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STUDY REGARDING THE INFLUENCE OF CALORIE RESTRICTION OVER LABORATORY ANIMALS BIOLOGY AND BEHAVIOR (SUMMARY OF Ph.D. THESYS)

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INTRODUCTION

Calorie restriction is a diet based on a low calorie levels. How low has to be this level, it’s necessary an unbiased comparison between the subject’s previous calorie intake to the physiological necessary of the subject’s species or at least to a different individual of the same species with a similar body mass index. This type of diet excludes malnutrition, being absolutely necessary to maintain a normal intake of vitamins, minerals and amino acids.

This diet can be successfully applied to a wide range of beings, starting with yeasts, nematodes, flies (the species being used as major experimental models to study the calorie restriction) finishing with mammals, such as horse, cow, dog, etc., and having as a result the delaying of ageing symptoms, the prevention of conditions caused by obesity and probably tumor growth.

Today, calorie restriction is considered the most safe method to extend the mean life span (mean number of years that a certain animal should live) and maximum life span (maximum number of years that a certain animal should live). Also, calorie restriction prolongs the number of years that an animal might live without suffering of chronic diseases.

Although it has been yet to establish with certainty the validity of this theory in primates, there are very solid proofs to suggest that calorie restriction may counter the risk factor which occur with old age in Maccacus rhesus and Homo sapiens.

The selection of this Ph.D. thesis subject was based on a careful and profound domain evaluation, being reviewed the latest scientific articles. The experimental protocols which were applied in the second part of this thesis are accordingly with those used at this moment in the most modern laboratories, the implementing and the specialization in these techniques being one of the main purpose of the experiments comprised in this thesis. The experiments were undertaken at the USAMV Cluj-Napoca, and the chosen subject has an interdisciplinary character, following aspect of normal and pathological physiology and morphological pathology.

Key words: calorie restriction, diet, life span.
AIM AND OBJECTIVES

The aim of this thesis is to emphasize the applicability and the impact of calorie restriction (CR) phenomenon in laboratory animals (Wistar rats and Swiss mice) and the investigation of biological variances (behavior, hematologic, biochemical and histologic) following CR.

The research was orientated on three main directions, as following:

1. the behavioral evaluation of individuals included in the experiments, subjected to various levels of CR;
2. the evaluation of some hematological and biochemical parameters of individuals included in the experiments, subjected to various levels of CR;
3. the evaluation of the chronic CR impact over the brain cellular components in individuals included in the experiments, subjected to various levels of CR.

The objectives of the first research direction are:

1. to emphasize the acute CR impact on behavior and memorizing and learning capacities in Wistar rats by their testing with \"T\" shaped labyrinth for swimming and radial arm maze;
2. to emphasize the chronic CR impact on behavior and memorizing and learning capacities in Wistar rats by their testing with \"T\" shaped labyrinth for swimming and radial arm maze;
3. to emphasize the acute CR impact on behavior and memorizing and learning capacities in Swiss mice by their testing with \"T\" shaped labyrinth for swimming and radial arm maze;
4. to emphasize the chronic CR impact on behavior and memorizing and learning capacities in Swiss mice by their testing with \"T\" shaped labyrinth for swimming and radial arm maze;
5. to emphasize the acute and chronic CR impact on behavior and memorizing and learning capacities in Wistar rats by their testing with forced swimming test;
6. to emphasize the acute and chronic CR impact on behavior and memorizing and learning capacities in Swiss mice by their testing with forced swimming test.

The objectives of the second research direction are:
1. to emphasize the acute CR impact on some hematological and biochemical parameters in Wistar rats;
2. to emphasize the chronic CR impact on some hematological and biochemical parameters in Wistar rats;
3. to emphasize the acute CR impact on some hematological and biochemical parameters in Swiss mice;
4. to emphasize the chronic CR impact on some hematological and biochemical parameters in Swiss mice;

The third research direction’s objective is:
1. to emphasize the chronic CR impact on some somatic-sensorial encephalic cortex cellular components in Wistar rats.

THESYS STRUCTURE

The Ph.D. thesis “The study regarding the influence of calorie restriction over laboratory animals biology and behavior” contains 192 pages and 8 chapters, being splinted in two parts, following the rules of writing.

The first part (chapters I, II, III, IV) contains 56 pages, representing 30% of the thesis, and summaries the actual level of knowledge in CR field of research and theory, the principles and action mechanisms, the CR in relation with ageing, memory capacity and learning behavior.

The second part (chapters V, VI, VII, VIII) spanned over 136 pages, represents 70% of the thesis, containing the personal researches undertaken between 2009-2013. Each chapter is structured in subchapters which represent the aim, the objectives, materials and methods, the results with the discussions over their novelty in comparison with other studies and the partial conclusions following the study.
RESEARCH RESULTS

Chapter 6, called "The evaluation of some blood parameters in laboratory animals subjected to various levels of acute calorie restriction" contains studies of:

1. The influence of acute calorie restriction over some hematological and biochemical parameters in Wistar rats.

   In the making of this study were used 23 Wistar rats, males, which were subjected for 30 days to various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, it has been harvested blood samples which were hematologically and biochemically analyzed. Following the statistical interpretation, it has been noticed that those two levels of CR induce a decrease in the leucocytes count and hypoglycemia, hipocholesterolemia, but an increase of total proteins and red blood cells count, in comparison with the control group, fed ad libitum.

2. The influence of various levels of acute calorie restriction over some hematological and biochemical parameters in Swiss mice.

   In the making of this study were used 24 Swiss mice, females, which were subjected for 30 days to various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, it has been harvested blood samples which were hematological and biochemically analyzed. Following the statistical interpretation, it has been noticed that those two levels of CR induce a decrease in the leucocytes count and hypoglycemia, hipocholesterolemia. On the other hand, it has been noticed on those groups an increase of total proteins and red blood cells count, in comparison with the control group, fed ad libitum.

3. The influence of various levels of chronic calorie restriction over some hematological and biochemical parameters in Wistar rats.

   In the making of this study were used 23 Wistar rats, females, which were subjected for 90 days to various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, it has been harvested blood samples which were hematological and biochemically analyzed. Following the statistical interpretation, it has been noticed that those two levels of CR induce a moderate decrease in the leucocytes count and hypoglycemia, hipocholesterolemia, but an
increase of total proteins and GGt, in comparison with de control group, fed *ad libitum*.

4. The influence of various levels of chronic calorie restriction over some hematological and biochemical parameters in Swiss mice

In the making of this study were used 24 Swiss mice, females, which were subjected for 90 days to various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, it has been harvested blood samples which were hematological and biochemically analyzed. Following the statistical interpretation, it has been noticed that those two levels of CR induce an decrease in the leucocytes count and hypoglycemia, hipocholesterolemia. On the other hand, it has been noticed on those groups a moderate increase of total proteins in comparison with de control group, fed *ad libitum*.

**Chapter 7**, called ``The influence of chronic calorie restriction over cellular component of sensorial-somatic encephalic cortex in Wistar rats``, included the usage of 23 Wistar rats, males, subjected to 90 days of various CR levels (25 and 40% less calories than the physiological necessary). In the end, we harvested the rats brain and made histological inclusions, stained with hematoxilin-eosin, PAS McManus and immune-histochemistry staining with anti-PCNA antibodies. Following the microscopically analyze of hematoxilin-eosin staining, there were noticed only slight and rare morfopathological modifications (gliosis, satelitosys and neuronal, astrocite and white matter vacuolization). Following the microscopically analyze of the PAS-McManus staining, it has been noticed that the rats from the second group (normally fed) had more lipofuscine inclusions than the other groups. Following the microscopically analyze of the immune-histochemistry staining, it has been noticed that the rats subjected to CR had more neuronal bodies and glial cells with positive reaction following anti-PCNA staining.

**Chapter 8**, called ```The influence of acute calorie restriction over learning and memorizing capacity in lab animals```, contains the following studies:

1. The influence of acute calorie restriction over learning and memorizing capabilities in Wistar rats

For this study it has been used 23 Wistar rats, males, subjected to 30 days of various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, we measured the body weight and their performances in ```T``` shaped labyrinth for swimming
and radial arms maze. The rats subjected to CR had the lowest gain in body weight than the control group. In the same time, these rats had the fastest times in solving the labyrinths than the control group.

2. The influence of acute calorie restriction over learning and memorizing capabilities in Swiss mice

For this study it has been used 24 Swiss mice, females, subjected to 30 days of various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, we measured the body weight and their performances in "T" shaped labyrinth for swimming and radial arms maze. The rats subjected to CR had the lowest gain in body weight than the control group. In the same time, these rats had the fastest times in solving the labyrinths than the control group.

Chapter 9, called "The influence of chronic calorie restriction over learning and memorizing capacities in laboratory animals", contains the following studies:

1. The influence of chronic calorie restriction over learning and memorizing capabilities in Wistar rats.

For this study it has been used 24 Wistar rats, females, subjected to 90 days of various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, we measured the body weight and their performances in "T" shaped labyrinth for swimming and radial arms maze. The rats subjected to CR had the lowest gain in body weight than the control group. In the same time, these rats had the fastest times in solving the labyrinths than the control group.

2. The influence of chronic calorie restriction over learning and memorizing capabilities in Swiss mice.

For this study it has been used 24 Swiss mice, females, subjected to 90 days of various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, we measured the body weight and their performances in "T" shaped labyrinth for swimming and radial arms maze. The rats subjected to CR had the lowest gain in body weight than the control group. In the same time, these rats had the fastest times in solving the labyrinths than the control group.

3. The evaluation of acute and chronic calorie restriction over Wistar rats behavior in forced swimming test.

For this study it has been used 24 Wistar rats, females, subjected to 90 days of various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, we tested the rats’ reaction in the forced swimming test device. Both in the acute faze of
CR (at 30 days) and in the chronic faze (at the 90 days), the rats subjected to the lowest level of CR (40%) had the lowest immobility time than the control group.

4. The evaluation of acute and chronic calorie restriction over Swiss mice behavior in forced swimming test.

For this study it has been used 24 Swiss mice, females, subjected to 90 days of various levels of CR (25 and 40% less calories than the physiological necessary). At the end of the study, we tested the rats reaction in the forced swimming test device. Both in the acute faze of CR (at 30 days) and in the chronic faze (at the 90 days), the rats subjected to the lowest level of CR (40%) had the lowest immobility time than the control group.

GENERAL CONCLUSIONS

1. The Wistar rats subjected to acute CR of 25% had shown better reaction times and superior memorizing capacity than the individuals from the other groups following the testing with ‘’T’’ shaped labyrinth and radial arms maze.

2. The Swiss mice subjected to acute CR of 25% had shown better reaction times and superior memorizing capacity than the individuals from the other groups following the testing with ‘’T’’ shaped labyrinth and radial arms maze.

3. The Wistar rats subjected to chronic CR of 25% had shown better reaction times and superior memorizing capacity than the individuals from the other groups following the testing with ‘’T’’ shaped labyrinth and radial arms maze.

4. The Swiss mice subjected to chronic CR of 25% had shown better reaction times and superior memorizing capacity than the individuals from the other groups following the testing with ‘’T’’ shaped labyrinth and radial arms maze.

5. The Wistar rats subjected to acute and chronic CR of 40% had shown the lowest immobility time than the control group following the testing with the forced swimming test.
6. The Swiss mice subjected to acute and chronic CR of 40% had shown the lowest immobility time than the control group following the testing with the forced swimming test.

7. Acute CR of 25% and 40% had a hypoglycemic, lipid and white blood cells count lowering effect in Wistar rats. But all the measured parameters fell between the physiological limits.

8. Acute CR of 25% and 40% had a hypoglycemic, lipid and white blood cells count lowering effect in Swiss mice. But all the measured parameters fell between the physiological limits.

9. Chronic CR of 25% and 40% had a hypoglycemic, lipid and white blood cells count lowering effect in Wistar rats. But all the measured parameters fell between the physiological limits.

10. Chronic CR of 25% and 40% had a hypoglycemic, lipid and white blood cells count lowering effect in Swiss mice. But all the measured parameters fell between the physiological limits.

11. Chronic CR of 25% had a powerful positive effect over the intracellular reparatory processes from the sensorial-somatic encephalic cortex in Wistar rats.

**THEESIS INNOVATIVE ADN ORIGINAL CONTRIBUTIONS**

This thesis contains CR experiments on laboratory animals for the first time in Romania. Although CR theory is almost a century old, Romanian scientists didn’t pay any attention to this subject, but we managed to present a series of accessible means of research in this domain, using three procedural cognitive and behavioral tests (swimming "T" test, radial arms maze and forced swimming test), with immediate applicability, at least in the CR field of study. None the less, the experimental procedures used in this thesis could be successfully used in other domains, such as: pharmacology, biology, oncology, metabolic pathology, etc.

We have shown for the first time the intracellular effects induced by the chronic CR in adults Wistar rats, detectable by immune histochemical staining with anti-PCNA anti-body. In the same time, we have shown that the experimental models used in this thesis may be successfully used for the impact evaluation of the CR over the behavior and memorizing capacity in Wistar rats and Swiss mice.