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Ph.D. thesis

**Experimental balneology: the biological effects of medicinal water and mud samples
from the Carpathian Basin**

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Program leader: Prof. Dr. Gábor Kovács L.
Tutor: Dr. Csaba Varga



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I. INTRODUCTION

Our homeland is one of the leading powers in balneology due to its favourable geographical conditions. Hungarian baths, medicinal waters and muds are popular with both domestic and foreign tourists. Some of the spa guests attend the facilities looking for cure to their diseases. However, there is a growing number of guests who participate in various balneological treatments with the aim of recreation and relaxation. The treatments have an important role in the prevention of different diseases, therefore balneology is essential to prevention.

Balneology or the science of spas deals with the study and therapeutic use of medicinal waters, mineral waters and medicinal muds.

Spa waters and muds are the results of numerous geological, geothermal and hydrogeological processes. The geothermal conditions in the Carpathian Basin are unique. The geothermal gradient of the Pannonian Basin is one-and-a-half fold of the world average because the crust is 10 kilometres thinner than that of the neighbouring areas. In the development of mineral-medicinal waters and muds high temperature and high pressure play a key role, which results not only in positive therapeutic effects but can also lead to the formation of potentially harmful or toxic substances.

Hungary abounds in mineral and medicinal waters, which are categorized according to their inorganic components.

Medical muds (peloids) are fine granular materials having good water binding and heat retaining capacity, making them suitable for balneological treatments.

Peloids can be categorized in different ways. Based on their origins, natural (eupeloid) and artificial (parapeloid) can be distinguished. Another classification does not only involve the origins of a particular peloid, but also pays attention to the ratio of its organic and inorganic constituents.

In 2007 there were 5 medicinal muds on the Hungarian market: Makó, Kolop, Hévíz, Hajdúszoboszló and a medicinal peat (the Alsópáhok „Georgikon”).

Currently there are still 5 muds in circulation with a minor alteration: instead of Hévíz peloid “Neydharting” peloid was registered.

Medicinal mud treatments may be recommended for a wide range of diseases including locomotor diseases, gynaecological disorders, skin diseases, traumas and rheumatic disorders.

Two major types of peloid treatment can be differentiated: active and passive. Out of the two, passive treatment comprising peloid wraps and packs is more common.

Inhabiting microorganisms may have an equally important role in case of medicinal muds, medicinal waters and mineral waters. These microorganisms can be divided into two large categories. On the one hand, we can talk about autochthonous or natural microflora.

Microorganisms that get into the waters or peloids from the outside belong to the other group, termed allochthonous microflora. They have great importance as they may include quite a few pathogenic groups as well, and thus their presence in mineral waters, medicinal water and peloids is undesirable.

Balneoprevention is a novel discipline of balneology, a field of science with increasing importance. Balneoprevention has a dual function: to prevent disease development by means of balneological treatment and to identify the components present in waters and muds that pose a risk to human health.

We do not have exact information about the attendance of domestic spas and the number of treatments implemented. Our baths had a total attendance of 23-24,5 million in 2009. In 2008 the net income exceeded 50 billion forints. The number of therapeutic treatments was over 6,5 billion in 2012 and expenses covered by social insurance almost reached 4 billion forints.

2. AIMS OF THE STUDY

The aims of our study were in vivo and in vitro investigations of different peloids and medicinal waters on different biological endpoints and the microbiological risk assessment of muds. We wanted to get answers to the following questions:

1. Can the microbiological risk of the applied peloids be shown? Is there a significant difference in the number of indicator groups in these peloids?
2. Can soil toxicity tests from ecotoxicology be adapted for the the toxicological study of peloids?
3. Is it feasible to develop a procedure of single-cell-gel electrophoresis assay for in vivo and in vitro genotoxicological risk assessment of medicinal muds?
4. Can the genotoxicological risk of different medicinal water concentrates be shown by genotoxicity tests?
5. What experimental model can be used to investigate the UV-exposure related biological effects of spa water concentrates?

3. MATERIALS AND METHODS

Out of the five peloids certified in Hungary, two were examined in ecotoxicological and genotoxicological tests. The two samples were the Kolop and the Hévíz mud. Microbiological investigations were also carried out in these peloids. Moreover, two medicinal waters with high level of organic matter were studied using genotoxicological tests.

3.1 Description of investigated peloids and medicinal waters

3.1.1 Kolop mud

Kolop mud belongs to the group of inorganic muds, containing only a minimal amount of organic components. The main indications of Kolop mud treatment are spinal and limb joint complaints, gynaecological and dermatologic diseases.

3.1.2 Hévíz mud

Hévíz peloid is a kind of mixed mud containing a considerable amount (at least 20%) of organic detritus (peat) besides inorganic components (volcanic mud). This peloid is applied in rheumatic locomotor diseases, osteoporosis, soft tissue rheumatism, pre- and postoperative treatment of the joints and discs, and gynaecological conditions.

3.1.3 Kakasszék medicinal water

The water contains a significant quantity of organic compounds. The therapeutic suggestions of this spa water are locomotor diseases, post-traumatic disorders, chronic gynaecological inflammations and skin diseases.

3.1.4 Gyopárosfürdő medicinal water

The water of the Gyopárosfürdő spa belongs to alkali carbonate waters and is similarly rich in organic content. It may also be recommended for a wide range of diseases including locomotor diseases, gynaecological disorders conditions, urological diseases, skin diseases and neurological disorders.

3.2 Experimental methods

3.2.1 Microbiological study of peloids

Our investigation was performed in accordance with relevant chapters of the government order 201/2001. (X. 25.) and along with the FVM-ESzCsM-GKM decree 65/2004. (IV. 27.), based on which the presence of some indicator groups was shown.

3.2.1.1 Total plate count

Total plate count defines how many mesophilic heterotrophic microorganism colonies such as bacteria, yeast and mould fungi will grow within 48 hours at a temperature of 37 °C. Total plate count was decided from 1 ml of peloid suspension and related to 1 g of mud.

3.2.1.2 Total coliform number

The study of total coliform aims to show indicator bacteria whose presence points to recent faecal contamination. Our study was performed on Endo agar. Total coliform count was given for 1 g of peloid.

3.2.1.3 Enterococcus

Another indicator study for faecal pollution. However, the presence of Enterococci points to an earlier contamination here. Their study is executed on Enterococcus agar. Enterococcus was given in 1 g peloid.

3.2.1.4 Clostridium count

In this study anaerobic sulphate-reducing bacteria were examined. During our investigation was used an ready-to-use SPS agar. Clostridium count was given for 1 g of peloid in this study.

3.2.2 Ecotoxicological tests

During our work two ecotoxicological tests were applied: one of them was the earthworm test and the other was the white mustard seedling growth test.

3.2.2.1 White mustard seedling growth test

First, the examination was carried out in two steps. Peloid extract was prepared from the samples using distilled water, then the experiment was conducted on the total amount of the peloid as well. The number of germinating seeds was observed and the length of roots was measured.

3.2.2.2 Eisenia test

During the test lethality, body mass alteration and reproductive capacity were used as endpoints. Both the Kolop and the Hévíz samples were tested on *Eisenia*. A pre-experiment was also done with the Hévíz mud.

3.2.3 Genotoxicological study

3.2.3.1 Investigation of medicinal muds with single-cell-gel electrophoresis assay (comet assay)

During our study the *in vitro* and *in vivo* DNA-damaging effects of peloid samples (Hévíz, Kolop) were examined using the single cell comet assay on mammal lymphocytes (human and Long Evans rat) and *Eisenia fetida* coelomocytes.

3.2.3.2 Genotoxicological study of medicinal water and thermal water concentrates and their possible role in protection from UV radiation

In our study, the potential genotoxic effects of Kakasszék and Gyopárosfürdő medicinal waters and their possible role in protection from UV exposure were investigated. 1000-fold concentrates were made from the tested medicinal and thermal waters. UV irradiation was performed using a germicidal lamp.

3.2.3.2.1 Study of medicinal water extracts in Salmonella Ames test

The essence of the method: Genetically modified, histidine auxotrophic mutant *Salmonella typhimurium* TA strains were used. If the sample is mutagenic, reverse mutation occurs and revertants grow on the minimal glucose medium containing a minimal amount of histidine.

During our investigations the potential genotoxic effects of the Kakasszék medicinal water concentrate and its role in protection from UV exposure (both with and without incubation) were tested.

3.2.3.2.2 Study of medicinal water concentrates with single cell comet assay

In this study, the effects of two concentrates (Kakasszék and Gyopárosfürdő) on keratinocytes (HaCat) were tested by comet assay. First, the UV sensitivity of cells was surveyed, then potential genotoxic effects and the role of the extracts in protection from UV radiation were investigated. Also, different exposure and incubation times were used during UV exposure.

4. RESULTS

4.1 Results of hygienic microbiological investigations

The average total plate count of the Hévíz peloid was 2794 colonies/g and in the Kolop mud it was 372 colonies/g. The presence of Coliforms and Enterococci could not be confirmed in either peloid sample. The average *Clostridium* count was 800 colonies/g in the Hévíz sample.

4.2 Results of ecotoxicological tests

4.2.1 White mustard seedling growth test

In the investigation of the peloid extracts there was no remarkable significant difference between either the Kolop or the Hévíz mud sample and the control. While the Hévíz peloid did not show any significant effects during the direct study of the peloids, the Kolop mud inhibited root elongation and this effect was statistically significant.

4.2.2 Results of the Eisenia test

Extreme mass loss and no cocoon (egg) production were detected that can be explained by the inadequately low organic content of the peloid. That is why in the present test the peloids and artificial soil used as control were mixed with horse dung.

In the investigations after the pilot study neither lethality nor significant difference from the control in mass gain was detected.

The only significant difference could be shown in the number of cocoons developed in the Kolop sample. No such divergence was found in the Hévíz sample.

4.3 Results of genotoxicity studies

4.3.1 Results of study of peloids by comet assay

While in human lymphocytes no considerable difference was observed between the negative control and Kolop peloid, the DNA damaging potential of Hévíz mud was significant ($p < 0,001$).

In the case of rat lymphocytes neither the Hévíz nor the Kolop sample showed significant difference from the negative control.

The examination of *Eisenia fetida* coelomocytes in the Kolop and Hévíz samples yielded well assessable results in both cases. No significant difference was found between the Kolop peloid and the negative control, as opposed to the Hévíz sample where there was a significant alteration ($p < 0,001$).

4.3.2 Studies on medicinal water concentrates in the Ames test

Based on our results it can be established that the Kakasszék medicinal water extract shows genotoxic effects (frameshift mutation or base-pair substitution mutation) in neither strain.

4.3.3 Results of comet assay on keratinocytes

During the study of UV sensitivity it could be proved that even the shortest (10 s) UV irradiation can cause significant DNA damage. Furthermore it was also confirmed the extent of the resulting damage is directly proportional to the time of irradiation.

In our subsequent investigations the genotoxic effects of medicinal water concentrates were tested. In the case of Gyopárosfürdő water, genotoxic effects can clearly be excluded. In contrast, genotoxicity cannot be either ruled out or confirmed in the case of the Kakasszék sample as the investigations produced results for both.

In our further studies the possible role of the extracts in protection from UV radiation was examined. Here, different UV exposure and incubation times (0,5; 1; 1,5; 2; 2,5 hours) were used.

From our results, we can say that protective effect could be verified with both samples, but in different condition. In the case of Kakasszék water protective effect could only be shown when cells were incubated in the medicinal water concentrates for at least 2-2.5 hours, following a 30 second irradiation.

In contrast, with the Gyopárosfürdő sample the positive effect was verifiable after a shorter (half an hour) incubation period as well.

As a last step, we investigated if the medicinal water concentrate and the length of the incubation time, or the two (factors) jointly play a role in the development of protective effect. It could clearly be confirmed that incubation time itself is not sufficient for the DNA-repair mechanism.

5. DISCUSSION

Spa bathing and balneology form an integral part of Hungarian history and culture. We consider it necessary to corroborate the importance of the waters and muds applied by appropriate, accurate scientific data. Only few countries in the world possess such an amount of mineral-medicinal water and peloid as Hungary and the Carpathian Basin. During our work a few possible biological effects were investigated using different endpoints. Primarily various toxicity and mutagenicity test were done, by the modification of which photobiological importance of the medicinal water concentrates was also examined, using endpoints. Our investigations were complemented with microbiological tests as well.

In our hygienic microbiological investigations the total plate count, total coliform, Enterococcus and Clostridium count were determined.

The total plate count was much higher in the Hévíz mud than in Kolop peloid, moreover the presence of Clostridium could be confirmed in the Hévíz peloid. The substantial divergence between the two muds can be derived from the different amount of organic matter. The organic matter content of Hévíz peloid is much more considerable than that of Kolop. This high level of nutrient content provides optimal ideal circumstances for the pullulation of saprophytic microorganisms (fungi and bacteria). In the case of both muds, total plate count exceeded the thresholds for drinking water and mineral waters prescribed by law.

The presence of coliform and enterococcus bacteria could not be shown in either mud, which implies that no faecal contamination affected the peloids. In the case of Hévíz peloid the circumstances of mud formation explain Clostridium presence. The mud contains a substantial amount of organic matter (peat), which develops in bogs, in an environment that favors the reproduction of Clostridium species.

In the European Union there are no uniform rules for the microbiological specifications of peloids. In Dinberger's work proposes to use the regulations for the microbiological purity of cosmetic products for peloids as well. Thus, total plate count in peloids is determined as a maximum of 1000 colonies per gram. In our study this criterion was fully met only in the Kolop peloid and not in the Hévíz sample.

The results of the investigations raise the question whether peloids are safe for domestic use. Therefore, we consider it important to develop rules, regulations and testing methods that clearly specify the microorganisms to be tested and control home mud treatment.

Thereafter, we applied ecotoxicological and genotoxicological tests to investigate different peloid and medicinal water samples in *in vivo* and *in vitro* studies.

First the white mustard seedling growth test was carried out, in which the samples were tested as both mud extract and full peloid. These tests produced significant differences only with the Kolop sample. Our next ecotoxicological study was the Eisenia test. These investigations produced significant differences in the case of the Kolop peloid again. The reproductive capacity of the earthworms was significantly lower compared with the control.

The difference observed with the Kolop peloid can not simply be explained by nutrient deficiency, since germination and root growth do not require external nutrients. Moreover, the difference found in the reproduction of the *Eisenia* worms is not caused by low organic content either, as it would have resulted in a substantial divergence in body mass as well. Thus, from the results it can be concluded that the Kolop peloid may include compounds which can adversely affect certain life processes of living beings.

Thereafter, the muds were classified genotoxicologically, based on the tests performed on rat and human lymphocytes and *Eisenia* coelomocytes. Hévíz mud caused significantly higher DNA damage than the negative control both in the human lymphocytes and the coelomocytes. Of course, it is not possible to draw a parallel between the results of the two species, as annelids were exposed for longer (3 weeks) and their whole body was in contact with the components of the mud.

The difference between the three species can naturally be explained by the dissimilarities in genetic backgrounds as well as in the quality and speed of DNA repair mechanisms.

In our further studies the genotoxic and protective effects of medicinal water extracts were tested (Ames test, comet assay). In the Ames test, the mutagenic and protective effect of Kakasszék medicinal water was studied with and without preincubation (8 hours), with and without metabolic enzymes on two *Salmonella* strains (TA98 and TA100).

Based on the results it can be said that the Kakasszék sample does not or only includes a small amount active ingredients which would facilitate the survival of bacteria. Neither does it comprise components that would prove mutagenic in the *Salmonella* test.

In later studies, the effects of two organic-rich medicinal water concentrates (Kakasszék and Gyopárosfürdő) were tested on keratinocytes (HaCat) by comet assay.

First the UV sensitivity of cells was determined using different exposure times. Our results clearly proved the assumption that UV radiation time and the extent of DNA damage are directly proportional.

Afterwards, the genotoxic effects of the waters were examined, using exposure and incubation times of different length.

While concerning the genotoxic effects of Kakasszék medicinal water concentrate conflicting results were obtained, with the Gyopárosfürdő sample the genotoxic effects on keratinocytes can clearly be excluded.

In the end the potential role of waters (Kakasszék and Gyopárosfürdő) in protection from UV radiation was tested. In the case of the Kakasszék sample it was observed that, using incubations of sufficient length (2-2,5 hours), the concentrate facilitates the function of repair mechanisms. The study of Gyopárosfürdő water gave similar results, with even a shorter incubation time being effective.

These results did not clearly show whether the concentrate and/or the incubation time plays key role in the function of repair mechanisms, so we also examined this issue. By this study, it could be proved that the presence of water concentrates is more essential to the repair of DNA damage than incubation time.

By the study the occurrence of compounds with an important role in the photobiological activity of waters and in protection against UV exposure could indirectly be confirmed.

There are no data in either international, or national literature as to similar experiments having been performed. Only one study deals with the effects of UV radiation and peloids on the human skin. In the present study it was established that treatment and the structural transformation of the skin generates and affects the apoptotic processes of epidermal cells. Furthermore, it increases the melanin content of the cells, which has a role in the neutralization of free radicals.

It would be necessary to clarify the presence of organic substances in waters and muds exactly, primarily by using analytical test methods and then examine both their toxicological and protective roles respectively. In medicinal thermal waters and peloids, these investigations could confirm the presence of compounds that enhance the efficiency of both therapy and balneoprevention. Constituents with positive effects, in turn, could serve as ingredients for remarkable therapeutic and preventive products (creams and solutions).

By means of our study a considerable amount of data was collected using different biological endpoints.

These results and data are necessary for balneology to become a more exact, evidence based area of science.

6. SUMMARY OF NOVEL FINDINGS

Hygienic microbiological, ecotoxicological and genotoxicological investigations of peloids:

Total plate count was found considerable in both muds.

The presence of faecal pollutants could not be confirmed from either peloid.

In the case of the Hévíz sample substantial *Clostridium* presence could be shown.

Acute toxic effect was excluded in both muds, furthermore Kolop mud proved to inhibit root elongation and earthworm reproduction.

The genotoxic effects of Kolop mud on *Eisenia* coelomocytes and human lymphocytes were verified by comet assay.

A new model was developed for the genotoxicological study of peloids with lymphocytes.

Genotoxicological study of medicinal water concentrates and their potential role in protection against UV irradiation:

In Salmonella Ames test the mutagenic effect of the Kakasszék medicinal water concentrate was excluded and no protective effect against UV exposure was found.

In the case of the Kakasszék concentrate the possibility of genotoxic effects could not clearly be ruled out. In contrast, protective effect was confirmed to develop on human cells after a sufficient incubation time.

With Gyopárosfürdő medicinal water concentrate, the potential DNA damaging effects were clearly excluded. Moreover, the applied water concentrate was confirmed to facilitate the function of repair mechanisms, with a sufficient incubation period.

It was proved that without concentrates the applied incubation times alone are not enough to repair strand breaks occurring in DNA.

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