

## Viewpoint 1

The clinical signature of vitiligo is an acquired, non-contagious idiopathic loss of constitutive pigment from the skin which can basically occur on any part of the body at any time in life, affecting both sexes equally (1,2). The worldwide incidence ranges from 0.5% to 1% (1). The cause of this ancient disease is yet unknown. All of the 35–45% of documented cases have other family members with vitiligo (2). The course for the individual patient is unpredictable. Diagnosis is easy for the experienced eye. The classical lesion presents single or multiple well-circumscribed chalk white maculae of different sizes which can occur anywhere on the skin and mucosa. Importantly, vitiligo can mimic other leukodermas. Examination by Wood's light (UV-A 351 nm) provides a useful tool for the correct diagnosis due to a distinct autofluorescence of vitiligo lesions (2).

It is still under debate, why and how the functionality of the entire epidermal unit is affected in this disease. It is also not clear whether melanocytes of other tissues (eyes and ear) are also targeted in this disease.

Several hypotheses have been put forward but none of them can conclusively explain the plethora of clinical and basic scientific data (1–4).

This Viewpoint essay will focus on the role of epidermal  $H_2O_2$  in the pathogenesis of vitiligo.

### Oxidative stress via $H_2O_2$ and peroxynitrite in vitiligo: what are the facts?

Generation of  $H_2O_2$  is a physiological process in all cells due to several metabolic pathways. Figure 1 summarizes the epidermal sources currently identified (2,5). On the one hand,  $H_2O_2$  in  $10^{-6}$  M concentrations is an important signal in control of many processes including transcription, while, on the other hand, the same reactive oxygen species in  $10^{-3}$  M concentrations can have deleterious effects.

Under normal conditions, this redox balance is under fine control via various enzymes including catalase, thioredoxin reductase/thioredoxin, thioredoxin peroxidases, glutathione/glutathione reductase and glutathione peroxidase to provide cellular homeostasis. In the case of short-term  $H_2O_2$  accumulation, methionine residues in protein and peptide structures can be oxidized to methionine sulfoxide which is repaired by methionine sulfoxide reductases A and B (MSRA&B) (5).

To date, there is ample evidence that vitiligo affects the entire epidermis. Convincing data support the participation of keratinocytes and Langerhans cells besides the loss of functioning melanocytes (4,5,6). Various degrees of intra-

**Table 1.** Confirmed sources for epidermal accumulation of  $H_2O_2$  and peroxynitrite in vitiligo

Increased epidermal monoamine oxidase A activities (52)
Increased NADPH oxidase activities from neutrophils and macrophages of the perilesional infiltrate (53)
Increased epidermal TNF- $\alpha$ levels (2,49,50)
Increased photo-oxidation of epidermal 6-biopterin and sepiapterin (55)
Increased epidermal-inducible nitric oxide synthase (2)
Increased peroxynitrite formation ( $NO + O_2^- \rightarrow$ peroxynitrite) (56)
Increased estrogen-/progesterone-mediated $H_2O_2$ generation (56)
Perturbed phenylalanine hydroxylase activity via 7BH <sub>4</sub> due to deactivation of 6BH <sub>4</sub> recycling (23–25)
Epidermal xanthine oxidase activity yields $H_2O_2$ which oxidizes the product uric acid in vitiligo forming allantoin (6)

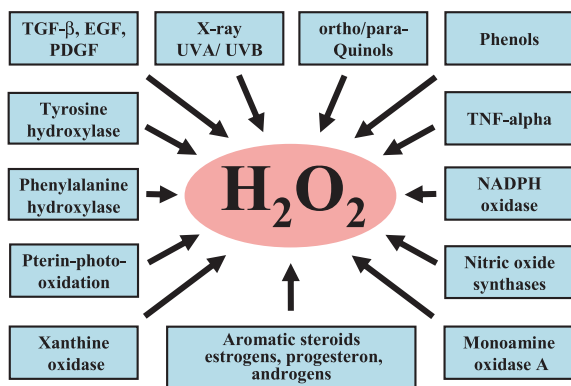
cellular vacuolation and debris in all epidermal cells have been documented (7–9). Recently, the vacuolation was attributed to  $H_2O_2$ -mediated lipid peroxidation (4). Even under *in vitro* conditions, this oxidative stress continues in epidermal melanocytes and keratinocytes established from patients with vitiligo (4,10,11). Very early it was shown that patients with acute vitiligo have low epidermal catalase levels and protein expression in their entire epidermis (12), although the expression of catalase mRNA is unaltered (10,12). This observation formed the basis for a long ongoing research.

Major proof for  $H_2O_2$ -mediated stress in vitiligo was accomplished by measuring epidermal concentrations of  $H_2O_2$  in the  $10^{-3}$  M range *in vivo* as well as its oxidation product, e.g. methionine sulfoxide from methionine, using Fourier Transform Raman spectroscopy (10,14).

The oxidative stress theory via  $H_2O_2$  is further supported by the identification of a number of endogenous sources leading to accumulation of epidermal  $H_2O_2$  in vitiligo (Table 1). Exogenous sources from our daily life including UV and X-rays, butyl and other phenols as well as quinones can also contribute to the pool (Fig. 1). In this context, it was shown that oxidative stress results in a reduced expression of MITF under *in vitro* conditions (15). The main question remains, what are the consequences of mM  $H_2O_2$  concentrations in the epidermis of patients with vitiligo?

### $H_2O_2$ affects the epidermal anti-oxidant defense machinery and its repair mechanisms

- $H_2O_2$  concentrations in the mM range deactivate catalase due to oxidation of the porphyrin ring as well as methionine and tryptophan residues in the structure of the enzyme-active site and the cofactor NADPH-binding site (16–18). The net result is a decrease in catalase activities which have been documented in vitiligo (12,17).



**Figure 1.**  $H_2O_2$  generation in the human epidermis.  $TNF-\alpha$  indirectly leads to  $H_2O_2$  formation via the induction of manganese superoxide dismutase (2,49,50).  $TGF-\beta$ , EGF and PDGF have been reported to generate  $H_2O_2$  (51). Epidermal monoamine oxidase A activities generate  $H_2O_2$  (53). NADPH oxidase activities from neutrophils and macrophages generate  $H_2O_2$  (53). Photo-oxidation of epidermal 6-biopterin and sepiapterin yields  $H_2O_2$  generation (55). Nitric oxide synthases can spontaneously foster the synthesis of  $H_2O_2$  in the absence of the substrate L-arginine (2,55). Generation of  $H_2O_2$  by oestrogen and progesterone has been demonstrated (57). Xanthine oxidase degrades purin bases to uric acid and this step generates  $H_2O_2$  (Shalbaf, unpublished results). Phenols, *o*- and *p*-quinols as well as UV-A/UV-B and X-rays can generate  $H_2O_2$  in the mM range (10,55).

- The same mode of action has been recognized for thio-redoxin reductase due to oxidation of the enzyme-active site and the NADPH cofactor-binding site (18). Low enzyme activities have been shown in vitiligo (19).

- Oxidation of methionine sulfoxide reductase A leads to compromised protein repair after oxidation of methionine residues to methionine sulfoxide. Low epidermal enzyme levels and activities have been demonstrated in vitiligo (5,20,21).

In summary,  $H_2O_2$  affects its entire degradation machinery due to oxidation of methionine, tryptophan and cysteine/selenocysteine residues in the structure of these enzymes.

### $H_2O_2$ affects catecholamine, serotonin and nitric oxide synthesis as well as melanogenesis via the essential cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (6BH<sub>4</sub>)

Oxidation of the essential cofactor and its 7-isomer by  $H_2O_2$  to 6- and 7-biopterin forms the basis for the observed characteristic fluorescence upon Wood's light examination in Refs (22,23). Consequently, this oxidation affects all cofactor-dependent mechanisms including tyrosine hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase and the nitric oxide synthases (24).

Deactivation of pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR), which are both involved in 6BH<sub>4</sub> recycling are deactivated by  $H_2O_2$

due to the oxidation of active site tryptophan and methionine residues consequently compromising the homeostasis for this very important cofactor (25,26).

Overproduction of the 7(R/S) isomer has a twofold effect on L-phenylalanine turnover. The enzyme is inactivated by the 7(S)-isomer and by  $H_2O_2$ , fostering accumulation of epidermal L-phenylalanine concomitant with decreased L-tyrosine levels (22,27–29). Increased epidermal phenylalanine levels have been documented in patients with vitiligo by *in vivo* FT-Raman spectroscopy (27).

### $H_2O_2$ compromises the epidermal cholinergic signal

Earlier Iyengar reported high acetylcholine levels in the epidermis of patients with vitiligo (30). The result was recently confirmed by our group and it can be explained by  $H_2O_2$ -mediated deactivation of the enzyme-active site of both acetylcholinesterase and butyrylcholinesterase. In addition, the tetramerization domain as well as the EF-hand calcium-binding domain is affected in the latter enzyme (13,30–33). Interestingly, high levels of epidermal acetylcholine have also been proposed contributing to the pruritus in patients with acute vitiligo (13,34). These results are also in agreement with impaired sweating in these patients (36).

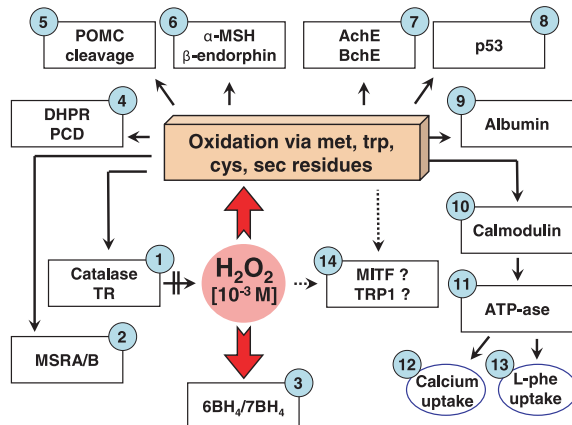
### $H_2O_2$ affects POMC-peptide levels and function

Earlier Graham *et al.*, reported low epidermal  $\alpha$ -MSH levels in vitiligo (36). These results have been reconciled in the context of  $H_2O_2$ -mediated oxidation by affecting POMC cleavage via the prohormone convertases as well as the structure and function of POMC-derived peptides including  $\alpha$ -MSH and  $\beta$ -endorphin (37).

### $H_2O_2$ affects the structure of epidermal albumin and alters epidermal calcium homeostasis

Due to the oxidation of tryptophan residues in the sequence of albumin by  $H_2O_2$  epidermal albumin levels are severely affected in vitiligo (14). Albumin has many crucial functions including control of calcium homeostasis. In this context, it is noteworthy that  $H_2O_2$ -mediated oxidation affects also all four calcium EF-hand-binding domains of calmodulin (38). Both calmodulin- and calmodulin-activated epidermal ATPase activities are indeed very low in the skin of patients with vitiligo (38). Here, it is tempting to attribute at least in part the earlier documented impaired calcium uptake in vitiligo (39,40). Considering that the uptake of L-phenylalanine requires calcium, this assumption is supported by the defective L-phenylalanine uptake in epidermal melanocytes and keratinocytes from these patients (39–41).

A summary of all data supporting the effect of  $H_2O_2$ -mediated stress is presented in Fig. 2.



**Figure 2.** Documented effects of  $10^{-3}$  M  $H_2O_2$  concentrations on cellular homeostasis in acute vitiligo.  $H_2O_2$  deactivates (1) catalase and thioredoxin reductase via oxidation of target residues increasing in turn the  $H_2O_2$  pool (17,18). Both repair enzymes for methionine sulfoxide (2) MSRA/B are a target for  $H_2O_2$ -mediated oxidation themselves (6) (3)  $H_2O_2$  oxidizes the cofactor  $6BH_4$  and its isomer  $7BH_4$  (23,28,54) and deactivates (4) both recycling enzymes for the cofactor again via target residues (25,26). By the same mechanism are (5) POMC cleavage via the prohormone convertases, the (5) POMC-derived peptides  $\alpha$ -MSH and  $\beta$ -endorphin (38), (6) the turnover of acetylcholine via acetylcholinesterase (AChE) as well as butyrylcholinesterase (BChE) (14,32–34), (7) p53 (Aben Eloof *et al.*, unpublished results) and (8) albumin affected (15). The calcium homeostasis is greatly impaired due to  $H_2O_2$ -mediated oxidation of (10) calmodulin (38) which in turn fails to activate (11) ATPase (38) leading to an impaired (12) calcium- and (13) L-phenylalanine uptake (28,29,39–41,57). Whether the effect on (14) MITF and TRP1 (57) is directly mediated by  $H_2O_2$  or also based on structural alterations due to oxidation of target residues is currently still under investigation. Note: MITF has 28 potential target residues, while TRP1 has 19 (Gibbons NCJ, unpublished results).

### ***In vivo* evidence for the involvement of $H_2O_2$ in the pathogenesis of vitiligo**

One important proof for the concept is the recovery of many affected pathways in association with cessation of the active disease and repigmentation of the affected individual after reduction in/removal of epidermal  $H_2O_2$  by a topical pseudocatalase PC-KUS (42) (Fig. 3). Reduction can be achieved by a synthetic catalyst that oxidizes  $H_2O_2$  to  $O_2$  and  $H_2O$ , thus mimicking the reaction catalysed by natural catalase. The active chemical catalyst is a low-dose narrow-band UV-B-activated bis- $Mn^{III}$ -(EDTA) $_2$ -( $HCO_3^-$ ) $_2$  complex (2,10,42).

Taken together, nowadays, there is ample evidence for  $H_2O_2$ -mediated oxidative stress in the entire epidermis of patients with vitiligo affecting many different systems including calcium homeostasis. The effects are concentration dependent. While  $10^{-3}$  M concentrations are deleterious,  $10^{-6}$  M concentrations can be of great benefit (6). Several publications support a genetic association of the catalase gene with vitiligo susceptibility (43,44). As these patients are constantly combating with epidermal oxidative



**Figure 3.** Proof of the concept. Repigmentation of facial vitiligo after reduction in/removal of epidermal  $H_2O_2$  by low-dose NB-UV-B-activated pseudocatalase PC-KUS: (a) before, (b) after.

stress via  $H_2O_2$  and peroxynitrite, it is tempting to ask the question why there is no increased risk of skin cancer (46,47). In the absence of apoptosis, how do they cope with DNA repair in the presence of these high epidermal  $H_2O_2$  levels (4)? (M. Shalbaf, unpublished results). Could increased functioning  $p53^{w/w}$  as reported earlier be a major player in the scenario (48)? Could  $H_2O_2$  and/or peroxynitrite alter the immune response and induce autoimmunity? It will be exciting in future to put the hierarchy of events in order.

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## Viewpoint 2

The rise and fall of vitiligo involves an understanding of virtually every aspect of the melanocyte. When studying vitiligo aetiology, one comes across cell biology, genetics, biochemistry and immunology, even microbiology. The genetic predisposition to vitiligo is almost a given. In certain communities, the disease frequency can run up to more than 20% and for most patients, it is completely clear that vitiligo ‘runs in the family’, with more than 25% of patients assigning their disease to hereditary factors (1,2).

With major studies underway to identify genes unequivocally associated with vitiligo, the next step is to understand the biology of depigmentation. First and foremost, it was important to establish that melanocytes are lost from vitiligo lesions as the disease progresses, and that depigmentation is not merely a process whereby melanogenesis is inhibited (3). Secondly, an understanding of the interaction between melanocytes and other epithelial cells comes into play. There is the possibility that melanosome transfer to neighboring keratinocytes is inhibited, which should definitely impact on skin colour (4). There is also an as of yet incompletely understood intimate relationship between melanocytes and Langerhans cells, where one can almost disguise as the other in appearance and function (Fig. 1).

Knowing melanocytes as an immunologic entity within the skin has helped us to understand that melanocytes are ideal targets for an autoimmune response. After all, melanocytes produce cytokines that draw attention to themselves (5,6). They can phagocytize, and can process and

present antigens in the context of MHC class II as one would normally expect a professional antigen-presenting cell to do (7,8). These are not routine tasks, as we see melanocytes express HLA-DR specifically in vitiligo, and in melanoma (9–11). But is that not exactly when it is time to intervene? Exactly when melanocytes are involved in the disease with no easy cure in sight?

The properties of melanocytes that make them legitimate members of the skin immune system are often lost from the general sphere of attention as the focus is placed on the incredible task of melanization. Of course, their responsibility for skin colour puts melanocytes in the limelight, and helps protect us from harmful effects of UV irradiation (12,13). It also defines our ethnic heritage and is thereby a visible part of our identity (14). And many factors identified as precipitating factors in vitiligo, play into the biochemical process called melanogenesis, including overexposure to UV by stimulating melanization (15). Melanization is not so safe a process for melanocytes to begin with, unless it is contained with flawlessly formed melanosomes and none of those toxic intermediates ever comes in touch with the cells’ cytoplasm (16).

Other obvious precipitating factors are those known to cause ‘occupational vitiligo’, which can be oversimplified as a process of competitive inhibition with melanin precursors that melanogenic enzymes can convert into toxins within melanocytes only (17). Or even wounding or bruising, possibly involving inflammation followed by postinflammatory consequences that touch on melanization (18).