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Molecular effects of 1-naphthyl-methylcarbamate and solar radiation exposures on human melanocytes

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Abstract

Carbaryl (1-naphthyl-methylcarbamate), a broad-spectrum insecticide, has recently been associated with the development of cutaneous melanoma in an epidemiological cohort study with U.S. farm workers also exposed to ultraviolet radiation, the main etiologic factor for skin carcinogenesis. We hypothesized that carbaryl exposure may increase deleterious effects of UV solar radiation on skin melanocytes. This study aimed to characterize human melanocytes after individual or combined exposure to carbaryl (100 μ M) and solar radiation (375 mJ/cm²). In a microarray analysis, carbaryl, but not solar radiation, induced an oxidative stress response, evidenced by the upregulation of antioxidant genes, such as *Hemeoxygenase-1 (HMOX1)*, and downregulation of *Microphthalmia-associated Transcription Factor (MITF)*, the main regulator of melanocytic activity; results were confirmed by qRT-PCR. Carbaryl and solar radiation induced a gene response suggestive of DNA damage and cell cycle alteration. The expression of *CDKN1A*, *BRCA1/2* and *MDM2* genes was notably more intense in the combined treatment group, in a synergistic manner. Flow cytometry assays demonstrated S-phase cell cycle arrest, reduced apoptosis levels and faster induction of cyclobutane pyrimidine dimers (CPD) lesions in carbaryl treated groups. Our data suggests that carbaryl is genotoxic to human melanocytes, especially when associated with solar radiation.

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Supplementary data

Figure S1 shows the emission radiation spectrum of the solar simulator used in the melanocyte irradiation assays.

Figure S2 shows that the microarray gene expression profile is clustered based on treatment, and importantly, that there are great similarities between groups treated with solar radiation; between groups treated with carbaryl; and between vehicle and non-treated control groups. Moreover, there is a group of genes with an expression profile unique to samples from the combined treatment group.

Keywords

Carbaryl; 1-naphthyl-methylcarbamate; melanocytes; microarray; solar radiation; melanomagenesis

Introduction

Carbaryl (1-naphthyl methylcarbamate, CAS 63–25-2), also known by the commercial name of Sevin®, is a broad-spectrum carbamate insecticide widely used in agriculture and in the domestic environment (CALEPA, 2014). Occupational exposure is the main concern regarding this pesticide. Recently, a cohort epidemiological study with a four year follow-up of agricultural workers demonstrated a significant association between the development of cutaneous melanoma and the use of carbaryl (56 exposure days; OR = 1.7; CI 95%, 1.1–2.5; trend $p = 0.013$) (Dennis et al., 2010). Additionally, a dose-response relationship was suggested between carbamate pesticides in general and prevalence of cutaneous melanoma. No linear relationship was found between sun exposure and melanoma development in that study cohort. Another publication from the same epidemiological study, with a two-year follow-up and fewer cases of cutaneous melanoma, also has indicated increased risk of melanoma associated to carbaryl exposure (Mahajan et al., 2007).

Carbaryl genotoxicity is reported as positive in several *in vitro* assays with different human cell lines (Bigot-Lasserre et al., 2003; Delescluse et al., 2001). Carbaryl 100µM was reported to induce oxidative stress and high levels of DNA damage in human lymphoblastoid cells, and these results were confirmed in HepG2 cells; apparently, its genotoxic potential was indirect, exerted by reactive metabolites resulting from its biotransformation, as the genotoxic activity was demonstrated in CYP1A1-transfected cells, but not on the parental cell line (Delescluse et al., 2001). When exposed to carbaryl via the diet, in doses up to 4000 ppm for 180 days, heterozygous p53 knockout mice did not develop tumors and carbaryl was considered non-genotoxic (Bigot-Lasserre et al., 2003). Overall, the weight of evidence indicates that carbaryl is not an *in vivo* genotoxic agent (European Food Safety Authority, 2006).

In parallel, ultraviolet (UV) radiation is the main etiological factor to the development of skin tumors, squamous cell carcinoma, basal cell carcinoma and cutaneous melanoma, being classified as a human carcinogen group I by IARC (1992). Melanoma skin cancer is one of the fastest growing malignancies in incidence worldwide, with one of the worst prognoses (De Santis et al., 2014). It is well known that UVB radiation induces DNA damage directly in epidermal cells, leading to mutagenesis; UVA radiation induces indirect genotoxicity through reactive oxygen species (ROS) and oxidative stress, and eventually leads to mutations that can trigger the carcinogenic process (de Gruijl, 2000). Although less abundant, UVB radiation is considered to be more carcinogenic than UVA, inducing formation of cyclobutane pyrimidine dimers (CPDs) and 6,4-photoproducts (6–4PP) (Matsumura and Ananthaswamy, 2002). CPD lesions are the most difficult to be repaired and, among them, dimers formed between adjacent cytosines (C-C) or between thymine and cytosine (T-C) are considered the most mutagenic (Marrot and Meunier, 2008). UVA

radiation is an important oxidative stress inducer in epidermal cells through single and/or double DNA strand breaks and formation of 8-oxo-7,8-dihydroguanine (8-oxo-dG), which is strongly associated with cutaneous carcinogenesis due to generation of high genomic instability (von Thaler et al., 2010; Ridley et al., 2009). It is noteworthy that melanocytes are slowly proliferating cells that persist in the epidermis for decades, and therefore are exposed to high cumulative levels of UV-induced ROS. In fact, the role exerted by ROS in melanomagenesis is well characterized in the literature (Wittgen and van Kempen, 2007).

Moreover, UVA radiation has been investigated with regard to its ability to intensify the susceptibility to carcinogenesis induced by UVB or by low doses of environmental chemicals, resulting in significant synergistic scenarios (Burke and Wei, 2009). These are called photosensitization reactions, where chemicals are capable of amplifying UV deleterious effects, leading to formation of DNA adducts and/or to induction of oxidative stress and damage (Marrot and Meunier, 2008).

Although comprehensive and well designed, the epidemiological study published by Dennis et al. (2010) has certain limitations. For example, it could not accurately estimate the UV dose and the accumulated carbaryl dose to which workers were exposed. Consequently, it is insufficient to determine each factor's direct contribution in the development of melanoma tumors found in that study. Therefore, we aimed to characterize human melanocytes after individual or combined exposure to carbaryl (100 μ M) and solar radiation (375 mJ/cm²). The following parameters were analyzed: global gene expression (microarray), cell growth curve (Trypan Blue cell viability assay), genotoxicity (CPDs and 8-oxo-7,8-dihydroguanine quantification) and cell cycle evaluation. Our hypothesis is that carbaryl is associated with initiation of melanocyte transformation and that this event occurs synergistically with solar radiation.

MATERIAL AND METHODS

Cell culture

This study was approved by the Ethics Committee of the School of Pharmaceutical Sciences – University of Sao Paulo (Process n. 943/09).

Experiments were performed with human primary skin melanocytes, extracted from the foreskin of two unrelated Caucasian donors (4- and 6-years of age) as described by Pennacchi et al. (2015). Melanocytes were cultured with 254CF medium supplemented with calcium chloride 0.2 M and Human Melanocyte Growth Supplement (HMGS, Gibco, Invitrogen Cell Culture, USA), with the addition of Ampicillin and Streptomycin 100mg/L. After plating, cells were maintained in a humidified incubator at 5% CO₂ and 37°C. Cells were cultured separately and pooled with equal proportions of both donors at the time of assay plating, in an effort to increase genetic diversity.

Treatment and irradiation

Twenty-four hours after plating, cells were at 80% confluency and were subjected to the following experimental treatment groups: Group 1: No treatment; Group 2: Irradiation and

no treatment; Group 3: Carbaryl treatment; Group 4: Irradiation and carbaryl treatment; Group 5: Vehicle treatment; Group 6: Irradiation and vehicle treatment.

Treatment regimen consisted of melanocyte incubation with carbaryl 100 μM (CAS No. 63–25–2; Sigma-Aldrich, St Louis, USA) for 6, 24, 48 or 72 hours (depending on the analysis performed) after single dose exposure to 375mJ/cm² of solar radiation using a solar simulator (SS2.5kW, Sciencetech Inc., Ontario, Canada) with a global air mass filter (A.M 1.5G, Sciencetech Inc, Ontario, Canada); duration of radiation exposure was 16 seconds, with a potency of 0.9kW. For the irradiation assays, culture medium was replaced by PBS buffer without Ca²⁺ or Mg²⁺ (PBS-A). All experiments were performed in triplicates.

The solar simulator lamp profile using the AM 1.5G filter, which lets through UVB, (280–320 nm), UVA (320–400 nm), visible light (400–700 nm) and infrared (700–1000 nm) irradiations, is similar to the mean global solar radiation that reaches the surface of the earth in the USA region.

Trypan Blue viability and cell growth assays

Cells were cultured in 24-well plates, 3×10^4 cells/well, and after 24, 48 and 72 hours of treatment, cells were trypsinized, resuspended in PBS-A with 3% fetal bovine serum and incubated with Trypan Blue 0.4% (Sigma-Aldrich, St. Louis, MO, USA) for 3 minutes. Cell counting was performed in a Neubauer chamber using a light microscope after 24, 48 and 72 hours of treatment. Cell viability was assessed by cell counting excluding blue stained cells. Cell growth was assessed by summing numbers of viable and non-viable cells.

Mechanisms of cell death characterization by flow cytometry

Cells were cultured in 6-well plates, 20×10^4 cells/well and, after 24 hours of treatment, cells were trypsinized, resuspended in binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) with 3% Annexin-APC (BD Biosciences, San Jose, CA, USA) and incubated for 20 minutes at room temperature, in the dark, for labelling of apoptotic cells. Subsequently, a Propidium Iodide (PI, Life technologies, USA) solution (8 $\mu\text{g/mL}$) was added to the cell suspension for labelling of exposed DNA (necrotic cells). Cells were further analysed by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA) using FlowJo software (Tree Star Inc., Ashland, OR, USA). Cisplatin (90 μM) was used as positive control for apoptosis induction (Liu et al., 2006) and DMSO (10%) was used as positive control for necrosis induction.

Cell cycle evaluation by flow cytometry

Cells were cultured in 6-well plates, 20×10^4 cells/well, and after 48 hours of treatment, cells were trypsinized, resuspended in lysis buffer (Triton X-100 0.1%, Trisodium Citrate 0.1%) with Ribonuclease-A 10 mg/mL, and incubated for 30 minutes at 37°C. Subsequently, a PI solution (10 $\mu\text{g/mL}$) was added to the cell suspension, which was further analysed by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA) using FlowJo software (Tree Star Inc., Ashland, OR, USA). Cell cycle evaluation was performed by quantification of DNA content measured as the intensity of PI fluorescence.

8-oxo-7,8-dihydroguanine quantification

Cells were cultured in 100 mm plates, 10^6 cells/plate. Labelling with primary antibody, anti-8-Hydroxiguanine (8-oxo-dG) MAbs (2E2 Clone - RandD Systems Inc., Minneapolis, MN, USA), was performed according to the manufacturer's protocol with some adaptations. Briefly, after 1 and 24 hours of treatment, cells were trypsinized, fixed in ethanol 70% and stored at -20°C . Cells were rehydrated with PBS-A followed by incubation with Ribonuclease-A 10 mg/mL for 1 hour and incubation with HCl 1.5 M for 10 minutes. Cells were incubated with 3% bovine serum albumin (BSA) solution to reduce non-specific antibody binding, followed by 1.5-hour incubation with anti-8-oxo-dG antibody. Cells were washed with PBS-A and incubated with secondary fluorescein-labeled anti-mouse IgG antibody (FI-2000, Vector Laboratories, Burlingame, CA, USA). Analysis was performed by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA) using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cyclobutane pyrimidine dimer (CPD) quantification

Cells were cultured in 100 mm plates, 10^6 cells/plate. CPD quantification was performed according to the protocol described by Greinert et al. (2000). Briefly, after either 1 or 6 hours of treatment, cells were trypsinized, fixed in ethanol 70% and stored at -20°C . Cells were rehydrated with PBS-A followed by incubation with Triton X-100 0.5% for 10 minutes and then incubation with HCl 1.5 M for 30 minutes. Cells were incubated with 3% bovine serum albumin (BSA) solution to reduce non-specific antibody binding, followed by 1.5-hour incubation with primary anti-thymine dimer antibody (KTM53 clone - Kamiya Biomedical Company, Seattle, WA, USA). Cells were washed with PBS-A and incubated with secondary fluorescein-labeled anti-mouse IgG antibody (FI-2000, Vector Laboratories, Burlingame, CA, USA). Analysis was performed by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA) using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Microarray analysis

Cells were cultured in 100 mm plates, 10^6 cells/plate. After 6 hours of treatment, total RNA was isolated using the Qiagen RNeasy kit following the manufacturer's protocol, including the addition of DNase I. RNA quality was verified using a Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). Gene expression analysis was conducted using Agilent Whole Human Genome 4x44 multiplex format oligo arrays (014850; Agilent Technologies, Santa Clara, CA, USA) following the Agilent 1-color microarray-based gene expression analysis protocol. Cy3 labeled cRNA was produced according to the manufacturer's protocol, fragmented and hybridized for 17 hours in a rotating hybridization oven. Slides were then washed and scanned with an Agilent Scanner (Agilent technologies, Santa Clara, CA, USA). Data were obtained using the Agilent Feature Extraction software (v9.5), using the one-color defaults for all parameters, performing error modeling, and adjusting for additive and multiplicative noise. The resulting gene expression data was processed and analyzed using Partek Genomics Suite (Partek® Genomics Suite software, version 6.6 beta, Copyright © 2009, Partek Inc., St. Louis, MO, USA). One-way ANOVA ($p < 0.05$, FDR corrected) was performed in order to compare carbaryl and/or solar radiation

treated groups with the vehicle control, identifying differently expressed genes using a cut off of 1.5 fold change. Ingenuity Pathway Analysis software (IPA, Qiagen, Hilden, Germany) was used to investigate differentially affected pathways relevant to melanocyte damage induced by solar radiation and carbaryl treatment. Non-treated control and solar irradiated control (no chemical treatment) groups were not included in the pathway analyses.

Real time polymerase chain reaction validation assay

In order to validate the microarray gene expression data, we performed quantitative real-time PCR using the TaqMan® gene expression assay and Gene Expression Master Mix (Applied Biosystems, Foster City, USA), according to the manufacturer's protocol. Briefly, cDNA samples were amplified in MicroAmp Optical 96-well plates, in 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The resulting fluorescence was detected using 7500 Real-Time PCR System software (Applied Biosystems, Foster City, USA). *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) gene was used as the reaction endogenous control. Samples were analyzed by cycle threshold (Ct) comparison in relation to *GAPDH* expression, using the 2^{-Ct} formula (Livak & Schmittge, 2001). The resulting 2^{-Ct} was then converted to a base 2 logarithm (Quackenbush, 2002). TaqMan probes used were *CDKN1A* (Hs00355782_m1), *CCNE1* (Hs01026536_m1), *GADD45A* (Hs00169255_m1), *GADD45B* (Hs04188837_g1), *EXO1* (Hs01116195_m1), *BRCA1* (Hs01556193_m1), *BRCA2* (Hs00609073_m1), *MITF* (Hs01117294_m1), *MDM2* (Hs00242813_m1), *PCNA* (Hs00427214_g1), and *HMOX1* (Hs01110250_m1).

Statistical analysis

We performed Analysis of Variance (ANOVA) followed by Dunnett's test in order to evaluate differences between treated groups and the control group, and ANOVA followed by Tukey's test in order to verify which groups differ from one another ($p < 0.05$). These analyzes were performed using GraphPad Prism 5 software (GraphPad, San Diego, California, USA). Lethal concentration was determined using Statistica software (Statsoft Inc., Tulsa, OK, USA) through non-linear regression analysis (CI 95%). In order to determine if there was a synergistic association between carbaryl treatment and solar radiation with regard to the relative RT-PCR gene expressions, the Student t-test was applied considering a non-orthogonal contrast.

RESULTS

Carbaryl and solar radiation cytotoxicity

Based on Trypan Blue viability assay, the carbaryl lethal concentration of 50% (LC₅₀) in primary human melanocytes was 339 μ M, 251 μ M and 218 μ M after 24, 48 and 72 hours of treatment, respectively. The concentration of 100 μ M was selected for the subsequent assays as it allows approximately 80, 70 and 60% of viability after 24, 48 and 72 hours of treatment, respectively. Solar radiation cytotoxicity was also evaluated by the Trypan Blue assay. The 375 mJ/cm² dose was the highest dose tested that did not alter cell viability or cell morphology even with carbaryl concomitant treatment (data not shown).

Microarray data

Sample quality and number of altered genes—After removing experiment batch effect and performing ANOVA analysis, we found 5934 significantly altered genes. Using a cut off level of 1.5 fold change, we restricted this number to 1478; carbaryl treatment alone and solar radiation alone induced 284 and 459 significantly altered genes, respectively. In the combined treatment group, we found 1230 significantly altered genes, a number which corresponds to 2.68 times more genes than the sum of both individual treatments, suggesting a possible synergistic effect between the two factors.

IPA reveals DNA damage response, altered cell cycle regulation, oxidative stress and endoplasmic reticulum stress—IPA allowed investigation of alterations in biological pathways in carbaryl and/or solar radiation treated groups as compared with the DMSO-treated group (vehicle control). After performing a comparison analysis between the carbaryl alone (Carb), the UV alone (UV) and the carbaryl plus UV (Carb UV) groups with regard to significantly altered canonical pathways (Table 1), we found an important DNA damage response. This effect was evidenced by relevant canonical pathways, such as *GADD45 (Growth arrest DNA damage-induced genes) signaling* and *Molecular mechanisms of carcinogenesis*, which presented higher statistical strength (lower *p*-values) in the combined treatment group, when compared to individual exposures to Carbaryl and solar radiation. We also found a number of genes with altered expression directly related to DNA repair, such as the *Breast Cancer 1 and 2 (BRCA1 and BRCA2)*, *Exonuclease-1 (EXO1)* and *Proliferating Cell Nuclear Antigen (PCNA)* genes.

IPA showed canonical pathways related to cell cycle regulation, e.g. *Estrogen-mediated S phase entry* and *Cell cycle: G1/S Checkpoint regulation*, presenting higher statistical strength (lower *p*-values) in the combined treatment group than in the individual treatment groups. These pathways revealed an overall imbalance in cell cycle regulation. Genes found to be significantly differentially expressed in these pathways included *v-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC)*, *Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A or p21)*, *Cyclin E1 (CCNE1)*, *Cell Division Cycle 25A (CDC25A)*, and the E2F transcription factors - *E2F2*, *E2F5*, *E2F7* and *E2F8*.

Moreover, the oxidative stress pathway *NRF2-mediated oxidative stress response* was found to be significantly altered in carbaryl treated groups. Most upregulated genes in this pathway encode for Heat Shock Proteins (HSP), i.e. *hemeoxygenase-1 (HMOX1 or HSP32)*, *HSP40 (DNAJC3, DNAJB9, DNAJB4)* and *HSP70 (HSPA5)*, in addition to genes involved with glutathione metabolism, i.e. *glutamate-cysteine ligase modifier subunit (GCLM)* and *glutathione peroxidase 5 (GPX5)* and the downregulated *FOS (FBJ murine osteosarcoma viral oncogene homolog)* gene. Furthermore, this pathway includes the upregulated *HERPUD* gene, which, in addition to HSPA5, is also present in the endoplasmic reticulum stress pathways, namely *unfolded protein response* and *endoplasmic reticulum stress pathway*.

qRT-PCR validation confirms microarray data and reveals evidence of synergistic and additive effects between Carbaryl and solar radiation

In order to verify the microarray data, we performed qRT-PCR for genes we considered critical for the evaluation of carbaryl and solar radiation effects. The relative expression of these genes are shown in Figure 1. The microarray data was consistent with qRT-PCR for most genes evaluated. We found an additive effect between carbaryl and solar radiation in relation to *CCNE1* and *GADD45B* genes, and a synergistic effect in relation to *BRCA1*, *BRCA2*, *CDKN1A* and *MDM2* genes. Upregulation of *PCNA*, *GADD45A* and *EXO1* genes appears to be exclusive to solar radiation, once there is no statistical difference between the solar radiation-exposed groups. Likewise, downregulation of *Mitf* and upregulation of *HMOX1* are exclusive to carbaryl treatment, once there is no significant difference between carbaryl treated groups. Thus, we confirmed an accentuated response in the combined treatment group in relation to some DNA damage response genes. Solar radiation did not induce an oxidative stress response.

Carbaryl induces cell cycle arrest at the S phase especially when combined with solar radiation

Table 2 shows that, at 24 hours of treatment, there are no significant differences between treatment groups. At 48 hours of treatment, in the group treated with carbaryl and solar radiation, there was a significant reduction in the percentage of cells in G0/G1 phase and a significant increase in S phase, while the percentage of G2 phase cells remained unaltered. These results demonstrate that cells in this experimental group appear to be either delayed or arrested in S phase. In the carbaryl-treated group, there is also an increase in the percentage of cells in S phase. At 72 hours, the decrease in the number of G0/G1 cells is maintained in the combined treatment group and, although not significantly altered, there is a slight increase in S phase cells. Results suggest that cell cycle arrest was more pronounced in the combined treatment group.

Carbaryl induces cell growth inhibition after 24, 48 and 72 hours of treatment

Figure 2 shows the cell growth curve of human melanocytes treated with carbaryl and/or solar radiation analyzed by the Trypan Blue assay. It is possible to observe that in all experimental groups there is a linear cell growth over time, with the exception of carbaryl treated groups, where the total number of cells remains almost unaltered. The reduction in the cell proliferation rate was significant. In the UV treated group, there is a significant reduction in the number of cells at 48 hours of treatment; however, cell proliferation clearly recovers at 72 hours of treatment in this group. It can be inferred that the increase in S phase cells in carbaryl treated groups is not related to a putative increase in the cell proliferation rate, confirming that the insecticide does cause cell cycle arrest.

Carbaryl inhibits apoptosis after 24 hours of treatment

In order to evaluate differences in the mechanisms of cell death induction between treatments, we performed a flow cytometry assay for the detection of phosphatidylserine exposure by Annexin V labelling. In figure 3, it is possible to note a significant reduction in the number of cells labelled for Annexin V only and cells labelled both for Annexin V and

PI. Results suggest there is a reduction in apoptosis and late apoptosis levels in carbaryl treated groups, but not in the group treated with solar radiation only, where there was an increase in the number of necrotic cells.

The absence of significant levels of apoptotic cell death in the group treated with solar radiation only is in agreement with data published by Marrot et al. (2005). Those authors demonstrated that Caucasian human melanocytes present limited cell death, mainly by apoptosis, even when exposed to high doses of solar radiation (1200 mJ/cm² UVB and 11000 mJ/cm² UVA). Apoptosis inhibition in carbaryl treated groups is surprising, considering the unaltered levels of necrotic cell death, the induction of S-phase cell cycle arrest and the inhibition of proliferation.

UV-induced CPD lesions are formed faster in the presence of Carbaryl

The CPD detection assay is an important tool for evaluating genotoxicity in epithelial cells, as these lesions are highly mutagenic and typically induced by UVB radiation (Marrot et al., 2010). After 1 hour of treatment, there is an increase in the amount of CPDs in the carbaryl and solar radiation combined treatment group and, after 6 hours of treatment, there is an equal response between the combined treatment group and the solar radiation group (Figure 4). These results suggest that lesions are formed faster in the first group. Greinert et al. (2000) irradiated keratinocytes with UVB 20 mJ/cm², which is close to the dose used in our experiments, if we consider that UVB corresponds to 5% of the solar radiation. Those authors detected CPD lesions immediately after irradiation, whereas 40% of lesions were repaired after 6 hours and the lowest levels of CPDs were detected after 50 hours. Hence, we considered it was reasonable to evaluate the presence of CPDs 1 and 6 hours after treatment with carbaryl and solar radiation. Although these lesions are typically induced by UVB radiation, it is interesting to note the significant increase in the group treated with carbaryl and solar radiation after 1 hour-treatment, indicating that the insecticide facilitates the formation of DNA dimers.

Carbaryl treatment induces a trending increase in 8-oxo-dG formation

In Figure 5, it is possible to observe that, after 1 hour of treatment, there are no differences in the detection of 8-oxo-dG lesions. After 24 hours, there is a non-significant increase in the amount of 8-oxo-dG lesions in the group concomitantly treated with carbaryl and solar radiation, which is consistent with oxidative stress induction in this experimental group. Although these lesions are typically induced by UVA radiation, they were not detected in the group treated with solar radiation only. The choice of treatment durations of 1 and 24 hours for the detection of oxidative lesions were based on previous studies from the literature that used a similar cell culture experimental design (Zhang et al., 2004; Zhou et al., 2012).

DISCUSSION

The solar radiation dose of 375 mJ/cm² used to irradiate human melanocytes in this study was considered subtoxic, since it did not induce an oxidative stress response measured by the expression of *HMOX1*, which is expected to be upregulated in human melanocytes after higher doses of UV radiation (Marrot et al., 2008). Although subtoxic, the solar radiation

dose used in this study was effective in eliciting a robust biological response, as evidenced by the UV signature response found in all irradiated groups, independently of concomitant chemical treatment. This response included a significant upregulation of *CDKN1A* and *PCNA* genes, which are known to be part of the cellular response to UV radiation (Fotedar et al., 2004). The protein encoded by the *PCNA* gene is necessary for DNA repair by nucleotide excision (NER), which is the main repair mechanism of radiation-induced lesions, such as CPDs (Wood, 1996). Likewise, we found that *EXO1* was induced by solar radiation, and this gene is known to be involved in NER in the DNA damage response to UV radiation (Sertic et al., 2011). On the other hand, carbaryl treatment induced oxidative stress response, cell cycle arrest and apoptosis inhibition when administered alone or in combination with solar radiation, and may result in the induction of DNA lesions in human melanocytes even in the absence of coadjuvant factors. The combined exposure to carbaryl and solar radiation was found to accentuate some carbaryl-induced responses, such as significant increase in *BRCA1* and *BRCA2* gene expression.

In this study, we tested the hypothesis that there is a synergistic effect between carbaryl and solar radiation exposures. We found a synergistic effect with regard to *BRCA1*, *BRCA2*, *CDKN1A* and *MDM2* gene expression, as well as an overall transcriptomic response as evidenced by an increase in the total number of significantly altered genes in the microarray. Regarding *CCNE1* and *GADD45B* gene expression, we found an apparently additive effect. Considering all other parameters analyzed, carbaryl treatment effects were not significantly exacerbated by solar radiation, or vice-versa. Hence, it was not possible to unequivocally confirm a photosensitization reaction in melanocytes from the combined treatment group.

Carbaryl-induced S-phase cell cycle arrest, especially when combined with solar radiation, seems to be closely related to the activation of DNA repair system evidenced by a significant upregulation of genes closely related to DNA double strand breaks, i.e. *BRCA1* and *BRCA2* (Roy et al., 2011), which were less intensely upregulated in the groups singly treated with carbaryl or solar radiation. Downregulation of *GADD45B* by treatment with carbaryl alone or in combination with solar radiation can be associated with a deficient cell cycle arrest and impairment of DNA repair (Higgs et al., 2010). Upregulation of *GADD45A* in melanoma cells results in inhibition of apoptotic cell death (Fayolle et al., 2008), thus this gene could be associated with inhibition of apoptosis in the carbaryl treated melanocytes. Upregulation of *MDM2* is consistent with cell cycle arrest, mainly in the combined treatment group, reflective of its role in the induction of S-phase cell cycle arrest and checkpoint activation in the presence of genotoxic stimuli (Deb et al., 2014). Moreover, a number of altered genes from the qRT-PCR and the microarray data corroborates with a G1-S transition in the carbaryl treated groups, e.g. upregulation of *CCNE1* (Möröy and Geisen, 2004); downregulation of *MYC* (Bretones et al., 2015); upregulation of the transcription factors *E2F2* and *E2F7*, and downregulation of *E2F5* (Lammens et al., 2009); and upregulation of *USP37* (*ubiquitin specific peptidase 37*) (Huang et al., 2011).

Our results are in agreement with data from the literature that have demonstrated carbaryl oxidant activity (Delescluse et al., 2001). In the carbaryl treated groups in this study, oxidative stress induction was evidenced by the increased expression of important antioxidant genes, such as *HMOX1*, *Heat Shock Protein* and glutathione metabolism related

genes, suggesting activation of the Nrf2 antioxidant pathway. In fact, Jian et al. (2011) reported that *HMOX1* gene expression protects melanocytes from cell death induced by oxidative stress through this antioxidant pathway. Marrot et al. (2007) reported that activation of the Nrf2 pathway led to upregulation of *HMOX1* and *GCLM* genes in human melanocytes. Curiously, those authors demonstrated that UVA radiation alone was more efficient in the activation of the Nrf2 pathway than total solar radiation. Subsequently, Kokot et al. (2009) showed that low dose UVB radiation (10 mJ/cm²) was capable of suppressing the Nrf2 pathway, as well as the activation of the *HMOX1* gene in melanocytes *in vitro*. This mechanism could explain the absence of detectable levels of oxidative stress in the group treated with solar radiation only, while it is remarkable that in combination with carbaryl, UVB radiation was not sufficient to suppress *HMOX1* expression. In addition, downregulation of *FOS* gene in the carbaryl treated groups, which was found in the microarray data, is also consistent with Nrf2 activation, as the c-fos protein is capable of negatively regulating antioxidant response elements in order to balance the expression of antioxidant enzymes (Jaiswal, 2004).

Regarding glutathione metabolism genes, *GCLM* is one of the key-elements for the synthesis of this protein, whose antioxidant function is highly dependent on reactions catalyzed by glutathione peroxidases (Lu, 2013). Jung and Kwak (2010) demonstrated that the antioxidant activity of glutathione peroxidases occurs through direct neutralization of ROS, and their deficiency in knock-out mice induces endothelial and cardiomyocytes abnormalities due to high levels of oxidative stress. Thus, upregulation of *GCLM* and the peroxidase *GPX5* found in the microarray data in carbaryl treated groups corroborates with carbaryl induced oxidative stress.

With regard to HSP genes, our data are once again in agreement with Delescluse et al. (2001), which also reported significant upregulation of *HSP70* induced by carbaryl insecticide (124 µM) in human lymphoblastoid cells. A review published by Jonak et al. (2009) described that upregulation of *HSP70*, which is not constitutively expressed in melanocytes, protects epidermal cells from death induced by UVB radiation, suggesting a mechanism for cell death inhibition in carbaryl treated groups. Moreover, HSP40 was reported to be a chaperone of HSP70, hindering its degradation and enabling resistance to oxidative stress-induced cytotoxicity (Kim et al., 2008).

Furthermore, in relation to melanocyte activity, the *MITF* gene is the most important to be considered. This transcription factor is the main regulator of melanocytic proliferation and differentiation, and it is responsible for a great variety of functions, such as DNA damage inhibition, differentiation and proliferation (Levy et al., 2006; Steingrímsson et al., 2004). *MITF* can also regulate genes associated with cell cycle control, such as *CDK2* (*cyclin dependent kinase 2*), *CDKN1A* (*cyclin-dependent kinase inhibitor 1A*) and *CDKN2A* (*cyclin-dependent kinase inhibitor 2A*), and genes associated with cell survival, such as *BCL2* (*B cell CLL/lymphoma 2*) and *HIF1A* (*hypoxia inducible factor 1, alpha subunit*) (Cheli et al., 2009). The decrease in *MITF* expression in carbaryl treated groups is in agreement with carbaryl-induced oxidative stress, as this gene has already been reported as downregulated after ROS induction in melanocytes (Jiménez-Cervantes et al., 2001). Moreover, several studies have related MITF activity to melanocytes homeostasis and

melanomagenesis (Goding, 2013; Wellbrock & Arozarena, 2015). Although MITF alone cannot be responsible for malignant transformation of melanocytes, it may contribute to various tumor-related pathways, such as beta catenin translocation (Delmas et al., 2007), E-cadherin expression (Kim et al., 2013) and BRAF pathway activation (Wellbrock & Arozarena, 2015). In melanoma cells, downregulation of *MITF*, together with cell cycle arrest, is indicative of tumor progression (Goding, 2013; Chapman et al., 2013; Ohanna et al., 2013). Thus, we can suggest *MITF* downregulation by carbaryl treatment may be related to tumorigenesis.

Detection of CPD lesions and 8-oxo-dG oxidative lesions evaluated by flow cytometry are extremely important in the context of carbaryl genotoxicity evaluation, as they are potentially mutagenic DNA lesions. CPD lesions, more specifically thymine dimers, were increased in the group concomitantly treated with carbaryl and solar radiation after 1 hour of treatment, indicating that these lesions are formed faster than in the group treated with solar radiation only. Carbaryl induced an apparent increase in the 8-oxo-dG lesions that is consistent with oxidative stress, although it was not statistically significant.

Results presented herein strongly indicate that carbaryl is an inductor of oxidative stress, potentially genotoxic and a carcinogenesis-initiating agent. In fact, ROS can be involved in all steps of carcinogenesis, including initiation, which requires the induction of non-repaired DNA damage and subsequent fixation of mutation (Valko et al., 2006). It is noteworthy that ROS can also cause DNA double strand breaks and oxidatively generated clustered DNA lesions (OCDLs), which are more complex lesions and represent great challenge for the repair system (Kryston et al., 2011). These lesions were not evaluated in this study, but they would be consistent with cell cycle arrest, autophagy induction and induction of repair genes such as, *BRCA1* and *BRCA2*.

The purpose of this study was to investigate the effects of acute exposure to carbaryl and solar radiation. Overall, data obtained from the different parameters analyzed are consistent and suggest that carbaryl treatment induces oxidative stress and DNA damage in human melanocytes treated for 6 hours, indicating that this exposure may be related to melanomagenesis. In the context of agricultural workers, exposure to the insecticide and to the sun can occur frequently, and at much higher doses than the ones used in this study. Considering the results presented in this study, chronic exposure to these two factors is potentially genotoxic and carcinogenic. As previously discussed, UV radiation can act both as a carcinogenesis initiating and promoting agent. Thus, it is probable that exposure of agricultural workers to this insecticide and, commonly, to solar radiation is potentially associated to the increase in cutaneous melanoma incidence. Additional studies should investigate induction of melanocyte transformation by carbaryl after long-term exposure and reevaluate the risk of cutaneous melanoma development associated to this pesticide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 –.

Relative gene expression validation by qRT-PCR – comparison between microarray and qRT-PCR data. Data generated in experimental triplicate, analyzed by ANOVA followed by Dunnett's test to evaluate differences between treated groups and the control group, and ANOVA followed by Tukey's test to verify which groups differ from one another (* $p < 0.05$, ** $p < 0.001$). Results are expressed as fold change (mean $\pm$ standard deviation) relative to DMSO treatment (vehicle control). Groups identified by the same letter do not differ significantly. Circled asterisks indicate a synergistic response evaluated by Student t-test for the non-orthogonal contrast ($p < 0.001$), between Carbaryl and UV radiation treatments. UV – treated with DMSO 0.07% and 375 mJ/cm² solar radiation; Carb UV – treated with carbaryl 100 μ M and 375 mJ/cm² solar radiation; Carb – treated with carbaryl 100 μ M; DMSO – treated with DMSO 0.07%.

Figure 2 –.

Cell growth evaluation - cell counting after 24, 48 and 72 hours of treatment. Data was generated in triplicate, analyzed by Data generated in experimental triplicate, analyzed by ANOVA followed by Dunnett's test to evaluate differences between treated groups and the control group (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$). DMSO UV – treated with DMSO 0.07% and irradiated with solar radiation 375 mJ/cm²; Carb UV – treated with carbaryl 100 μ M and irradiated with solar radiation 375 mJ/cm²; Carb – treated with carbaryl 100 μ M; DMSO – treated with DMSO 0.07%; and Ctrl – non-treated group.

Figure 3 –.

Evaluation of cell death mechanisms by flow cytometry analysis – human melanocytes treated for 24 hours. Data was generated in triplicate, analyzed by ANOVA followed by Tukey's test, and compared to the DMSO control group (* $p < 0.05$, ** $p < 0.001$). DMSO UV – treated with DMSO 0.07% and irradiated with solar radiation 375 mJ/cm²; Carb UV – treated with carbaryl 100 μ M and irradiated with solar radiation 375 mJ/cm²; Carb – treated with carbaryl 100 μ M; DMSO – treated with DMSO 0.07%; and Ctrl – non-treated group.

Figure 4 –.

Quantification of cyclobutane pyrimidine dimers (CPDs): fluorescence absorbance median analyzed by flow cytometry after 1 and 6 hours of treatment. Data was generated in triplicate, analyzed by ANOVA followed by the Tukey's test, and compared to the DMSO control group (* $p < 0.05$, ** $p < 0.005$). DMSO UV – treated with DMSO 0.07% and irradiated with solar radiation 375 mJ/cm²; Carb UV – treated with carbaryl 100 μ M and irradiated with solar radiation 375 mJ/cm²; Carb – treated with carbaryl 100 μ M; DMSO – treated with DMSO 0.07%; and Ctrl – non-treated group.

Figure 5 –.

Quantification of 8-oxo-dG: fluorescence median absorbance analyzed by flow cytometry after 1 and 24 hours of treatment. Data was generated in triplicate, analyzed by ANOVA followed by the Tukey's test, and compared to the DMSO control group. DMSO UV – treated with DMSO 0.07% and irradiated with solar radiation 375 mJ/cm²; Carb UV – treated with carbaryl 100 µM and irradiated with solar radiation 375 mJ/cm²; Carb – treated with carbaryl 100 µM; DMSO – treated with DMSO 0.07%; and Ctrl – non-treated group.

Table 1 -

Significantly altered canonical pathways and the related altered genes in the different treatment groups.

Canonical Pathway	Treatment group		
	Carb	Carb UV	UV
Estrogen-mediated S-phase entry			
p-value	4.1e-2	1.55e-5	9.31e-2
Altered genes (mean fold change)	E2F5 (-1.98) ESR2 (-1.53)	CCNE2 (+2.32) CDC25A (+1.82) CDKN1A (+1.62) E2F2 (+2.20) E2F5 (-2.58) ESR1 (-2.06) ESR2 (-2.02) MYC (-1.65)	CDKN1A (+1.45) E2F2 (+2.07)
NRF2-mediated oxidative stress response			
p-value	1.66e-3	1.41e-1	2.76e-1
Altered genes (mean fold change)	DNAJB4 (+1.86) DNAJB9 (+2.37) DNAJC3 (+1.56) ENC1 (-1.77) FOS (-1.81) GCLM (+2.18) HERPUD1 (+1.72) HMOX1 (+2.46)	DNAJB4 (+1.89) DNAJB9 (+2.02) ENC1 (-2.07) FOS (-1.95) GCLM (+2.26) HMOX1 (+2.58) MAP3K5 (-2.68) PRKCA (-1.85) PRKCB (-1.55) PRKCE (-1.98) PRKCH (-1.69) PRKD1 (-1.73)	MAP3K5 (-1.68) PRKCA (-1.73) PRKCE (-1.82) PRKCH (-1.55) PRKD1 (-1.64)
GADD45 Signaling			
p-value	2.46E-1	3.38e-3	3.63e-1
Altered genes (mean fold change)	GADD45B (-2.21)	BRCA1 (+1.28) CCNE2 (+2.32) CDKN1A (+1.62) GADD45B (-1.67) PCNA (+1.62)	CDKN1A (+1.45)
Cell Cycle: G1/S Checkpoint Regulation			
p-value	5.59e-2	1.56e-3	1.5e-1
Altered genes (mean fold change)	E2F5 (-1.98) FOXO1 (-1.64) TGFB2 (+2.87)	CCNE2 (+2.32) CDC25A (+1.82) CDK6 (-1.88) CDKN1A (+1.62) E2F2 (+2.20) E2F5 (-2.58) HDAC9 (-2.95) MDM2 (+1.77) MYC (-1.65) TGFB2 (+2.25)	CDK6 (-1.79) CDKN1A (+1.45) E2F2 (+2.07)
Endoplasmic reticulum stress pathway			
p-value	3.42e-3	3.34e-1	3.93e-1
Altered genes (mean fold change)	DNAJC3 (+1.56) HSPA5 (+1.52) XBP1 (+1.63)	MAP3K5 (-2.68) XBP1 (+1.55)	MAP3K5 (-1.68)
Molecular mechanisms of carcinogenesis			
p-value	2.95e-1	4.26e-3	1.31e-2
Altered genes (mean fold change)	BCL2L11 (-1.41) E2F5 (-1.98) FOS (-1.81) FOXO1 (-1.64)	ARHGEF3 (+1.71) BBC3 (+1.66) BMPRI1B (-1.82) BRCA1 (+1.28)	BCL2L11 (-1.55) BMPRI1B (-1.99) CAMK2D (-1.67) CDK6 (-1.79)

Canonical Pathway	Treatment group		
	Carb	Carb UV	UV
	TGFB2 (+2.87) WNT6 (−1.50)	CCNE2 (+2.32) CDC25A (+1.82) CDK6 (−1.88) CDKN1A (+1.62) E2F2 (+2.20) E2F5 (−2.58) FNBP1 (−1.16) FOS (−1.95) FZD8 (+1.56) GNAQ (−1.28) HHAT (−1.64) ITGA2 (−2.48) MAP3K5 (−2.68) MDM2 (+1.77) MYC (−1.65) PRKAR2B (−1.53) PRKCA (−1.85) PRKCB (−1.55) PRKCE (−1.98) PRKCH (−1.69) PRKD (−1.73) TCF4 (−2.28) TGFB2 (+2.25) WNT6 (−1.83)	CDKN1A (+1.45) E2F2 (+2.07) GNAQ (−1.15) HHAT (−1.57) MAP3K5 (−1.68) PRKCA (−1.73) PRKCE (−1.82) PRKCH (−1.55) PRKD1 (−1.64) TCF4 (−2.03)
Unfolded protein response			
p-value	6.81e−5	1.32e−1	3.11e−1
Altered genes (fold change)	CEBPA (−1.71) DNAJB9 (+2.37) DNAJC3 (+1.56) HSPA5 (+1.52) HSPA6 (−1.65) XBP1 (+1.63)	DNAJB9 (+2.02) HSPA6 (−1.54) MAP3K5 (−2.68) PPARG (−1.75) XBP1 (+1.55)	CEBPA (+1.53) MAP3K5 (−1.68)
Cyclins and cell cycle regulation			
p-value	2.64e−1	3.68e−2	2.14e−1
Altered genes (mean fold change)	E2F5 (−1.98) TGFB2 (+2.87)	CCNE2 (+2.32) CDC25A (+1.82) CDK6 (−1.88) CDKN1A (+1.62) E2F2 (+2.20) E2F5 (−2.58) HDAC9 (−2.95) TGFB2 (+2.25)	CDK6 (−1.79) CDKN1A (+1.45) E2F2 (+2.07)
Other significantly altered genes			
	GPX5 (+2.14)	BRCA2 (+1.68) E2F7 (+1.54) E2F8 (+1.80) EXO1 (+1.96) GPX5 (+2.50) MITF (−1.64)	E2F8 (+1.80) EXO1 (+1.59)

p-values indicate the statistical significance of the altered canonical pathway for each treatment group in comparison to the vehicle control (DMSO).

Table 2 –

Evaluation of cell cycle phase at different time-points.

Time of treatment (h)	Treatment Group	Cell count (%)			
		G0/G1 phase	S phase	G2 phase	Necrosis
24	DMSO	56.93 ± 12.59	12.25 ± 5.02	25.80 ± 10.80	3.52 ± 3.17
	Carb	52.33 ± 12.07	18.15 ± 2.52	20.83 ± 0.33	6.62 ± 7.83
	Carb UV	43.52 ± 12.52	23.13 ± 10.18	26.30 ± 4.67	3.99 ± 4.01
	DMSO UV	60.63 ± 0.66	9.58 ± 0.49	25.52 ± 3.18	2.99 ± 2.83
48	DMSO	56.72 ± 5.83	11.06 ± 2.77	29.35 ± 5.03	1.96 ± 1.38
	Carb	49.68 ± 5.35	16.33 ± 2.02*	28.85 ± 7.66	4.09 ± 5.73
	Carb UV	45.76 ± 7.88*	16.65 ± 4.27*	33.70 ± 8.16	3.11 ± 3.58
	DMSO UV	52.99 ± 6.36	11.77 ± 1.68	31.41 ± 6.86	2.81 ± 3.09
72	DMSO	61.12 ± 3.36	10.35 ± 3.85	24.52 ± 5.42	2.42 ± 2.00
	Carb	54.14 ± 4.00	17.20 ± 6.24	25.56 ± 3.79	1.59 ± 0.25
	Carb UV	49.31 ± 6.90*	19.38 ± 8.26	27.03 ± 2.39	2.44 ± 1.14
	DMSO UV	59.40 ± 1.75	11.64 ± 2.72	24.83 ± 1.35	2.32 ± 2.36

Data from 24h, 48h and 72h were generated in experimental duplicates, quintuplicates and triplicates, respectively. No difference was observed between non-treated control group and the vehicle control (data not shown). 48h and 72h data were analyzed by ANOVA followed by Dunnett's test to evaluate differences between treated groups and the vehicle control group (*p<0.05). Results are expressed as mean ± standard deviation.

DMSO UV – treated with DMSO 0.07% and 375 mJ/cm² solar radiation; Carb UV – treated with carbaryl 100 µM and 375 mJ/cm² solar radiation; Carb – treated with carbaryl 100 µM; DMSO – treated with DMSO 0.07%.