

EFFECT OF NOVOBIOCIN ON REPAIR OF HYDROGEN
PEROXIDE AND UV-INDUCED DNA DAMAGE IN
TUMOUR CELL LINES BY MODIFIED COMET ASSAY

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Abstract

In the present study, a modified comet assay for treatment and extended periods of post-incubation of cells embedded in agarose on microscope slides was used. The human prostate adenocarcinoma PC3 cell line and colon adenocarcinoma HCT116 cell line were used. The survival of PC3 cells in these conditions was evaluated up to 48 h post-incubation after treatment, showing high percentage of viable cells and proliferative activity. The repair of DNA strand breaks induced by hydrogen peroxide and UV light in PC3 and HCT116 cell lines was evaluated for periods of 1 h and 24 h of post-incubation in complete culture medium. The effect of the DNA Polymerase theta inhibitor novobiocin was also investigated. The PC3 cell line showed decrease in the induced DNA strand breaks at 24 h of post-incubation for both agents, indicating DNA repair process. In the HCT116 cell line, a similar decrease in DNA strand breaks was observed only in absence of novobiocin in the post-incubation medium. This could suggest involvement of DNA Polymerase theta in the repair process of the HCT116 cell line. Our results indicated the utility of the modified comet assay to study the effect of DNA repair inhibitors on the action of chemotherapeutic agents.

Key words: comet assay, DNA repair, hydrogen peroxide, UV light, novobiocin, tumour cell lines

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Introduction. Chemotherapy approaches include use of DNA-damaging agents, which induce various types of lesions, such as base modifications, single-strand and double-strand breaks. The combined administration of chemotherapeutics and DNA repair inhibitors can improve the therapeutic index in the treatment of cancer [1]. The comet assay is a highly sensitive and rapid quantitative technique used to detect DNA damage and repair. The assay allows direct measurement of DNA strand breaks in eukaryotic cells, based on the amount of DNA migrating from the cell nucleus during electrophoresis, forming a characteristic comet-like image. This method is widely used to measure DNA damage in acute and chronic exposure to genotoxic agents, as well as in studies with human blood samples [2].

To assess the kinetics of DNA repair (decrease in the level of DNA breaks) by comet assay, new approaches are also being applied, in which cells are treated in vitro, after embedding in low-melting agarose and spreading on microscope slide. Following the treatment, the slides are incubated in a complete culture medium without a DNA damaging agent for 1–3 h in order to permit DNA repair. This modified method was used successfully to study the repair capacity in peripheral blood lymphocytes of breast cancer patients [3] and doxorubicin treated male Wistar Kyoto rats [4]. Alternatively, the cells can be treated with a damaging agent under standard conditions and allowed to repair DNA damage after being embedded in a gel and placed in a humid chamber [5]. In these studies, the decrease in DNA strand breaks is examined for shorter time intervals, up to 3 h after DNA damage. In the present study, we aimed to extend the incubation time and evaluate the possibilities to apply this approach for in vitro studies of chemotherapeutic agents in combination with DNA repair inhibitors. The advantages of the new approach are the possibility for observation upon longer periods, shortening the analysis time and greater reproducibility of the experimental conditions.

Material and methods. Cell culture. Human cell lines from prostate adenocarcinoma (PC3) and colorectal carcinoma (HCT116) were cultivated under standard conditions in DMEM medium with 10% foetal bovine serum and antibiotics, in a humidified atmosphere with 5% CO₂ at 37 °C, to approximately 90% confluency. For the experiments, the cells were detached by trypsinization and resuspended in complete DMEM medium to a density of 1×10^6 cells/ml.

Modified comet assay. In the present work, a modified comet assay was used to study the effect of DNA Polymerase theta (POLQ) inhibitor novobiocin (NVB) on DNA repair after treatment with two DNA-damaging agents (H₂O₂ and UV light) in PC3 and HCT116 cell lines. Briefly, the cell suspension was mixed with 0.7% low-melting agarose (LMP) and spread on microscope slides. After solidification, the slides with encapsulated cells were treated for 5 min with 60 μ M H₂O₂ or exposed to 20 J/m² (at rate 8.3 J/(m²/s)) UV light, on ice. Control slides were also included. Then the slides were incubated at 37 °C and 5% CO₂ in complete culture medium, with or without 100 μ M NVB for 1 h and

24 h. After the post-incubation, the slides were placed in lysis buffer and further processed as in the alkaline comet assay (SINGH et al. [6]). Silver nitrate staining was performed according to NADIN et al. [7]. The cell viability was assessed for a period of 1–48 h. For viability evaluation, the slides were incubated for 5 min in 0.5% methylene blue solution, and then washed with distilled H₂O. The slides were photographed on a Carl Zeiss Jena 424320 microscope with a 14MP HDMI digital camera. The free software, Comet Score (<http://rexhoover.com/>) was used to measure the comet parameters per 100 cells. The results are presented as “Olive Tail Moment” (OTM – % DNA in the tail multiplied by the length between the centres of the head and tail). Evaluation of the comets was also done by visual scoring of 100 cells per sample [4].

Statistical analysis. Data are presented as mean $\pm$ SD from 100–200 cells per sample. The treatments were compared using One-way ANOVA followed by Tukey’s test, with $p < 0.05$ being considered the lowest level of statistically significant difference (GraphPad Prism 8.1 software package).

Results. In the present study, a modification of the comet assay was tested

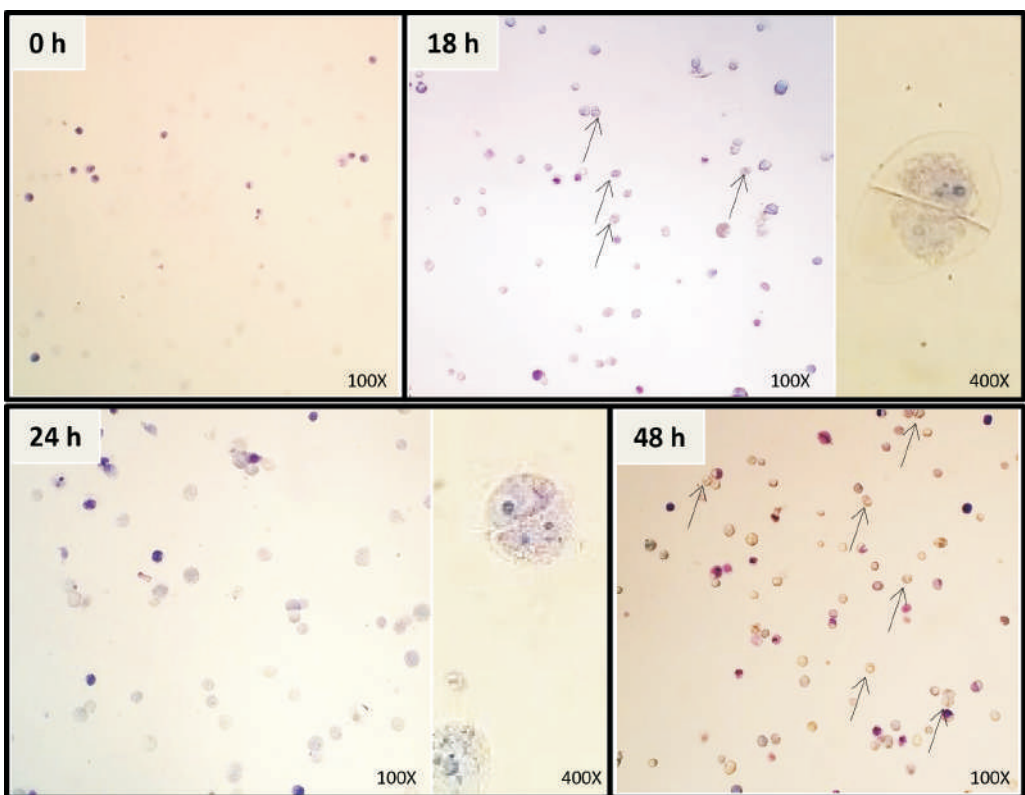


Fig. 1. PC3 cells encapsulated in agarose gel and incubated for 0, 18, 24 and 48 h in complete medium, observed at 100 $\times$ and 400 $\times$ magnification

to assess the DNA damage and repair after exposure to DNA damaging agents and post-incubation for longer periods of time (24 h). First, it was necessary to check whether the cells survive in the experimental conditions for 24–48 h. As can be seen in Fig. 1, high percentage of PC3 cells embedded in agarose are still alive (not stained in blue) at 48 h post-incubation in complete medium. Moreover, characteristic images of recently divided cells are clearly visible after 18 and 48 h of incubation, indicated by arrows (Fig. 1, and detail).

The observed DNA strand breaks after 24 h of post-incubation are significantly lower, in relation to the observed after 1 h in PC3 cell line, for both genotoxic agents (Fig. 2A). Also, the level of strand breaks at 24 h is similar to the control levels, indicating efficient DNA repair. No increase in the OTM was found in control cells incubated for up to 48 h in complete medium. The representative comet images were shown in Fig. 2C. The presence of NVB dur-

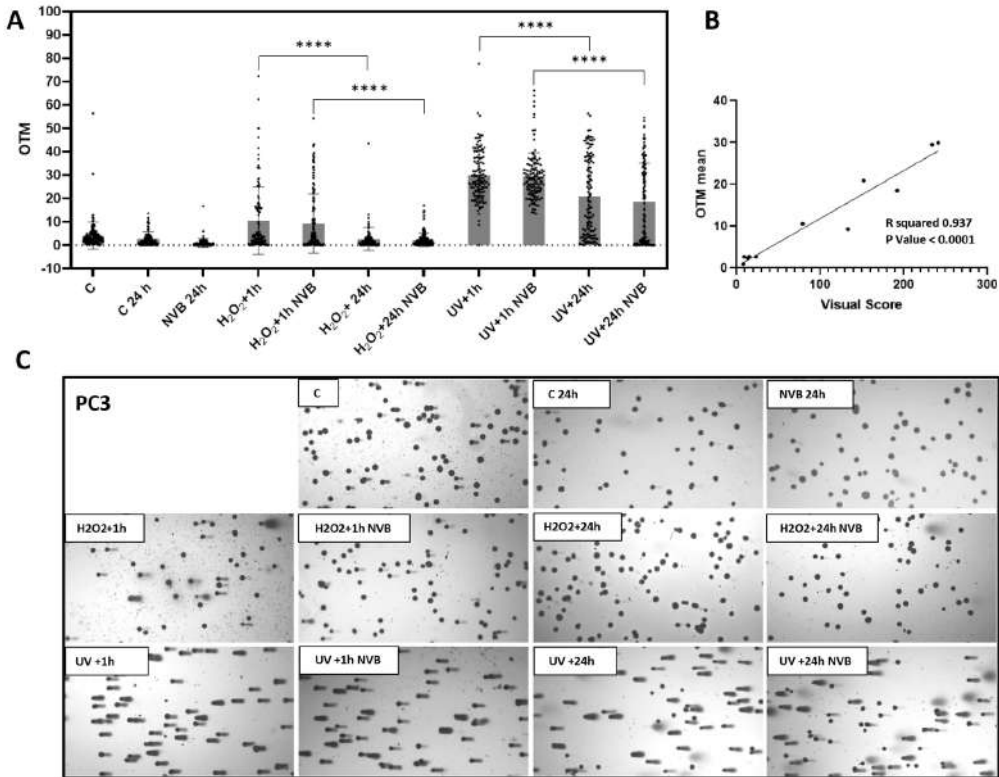


Fig. 2. A) DNA damage in the modified comet assay of PC3 cells treated with 60 μM H₂O₂ for 5 min or irradiated with 20 J/m² UV light. The recovery times after irradiation are indicated. Data are presented as mean $\pm$ SD from measurements of 100 cells. Significantly different compared with one-way ANOVA followed by Tukey's test, * p < 0.05, *** p < 0.001, **** p < 0.0001. B) Correlation of the Comet Score software measured OTM and visual score. C) Representative images of the performed comet assay

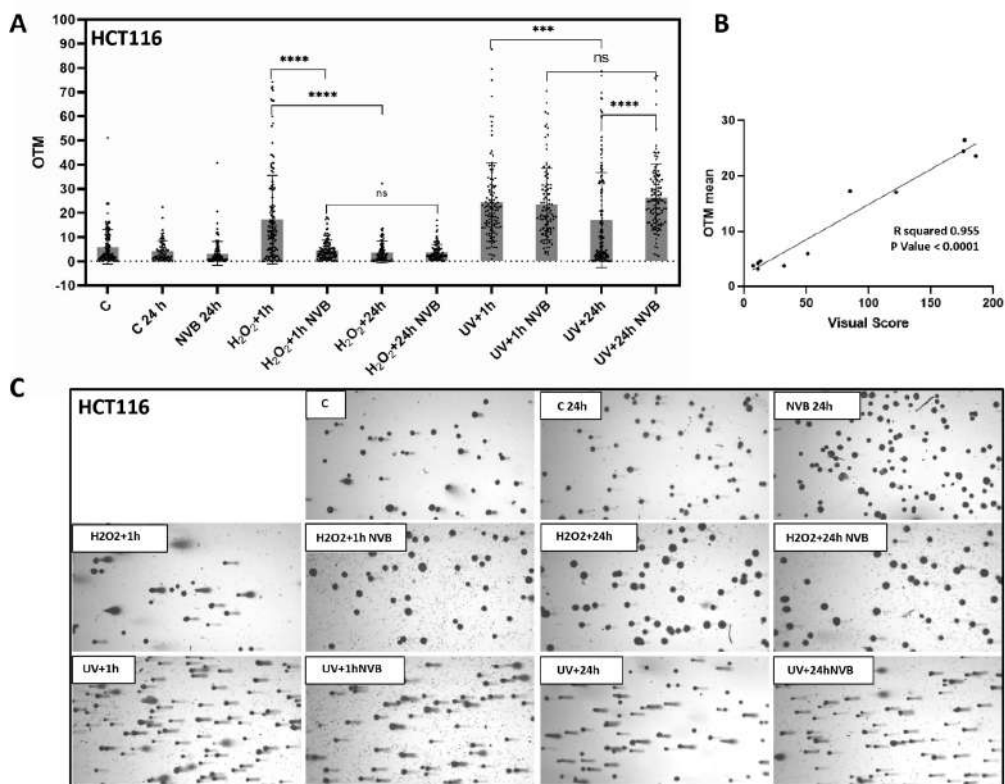


Fig. 3. A) DNA damage in the modified comet assay of HCT116 cells treated with 60 μM H_2O_2 for 5 min or irradiated with 20 J/m^2 UV light. The recovery times after irradiation are indicated. Data are presented as mean $\pm$ SD from measurements of 100 cells. Significantly different compared with one-way ANOVA followed by Tukey's test, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. B) Correlation of the Comet Score software measured OTM and visual score. C) Representative images of the performed comet assay

ing the post-incubation period did not influence the DNA damage repair in this cell line, as indicated by the similar levels of DNA strand breaks with the cells post-incubated without NVB for the corresponding period of time (Fig. 2A).

In HCT116 cell line, the hydrogen peroxide and UV-induced strand breaks decreased at 24 h, in relation to 1 h post-incubation in absence of NVB (Fig. 3A). In presence of NVB, the observed level of hydrogen peroxide-induced DNA strand breaks after 1 h post-incubation was significantly lower in relation to the corresponding incubation without NVB, and remained at the same level after 24 h. Whereas, the level of UV-induced DNA strand breaks with NVB was comparable to those without NVB after 1 h post-incubation, but did not decrease at 24 h (Fig. 3A). There is a good correlation between the OTM and the visual score for the two studied cell lines (Fig. 2B and 3B).

Discussion. To study the effect of DNA damage inducing chemotherapeutic agents and DNA repair inhibitors in different tumour cell lines, it is necessary to assess the kinetics of repair over longer periods of time. In the present study, a modified comet assay method was performed, in which the cells were embedded in an agarose gel, then treated and post-incubated for periods of time till 48 h under standard conditions. Our results indicated that the cells embedded in agarose remained viable in these conditions and can divide (Fig. 1). This finding is in accordance with a previous report showing increase and doubling in the size of the comet head area in PC3 cells, which is indicative for proliferative activity during the incubation period [8].

Based on the observed high percentage of viable cells after the periods of post-incubation used, we conducted a pilot study to evaluate the decrease of DNA strand breaks induced by hydrogen peroxide and UV light in tumour cell lines for periods of 1 h and 24 h. As expected, the induced DNA damage decreased over the 24 h in PC3 cells, indicating ongoing repair. Similar results were shown after treatment with hydrogen peroxide in HepG2 cells in the standard protocol for comet analysis to assess DNA repair [9]. The repair in our study was unaffected by the inhibitor of POLQ in PC3 cells, indicating insignificant involvement of this polymerase for repair of hydrogen peroxide and UV-induced damage in this cell line (Fig. 2). In contrast, the UV-induced damage in HCT116 remained unrepaired till 24 h of post-incubation with NVB, suggesting involvement of POLQ in the process. Although the Nucleotide Excision Repair (NER) is the main repair pathway for UV-induced damage, POLQ was shown to protect against replication stress promoting error-prone Translesion Synthesis (TLS) through UV lesions [10]. Also, MATSUNO et al. [11] reported that HCT116 cell line relies on POLQ-dependent mechanism, instead of Homologous Recombination (HR), for repair of replication stress induced DNA double-strand breaks, because of its mismatch repair deficiency.

The observed lower level of DNA strand breaks in 1 h post-incubation with NVB after hydrogen peroxide treatment in HCT116 cells could be a result of complete repair. On the other hand, this could indicate a delay in DNA repair initiation. Such a delay in DNA repair initiation was observed in comet assay after in vitro treatment with hydrogen peroxide in peripheral blood lymphocytes of breast cancer patients [3]. Oxidative DNA damage is repaired mostly by base excision repair (BER), which involves formation of DNA strand-break intermediate metabolites. Thus, the observed strand breaks in comet assay could include direct strand breaks and BER intermediates, resulting from ongoing repair. POLQ was shown to play a backup role in BER, with its 5'-deoxyribose phosphate lyase activity, which is crucial for removing the generated 5'-dRP fragment [12]. As the mismatch repair deficiency of HCT116 cell line limits the DNA repair by HR [11], the inhibition of POLQ could have greater impact in this cell line.

Conclusion. Our results indicated the utility of the modified comet assay used to study the effect of DNA repair inhibitors. Moreover, the possibility of longer incubation established in this study is particularly important for assessing the effect of DNA repair inhibitors on the action of chemotherapeutic agents. The advantages of the new approach are a reduction in the analysis time and greater reproducibility of the experimental conditions, to obtain more precise results. The proposed modified comet assay could serve as a basis for future experiments in which both the activity of the genotoxic agent and the DNA repair capacity of a specific cell line can be evaluated simultaneously.

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