Robb EL, Salway KD, Stuart JA. Mitochondrial redox metabolism: Aging, longevity and dietary effects. Mech Ageing Dev 2010; 131: 242-52. http://dx.doi.org/10.1016/j.mad.2010.02.005

[11] Barja G. Aging in vertebrates, and the effect of caloric restriction: a mitochondrial free radical production — DNA damage mechanism? Biol Rev 2004; 79: 235-51. http://dx.doi.org/10.1017/S1464793103006213

[12] Gredilla R, Barja G. Minireview: The role of oxidative stress in relation to caloric restriction and longevity. Endocrinol 2005; 146(9): 3713-17. http://dx.doi.org/10.1210/en.2005-0378

[13] Rahman J, Biswas SK, Kode A. Oxidant and antioxidant balance in the airways and airways discuss. Eur J Pharmacol 2006; 12(1): 222-39. http://dx.doi.org/10.1016/j.ejphar.2005.12.087

[14] Mak JC, Chan-Yeung MM. Reactive oxidant species in asthma. Curr Opin Palm Med 2006; 12(1): 7-11. http://dx.doi.org/10.1097/01.mcp.0000198067.50457.71

[15] Mongkolsuk S, Helmann JD. Regulation of inducible peroxide stress responses. Mol Microbiol 2002; 45: 9-15. http://dx.doi.org/10.1046/j.1365-2958.2002.03015.x

[16] Forman HJ, Fukuto JM, Torres M. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. Am J Physiol Cell Physiol 2004; 287: C246-C256. http://dx.doi.org/10.1152/ajpcell.00516.2003

[17] Covarrubias L, Hernández-García D, Schnabel D, Salas-Vidal E, Castro-Obregón S. Function of reactive oxygen species in animal development: Passive or active? Dev Biol 2008; 320: 1-11. http://dx.doi.org/10.1016/j.ydbio.2008.04.041

[18] Bozhkov AI. Low-calorie diet as a model to increase life expectancy and the study of mechanisms of aging. Uspechy gerontologii (Russian) 2001; 8: 89-99.

[19] Masoro EJ. Subfield History: Caloric Restriction, Slowing Aging, and Extending Life. Science's SAGE KE (26 February 2003). http://sageke.sciencemag.org/cgi/content/full/sageke; 2003/8/re2.

[20] Weindruch R, Kayo T, Lee CK, Prolla TA. Gene expression profiling of aging using DNA microarrays. Mech Ageing Dev 2002; 123: 177-93. http://dx.doi.org/10.1016/S0047-6374(01)00344-X

[21] Bozhkov AI, Menzyanova NG. Calorie restricted diet induce alternative Pathways of lipid metabolism for support of proliferative processes in regenerating liver. Adv Gerontol 2009; 3(3): 440-7. http://dx.doi.org/10.1134/S2079057011010048

[22] Bozhkov AI, Dlubovskaya VL, Dmitriev YuV, Meshaikina NI, Maleev VA, Klimova EM. Supposed Role of "Metabolic Memory" in Formation of Response Reaction to Stress Factors in Young and Adult Organisms. Adv Gerontol 2011; 1(1): 63-1.

[23] Jonson D, Lardy H. Methods in enzymology. New York; 1987; vol. 10: p. 94.

[24] Chance B, Williams GR. The respiratory chain and oxidative phosphorylation. Adv Enzymol 1956; 17(1): 65-134.

[25] Lemeshko V. Effect of thyroxine on the NADH-cytochrome c reductase activity of microsomes and the outer membrane of liver mitochondria of rats of different age. Biochemistry (Moscow) 1981; 46(10): 1807-14.

[26] Bauman DE, Brown RE, Davis CJ. Pathways of fatty acid synthesis and reducing equivalent generation in mammary gland of rat, sow and cow. Arch Biochem Biophys 1970; 140(1): 237-44. http://dx.doi.org/10.1016/0003-9861(70)90028-7

[27] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70: 158-69.

[28] Younes M, Schlichting R, Siegers C-P. Glutathione-S-transferase activities in rat liver: effect of some factors influencing the metabolism of xenobiotics. Pharmacol Res Communs 1980: 12(2): 115-28. http://dx.doi.org/10.1016/S0031-6989(80)80069-5

[29] Carlberg I, Mannerviek B. Glutathione reductase levels in rat brain. J Biol Chem 1975; 250: 5475-80.

[30] Buege A, Aust SD. Microsomal lipid peroxidation. Methods in Enzymology, Part C: Biological Oxidations 1978; 52: 302-10.

[31] Flercher BL, Dillard CJ, Tappel AY. Measurement of fluorescent lipid peroxidation products in biological systems and tissues. Anal Biochem 1973; 52: 1-9. http://dx.doi.org/10.1016/0003-2697(73)90327-8

[32] Miller GL. Protein determination for large numbers of samples. Anal Chem 1959; 31(5): 964-66. http://dx.doi.org/10.1021/ac60149a611

[33] Bozhkov AI, Nikitchenko YuV, Sidorov VI, Kurguzova NI, Klimova EM. Hormesis Model of Metabolic Memory and Possibility of Regulation of the Rate of Aging. Problems Ageing Longevity 2012; 21(N3): 245-54.

Received on 24-12-2012 Accepted on 07-01-2013 Published on 31-03-2013

DOI: http://dx.doi.org/10.6000/1929-5634.2013.02.01.4