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Q10 and Xenobiotics Induce Nitration of Dicarbonyl –Xylulose Reductase (DCXR) via H_2O_2/ONOO⁻ in Human Skin

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ABSTRACT

Till today, there is a clear evidence for massive H_2O_2/ONOO⁻ - mediated stress in the epidermal compartment of vitiligo. It has been demonstrated that this accumulation originates from numerous cellular (endogenous) and environmental sources. There are convincing results that these reactive species induce oxidation of many amino acids of various proteins/peptides which lead to structural modification, that can be a reason for partial or complete loss of functionality. It was shown that hydroquinone (HQ) has been utilised in topical preparations by Dermatologists in skin lightening and depigmentation of widespread vitiligo treatment. It was decided to investigate whether these patients exhibit quinone reduction via dicarbonyl xylulose reductase (DCXR) as this enzyme reduces quinones and xenobiotics. The results show that DCXR is markedly decreased and affected by H_2O_2 and ONOO⁻. The results were demonstrated by utilising *in situ* immunofluorescence labelling, Western blot, and FT- Raman spectroscopy.

INTRODUCTION

Vitiligo disease is a disorder affecting 0.6-2.28% of the whole world population (Krüger and Schallreuter, 2012). The cause of the disease is still not discovered; however, there is a confirmed *in vivo* and *in vitro* evidence for oxidative/nitrative stress throughout the epidermal compartment as well as in the system of the vitiligo patients (Schallreuter *et al*., 2008a; Salem *et al*., 2009; Schallreuter *et al*., 2012a/b). Many years ago, it was shown by Anderson *et al*., (2003) as well as Schallreuter *et al*., (2006a) using Comet assay that semiquinone radicals mediate damage in blood lymphocytes. Hydroquinone (HQ) has been used for 50 years in topical preparations by Dermatologists for the treatment of skin lightening and for depigmentation of widespread vitiligo (Arndt and Fitzpatrick, 1965). 2% HQ that represents a concentration of $182×10^{-3}$M has the ability to generate H_2O_2 and the para-quinone, 1,4-benzoquinone in the epidermis. Moreover, several publication demonstrated that 45% of HQ metabolites were detected in the urine even 24 hours following topical application (Wester *et al*., 1998; Foppoli *et al*., 2005). Because of expected side effects of HQ, the regulatory agencies in the USA reduced its concentration in creams to 2% (Bentley-Phillips and Bayles, 1975; FDA, 1982). Interestingly, in Europe, this compound was officially prohibited in skin creams since 2001 (EC, 2000). Moreover, in the USA, this ban has been applied later (FDA, 2006). Coenzyme Q10-quinol is a natural analogue of HQ.

This enzyme acts as electron transfer cofactor in the respiratory electron transport chain in the mitochondria. Nearly 2% of these electrons migrate from Coenzyme Q10-quinol and bind with oxygen to produce superoxide anion radical ($O2^-$) which converts to H_2O_2 spontaneously or catalysed by mitochondrial Mn-superoxide-dismutase (Nordberg and Arner, 2001). HQ is oxidised to its semiquinone radical then oxidation to 1, 4-benzoquinone. Coenzyme Q10 is oxidised to Coenzyme Q10-quinol and semiquinone radical followed by a second oxidation step giving Coenzyme Q10-quinone with H_2O_2 as a by-product (Ernster and Dallner, 1995; Wester *et al.*, 1998; Foppoli *et al.*, 2005; Schallreuter *et al.*, 2008b) (Scheme 1).

It was shown that high concentrations (3-30 × 10^{-3}M) of this cofactor, as in the current cosmetic creams , (Boyle and Kennedy, 1986; Bucks *et al.*, 1988) produce both quinones and H_2O_2, leading to oxidative stress in susceptible individuals especially aged and fair skin as well as in patients susceptible to vitiligo, where the degradation mechanisms are lower or absent (Schriner *et al.*, 2005). This suggestion is supported and backed up by low catalase levels in the mitochondria with increasing age and by H_2O_2-mediated deactivation of epidermal and mitochondrial catalase in vitiligo (Schriner *et al.*, 2005; Dell'Anna and Ottaviani, 2006; Maresca *et al.*, 2006). There is convincing evidence in vitiligo that both $6BH_4$ as well as TR/T are undergone to H_2O_2-mediated oxidation which followed by alteration in its function including reduction of quinone (Rokos *et al.*, 2002).

The main aim of the current study is to provide a novel results on dicarbonyl L-xylolose reductase (DCXR) expression since this enzyme detoxifies xenobiotics including quinones.
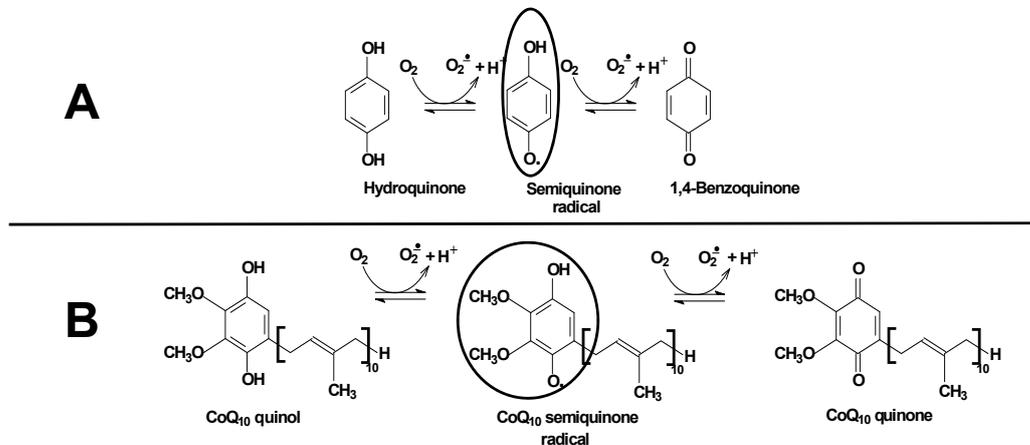
Scheme 1. Reaction pathways of the oxidation of quinols to quinones. A) HQ is oxidised to its semiquinone radical followed by oxidation to 1,4-benzoquinone. During the reaction pathway single electrons escape to produce $O_2^{\bullet-}$ which in turn disproportionates to H_2O_2 plus O_2. B) Coenzyme Q10 is oxidised to Coenzyme Q10-quinol and semiquinone radical followed by a second oxidation step yielding Coenzyme Q10-quinone with H_2O_2 as a byproduct as described in A The Coenzyme Q 10-quinol is a natural analogue of HQ.

Materials and Methods
Human Skin Biopsies

For *in situ* investigation, we used 3mm full skin punch biopsies taken under local anaesthesia after written consent as described in detail (Salem *et al.*, 2009).

For the experiments, we obtained biopsies from 4 healthy control (skin phototype III, Fitzpatrick classification) (Fitzpatrick, 1988) and from 7 untreated patients with vitiligo (lesional and non lesional skin, skin photo type III). Moreover, we included 4 patients

(lesional and non lesional skin, skin photo type III) after reduction of epidermal H_2O_2 with a low dose narrowband UVB activated pseudocatalase PC-KUS (Schallreuter *et al*., 2008b). In addition, we included biopsies from repigmenting skin of 3 patients. All patients and controls had skin photo type III. The study was approved by the local ethics committee in 1999 and was in agreement with the principles of the Helsinki declaration.

In vivo FT-Raman Spectroscopy:

FT-Raman spectra were measured using a Bruker RFS 100/S spectrometer (Bruker Karlsruhe, Germany). All peaks of interest were repeated prior to the scan of the skin with standard solutions to ensure that the assigned wave number was correct. The *in vitro* measurement of H_2O_2 gave a peak at $878cm^{-1}$ reflecting the O-O stretch. The measurement of solid L-phenylalanine (ICN Biomedicals Inc.) gave rise to a strong peak at $1004 cm^{-1}$ which is in agreement with ring breathing for mono-, meta- or 1, 3, 5-substituated benzenes such as phenylalanine (Cartaxo *et al*., 2010). In addition, we controlled the presence of N-formyl kynurenine/kynurenine as indicators for tryptophan oxidation and the formation of methionine sulfoxide (Rokos *et al*., 2008; Schallreuter *et al*., 2012a).

Preparation of Cryosection:

The tissue was embedded in optimal cutting temperature compound (OCT) (Sakura, RA lamb, Eastbourne, UK), snap frozen in liquid nitrogen, and stored at -80˚C until further use. 3-4μm sections were cut using a Lucia CM 1800 or Lucia CM 3050 S cryostat. Slides were stored for further use at -82˚C.

Determination of Protein Concentration:

Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hemel Hempstead, UK) according to the manufacturer's protocol.

In situ Immune Fluorescence Studies:

For immuno-reactivity studies, the experiment was performed as described in details (Salem *et al*., 2009). The primary polyclonal rabbit DCXR antibody (dilution 1:50, ab82303, abcam, Cambridge, UK),

mouse monoclonal anti-NKIbeteb (dilution 1:50, Biosciences Cambridge, UK), and mouse monoclonal anti-3-nitro-tyrosine (clone 1A6, dilution 1:25, Upstate, Dorset, UK) were used.

Quantification and Statistical Analysis of Immune Fluorescence Data:

Quantification was calculated by using WCIF image J (NIH) supplied on line by (http://rsb.info.nih.gov/ij/index.html) as described in (Salem *et al*., 2009) The mean of the calculated values were used for documentation ± SD.
*** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns not significant $p>0.05$.

SDS-Page and Western Blot Analysis :

Utilising the standard method for SDS-PAGE and Western blotting, polyvinylidene difluoride membrane as described in details (Salem *et al*., 2009). The primary antibody DCXR, rabbit polyclonal antibody (ab82303, abcam, Cambridge, UK, dilution of 1:3000 in 2.5% milk) and GAPDH as a marker for equal protein loading. The secondary rabbit anti-rabbit IgG antibody at a 1:1000 dilution (DakoCytomation, Glostrup, Denmark).

Quantification and Statistical Analysis of Western Blot Data:

Quantification was performed by using Image J-version supplied by NIH on line, (http://rsb.info.nih.gov/ij/index.html).

Results

In vivo FT-Raman spectroscopy confirms the presence of epidermal H_2O_2 in association with 5-OH-tryptophan (5-OH-trp), methionine sulfoxide (Met-S=O), and N-formyl kynurenine/kynurenine (NFK/K) in facial vitiligo after the use of Q10 containing cream :

The *in vivo* spectra of lesional skin revealed in 2 patients a peak for H_2O_2 in their facial vitiligo. Figure 1 presents the spectrum. Given that FT-Raman analyses only detects concentrations in the $10^{-3}M$ range, we can conclude that Q10 triggered depigmentation is true vitiligo. To the best of our knowledge, these *in vivo* data prove for the first time the presence of this ROS in association with Q10 containing cream.
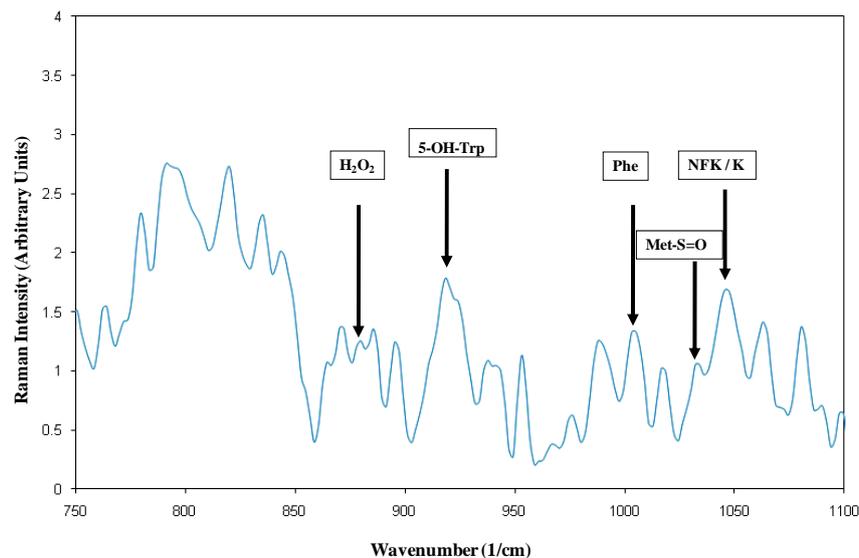
Fig. 1. *In vivo* **FT-Raman spectrum from facial Q10 triggered depigmentation.** *In vivo* FT-Raman spectroscopy confirms accumulation of H_2O_2 and its oxidation products in mM range represented by peaks for H2O2 at 877cm-1, phenylanine at 1003 cm-1, methionine sulfoxide (Met-S=O), OH-Tryptophane (5-OH-Trp), N-formyl kynurenine / kynurenine (NFK / K).

Significantly Reduced *in situ* Expression of DCXR in Vitiligo :

Epidermal DCXR protein expression is seen throughout all epidermis. The enzyme shows strong granular distribution in the cell membrane and some cytosol. DCXR expression is significantly ($p<0.005$) lower in both non-lesional and lesional skin of untreated patients (n=7), compared to healthy controls (n=5) (Figures 2(a) & 2(b)). After reduction of epidermal H_2O_2 levels with low dose narrowband of UVB (NB-UVB) (311nm) activated propseudocatalase PC-KUS (Schallreuter *et al.*,2008b), protein expression increases, but it still is significantly lower in both non-lesional and lesional skin giving approximately 80% expression in repigmenting skin (n=3) compared to healthy control skin (Figures 2(a) & 2(b)).

Western Blot Confirms Decreased Expression of DCXR in Vitiligo Lesional and Non Lesional Skin:

The results confirm significantly reduced DCXR expression in both non-lesional and lesional skin compared to healthy normal controls (Figures 2(c) & 2(d)).

Clearly, from these data it appears that epidermal DCXR protein levels are severely affected by H_2O_2 in the skin of these patients. We see significantly lower expression in both non-lesional and lesional and even in repigmented skin.

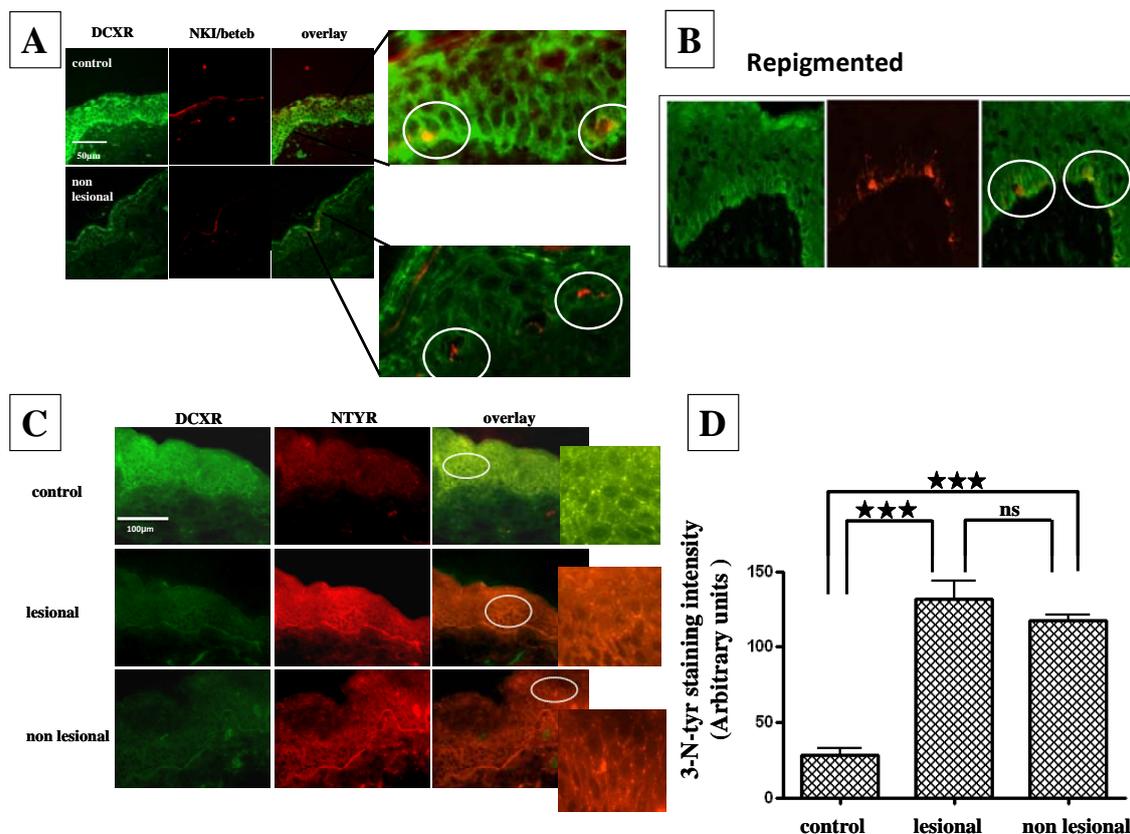**Normal Human Epidermal Melanocytes and Keratinocytes Express DCXR *in situ* :**

For this purpose, we used double immuno-fluorescence labelling with NKI-beteb and DCXR. The results show the presence of the enzyme in melanocytes of healthy control skin (Figure 3(a)), while it is absent in melanocytes of non lesional skin of vitiligo (Figure 3(a)). However, in repigmented skin we can see a weak yellow colour after overlay, indicating that there is a little increase in the protein expression in melanocytes but it does not return to normal levels (Figure 3(b)).

***In situ* Evidence for Nitrated DCXR in Vitiligo**:

As ONOO$^-$ is present in active vitiligo (Salem *et al.,* 2009). Significantly, our results show higher 3-N-tyrosine (NTYR) expression in vitiligo skin which confirms the presence of ONOO$^-$ *in situ* (Figures 3(c) & 3(d)).
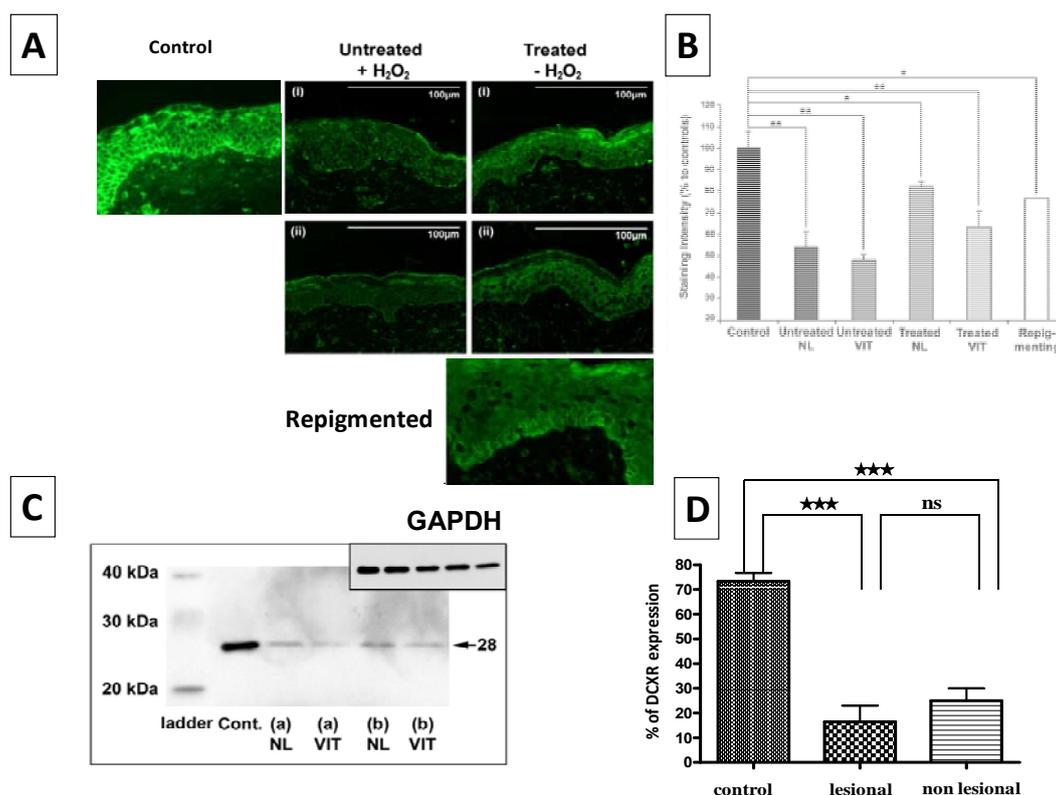
Fig. 2. Significantly reduced epidermal DCXR protein expression in vitiligo. A) Epidermal DCXR immunofluorescence labelling in healthy control skin and of patients with vitiligo non-lesional (i) and lesional (ii) skin of untreated (+ H$_2$O$_2$), after treatment with NB-UVB activated pro-pseudocatalase PC-KUS (-H$_2$O$_2$) (Schallreuter *et al.*, 2008b; Salem *et al.*, 2009). N.B. The enzyme is expressed throughout the entire epidermis, supporting the involvement of keratinocytes and melanocytes in vitiligo. Pictures were taken at a magnification x400. **B)** Image analysis of the staining intensity for DCXR (arbitary units) reveals a significant reduction in the epidermal expression in patients with vitiligo compared to healthy controls. Data are based on *in situ* immuno fluorescence staining in healthy controls (n=4) compared to the non-lesional (NL) and lesional (VIT) skin of patients with progressive (untreated, n=7) and stable (treated, n=4) and repigmenting vitiligo (n=3) vitiligo. * p<0.025, ** p<0.005 (students't-test). **C)** Western blot analysis shows reduced expression of epidermal DCXR in non-lesional (NL) and lesional (VIT) cell extracts from suction blister tissue in patients with vitiligo compared to healthy controls (Cont), the predicted size of 28kDa. Equal protein loading is confirmed via GAPDH (insert), n=2 controls and 3 patients with vitiligo. **D)** Image analysis confirms significantly decreased enzyme expression.

Fig. 3. Absent *in situ* DCXR expression in epidermal melanocytes of non lesional skin corresponds with low enzyme expression in epidermal keratinocytes. A) *In situ* expression of FITC-labelled DCXR (green) in epidermal melanocytes with TRITC-labelled NKIbeteb (red) indicates the presence of DCXR in the overlay by a strong yellow colour in healthy control skin which is absent in non lesional melanocytes in vitiligo epidermis (magnification 400x). **B)** Epidermal melanocytes regain some enzyme protein expression in repigmenting skin as shown in yellow after overlay. (FITC-labelled DCXR (green) and the TRITC- labeled melanocyte specific protein NKI beteb (red)). Pictures were taken at a magnification x400. **C)** *In situ* expression of ONOO⁻ in lesional (n=4) and non lesional skin (n=4) as shown by TRITC – labelled 3-nitro-tyrosine (red) is significantly higher compared to control skin (n=2). The high ONOO⁻ expression correlates with low FITC – labelled DCXR expression (green). The overlay of both chromophores is not ideal due to the presence of high and very low expression of high ONNO⁻ and very low DCXR expression. Pictures were taken at a magnification x400. **D)** Image analysis of *in situ* 3-nitro tyrosine expression as foot print of ONOO⁻ reveals significantly higher epidermal levels for this RNS in both lesional and non lesional vitiligo.

Discussion

It was documented by many scientists that epidermal and systemic oxidative stress are present in vitiligo (Salem *et al.*, 2009; Schallreuter *et al.*, 2012a/b). Ogawa *et al.*, (2006) and Schallreuter *et al.*, (2006a/b) demonstrated that in normal conditions, the human epidermis exhibits high levels of anti-oxidant enzymes such as catalase, glutathione reductase/glutathione peroxidase, thioredoxin reductase /thioredoxin /thioredoxin peroxidase, and methionine sulfoxide reductases A and B (B1, B2, B3) to keep the redox homeostasis under control. These enzymes balance the production of H$_2$O$_2$/OH$^-$ in response to UVB-irradiation and the production of singlet oxygen/OH$^-$ in response to UVA light in addition to other environmental and endogenous ROS/radicals (Schallreuter and Wood, 2001; Maresca *et al.*, 2006). It was documented that in vitiligo the redox balance is severely compromised mainly due to loss of functionality of catalase, thioredoxin reducatse/thioredoxin, glutathione peroxidase, and the repair enzymes MSRA/B (for review see Schallreuter *et al.*, 2008a; Schallreuter *et al.*, 2012a/b). Production of reactive oxygen species (ROS) by Coenzyme Q10 quinol and HQ has been established previously by several scientific researchers (Shigenaga *et al.*, 1994; Anderson *et al.*, 2003; Schallreuter *et al.*, 2006a). The oxidation of dihydroquinols via semiquinone radical intermediates to quinones involves two single electron transfer reactions to molecular oxygen (O$_2$) producing two superoxide anion radicals (O$_2^{\bullet-}$) which in turn disproportionate to H$_2$O$_2$ and O$_2$ via the superoxide dismutases (Nordberg and Arner, 2001). Consequently, topical applications of formulations containing high concentrations (mM) of coenzyme Q10 quinol and HQ can lead to H$_2$O$_2$ -mediated oxidative stress in the human epidermis (Scheme1). On the other hand, this coenzyme at low concentrations in cell plasma membranes acts as an antioxidant via inhibiting lipid peroxidation (Ernster and Dallner, 1995). This is why it

has been used worldwide as an oral antioxidant supplement. However, oral administration of radio-labelled Coenzyme Q10-quinol showed only very little gastrointestinal absorption (Beyer, 1990), questioning the efficacy of this compound. Topical formulations containing HQ have been utilised in Dermatology skin lightening and for vitiligo treatment for more than 40 years (Arndt and Fitzpatrick, 1965). Despite its short lifetime in the human epidermis, continuous application of this compound leads to loss of melanocytes functionality, hence, skin hypopigmentation and permanent loss of the inherited skin colour (Arndt and Fitzpatrick, 1965). In this research work, we investigated the effect of quinones on epidermal DCXR expression. Our results identified that patients with vitiligo in the presence of massive oxidative/nitrative stress via H$_2$O$_2$ and ONOO$^-$ lose protein expression and enzyme function. This severe redox imbalance is clear upon structural examination of the depigmented epidermis of patients with active disease, with extensive vacuolisation in all epidermal cells (lipid peroxidation) and loss of functioning melanocytes (Tobin *et al.*, 2000). Recently, these findings as well as the white and grey hair in these patients have been reconciled in the light of massive (10^{-3}M) H$_2$O$_2$ accumulation in the epidermis and in the hair of individuals with active disease (Tobin *et al.*, 2000; Salem *et al.*, 2009; Wood *et al.*, 2009; Schallreuter *et al.*, 2012a/b). The removal of H$_2$O$_2$ from the epidermis of these patients with a topical supplementation of a NB-UVB$_{311nm}$ activated pro-pseudocatalase PC-KUS results in correction of the redox balance in the skin of these patients, followed by repigmentation of lesional skin and in some cases even hair colour restore (Schallreuter and Elwary, 2007a; Schallreuter *et al.*, 2008a; Schallreuter *et al.*, 2012a/b; Schallreuter *et al.*, 2013).

The redox balance can be monitored *in vivo* by FT – Raman spectroscopy. Moreover, it is also possible to monitor the systemic redox status of the individual

patients (Hasse *et al.*, 2004; Schallreuter *et al.,* 2006a). It has been shown that di-hydropteridin reductase (DHPR) activity in blood is an excellent marker for systemic H_2O_2-levels (Hasse *et al.*, 2004). Reduction of epidermal H_2O_2 via a topical supplementation of the activated pro-pseudocatalase PC-KUS is associated with normalization of decreased systemic DHPR levels (Hasse *et al.*, 2004). Based on these observations, it can be concluded that the origin of the systemic oxidative stress in these patients originates in the epidermal compartment and it is transferred to the system (Hasse *et al.*, 2004; Schallreuter *et al.*, 2006a). Moreover, the impaired redox-balance is not just affecting one isolated system, it will affect many systems including immune response and its signalling at the same time (Schallreuter *et al.*, 2012a/b). However, the approach to reduce the epidermal ROS/RNS overload in association with stabilising progressive vitiligo and skin repigmentation proves the validity of the scientific data in this disease (Schallreuter *et al.*, 2012a/b) . Taking into consideration that many proteins and peptides, receptors, co-factors etc., can be subject to H_2O_2- mediated oxidation and $ONOO^-$ - mediated nitration. This present work reveal for the first time significantly decreased *in situ* protein expression of DCXR, in vitiligo lesional and non lesional skin compared to age and skin photo type matched healthy control skin (Fitzpatrick, 1988). We demonstrate that the epidermal enzyme is subject to nitration by using 3-nitro tyrosine as the foot print for $ONOO^-$ (Salem *et al.*, 2009).

Taken together, we here identified severe quinone and xenobiotics sensitivity in the epidermal compartment of patients with vitiligo. These data confirm and extend for earlier observation in peripheral lymphocytes of patients with this disease (Ernster and Dallner, 1995; Schallreuter *et al.*, 2006a). Based on these results, it is recommended that this patient group should avoid Q10 containing cosmetics as well as systemic supplementation of Q10.

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