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dr hab. Teresa Małecka-Massalska  
Head of the Chair and Department of Human Physiology  
Medical University of Lublin  
20-080 Lublin, Radziwiłłowska Street 11

### **Evaluation of the doctoral dissertation**

**of doctoral student Hussein Bae Khudhur entitled: "Studies on the effect of bovine milk casein and whey proteins on cadmium toxicity in rats" presented to the Scientific Council of the Faculty of the Veterinary Medicine, University of Life Sciences in Lublin. The Supervisor of the presented dissertation: Jose Luis Valverde Piedra, DVM, Ph.D. Assoc. Prof. Department of Preclinical Veterinary Science Sub Department of Pharmacology, Toxicology and Environmental Protection, Co-advisor: Sylwia Shymanczyk, PhD Eng. Department of Animal physiology.**

Pollution of the environment by cadmium (Cd) caused by its release from natural and industrial sources is a growing health problem due to the negative effect on the structure and function of many human and animal organs.

As is commonly known, Cadmium (Cd) is a mineral element highly toxic to humans and animals, even at low concentrations. Cadmium (group IIB of the periodic table of elements) is a heavy metal posing severe risks to human health. Up to this day, it could not be shown that cadmium has any physiological function within the human body. Therefore interest has risen in its biohazardous potential. As first described by Friedrich Stromeyer (Göttingen, Germany) in 1817, cadmium intoxication can lead to kidney, bone, and lung damage.

Cadmium is regularly found in ores together with zinc, copper and lead. Therefore volcanic activity is one natural reason for a temporary increase in environmental cadmium concentrations. This highly toxic mineral element is also widely used in industrial processes,

e.g.: as an anticorrosive agent, as a stabilizer in PVC products, as a colour pigment, a neutron-absorber in nuclear power plants, and in the fabrication of nickel-cadmium batteries.

The environmental pollution with cadmium and its devastating impact on the organisms of humans and animals is a big problem in Iraq. The most dangerous characteristic of cadmium is that it accumulates throughout a lifetime due to its long biologic half-life (more than 30 years). Prolonged low level exposure leads to excessive accumulation in certain tissues, especially in the kidney. The toxicity of cadmium depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of the exposed individuals.

The treatment strategies in the case of cadmium poisoning include mainly chelating and antioxidant therapies but are burdened with numerous undesirable symptoms. Therefore, in many countries, researchers are looking for substances of natural origin that could potentially be used in such therapies without causing side effects. Among such substances, milk protein products, whey proteins and casein products seem to have promising properties which provide specific health benefits beyond their basic nutritional value.

The work presented for the evaluation has the character of a dissertation, developed according to generally accepted principles. The work carried out under the direction of Assoc. Professor Jose Luis Valverde Piedra Ph.D. and Co-Advisor Sylwia Shymanczyk PhD, has 238 pages, divided into 8 chapters. It contains 60 figures, 4 tables in chapter IV, 16 tables in chapter V, 30 tables in Appendices, and 377 items of English language literature.

The aim of the study was to investigate the possible role of bovine milk casein and whey proteins in alleviating cadmium toxicity or in lowering its accumulation by means of *in vitro* and *in vivo* studies.

In chapter I – “*Introduction*”, the doctoral student makes a general introduction to the problem of cumulation of cadmium in the environment, cadmium poisoning, emphasizing the importance of the problem.

In chapter II, the doctoral student has presented a comprehensive review of literature. This chapter contains 8 subsections dealing with: cadmium effects on ecosystem, cadmium pollution in Iraq, cadmium toxicity and epidemiology, cadmium toxicokinetic, mechanism of toxic effects, cadmium toxicity effects, cadmium histopathological effects and milk proteins.

In the subsection dealing with the influence of cadmium on the environment, the author lists the main sources of pollution identifying nonferrous metal mining and refining, manufacture and application of phosphate fertilizers, fossil fuel combustion, waste incineration and disposal as the main anthropogenic sources of diffuse cadmium pollution in the environment.

The doctoral student rightly observes that the cadmium absorbed by the organic matter of the soil is extremely dangerous because Cd can accumulate in animals that are dependent upon the plants for survival, especially when they eat large quantities of the plants. Cows may have large amounts of Cd in their kidneys due to that. In next subsections of this chapter the problem of cadmium pollution in Iraq and epidemiology are clearly described and explain the need of research undertaken by the author.

In the further part of the literature review, the doctoral student focused on cadmium toxicokinetic, including mechanisms leading to absorption, distribution, excretion and toxicity of cadmium.

Most literature data comes from studies on rodents. The oral exposure of cadmium in laboratory animals has common findings in many studies, which are represented either by the decrease or increase of body weight and the difference between these findings vary according to the route of administration, dose, chemical form, duration, species, sex, age and nutritional status. The author also describes histopathological signs of cadmium toxicity focusing mainly on the effect of cadmium on kidney and liver.

Last section of Literature Review deals with milk proteins and their possible role in the process of protection against the toxic effect of cadmium. The doctoral student has indicated that, according to Elias, *et al.*, 2008, the hydrolysates or the purified peptides from milk proteins have antioxidant properties, and thus they have been receiving increasing attention in the recent years. Bovine milk proteins are divided into 2 groups, casein and whey proteins which became the subject of experiments undertaken in this work.

Chapter III contains a description of the aim of the undertaken research.

In Chapter IV the materials and methods are described. The research was logically planned, and the research methodology was described in detail. Two different sets of the experiments were performed to achieve the answer to the hypothesis that bovine milk proteins

may protect the organism against cadmium toxicity. The first set of studies comprised *in vitro* studies and the second set comprised *in vivo* studies on rats. Moreover, this chapter provides an exact scheme of the *in vitro* study (Figure 1) that clearly explains the course of the experiment and tables (Table 3 and 4) containing the design of the experiments on animals.

*In vitro* studies were performed on mouse fibroblast line L929 (ATCC CCL-1) by the use of a unique technique - ECIS (Electric Cell-substrate Impedance Sensing). The *in vitro* ECIS experiment was conducted to examine an intermediate-term exposure (20-25h) of different Cd concentration, in order to observe the fibroblast cell behavior in the presence or lack of casein and whey peptides in the medium.

The *in vivo* study was conducted on 72 male and female albino Wistar rats randomly divided into six groups. (T1 - Cd group; +ve control) received CdCl<sub>2</sub> and control diet, (T2 - -ve control) the group received filtered water and control diet, (T3 - Casein+CdCl<sub>2</sub>) the group received Cd and a casein based diet, (T4 - Casein) the group received filtered water and a casein based diet, (T5 - Whey Proteins+CdCl<sub>2</sub>) the group received Cd and a whey proteins based diet, (T6 - Whey Proteins) the group received filtered water and a casein based diet. The CdCl<sub>2</sub> was administrated in drinking water (19.4 mM/L) for 10 weeks. All of rats received a basal diet during the first five weeks of the experiment but later they received different feeding treatments.

The doctoral student carried out a statistical analysis using generally accepted methods.

The results are described in chapter V. This chapter contains two subsections: the first one describing *in vitro* tests and the second concerning animal studies.

The doctoral student clearly described the obtained results. In the ECIS assay author indicated that higher Cd concentration (6.2 μM), caused sharp decrease of impedance in time, which is an indication of cell death. While earlier addition of casein and whey proteins peptides to the culture increased markedly the impedance value and protected from Cd toxicity. The doctoral student stated that Cd could bind the milk peptides present in the medium avoiding its interaction with the fibroblast cell membrane. Moreover, in the microscopic evaluation of time-dependent cytotoxicity, gradual morphological changes were observed and they were represented in early cell shrinkage, and round or oval-shaped

appearances of fibroblast cells, which were the most obvious effects observed in the case of fibroblast treated with cadmium. In contrast, the author didn't noticed these types of morphological changes in fibroblast cultures treated with casein and whey protein peptides, which was interpreted by the doctoral student as prophylactic activity against cadmium cytotoxicity. The whey protein peptides revealed better prophylactic activity against cadmium fibroblast cytotoxicity as compared with casein peptides. The promising results of *in vitro* study justify the *in vivo* experiments on animals.

In the *in vivo* study, the doctoral student demonstrated that administration of cadmium resulted in disturbance of serum calcium, cholesterol and triglyceride concentration, which increased significantly mainly in female rats receiving Cd and the control diet, while in rats exposed to Cd and fed with casein or whey protein based diet the cholesterol and triglyceride levels were not affected. The author also noticed relatively lower accumulation of cadmium in liver, and increased Cd elimination via hair and feces in rats fed with casein and whey protein+Cd in both sexes.

The results obtained by the doctoral student constitute an interesting set of data that allowed him to draw a general conclusion that casein and whey proteins supplemented diet is a good strategy to reduce cadmium toxicity since they influence the absorption, distribution and elimination of cadmium in rats.

In the "Discussion" chapter, the author thoroughly discusses the obtained data, presenting them against the background of results obtained by other authors.

The work presented for evaluation is written according to the standard, contains all the necessary chapters, correctly set goals and corresponding conclusions. The author has proved that he can set a goal, choose material and research methods. The literature collected by the doctoral student is extensive.

The results obtained have been well described and, in my opinion, they are promising. Nevertheless, the text needs minor changes:

1. In chapter III: there is no need to re-justify the undertaken research, the aim of the study could be presented in more concise form, avoiding additional citations that can be included in the literature review.

2. It would be more understandable to put tables in the right place in the text instead of Appendices.
3. Minor punctuation errors such as a double space or no space, periods instead of commas, typos, unclosed quotes.

The above shortcomings do not diminish the value of the work and should be treated as benign guidance to be included before publication. The PhD thesis of the doctoral student Hussein Bae Khudhur is logically planned, based on the important literature references and methodological assumptions. I highly assess the overall level of the dissertation. The doctoral student showed the ability to conduct scientific research, good knowledge of the literature and its critical use. The doctoral student showed high level of scientific maturity in the selection and analysis of scientific sources, which makes this dissertation a huge potential for publication. The research was planned and carried out in a way that I rate very highly. The obtained results, their scientific and innovative potential, make this work exceptionally scientifically valuable.

In my opinion the work presented for review meets all the requirements set out in the Act on Degrees and Scientific Titles for PhD dissertations. It corresponds to the conditions specified in art. 13 of the Act of 14 March 2003 on academic degrees and academic title, and on degrees and title in the field of art. and the enactment of the Ministry of Science and Higher Education of 26 September 2016 with latter changes reasons.

For the above I am asking the Scientific Council of the Faculty of the Veterinary Medicine, University of Life Sciences in Lublin for acceptance of "Studies on the effect of bovine milk casein and whey proteins on cadmium toxicity in rats" dissertation and for the author's, doctoral student Hussein Bae Khudhur, admission to the further stages of the doctoral thesis.

Due to the high scientific and innovative value, the dissertation of the doctoral student Hussein Bae Khudhur deserves, in my opinion, to be honored and awarded.

dr hab. Teresa Małecką-Massalska