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Original article

The key role of UVA-light induced oxidative stress in human Xeroderma Pigmentosum Variant cells

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ABSTRACT

The UVA component of sunlight induces DNA damage, which are basically responsible for skin cancer formation. Xeroderma Pigmentosum Variant (XP-V) patients are defective in the DNA polymerase pol eta that promotes translesion synthesis after sunlight-induced DNA damage, implying in a clinical phenotype of increased frequency of skin cancer. However, the role of UVA-light in the carcinogenesis of these patients is not completely understood. The goal of this work was to characterize UVA-induced DNA damage and the consequences to XP-V cells, compared to complemented cells. DNA damage were induced in both cells by UVA, but lesion removal was particularly affected in XP-V cells, possibly due to the oxidation of DNA repair proteins, as indicated by the increase of carbonylated proteins. Moreover, UVA irradiation promoted replication fork stalling and cell cycle arrest in the S-phase for XP-V cells. Interestingly, when cells were treated with the antioxidant N-acetylcysteine, all these deleterious effects were consistently reverted, revealing the role of oxidative stress in these processes. Together, these results strongly indicate the crucial role of oxidative stress in UVA-induced cytotoxicity and are of interest for the protection of XP-V patients.

1. Introduction

Ultraviolet (UV) light is the most harmful and mutagenic component of the solar radiation spectrum [1]. It is classified in three wavelength ranges: UVC (200–280 nm), UVB (280–320 nm) and UVA (320–400 nm) light [2]. Only UVB and UVA pass through the ozone layer in the stratosphere, with UVA representing 95% of the total UV energy that reaches the Earth's surface [3]. These UVA wavelengths also penetrate more deeply into the human skin, where they may trigger cell damage in membranes, lipids, proteins and DNA. These processes may have deleterious consequences for human health, such as skin aging and various types of skin cancer, with melanoma as the most severe [3–5]. As a result, UV radiation was classified as a class I carcinogen by the World Health Organization (WHO) [6]. However, the role of UVA light in the mechanisms by which UVA induces mutations and skin cancer are still under debate [3,7,8].

Despite the low energy of the photons in UVA light (3.1–3.9 eV), these photons can be absorbed by the DNA molecule and chromophores present in skin cells. Cyclobutane pyrimidine dimers (CPDs) are the main lesions induced by direct excitation of DNA bases by UVA photons [9–12], although there is also evidence for the formation of pyrimidine (6 − 4) pyrimidone photoproducts (6–4PPs) [12,13]. These photoproducts generate distortions on the DNA double helix, causing physical barriers to transcriptional polymerases (RNA polymerase) as well as to DNA replication polymerases. The distortions block these processes, which are essential for normal cell metabolism [14]. At the same time, the absorption of UVA photons by chromophores through photosensitization reactions leads to the formation of reactive oxygen species (ROS) [15]. This process causes DNA damage through indirect mechanisms [16–19]. Recently, singlet oxygen was demonstrated to form directly on DNA after UVA exposure [20]. UVA light induces various types of DNA damage by oxidation, including 8-oxo-7,8-dihydroguanine (8-oxoG), single and double strand breaks, abasic sites and crosslinks between DNA and proteins [21–23]. 8-oxoG is frequently used as a biomarker to identify oxidative stress [24], and these lesions, due to their structural characteristics, have been shown to be highly

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mutagenic [25].

DNA repair mechanisms have some specificity for the type of damage on the double helix. Oxidized DNA bases are normally repaired by specific glycosylases of the Base Excision Repair (BER), although Nucleotide Excision Repair (NER) proteins may also participate in the process. CPDs and 6–4PPs, which cause bulky distortions in the double helix, are repaired by NER [26].

Genetic defects in NER give rise to severe and rare syndromes, such as xeroderma pigmentosum (XP). The main clinical feature of XP patients is an increased frequency of skin cancer, with close to 10,000-fold greater incidence than the general population average in the sunlight-exposed area of the body [27,28]. XP mainly occurs due to defects in one of seven genes encoding NER proteins (XP-A to XP-G). However, some patients known as XP variant (XP-V) have normal NER activity but are defective in DNA polymerase eta (pol eta). Pol eta participates in the replication of UV-damaged DNA through the translesion synthesis (TLS) pathway. The main function of pol eta is to accurately replicate DNA damage, including CPDs [29]. Pol eta may also bypass oxidized lesions *in vitro* [3,30,31]. In the absence of pol eta, other TLS polymerases may bypass UV-induced lesions, but those enzymes are error-prone, enhancing mutagenesis after sunlight exposure and consequently leading to skin cancer [32–37].

Little is known about the effects of UVA light on cells from XP-V patients. In this context, this work investigated the detrimental effects of UVA irradiation in pol eta-deficient (XP-V) human cells to better understand how these wavelengths may affect XP-V patients. XP-V cells exhibited increased sensitivity to UVA irradiation, with effects including more pronounced genotoxic stress, replication fork stalling and cell cycle arrest. The search for the mechanisms involved in these increased effects revealed that UVA irradiation affected the capacity of these cells to remove DNA damage, possibly due to direct effects on repair proteins. Remarkably, the antioxidant N-acetyl cysteine (NAC) conferred protection to the cells, including recovering DNA damage removal and DNA replication. In addition, metabolic stress, by NADPH oxidase, was shown to participate in ROS production after exposure to UVA light in human cells, but mainly when pol eta activity is absent. This demonstrates that ROS, induced a few hours after UVA irradiation, are responsible for at least part of the deleterious effects of UVA-light exposure, especially in XP-V cells.

2. Results

2.1. XP-V cells are more sensitive to UVA-light than complemented cells

Cellular sensitivity to UVA exposure was evaluated for a set of human fibroblast cell lines derived from an XP-V patient with a complete absence of the pol eta protein and a wild type pol eta-complemented counterpart (XP-V and XP-V comp). The sensitivity of the XP-V comp cells was comparable to another control cell line (MCR5-SV) indicating this is a good control cell line for experiments with UVA light, as previously shown for UVC light [33]. Cell viability results using the XTT assay show that under environmentally relevant UVA doses (between 30 and 120 kJ/m^2), XP-V cells were more sensitive compared to XP-V comp cells (Fig. 1A), and to MRC5-SV cells (Supplementary Fig. S1A). The induction of nuclear fragmentation (indicative of apoptosis) by UVA irradiation was detected as sub-G1 content by flow cytometry. At the higher UVA dose and especially at later times (72 h), a higher number of XP-V cells were in sub-G1 compared with XP-V comp (Fig. 1B) and MRC5-SV cells (Supplementary Fig. S1B). These data indicate that environmentally relevant doses of UVA irradiation induce higher cellular death in XP-V cells compared with the control due to the pol eta deficiency, probably due to their inability to replicate UVA-induced DNA damage.

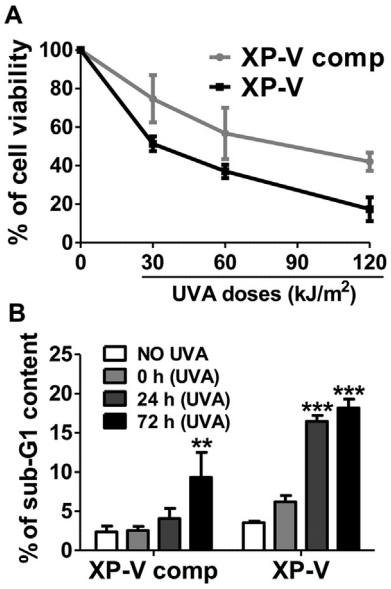

Fig. 1. XP-V cells are more sensitive to UVA irradiation. (A) Cell viability of XP-V or complemented cell lines by the XTT assay, measured 72 h after UVA exposure. The values represent the average of three independent experiments. (B) Sub-G1 content was detected at different times after 120 kJ/m^2 of UVA irradiation. The data represent the average of four independent experiments. Asterisks represent significant differences between the UVA-irradiated cells compared to the respective control non-irradiated cells. **p < 0.01 and ***p < 0.001 – ANOVA test.

2.2. UVA-irradiated XP-V cells exhibit slower DNA lesion repair

CPD formation and removal after UVA irradiation in XP-V and XP-V comp cell lines are shown in Fig. 2A. The XP-V comp was able to repair most CPDs during the three days after exposure. Surprisingly, however, pol eta defective cells seem to remove these lesions much less efficiently. As these cells are NER proficient [38,39], this difference was further investigated by HCR, which measures the capacity of these cells to repair DNA damage in a plasmid containing DNA lesions. In this case, UVC-irradiated plasmids were transfected into these cells, and the ability to express the luciferase was measured. The expression of this reporter gene correlates with the ability of the cells to repair UVC-induced lesions. The results clearly indicate that a small decrease in repair capacity is observed in UVA-irradiated complemented cells, indicating that UVA irradiation *per se* may affect the ability of these cells to remove CPD lesions (Fig. 2B). However, this effect is much stronger in XP-V cells. Although non-irradiated XP-V cells also exhibit a small reduction in HCR, UVA-irradiated XP-V cells seem to have completely lost their capacity to remove DNA lesions from the transfected plasmid (Fig. 2B). In these experiments, plasmid stability was evaluated by quantitative PCR, and the results indicate that there is no specific effect in pol eta deficient or UVA irradiated cells, although damaged plasmid (after UVC irradiation) did result in less plasmid in the cells (Supplementary Fig. S2). These results confirm the UVA-irradiated XP-V cells have a decreased capacity of DNA repair, compared control cells.

The defective repair in UVA-irradiated XP-V cells was also tested through EdU-based unscheduled DNA synthesis (UDS) assay, as described in materials and methods. As expected, the XP-V cells presented a similar percentage of cells containing EdU-foci to XP-V comp after UVC irradiation (Supplementary Fig. S3), indicating no difference of NER in these cells, as extensively demonstrated for the classical UDS assay [40,41]. However, UVA-irradiated XP-V cells presented a significantly lower percentage of cells containing EdU-foci when compared to the control cells (Supplementary Fig. S3). These data together demonstrate that XP-V presents reduced ability of repair after exposure

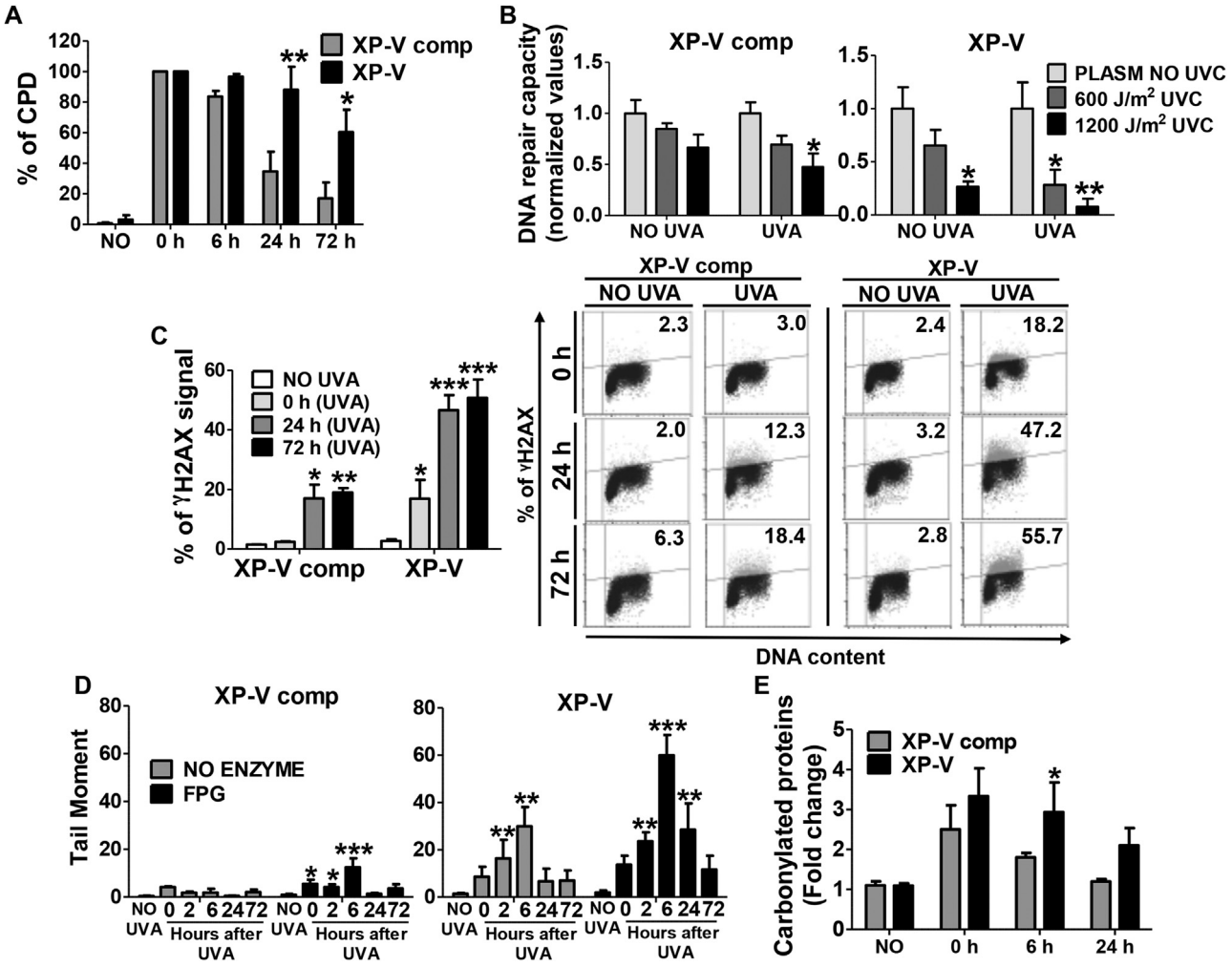

Fig. 2. XP-V cells exhibited a decreased DNA repair capacity. XP-V comp and XP-V cells were irradiated with 0 (NO UVA) or 120 kJ/m^2 of UVA. (A) The amount of CPD lesions was detected immunologically by Slot Blot. The values represent the average of three independent experiments. (B) The DNA repair capacity of those cells to repair UVC irradiated plasmid was measured by HCR. The values represent the average of three independent experiments. (C) Genotoxic stress was detected by immunological quantification of γH2AX levels and representative immunostaining of γH2AX at different points in the cell cycle. The values represent the average of four independent experiments. The UVA-induced oxidative stress was demonstrated by (D) the detection of strand breaks and FPG-SSB by the comet assay, and by (E) the quantification of carbonylated proteins 0, 6, 24 h after UVA irradiation. The data represent the average of three and four independent experiments, respectively. In A, the asterisks represent significant differences between XP-V and XP-V comp cells at the same experimental time. In B, C and E, the asterisks represent significant differences between UVA-irradiated cells compared to the respective non-irradiated control cells. *p < 0.05, **p < 0.01 and ***p < 0.001 – ANOVA test. In D, the asterisks represent significant differences between UVA-irradiated cells compared to the respective non-irradiated control cells. *p < 0.05 in 0 and 2 h (FPG) for XP-V comp – one-way ANOVA, **p < 0.005 in 2 h (NO ENZYME) for XP-V – Test T, **p < 0.01 and ***p < 0.001 for others – ANOVA test.

to UVA light.

To evaluate the genotoxic stress induced in cells exposed to UVA irradiation, phosphorylation of H2AX histone (Ser-139, γH2AX) was quantified by flow cytometry. Significant increased levels of γH2AX were observed in XP-V cells when compared to non-irradiated control cells (Fig. 2C), indicating occurrence of a genotoxic stress.

The alkaline comet assay allows the detection of single- and double-stranded breaks as well as alkali-sensitive sites (here generally named SSB). This is measured as the tail moment of single cells. When FPG is added to the assay, oxidized DNA damage (or FPG-sensitive sites, FGP-SS) can also be detected. Using this assay, no significant difference was observed in SSB and FPG-SS induction immediately after UVA irradiation (0 h) (Fig. 2D and Supplementary Fig. S4). However, SSB and FPG-SS were much more pronounced 2–6 h after in UVA-irradiated XP-V cells compared to XP-V complemented cells (Fig. 2D). Interestingly, these two classes of lesions are mostly detected 6 h after UVA irradiation, indicating that these are not direct products of exposure. These results also indicate that UVA-irradiated XP-V cells are under much stronger oxidative and genotoxic stress than cells containing pol eta.

Previous reports indicate that UVA light may cause extensive protein oxidation, consequently reducing DNA repair by damaging NER essential proteins [42]. This was investigated by evaluating the levels of carbonylated proteins induced in each condition. In fact, as shown in Fig. 2E, no significant difference of carbonylated protein induction was observed 0 h upon UVA irradiation. But 6 h later, UVA irradiation leads to increased levels of carbonylated proteins especially in XP-V cells. These findings indicate that slower removal of lesions may be explained, at least in part, by protein oxidation after UVA irradiation.

2.3. UVA-induced DNA damage results in replication fork stalling and cell cycle arrest in pol eta-deficient cells

Given that pol eta is the main polymerase involved in UVC-induced lesion bypass, cells deficient in this enzyme exhibit DNA replication stalling and S-phase arrest after UVC irradiation. Therefore, the effects of UVA irradiation on replication fork stalling and cell cycle profiles were investigated by the fiber assay and flow cytometry. An average 5-chloro-2′-deoxyuridine (CldU)/5-iodo-2′-deoxyuridine (IdU)

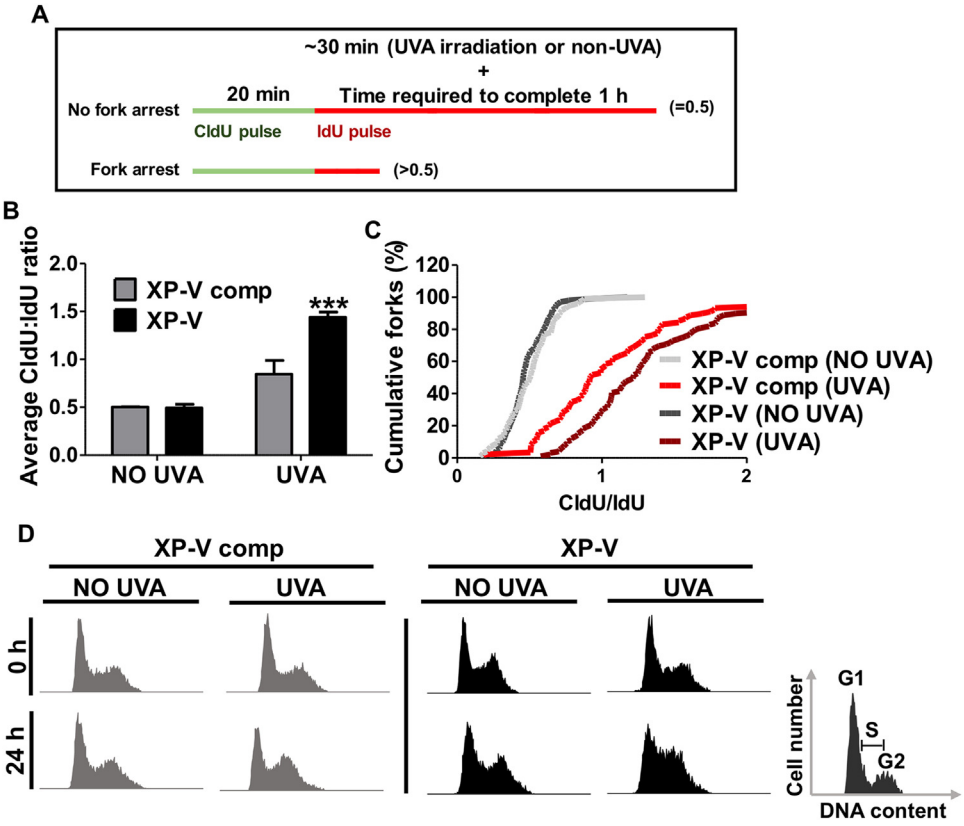

Fig. 3. UVA-induced DNA damage caused replication fork stalling and cell cycle arrest in XP-V cells. XP-V comp and XP-V cells were irradiated with 120 kJ/m^2 of UVA. (A) Schematic representation of the fiber assay. (B) CldU/IdU average incorporation ratio and (C) cumulative fiber distribution in UVA-irradiated cells indicating fork stalling, mainly in XP-V cells. The values showed in (B) and (C) represent the average of two independent experiments. Asterisks represent significant differences between the UVA-irradiated compared to the respective non-irradiated cells. ***$p < 0.001$ – ANOVA test. (D) Cell cycle profiles were evaluated by flow cytometry. The histograms illustrate representative results from four independent experiments.

(CldU:IdU) incorporation rate of 0.5 represents no fork arrest under the conditions of this experiment, with higher rates indicating fork arrest (Fig. 3A). The fiber assay analyses clearly show that UVA irradiation led to a significant increase in the CldU/IdU incorporation rate, only in pol eta-deficient cells, with no effect observed for control cells (Fig. 3B) or cumulative fork progression (Fig. 3C). These results indicate that UVA-induced DNA damage compromises the replication fork elongation in XP-V cells. Accordingly, these cells also displayed a strong S-phase arrest 24 h after UVA irradiation, an effect not observed in control cells (Fig. 3D).

2.4. *UVA-induced redox process has strong and harmful effects in XP-V cells*

The observation that UVA irradiation resulted in DNA damage by oxidative stress (Fig. 2D) and protein carbonylation (Fig. 2E), which may be responsible for the reduced DNA repair capacity, raised the question of the role of ROS in the deleterious effects of UVA-irradiated cultured cells. This was tested by adding the antioxidant NAC to the cell cultures. In these conditions, XP-V cells exhibited significantly improved cell viability when NAC was added, whereas no change was observed for control cells (Fig. 4A and Supplementary Fig. S5). However, a significant increase in cell survival (clonogenic assay) was observed for both cell lines following NAC treatment after UVA irradiation. Interestingly, both cell lines presented increased cell plating efficiency, even if not irradiated (Supplementary Fig. S6A and S6B Figure). Corroborating these results, NAC significantly decreased UVA-induced sub-G1 content in both cell lines (Fig. 4B).

The protective effect of NAC was also observed for UVA-induced protein oxidation in both cell lines (Fig. 5A), which was also reflected in improvements in the cells' capacity to repair UVC-irradiated transfected plasmids, as detected by HCR (Fig. 5B). This effect was significant for XP-V cells, and NAC significantly increased the capacity of CPD removal, especially in UVA-irradiated XP-V cells (Fig. 5C). The SSBs and FPG-SS DNA lesions were clearly reduced by NAC in UVA-irradiated XP-V cells (Fig. 5D). Moreover, corroborating the improvement in DNA lesion repair, NAC significantly reduced γH2AX levels in XP-V cells (Fig. 5E).

The effect of UVA on fork replication stalling was also investigated in the presence of NAC. The DNA fiber assay data indicate a clear improvement in fork elongation (CldU/IdU ratio) in NAC-treated UVA-irradiated XP-V cells. In fact, the results showed an almost complete recovery of DNA replication, indicating that fork stalling cannot be attributed to the remaining CPDs in XP-V cells (Fig. 6A, B and C). The effect of NAC on protecting cells from experiencing reductions in DNA synthesis was further analyzed by checking the S-phase arrest in UVA-irradiated cells. In these experiments, cells were also incubated with nocodazole, which blocks mitosis progression, thus preventing cells from initiating a second round of the cell cycle. The results clearly show that although UVA irradiated XP-V cells presented a strong S-phase arrest (not observed for normal cells), adding NAC to the cells recovered their ability to progress through the S-phase (Fig. 6D). These results confirm that protecting XP-V cells from oxidative stress allows them to better replicate their genome.

Additionally, the participation of ROS generated by metabolic stress upon UVA was tested through quantification of mitochondrial superoxide and diphenyleneiodonium (DPI) treatment that inhibit NADPH oxidase, a potential generator of ROS after UVA irradiation. In fact, the mitochondrial superoxide is significantly increased 6 h after UVA exposure in both cell lines, however the increase was more prominent in XP-V cells (Supplementary Fig. S7). NAC treatment significantly reduced the induction of superoxide in both cell lines. On the other hand, significant increase of cell survival in XP-V cells irradiated with UVA and treated with 0.25 μM of DPI (Supplementary Fig. S8A and B). Also, significant decrease of FPG-SS was observed when DPI is added to cell media in both cell lines, however, the decrease was more evident in XP-V cells. These results confirm that metabolic stress, related to NADPH oxidase is, at least in part, responsible for the generation of ROS after

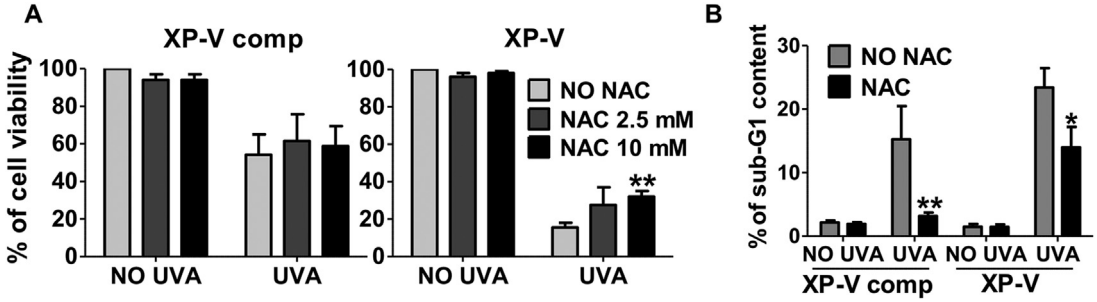

Fig. 4. NAC protects human cells from UVA irradiation. XP-V comp and XP-V cells were irradiated with 120 kJ/m^2 of UVA, and the protection provided by 10 mM NAC protection was evaluated by (A) the XTT assay. The values represent the average of three independent experiments. (B) sub-G1 content 72 h after treatment. The values represent the average of six independent experiments. Asterisks represent significant differences between UVA-irradiated cells and UVA-irradiated cells treated with NAC, within the same cell line. *p < 0.05 and ***p < 0.001 – ANOVA test.

UVA irradiation, mainly in the XP-V cells.

In order to confirm that the difference in the oxidative stress induction generated by UVA is due to absence of the pol eta, similar experiments were performed with normal (GO01) and XP-V (XP06GO) primary fibroblasts. In fact, XP06GO presented a significant increase of carbonylated protein after UVA irradiation, reduced when cells were treated with NAC (Supplementary Fig. S9A). In the comet assay, GO01 showed a significant increase of FPG-SS compared to their respective control (NO UVA + FPG), as well as significant decrease of DNA breaks and FPG-sensitive sites when these cells were treated with NAC and DPI (Supplementary Fig. S9B). However, the XP06GO cells showed a significant increase of DNA breaks after UVA, as well as FPG-SS, compared with pol eta proficient cells. Similar to SV40 transformed cell lines, the primary XP-V fibroblasts also showed significant reduction of DNA breaks and sites sensitive to FPG when NAC and DPI were added to the culture media. These results confirm that the absence of the pol eta is related to the lack of control against the redox processes.

3. Discussion

In recent decades, there has been a growing interest in unveiling the mechanisms of UVA-induced DNA damage, especially in determining the types of lesions responsible for skin aging and cancer. Importantly, because UVA photons damage DNA less efficiently than UVB and UVC irradiation, the potential of UVA to induce deleterious effects is often underestimated. We hypothesized that the molecular defects in XP cells could provide us with new clues on the real effects of UVA irradiation in human cells, which could help to understand how skin lesions are produced not only in these patients but also in the DNA repair-proficient population. Thus, this work investigated pol eta-deficient human cells as a model to understand the participation of UVA-induced damage in the phenotype of XP-V disease and its effects on human skin in general.

This work was performed with an XP-V cell line (TLS-deficient), derived from a patient with frameshift mutations on the *POLH* gene leading to the total absence of the pol eta protein [43]. This cell line was systematically compared with an isogenic cell line complemented

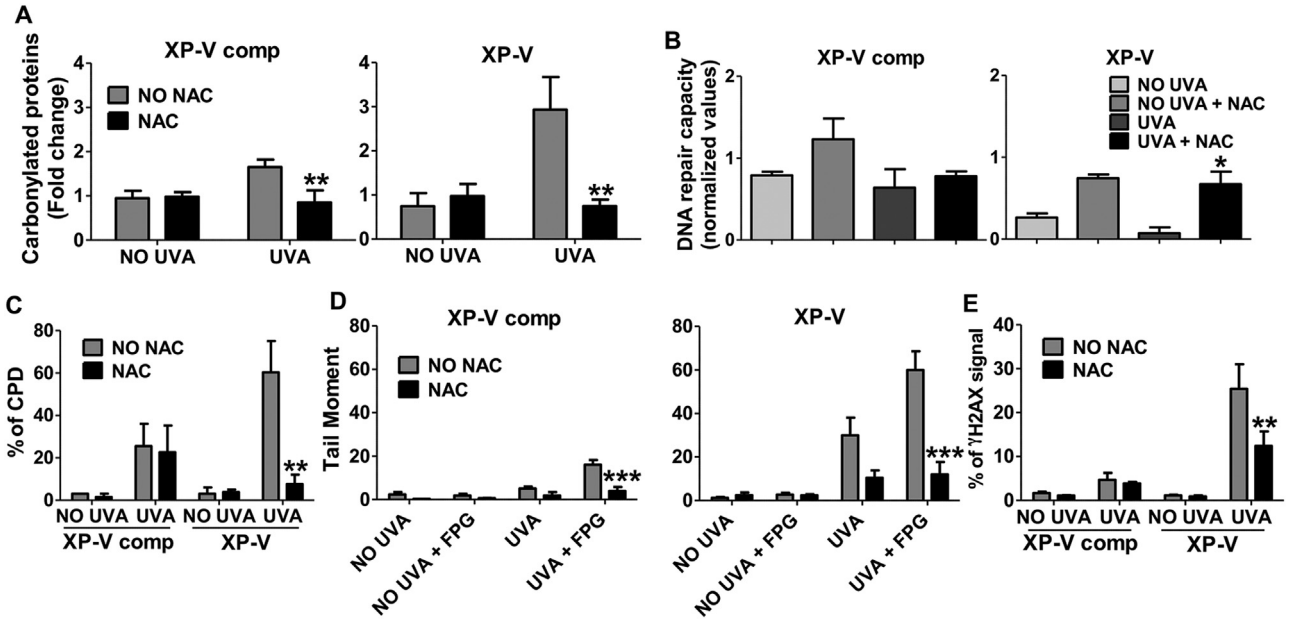

Fig. 5. NAC protection from UVA-induced oxidative stress resulted in decreases in DNA and protein damage. XP-V comp and XP-V cells were previously treated with NAC (10 mM), and upon UVA irradiation (120 kJ/m^2). (A) protein carbonylation 6 h after UVA irradiation. The values represent the average of four independent experiments. (B) repair capacity using UVC-irradiated (1200 J/m^2) plasmids. The values represent the average of three independent experiments. (C) the amount of CPDs 72 h after UVA irradiation. The values represent the average of three independent experiments. (D) the repair of strand breaks and oxidized bases (detected by FPG) by comet assays 6 h after UVA irradiation. The values represent the average of two independent experiments. (E) γH2AX levels after 24 h of UVA exposure. The values represent the average of four independent experiments. Asterisks represent significant differences between cells in the same experimental condition with or without NAC. *p < 0.05, **p < 0.01 and ***p < 0.001 – ANOVA test.

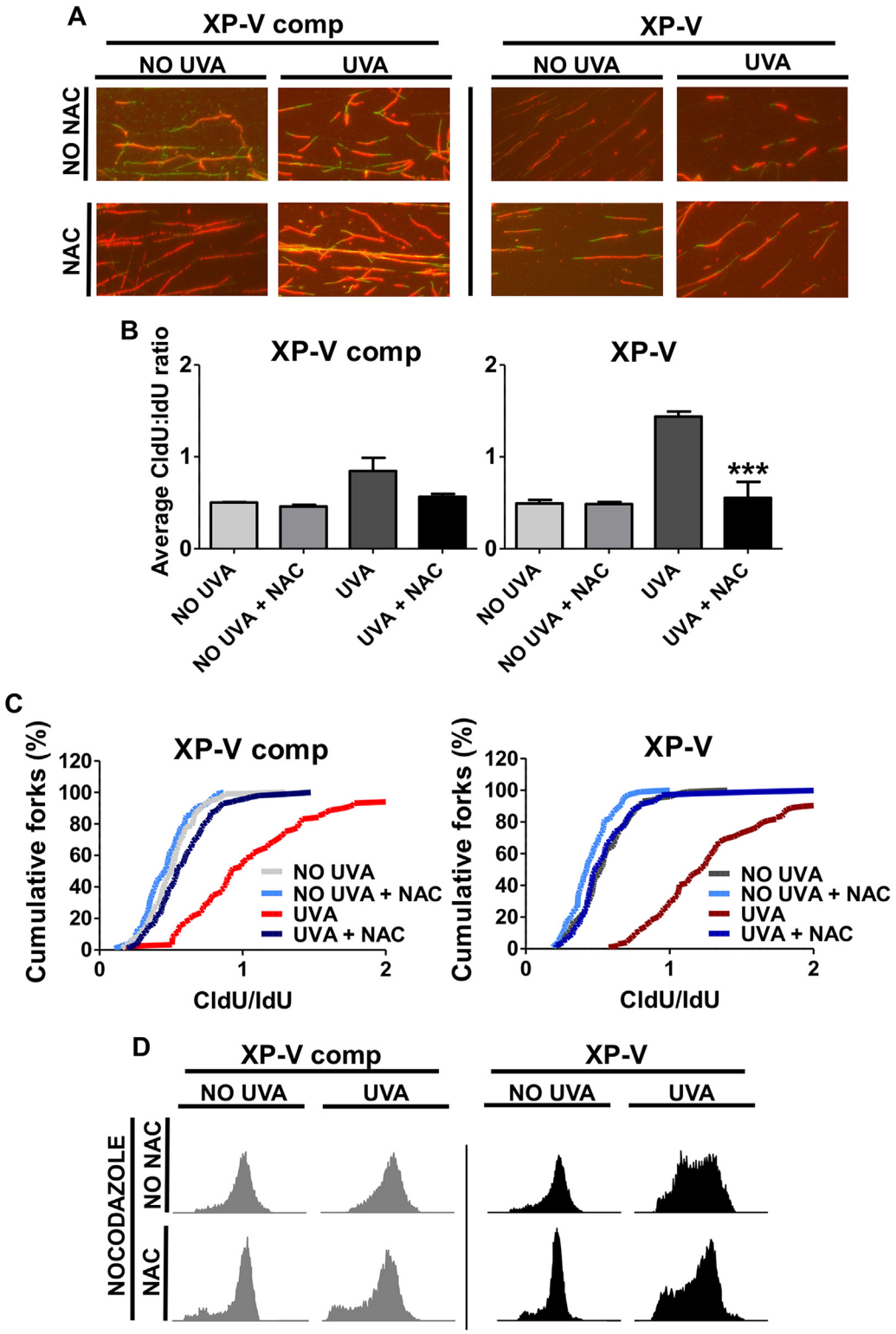

Fig. 6. NAC rescues cells from replication fork stalling and S-phase arrest following UVA irradiation. XP-V comp and XP-V cells were UVA-irradiated (120 kJ/m^2) in the presence (10 mM - NAC) or absence of NAC (NO NAC). (A) representative figures of DNA fibers in the different conditions from two independent experiments. (B) replication fork ratio and (C) cumulative fiber distribution, indicating fork stalling in XP-V cells, which is reduced in the presence of NAC. In both, the values represent the average of two independent experiments. (D) Cell cycle profiles were performed in the presence of nocodazole to avoid initiation of a second cycle. The histograms illustrate representative results from three independent experiments. Asterisks represent significant differences between cells treated with or without NAC in the same experimental condition. ***p $<$ 0.001 – ANOVA test.

with the wild type *POLH/XPV* gene. The complemented cell line expresses physiological levels of pol eta protein and is sensitive to UV as other normal cells [33]. UVA irradiation was employed with environmentally relevant doses, with the highest (120 kJ/m^2) corresponding to close to half an hour of exposure (depending on the season) under the sun in a tropical latitude, such as in the city of Sao Paulo, Brazil [44]. The TLS-deficient cells were unable to efficiently address UVA-induced DNA damage, as revealed by their increased sensitivity to irradiation, with effects including decreased cell viability and survival and increased apoptosis. Normal cells also showed sensitivity to UVA light, as previously reported for mammalian cells [45], although the effects were less prominent compared to XP-V cells. This increased sensitivity involved genetic stress, as the XP-V cells had increased levels of γH2AX. The phosphorylation of the core histone H2AX is normally taken as a double-strand break (DSB) marker [46], but chromatin remodeling due to the presence of DNA damage, or DNA repair, may also result in the formation of γH2AX [47]. The direct detection of DNA damage such as CPDs, SSBs (or alkaline sensitive sites) and oxidized

bases (FPG-SS) in UVA-irradiated cells confirmed the deleterious effects of these wavelengths on DNA, as previously reported [48,49]. Interestingly, however, XP-V cells not only exhibited increased levels of SSBs and FPG-SS but also seemed to experience a continuous oxidative stress after UVA-light exposure, as these types of lesions accumulate in the cells at least up to 6 h later. This is in agreement with previous work in which a late oxidative stress was detected in UVA-irradiated cells. This late oxidative stress was associated with the activation of NADPH oxidase and mitochondrial induced oxidative stress, which are potential cellular sources responsible for increasing the levels of ROS after UVA exposure demonstrated in keratinocytes [50,51]. The results shown here confirm that at least part of the effects observed after UVA irradiation of XP-V cells are reduced in the presence of DPI, a NADPH oxidase inhibitor. This inhibitor protected the cells from UVA, reducing the amount of FPG-SS on DNA. Moreover, the production of mitochondrial superoxide was observed, which was reduced in the presence o NAC. These data indicate that the deficiency of pol eta not only affects damage removal or replication but also generates metabolic cell stresses, which lead to further DNA, cellular and protein damage and which could be related to cell death and carcinogenesis [52,53].

General protein oxidation was also detected in these cells, with an increase in protein carbonylation, which alters the normal protein function and has an impact on cell signaling [54,55]. Protein carbonylation may explain the deficiency for the removal of CPDs observed in XP-V cells, and reduced repair capacity, despite the NER proficiency of these cells. Similar levels of carbonylated protein were also detected in normal and NER-deficient cells after UVA irradiation [13]. This oxidation may be responsible for the decreased capacity to repair UV-induced lesions, most likely NER. Indeed, recent research has provided evidence that NER can be impaired by UVA irradiation through the oxidation of essential proteins [42,56]. One of these proteins, RPA, seems to be particularly affected by oxidative stress generated by UVA light, and its depletion may limit the ability of NER to repair CPDs, 6–4 PPs and 8-oxoG in human keratinocytes [42]. Additionally, previous reports have indicated that NER may be decreased in XP-V cells after UVC light, and this is mainly observed in S-phase cells [57], however, by a different mechanism from that demonstrated here. In fact, limitations in the RPA pool can also impair NER because of its recruitment to stalled replication forks. This was demonstrated in mouse embryonic fibroblasts lacking the TLS polymerase activities of Rev3 and Rev1 [58], suggesting that the lack of pol eta could also have a similar effect. Thus, the results presented in this work basically confirm that UVA may reduce cells DNA repair capacity, probably due to protein oxidation, and extend these findings indicating these effects are more pronounced in pol eta deficient cells. The reduced NER functions in the XP-V cells may have important previously unsuspected clinical implications for patients, as sunlight exposure may combine DNA damage induced by UVB and UVA wavelengths. XP-V patients are highly prone to skin cancers, with some patients particularly susceptible to melanoma [59], which may be explained by these reduced TLS and NER activities.

Fork stalling has long been known to result from DNA damage (mainly induced by UVC, that is, pyrimidine dimers, CPDs and 6–4PPs) in XP-V cells, as cells from XP-V patients were initially described as deficient in their ability to replicate damaged templates [57,60]. In this work, we extended these conclusions for UVA-induced DNA damage, as fiber assays clearly indicate replication fork stalling in XP-V cells, resulting in persistent cell cycle arrest in the S phase for irradiated cells. Most likely, fork replication blockage generates single stranded structures, signaling for replicative stress in these cells. Moreover, the induction of γH2AX levels in XP-V cells upon UVA exposure may also reflect the replicative stress on these cells, in line with previous studies using UV-irradiation of mouse fibroblasts and MCF-7 cells [61]. H2AX is normally phosphorylated by kinases of the PI3K family (such as ATM, ATR, DNA-PK) [46], which may also be part of the signaling to trigger cell cycle checkpoints [62].

As UVA irradiation induces the formation of pyrimidine dimers on DNA, such as CPD, we expected that most of the outcomes described above would originate from these lesions, as previously observed for UVC irradiation. However, the contribution of oxidatively generated damage to the harmful effects induced by UVA in complemented and XP-V cells was demonstrated through the use of NAC as an antioxidant. NAC is a precursor of glutathione (GSH), which increases the intracellular antioxidant capacity and consequently protects the cells against the exacerbated production of ROS [48,63]. When previously added to XP-V cells, NAC promotes strong improvements in cell viability and survival to UVA-light, indicating the attenuation of oxidative stress effects in these cells. Similarly, direct measurement of DNA damage (such as SSBs and FPG-SS) and even protein carbonylation shows that NAC protects these cells from UVA irradiation. This is also reflected in an increase in the ability of cells to remove CPDs and repair UVC plasmids. Improved CPD removal is most likely related with the recovery of NER in the irradiated cells. The possibility that NAC could be acting through photoprotection alone has been discarded for the induction of pyrimidine dimers by UVB light [64], and this work also demonstrates NAC activity in UVC-irradiated plasmids by HCR. Due to the decrease of oxidized lesions and repair improvement, a reduction in γH2AX levels was also observed in NAC treated complemented and XP-V cells. Interestingly, the replication fork stalling and S-phase inhibition in UVA-irradiated XP-V cells were clearly reduced after NAC treatment. Previous reports indicate that oxidized lesions (mainly induced by singlet oxygen) obstruct the dynamics of DNA replication in normal, UVA-irradiated cells, independently of ATM or ATR sensors and checkpoints [65,66]. In fact, in a recent work we provide evidence of activation of the ATR/Chk1 pathway in DDR by UVA-induced oxidatively generated damage in pol eta deficient cells [67]. In this work, we found that NAC clearly contributed to restoring the movement of the replicating fork in UVA-irradiated XP-V cells. Similar findings were observed in cells deficient in homologous recombination subjected to oxidative stress, indicating that oxidatively induced lesions affect DNA replication [68]. The results presented here extend these findings, indicating that pol eta deficiency may compromise the elongation of DNA synthesis in UVA-oxidized templates, in agreement with previous *in vitro* experiments that show that pol eta is able to bypass an 8-oxoG damaged template [30]. Supporting the role of oxidatively generated DNA damage affecting DNA replication as photoproducts [69], much lower levels of SSB and FPG-SS were detected when NAC was applied to UVA-irradiated cells. In fact, the decrease in DNA fragmentation detected by the comet assay with NAC treatment had already been demonstrated for skin fibroblasts irradiated with UVA light [48,49]. Thus, DNA lesions generated by oxidative stress may also affect DNA replication in UVA irradiated XP-V cells. Alternatively, the rescue of DNA replication blockage by NAC may be related to the protection of protein oxidation, especially RPA [42], which is probably also a limiting factor for replication. However, the improvement of CPD removal in NAC treated cells can also partially explain the observed recovery of DNA replication.

The effects of oxidative stress induced by UVA on normal cells could also explain the appearance of lesions and skin aging over the long term. These findings are highly biologically relevant and confirm that skin mutagenesis and carcinogenesis are related not only to pyrimidine dimers but also to oxidative stress and protein modifications. Here, we have disclosed the role of UVA-induced oxidative stress in cells of patients with sensitivity to sunlight.

4. Conclusions

In summary, this work showed the deleterious effects of UVA light on XP-V cells. The search for mechanisms involved revealed the strong contribution of UVA-induced oxidatively generated damage. Protein oxidation reduced the DNA repair capacity in response to UVA-induced damage, and this was also more pronounced in XP-V cells. Moreover, UVA-induced DNA damage promoted replication fork stalling and S-

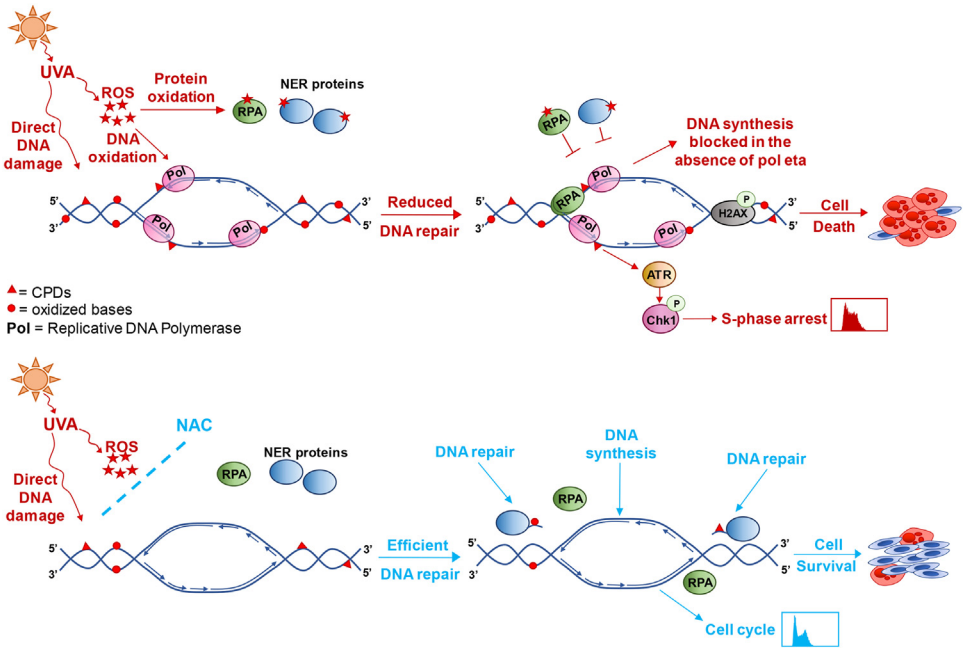

Fig. 7. Schematic representation of the deleterious effects of UVA-induced DNA damage in XP-V cells. UVA irradiation mainly affects XP-V cells through UVA-induced DNA damage (CPDs and oxidized bases) and carbonylated proteins arising from oxidative stress. Although XP-V cells are proficient in NER, an accumulation of lesions was detected, probably due to extensive protein oxidation (especially RPA and NER proteins) along with a reduced repair capacity. As a consequence, XP-V cells showed H2AX phosphorylation, blocked DNA synthesis, and consequently replication fork stalling. The participation of oxidative stress in these deleterious effects of UVA light was demonstrated with the antioxidant NAC. The protection afforded by NAC resulted in a decrease in oxidatively induced DNA damage and an improvement in CPD removal in XP-V cells, corroborating the hypothesis that protein oxidation disrupts NER. NAC protection was also reflected in the recovery of replication, the progression of the replication fork and cell cycle, and increased cell survival.

phase cell cycle arrest in the absence of pol eta, which was related to the oxidative stress in these cells. Interestingly, the use of an antioxidant resulted in significant protection against the detrimental effects of UVA. A schematic representation of the effects shown in this work is represented in Fig. 7. This work provides important information that may reflect the clinical phenotypes of XP-V patients, which could be, at least partially, due to UVA light. Moreover, in the absence of pol eta, we may simply amplify the damaging effects of UVA-light on human cells, revealing that the decrease in NER capacity may be involved in the phenotype of XP-V patient sensitivity to sunlight. Thus, the observed effects may also explain the effects of UVA-light on human skin in the general population, where the accumulation of sun exposure with age leads to skin aging and a high level of skin cancers.

5. Materials and methods

5.1. Cell lines and cell culture conditions

Three human SV40-transformed fibroblast lineages were used: the TLS-deficient cell line XP30RO (XP-V), the isogenic counterpart cell line complemented with the wild type *POLH/XPV* gene, XP30RO complemented clone 6 (XP-V comp), both kindly provided by Dr. Anne Stary and Dr. Patricia Kannouche (IGR, France) [33], and MRC5-SV. Two primary fibroblasts were also used, one normal (GO01) and one XP-V mutated (XP06GO) [70]. The cells were maintained in Dulbecco's Modified Eagle Medium High Glucose (DMEM, LGC Biotechnologies, Cotia, SP, Brazil), supplemented with 10% (transformed cells) or 15% (primary fibroblasts) fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA) and 1% antibiotic/antimycotic solution (0.1 mg/mL penicillin, 0.1 mg/mL streptomycin and 0.25 mg/mL fungizone - Life Technologies). The cells were cultivated under a humidified atmosphere with 5% CO_2 at 37 °C.

5.2. Cell lines' authentication

The cell lines had their exome recently (March 2017) sequenced, confirming their common origin and the mutation on the *POLH* gene, and complementation for this mutated allele in the complemented cells. Moreover, functional experiments, such as cell sensitivity to UVC, S-phase after DNA damage and TLS, all confirm the expected phenotypes. The last time these experiments were performed for phenotype confirmation were performed in August 2017.

Human cell lines employed in this work are part of a collection that was approved for use by the Ethical Committee for Research in Humans of the Institute of Biomedical Sciences, USP (Of. CEPSH 142.11).

5.3. UVA irradiation

Immediately before UVA exposure, the culture medium of cells was replaced by phosphate buffer solution (PBS) with calcium added. The experiments were performed with a UVA lamp (Osram Ultramed FDA KY10s, 1000 W) with a Schott BG39 filter (Schott Glass, Mainz, Rheinland-Pfalz, Germany) of 3 mm thickness that shuts off wavelengths lower than 320 nm, preventing cells from receiving UVB and UVC light [12]. As the lamp emission intensity was 0.058 kJ/m^2/s on average, higher doses of UVA (120 kJ/m^2) were delivered over approximately 30 min, and the temperature was kept constant, at 37 °C. The emissions were determined by a VLX3W radiometer (Vilber Lourmat, Torcy, Ile de France, France).

5.4. Cell treatments: nocodazole, N-acetylcysteine (NAC) and DPI

After UVA irradiation, PBS was replaced by cell media, supplemented with one of the following drugs (from Sigma Aldrich, St Louis, MO, USA): 1) nocodazole (0.1 μg/mL), 2) NAC (2.5 or 10 mM) before (16–18 h) and after UVA irradiation (until cell harvesting) and 3) DPI (0.1 and 0.25 μM), 1 h before UVA irradiation. These substances were diluted in culture medium at the specified concentrations, with treatment time varied among experiments.

5.5. Cell sensitivity (XTT, clonogenic assay and sub-G1 content)

To evaluate cell survival following UVA irradiation by XTT, 5×10^4 cells were plated in 35 mm plates. After 72 h, the culture medium was replaced by 500 μl of XTT solution diluted in PBS, according to the manufacturer's specifications (Roche Applied Science, Penzberg, Bayern, Germany). The cells were incubated at 37 °C to metabolize the tetrazolium for 1 h or until the controls reached an intense orange color. After this time, the formazan salt concentration was measured in the GloMax®-Multi Detection System (Promega, Madison, WI, USA) spectrophotometer at 490 nm and 750 nm. The ratio of absorbance values was used to calculate the percentage of cell survival using the non-

irradiated control sample as a reference.

For the clonogenic assay, 2×10^3 cells were plated in 60 mm diameter dishes, 16–18 h before UVA irradiation. Ten days later, the colonies were fixed with formaldehyde (10%) for 10 min and stained with crystal violet (1%, Sigma-Aldrich) for 5 min. Colonies with more than 50 cells were counted. Cell survival was calculated as the percentage of colonies formed after irradiation in relation to the non-irradiated control.

The sub-G1 content (corresponding to apoptotic cells) was assessed by flow cytometry. The cells (10^4) were harvested after UVA irradiation and stained with propidium iodide (PI), as previously described [60].

5.6. DNA damage induction (γH2AX, Slot Blot and Comet assay)

UVA-induced DNA damage was evaluated indirectly using a γH2AX antibody by flow cytometry. For the γH2AX assay, the cells (10^5) were collected 0, 24 and 72 h after UVA exposure and performed as previously described [71]. The Slot Blot assay was performed to directly detect CPDs. DNA was extracted from 10^6 cells [12], and the immunoblot assay was performed as described elsewhere [13]. The damage caused by oxidative stress was evaluated by the comet assay. A modified alkaline comet assay was performed using the formamidopyrimidine-DNA glycosylase from *E. coli* (FPG – BioLabs, Ipswich, MA, USA) [13]. For this assay, the cells (1.5×10^5) were harvested at different times after UVA irradiation, permeabilized, and incubated with 0.8 U of FPG for 30 min. The head/tail DNA ratio (tail moment) was scored from one hundred comets on each slide, analyzed using LUCIA Comet Assay™ software (Laboratory Image, Prague, Czech Republic).

5.7. Cell Cycle (Flow cytometry)

The cell cycle profiles after UVA irradiation were assessed by flow cytometry. At each timepoint, 10^5 cells were harvested from the plates, stained with PI, and analyzed as previously described [60].

5.8. Quantification of carbonylated proteins

Carbonylated proteins were measured as products of UVA-induced oxidative stress. After UVA exposure, 3×10^6 cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 20 mM NaCl, 1 mM $MgCl_2$, 0.1% SDS, protease inhibitor Cocktail Set II and III, Calbiochem, Merck, White House Station, NJ) with benzonase (0.25 U/μl, Novagen, Merck, Millipore) for 40 min, and then centrifuged for 10 min at 14,000 rpm at 4 °C. Proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein denaturation was achieved as previously described [13]. The proteins were separated with SDS polyacrylamide gel electrophoresis (Life Technologies). After the blocking step, the membranes were incubated overnight at 4 °C with DNPH antibody (1:1000, Sigma-Aldrich) followed by rabbit peroxidase (1:2000, Sigma-Aldrich) for 1 h at room temperature. The signal was detected using Luminata™ Forte Western HRP substrate (Millipore Corporation, Billerica, MA, USA) in Universal Hood III Gel Doc equipment (Bio-Rad, Hercules, CA, USA).

5.9. Host Cell Reactivation

Host cell reactivation (HCR) was performed to evaluate the DNA repair capacity of UVC-induced DNA lesions in UVA-irradiated cells. The protocol was adapted from previous work [71]. A luciferase expressing plasmid (pHIV-Luc) was irradiated with 600 and 1200 J/m^2 of UVC light using a germicide lamp. The pRenilla plasmid was used as an internal transfection control. The plasmids were co-transfected with Lipofectamine 3000 Transfection Reagent (Life Technologies) just after UVA irradiation (120 kJ/m^2). The luciferase activity was quantified after 48 h using the Dual-Glo® Luciferase Assay kit (Promega) according to the manufacturer's specifications, with detection performed in a GloMax®-Multi Detection System luminometer (Promega).

5.10. Replication fork stalling (Fiber Assay)

Replication fork progression after UVA irradiation was investigated using the DNA fiber assay, as previously described [72]. Briefly, 3×10^5 cells were plated in 35 mm dishes, and 16 h later, a pulse of 20 μM 5-chloro-2′-deoxyuridine (CldU, Sigma-Aldrich) was applied for 20 min. The cells were washed twice with PBS and then received a 5-iodo-2′-deoxyuridine pulse (IdU, 200 μM, Sigma-Aldrich) diluted in PBS during UVA irradiation (120 kJ/m^2), followed by dilution in culture medium for the remainder of the 1 h incubation. IdU incorporation was detected using mouse anti-BrdU (1:40, BD Biosciences, Franklin Lakes, NJ, USA) and the anti-mouse Alexa Fluor 594 secondary antibody. Rat anti-BrdU (1:40, Accurate Chemicals, Westbury, NY, USA) and anti-rat Alexa Fluor 488 secondary antibody were used for CldU detection. DNA fiber images were captured using fluorescence microscopy (Zeiss LSM-780 NLO, Oberkochen, Baden-Württemberg, Germany). The lengths of DNA tracts were analyzed using Zeiss LSM Image Browser software. The experiments were performed as two independent repeats, with at least 100 fibers measured for each sample.

5.11. Unscheduled DNA synthesis through EdU incorporation

Unscheduled DNA synthesis was evaluated through EdU incorporation using Click-iT® EdU Imaging Kits (Invitrogen, Waltham, MA, USA). The protocol was adapted from previous work [73] and performed as manufacturer's instructions. 6×10^5 cells were plated in 35 mm dishes on a coverslip and later irradiated with UVA light. After UVA irradiation, the cells were incubated with medium FBS-free with 10 μM of EdU for 6 h and then harvested. The glass slides were mounted with Fluoroshield™ with DAPI (Sigma-Aldrich). The images were captured by Leica DMC6200 camera (Leicam, Wetzlar, Hesse, Germany) and analyzed with CellProfiler software (www.cellprofiler.org). One hundred cells were captured from at least ten different fields. Only nuclei with 1 up to 50 foci were considered as those performing unscheduled DNA synthesis.

5.12. Quantification of mitochondrial superoxide

Mitochondrial superoxide quantification was performed using MitoSOX Red Mitochondrial Superoxide Indicator kit (Thermo Fisher). For this assay, 1.5×10^5 cells were harvested 6 h upon UVA irradiation. The cells were incubated with DMEM without red phenol (5% SBF) for 30 min in the dark. The fluorescence was measured by flow cytometry (Accuri, BD Bioscience).

5.13. Statistical Analysis

The statistical analyses were performed through GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Two-way ANOVA were used to evidence the significant difference in the experiments. Test T (followed by Tukey's Multiple comparison test) and one-way ANOVA were eventually used.

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Conflict of interest statement

None declared.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.12.012.

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