In vitro and in vivo identification of ‘pseudocatalase’ activity in Dead Sea water using Fourier transform Raman spectroscopy

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Balneotherapy with Dead Sea water has been reported as a successful treatment modality for psoriasis, atopic eczema and vitiligo, but the precise mode of action has escaped definition so far. The saturating salt concentration (346 g/litre) together with the unique UV spectrum have been suggested to trigger the release of pro-inflammatory and chemotactic mediators. The results of our study show for the first time a high content of transition metal ions (manganese, iron and copper) in Dead Sea water. Using in vitro Fourier transform (FT) Raman spectroscopy, we were able to identify ‘pseudocatalase’ activity by observing the decomposition of hydrogen peroxide (H2O2) over time by Dead Sea water. Since patients with vitiligo accumulate millimolar levels of H2O2 in their skin, we followed the degradation of H2O2 in vivo again utilizing the same technique. The results of this in vitro and in vivo study show for the first time a ‘pseudocatalase’ activity of Dead Sea water and provide evidence that the antioxidant properties of Dead Sea water bathing could play an important role in this unique treatment modality. Furthermore, the use of non-invasive in vivo FT-Raman spectroscopy introduces an excellent biomedical application in investigative dermatology. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Vitiligo is an acquired depigmentation disorder affecting 0.5–4% of the world population.1 Despite its early recognition, the aetiology is still unclear. Several hypotheses have been proposed, but none of them can satisfy the entire spectrum of this cosmetically disfiguring disorder.1,2 Nowadays there is convincing evidence for the presence of oxidative stress in the skin of patients with this disease.3–12 Extremely high concentrations of epidermal H2O2 together with low catalase levels have been identified in vitro and in vivo in this disorder.6,9,10,12,13 However, the missing catalase activities can be substituted with a low-dose narrowband UVB-activated pseudocatalase (PC-KUS) which consequently removes the high H2O2 levels from the skin of these patients, leading to initiation of repigmentation and to recovery from vacuolation in the epidermal cells.6,9–13

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The time course for repigmentation varies considerably, but repigmentation can even be achieved in long-term vitiligo.

The combination of solar radiation and sea water bathing at the Dead Sea is another successful treatment modality for various skin diseases, such as psoriasis, atopic eczema and also vitiligo.14–18 It is generally accepted that the high salt concentration (346 g/litre) contributes to the efficacy of the therapy by several mechanisms, such as the release of pro-inflammatory and chemotactic mediators.14–18 Recently the effect of high magnesium chloride (MgCl2) concentrations found in this water has been further elucidated.19 The authors were able to show that MgCl2 dramatically inhibits the antigen presenting capacity of Langerhans cells. Furthermore, the solar UV spectrum together with sea water bathing in the Dead Sea clearly contribute to the therapeutic response. In this context, an increased photosensitivity after salt bathing has been implicated.18,20–22 Solar radiation at the Dead Sea is filtered by an additional 400 m below sea level. Studies on UV intensities at the Dead Sea area showed that 9.4% of UVB and 3.5% of UVA are filtered out, yielding a unique environment in this region.21,22 The safety aspects of solar phototherapy have been investigated and discussed extensively.21,22 Based
on these studies, it has been suggested that the UVB range at
the Dead Sea could be a natural narrowband UVB therapy. The
Dead Sea water had been analysed earlier, but to the
best of our knowledge there are no data available on
soluble transition metal ions with antioxidant properties.\textsuperscript{14,23}
Therefore, the aim of this study was to explore in \textit{vitro} and
in \textit{vivo} whether Dead Sea water could potentially degrade
\( \text{H}_2\text{O}_2 \). The use of transition metals such as copper, iron and
manganese as catalysts has been reported earlier.\textsuperscript{12,13,24,25}

For this purpose, we utilized atomic absorption
spectroscopy for transition metal ion determinations and Fourier
transform (FT) Raman spectroscopy to follow the fate of
\( \text{H}_2\text{O}_2 \) in \textit{vitro} in solution and in \textit{vivo} in the epidermis of
patients with vitiligo and in healthy controls.

**EXPERIMENTAL**

**Atomic absorption spectroscopy**

Total transition metal determinations were carried out using
a Perkin-Elmer Model 1100 atomic absorption spectrometer.
Samples of Dead Sea water were diluted 1:20 with deionized,
distilled water and made up to 1 litre.

**FT-Raman spectroscopic analysis of \( \text{H}_2\text{O}_2 \)**

FT-Raman spectra were produced with a Bruker RFS
100/S spectrometer equipped with a liquid nitrogen-cooled
germanium detector. Sample excitation was accomplished
using an Nd:YAG laser operating at 1064 nm with a laser
power of 400 mW. Each spectrum was accumulated over
5 min with 300 scans and a resolution of 4 cm\(^{-1}\). Total \( \text{H}_2\text{O}_2 \)
was visualized as a well defined peak at 875 cm\(^{-1}\) based on
the oxygen–oxygen (O–O) stretch vibration.\textsuperscript{9,10} The results
are presented in arbitrary units. This method is sensitive for
concentrations down to the millimolar range.\textsuperscript{10}

**Evaluation of \textit{in vivo} and \textit{in vitro} FT-Raman spectra**

In order to evaluate individual components of the FT-
Raman spectrum, curve fitting analysis was utilized. The
Opus suite of programs provided with RFS 100/S was used
to effect the curve analysis with individual components
being fitted at a mixture of Lorentzian and Gaussian curve
profiles. The special region studied was fitted as close as
possible before computer iteration was employed producing
the final adjustments. Before curve fitting, in \textit{vivo} spectra
were normalized using the CH\(_2\) scissor mode at 2934 cm\(^{-1}\)
because it is insensitive to environmental changes in the
skin. It has the most consistent spectral intensity across
differing spectra of the same region. Owing to the variability
of the spectra from different people (i.e. relative amounts of
lipids, proteins, pigments, levels, etc.), only paired spectra
were used for direct comparison. The curve fitting approach
for evaluation of FT-Raman spectra of the skin was first
employed by Edwards \textit{et al.}\textsuperscript{24} in 1995 and since then has
been used in model stratum corneum lipid systems and also

![Figure 1](image.png)

\textbf{Figure 1}. Comparative \textit{in vitro} FT-Raman analysis of the efficacy of Dead Sea water and pseudocatalase (PC-KUS) on \( \text{H}_2\text{O}_2 \).

(A) Removal of \( \text{H}_2\text{O}_2 \) by Dead sea water (dashed line) after 24 h standardized to a distilled water control (solid line) \( \text{H}_2\text{O}_2 \)
concentration 0.13 m. (B) Rapid removal of a 30 times higher \( \text{H}_2\text{O}_2 \) concentration by pseudocatalase (PC-KUS). Note the complete
turnover after only 6 min. \( \text{H}_2\text{O}_2 \) concentration 4 m.
most recently for in vivo measurements of the depigmentation disorder vitiligo.\textsuperscript{10}

In vitro FT-Raman spectra were evaluated using the same principle with the exception that the spectra did not need to be paired.

**FT-Raman protocol to follow the fate of H$_2$O$_2$ in solution in vitro**

H$_2$O$_2$ degradation was studied over time in the presence of Dead Sea water and pseudocatalase, PC-KUS (Patent EPO 584171A), following the reduction of the O–O stretch peak of H$_2$O$_2$ at 875 cm$^{-1}$.\textsuperscript{10} It has been shown earlier that the detection limit of H$_2$O$_2$ was in the millimolar range.\textsuperscript{10}

**Patients**

Twenty-two English and German patients with vitiligo vulgaris (eight males and 14 females) with a mean age of 38.6 years (range 18–62 years) from the Institute for Pigmentary Disorders in Greifswald, Germany, were included in this study. The patients had photo skin type III, Fitzpatrick classification.\textsuperscript{26}

The onset of the disease in this group was at a mean age of 23.5 years (range 1–46 years). The duration of the disease process varied from 3 to 53 years with a mean age of 16.7 years.

**Clinical assessment by standardized photography**

For a valid clinical assessment of the repigmentation of the face, we utilized black and white photographs using a fixed frame assembly holding the camera (Pentax MZ-M with a 90 mm lens and an Ilford 828 glass filter) and a Kodak T-MAX 400 film. A flashlight with non-UV coated bulbs was also fixed (Bowens Esprit 500). This standardized technique ensures a good quality comparison of subsequent photographs and allows even in very fair skin the objective evaluation of affected areas.

**FT-Raman protocol for epidermal H$_2$O$_2$ removal in vivo in the skin of patients with vitiligo compared with healthy controls**

All in vivo measurements were taken at the same skin area of the distal inner right or left forearm.

Twenty-two patients were tested in vivo before treatment for epidermal H$_2$O$_2$ concentrations using FT-Raman spectroscopy. The results were compared with 15 healthy photo skin type matched controls (Fitzpatrick classification).\textsuperscript{26}

To test the effect of Dead Sea water alone on epidermal H$_2$O$_2$ concentrations, eight untreated patients were examined after 15 min of bathing and compared with the effect of a topical pseudocatalase cream ($n = 14$). In order to activate the manganese catalyst, the patients were exposed to the sun for 10 min either after the Dead Sea

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**Figure 2.** in vivo FT-Raman spectrum of the skin from a patient with vitiligo. (A) Full spectrum (range of wavenumbers from −1500 to 3500 cm$^{-1}$). (B) Normalized spectrum (range of wavenumbers from 750 to 1025 cm$^{-1}$). H$_2$O$_2$ at 875 cm$^{-1}$; phenylalanine at 1004 cm$^{-1}$.
bath only or after the application of pseudocatalase (PC-KUS). Written consent was obtained from each patient. This study was approved by the local Ethics Committees. The study was carried out at the DMZ-Mor Clinic in Ein Bokek, Israel, and at the University of Bradford, UK.

**Statistical analysis**

Statistical analysis was based on the Wilcoxon signed rank test for paired samples. Values $p < 0.05$ were significant.

**RESULTS AND DISCUSSION**

**Atomic absorption analysis of Dead Sea water identifies potential ‘pseudocatalase’ activity**

Soluble transition metal ions could potentially act together with hydrogen carbonate as pseudocatalases.\(^{13,25,27,28}\) Therefore, atomic absorption spectroscopy was utilized for detailed analysis of the Dead Sea water yielding the presence of manganese, iron and copper ions. The concentrations of these transition metal ions are presented in Table 1. These data suggested potential ‘pseudocatalase’ activity of the Dead Sea water.

**Table 1. Concentrations of transition metals in Dead Sea water**

<table>
<thead>
<tr>
<th>Transition metal</th>
<th>Concentration/ ppm</th>
<th>Concentration/ $10^{-6}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manganese (Mn$^{2+}$)</td>
<td>7140</td>
<td>13.0</td>
</tr>
<tr>
<td>Iron (Fe$^{2+}$)</td>
<td>1398</td>
<td>2.5</td>
</tr>
<tr>
<td>Copper (Cu$^{2+}$)</td>
<td>582</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Figure 3.** *in vivo* FT-Raman analysis of epidermal H$_2$O$_2$ in vitiligo. Solid line, before treatment; dashed line, after treatment with Dead Sea bathing for 15 min; dotted line, after treatment with pseudocatalase (PC-KUS).

**Dead Sea water removes millimolar concentrations of H$_2$O$_2$ in vitro**

In order to test this hypothesis, we utilized *in vitro* Raman spectroscopy to follow the degradation of H$_2$O$_2$ at 875 cm$^{-1}$.\(^{9,10}\) The *in vitro* result confirmed ‘pseudocatalase’ activity of the Dead Sea water. However, a comparative study of the rates of H$_2$O$_2$ removal by Dead Sea water and pseudocatalase (PC-KUS) showed a significantly slower turnover by Dead Sea water [Fig. 1(A) and (B)].

**Figure 4.** Comparative *in vivo* study of epidermal H$_2$O$_2$ removal in vitiligo ($n = 22$). (A) Percentage H$_2$O$_2$ before and after treatment with pseudocatalase (PC-KUS) ($n = 14$) compared with healthy controls ($n = 15$). (B) Percentage H$_2$O$_2$ before and after Dead Sea bathing for 15 min ($n = 8$) compared with healthy controls ($n = 15$). No epidermal H$_2$O$_2$ is detectable in the healthy controls.
distilled nor deionized water showed any ‘pseudocatalase’ activity on \( \text{H}_2\text{O}_2 \) removal. The results are shown in Fig. 1(A).

**Presence of millimolar concentrations of \( \text{H}_2\text{O}_2 \) in vivo in the epidermis of patients with vitiligo and its removal by Dead Sea water**

In order to substantiate ‘pseudocatalase’ activity of Dead Sea water in vivo, we first confirmed millimolar levels of epidermal \( \text{H}_2\text{O}_2 \) in untreated patients with vitiligo using FT-Raman spectroscopy.\(^9,10,12\) One example of a full and a normalized in vivo FT-Raman spectrum of the skin of one untreated patient with vitiligo is shown in Fig. 2(A) and (B). Next we tested in vivo the effect of \( \text{H}_2\text{O}_2 \) removal by Dead Sea water on the skin of patients with vitiligo (\( n = 8 \)) after 15 min of bathing in the Dead Sea and compared this efficacy with pseudocatalase (PC-KUS) (\( n = 14 \)). Since the capacity of \( \text{H}_2\text{O}_2 \) removal with pseudocatalase (PC-KUS) has been established previously, we used this formulation as an internal standard.\(^9,10,12\) One example of an in vivo FT-Raman spectrum for the effect of Dead Sea water and pseudocatalase (PC-KUS) on epidermal \( \text{H}_2\text{O}_2 \) is shown in Fig. 3.

Figure 4(A) and (B) demonstrate the percentage decrease of \( \text{H}_2\text{O}_2 \) after Dead Sea water and pseudocatalase (PC-KUS) for the entire patient group. These results confirm in vivo that Dead Sea water removes \( \text{H}_2\text{O}_2 \) from the skin of patients with vitiligo, although a comparison of the \( \text{H}_2\text{O}_2 \)-removing

![Figure 5](image_url)

**Figure 5.** Rapid initiation of repigmentation in vitiligo after removal of epidermal \( \text{H}_2\text{O}_2 \). (A) Time course of repigmentation in facial vitiligo using combined climatotherapy at the Dead Sea and pseudocatalase (PC-KUS) over 21 days. Duration of the disease, 20 years. (B) The same patient on day 47 after continuation of the treatment with narrowband UVB-activated pseudocatalase (PC-KUS) only.
capacity of the Dead Sea water and pseudocatalase cream alone demonstrates a significantly higher activity with pseudocatalase cream ($p < 0.0001 \pm $ SEM).

The positive therapeutic effect of the climate in the Dead Sea basin has been well established in the treatment of psoriasis, atopic eczema and vitiligo. The treatment protocol for these diseases usually lasts 28–42 consecutive days and the response rates are reported as high as 80%. The high mineral content of the Dead Sea has been considered a major factor in this successful treatment modality. Based on a detailed water analysis using atomic absorption spectroscopy, the study presented here suggested that the Dead Sea could have a potential 'pseudocatalase' activity owing to the presence of the transition metal ions manganese, iron and copper together with hydrogen carbonate. These inorganic complexes can undergo the catalase reaction. Since these complexes are not protein based, they are defined as pseudocatalases. In this context, it is noteworthy that manganese hydrogen carbonate and also iron and copper hydrogen carbonate effectively disproportionate $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$. A detailed kinetic analysis of $\text{H}_2\text{O}_2$ removal by manganese hydrogen carbonate established the precise mechanism for this catalase activity.

Here it is also noteworthy that microorganisms possess catalases with bis-manganese active sites which are more effective than the haem-containing enzyme in higher organisms. Based on this understanding, we have previously developed a pseudocatalase (PC-KUS) which is a bis-manganese-EDTA complex with hydrogen carbonate bridging ligands between the two manganese centres. This complex, at a concentration of $12 \times 10^{-6} \text{ M}$, catalyses $\text{H}_2\text{O}_2$ removal in the millimolar range. It is interesting that the manganese ion content of the Dead Sea water is $13 \times 10^{-6} \text{ M}$. However, despite the same concentration, the results of this study showed in vitro and in vivo a much slower 'pseudocatalase' activity compared with pseudocatalase (PC-KUS) (Figs 3 and 4). This discrepancy can be explained best by the more active bis-manganese-EDTA complex compared with manganese hydrogen carbonate in Dead Sea water. Furthermore, the incorporation of the bis-manganese complex into a base cream allows a more efficient and prolonged contact and delivery into the skin [Figs 3 and 4(A)]. The low 'pseudocatalase' activity of the Dead Sea water in vitro compared with the in vivo results supports complexation of the manganese ions with biological ligands in the skin, yielding higher 'pseudocatalase' activity than the inorganic complex alone.

Epidermal $\text{H}_2\text{O}_2$ stress has been established in vivo in patients with vitiligo. The effective removal of the millimolar levels of $\text{H}_2\text{O}_2$ from patients' skin can be achieved with a pseudocatalase (PC-KUS) which is incorporated into a cream base. Consequently, this patient group represented an ideal model to elucidate a potential antioxidant mechanism in the Dead Sea water. We have shown that the removal of epidermal $\text{H}_2\text{O}_2$ in patients with vitiligo coincides with successful repigmentation even in long-standing disease. One example of sequential facial repigmentation at the Dead Sea is shown in Fig. 5(A). The same patient is shown 28 days later after continuation of the treatment with narrowband UVB activated pseudocatalase (PC-KUS) [Fig. 5(B)].

**CONCLUSION**

This in vivo study on the skin of patients with vitiligo and healthy controls for the identification of epidermal $\text{H}_2\text{O}_2$ using non-invasive FT-Raman spectroscopy has led to new insight into the physiology and pathophysiology of the skin and underlines the importance of this technique for further investigations in the field of dermatology.

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