https://doi.org/10.31925/farmacia.2023.6.10 **ORIGINAL ARTICLE**

COMPARATIVE *IN VITRO* AND *IN OVO* STUDY OF THE CYTOTOXIC PROFILE OF NICOTINE FROM ELECTRONIC CIGARETTES *VERSUS* CHEWING GUM

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Abstract

This study aimed to assess and compare the cytotoxic and vascular effects of e-liquid used in electronic cigarette (e-liquid) and nicotine containing chewing gum (NCG) using *in vitro* models of keratinocytes and cardiomyocytes, as well as *in ovo* models using the chorioallantoic membrane of developing chicken embryos. In the *in vitro* experiments, both cell lines were exposed to e-liquid and NCG at similar and relevant concentrations for *in vivo* administration. The results demonstrated that e-liquid exerted a more pronounced cytotoxic impact when compared to NCG. E-liquid caused a significant decrease in cell viability (up to about 46%) and induced substantial alterations in cellular morphology and nuclei. This finding implies an elevated risk linked to the use of electronic cigarettes, emphasizing that the cumulative content of harmful substances in these devices, beyond nicotine, contributes to their potential adverse effects. Additionally, in the *in ovo* evaluation, e-liquid revealed alarming signs of vascular irritation, including haemorrhage, lysis and intravascular coagulation within the chorioallantoic membrane. These findings underscore the vascular and skin health risks associated with e-liquid, prompting a need for comprehensive investigation into the long-term consequences for users. As a conclusion, the study outcomes highlight that, *in vitro*, the e-liquid used in electronic cigarette exerts heightened toxic effects on both keratinocytes and cardiomyocytes in comparison to the impact observed with nicotine containing chewing gum.

Rezumat

Prezentul studiu și-a propus să evalueze și să compare efectele citotoxice și vasculare ale e-lichidului utilizat în țigări electronice (e-lichid) și guma de mestecat care conține nicotină (NCG) utilizând modele *in vitro* de keratinocite și cardiomiocite, precum și modele *in ovo* folosind membrana corioalantoică a embrionilor de pui. În experimentele *in vitro*, ambele linii celulare au fost expuse la e-lichid și NCG la concentrații similare și relevante pentru administrarea *in vivo*. Rezultatele au demonstrat că e-lichidul a exercitat un impact citotoxic mai pronunțat în comparație cu NCG. E-lichidul a provocat o scădere semnificativă a viabilității celulare (până la aproximativ 46%) și a indus modificări substanțiale în morfologia și nucleii celulelor. Această constatare indică un risc crescut asociat utilizării țigărilor electronice, evidențiind conținutul cumulativ de substanțe nocive din aceste dispozitive, nu doar de nicotină, care contribuie la potențialele lor efecte adverse. În plus, în evaluarea *in ovo*, e-liquid a indus semne alarmante de iritație vasculară, inclusiv hemoragie, liză și coagulare intravasculară în membrana corioalantoidiană. Aceste constatări subliniază riscurile pentru sănătatea vasculară și a pielii asociate cu lichidul din țigările electronice, accentuând necesitatea unei investigații ample a consecințelor pe termen lung pentru utilizatori. În concluzie, rezultatele studiului evidențiază că, *in vitro*, lichidul provenit de la țigările electronice exercită efecte toxice accentuate asupra keratinocitelor și cardiomiocitelor, comparativ cu impactul observat în cazul gumei de mestecat care conține nicotină.

Keywords: nicotine, cytotoxicity, nuclei structure, chorioallantoic membrane

Introduction

The consumption of tobacco and other smoking products has been documented from ancient times, but their use has not been widely spread among the population. With the advent of the 20th century, the use of tobacco increased considerably in the general

population as a result of the industrialization of cigarette production, as well as mass media promotion of smoking and the World War I [18]. In conjunction with the increase in tobacco consumption, certain pathologies also increased in prevalence, though the causative relationship between the two was not established until a few decades later. Thus, both the adverse effects on health and the development of addiction were revealed [21]. Tobacco industry opposition to these discoveries made it even more difficult for information to be provided and awareness to be raised among the general public [31].

Statistically, over a billion people smoke, and almost half of them will develop and die from diseases associated with smoking [12]. The main component of cigarettes is nicotine (Nic). It acts at the level of the nervous system, on nicotinic acetylcholine receptors [36]. Depending on the dose, the individual's sensitivity and the presence of tolerance, nicotine has both rewarding effects as well as adverse effects. Nicotine addiction involves complex mechanisms from multiple brain regions that process rewards and aversion [18]. The well-known neurological effects of nicotine can be accompanied by several other complications. Consequently, Nic can interact with nicotinic acetylcholine receptors at other levels, such as the keratinocytes of the skin and the inflammatory cells [25]. Initiating ganglionic transmission and subsequent enduring depression, Nic can induce diverse skin complications, encompassing premature aging, compromised wound healing, psoriasis, chronic dermatitis and potential tumour pathologies like melanoma. It is noteworthy that not only nicotine, but also numerous other substances in cigarettes, such as polycyclic aromatic hydrocarbons (PAHs), may contribute to these adverse effects, extending to the potential for seizures [1, 8, 27, 34]. In addition to nicotinic acetylcholine receptors, Nic also binds to cardiac β_2-receptors [5]. Moreover, in order to stimulate these receptors, nicotine can also lead to cardiovascular complications such as decreased coronary blood flow, myocardial remodelling, endothelial dysfunction and arrhythmias [10, 19, 26].

Besides conventional cigarettes, nicotine can also be found in a variety of pharmacological and non-pharmacological products. Electronic cigarettes became widely known in 2006, and in 2009 the World Health Organization introduced the term “Electronic Nicotine Delivery Systems” (ENDS) [7]. Media coverage of these devices has led to a rapid increase in their consumption globally, and with it, the emergence of potentially serious health risks. The toxic potential of ENDS has been assessed in recent studies, considering the increasing use of electronic cigarettes by adolescents and their use as an alternative to quitting smoking [15]. Nicotine is used in pharmacological products primarily for replacement therapy and the prevention of withdrawal symptoms. A variety of methods can be used to administer Nic, including pills, transdermal patches, nasal and also oral sprays or chewing gum [35]. Gum is the most frequently used method of administration because it produces a level of nicotine in the blood similar to that attained after smoking, is easy to administer and is quite inexpensive [16].

Although electronic cigarettes and chewing gum with nicotine are widely used among the general population, the negative health effects associated with these products remain unclear. For this reason, this study proposed the comparative evaluation of the cytotoxic potential of Nic from these sources, *in vitro*, at the level of keratinocytes and cardiomyocytes, and also *in ovo*, at the level of the vascular plexus.

Materials and Methods

Reagents

In the present study, nicotine was obtained from two different sources: unflavoured e-cigarette liquid and chewing gum marketed as a smoking cessation product. The other reagents used for the *in vitro* studies included: dimethylsulfoxide (DMSO), foetal calf serum (FCS), penicillin and streptomycin mixture, trypsin solution, phosphate buffer saline, 3-(4,5-dimethylthiazole-2-bromide)-yl)-2,5-diphenyltetrazolium) (MTT) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and Dulbecco′s Modified Eagle′s Medium (DMEM), were purchased from Sigma Aldrich and Merck KgaA (Darmstadt, Germany).

Human keratinocytes - HaCaT were obtained from CLS Cell Lines Service GmbH, and cardiomyocytes - H9C2(2-1) were obtained from ATCC (American Type Cell Collection, Manassas, VA, USA). Both cell lines were purchased frozen. The reagents had all of the necessary characteristics for the *in vitro* evaluation on cell cultures.

Cell culture

The current study utilized human keratinocytes - HaCaT and cardiomyocytes - H9C2(2-1) that were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin mixture. The cells were thawed according to the supplier's recommendations and then grown on T75 plates until the experiment was initiated. Cells were incubated under standard conditions throughout the experiment at 37°C and 5% CO_2.

Cellular viability evaluation

In order to evaluate the cytotoxicity e-liquid used in electronic cigarette (e-liquid) and nicotine containing chewing gum (NCG), the MTT cell viability determination method was applied, previously described in the literature [36]. The cells were detached from the T75 plate and cultured in 96-well plates at a confluence of 1×10^4 cells/well/200 μL. After reaching approximately 90% confluence, cells were stimulated with five concentrations of nicotine (0.1, 10, 25, 50 and 100 μg/mL) for 24 hours. The chewing gum was initially grinded in a mortar, in artificial saliva, for five minutes and subsequently placed in artificial saliva for three

hours. The time interval selected was based on the fact that chewing gum is normally kept in the mouth for a period of one to three hours so that nicotine releases gradually. Immediately after 24 hours, the medium from which the stimulation occurred was replaced with 100 μL of fresh medium and 100 μL of MTT reagent was added to each well. After incubating the cells for 3 hours, 10 μL/well of solubilisation solution was added, and the cells were maintained at room temperature for 30 minutes in the dark. Lastly, the absorbance was measured at 570 nm using a Cytation 5 device (BioTek Instruments Inc., Winooski, VT, USA). Using the absorbance of the samples, cell viability was calculated and compared with control cells that had not been treated. Data obtained was expressed as percentage (%) of viable cells normalized to control cells.

Cellular morphology

Microscopic evaluation was performed on cells cultured in 12-well plates at a confluence of 1 x 10^5 cells *per* well. After attaining the optimal confluence for the experiment (approximately 80%), the cells were treated for 24 hours with the five concentrations of e-liquid and NCG previously tested in the cell viability assay (0.1, 10, 25, 50 and 100 μg/mL). The cells were examined microscopically and then photographed using the Gen5TM Microplate Data Collection and Analysis Software (BioTek Instruments Inc., Winooski, VT, USA).

Immunofluorescence staining

To obtain a better understanding of the effects induced by the two types of nicotine, DAPI staining was applied to the nuclei of human keratinocytes and cardiomyocytes according to the previously described protocol [19]. Cells were cultured in 12-well plates, and after reaching confluence, they were stimulated with a concentration of both samples (50 μg/mL) for 24 hours. Following the stimulation period, the cells were washed with cold PBS and fixed by adding 4% paraformaldehyde and kept cold for 30 minutes. The cells were then washed again with cold PBS and permeabilized with Triton X 0.2% for 30 minutes at room temperature. Lastly, a blocking solution consisting of FCS and Triton X 0.01% was added, with which the cells were kept in contact for 30 minutes. To conclude the experiment, DAPI was added, at a dilution of 1:200, for a period of 20 minutes. Nuclei were photographed using an Olympus IX73 inverted microscope with a DP74 camera, and images were processed and analysed with CellSens software.

Hen's egg test - chorioallantoic membrane (HET-CAM)

By using chicken eggs and the chorioallantoic membrane as a biological model, the potential irritant effect was determined at the level of the vascular plexus. To prepare the eggs, the following steps were taken: I) the eggs were washed and disinfected with 70% (v/v) alcohol and incubated at 37°C and 47% humidity; II) on the 4th day of incubation, a small hole was made in the egg shell through which approximately 7 mL of albumen were extracted and III) on the 5th day of incubation, a small window was cut in the upper part of the egg to facilitate tracing the blood vessels. The eggs were then incubated until the day of the start of the experiment.

The experiment was conducted on the 10th day of incubation with 600 μL of negative control (water), positive control (sodium dodecyl sulphate 1%) and two samples - e-liquid and NCG (50 μg/mL). Chorioallantoic membrane was photographed before and after the application of the samples (T0 and T5), and three potential toxic effects were observed: haemorrhage (H), vascular lysis (L) and coagulation (C), noting the time at which they appeared. Based on the formula described previously, the irritation score (SI) was calculated as follows:

$$IS = 5 \times \frac{301 - H}{300} + 7 \times \frac{301 - L}{300} + 9 \times \frac{301 - C}{300},$$

where, IS = irritation score, H = the time at which the haemorrhage was observed, L = the time at which vascular lysis occurred and C = the time at which intravascular coagulation was established.

The images were taken and analysed using the Discovery v.8 stereomicroscope and the ZEN core 3.8 software.

Statistical analysis

Results are expressed as means ± SD (standard deviation), the one-way ANOVA test, followed by Dunett`s multiple comparison post-test being applied. The software used for the statistical analysis was GraphPad Prism version 9.4.0 for Windows (GraphPad Soft-ware, San Diego, CA, USA). The statistically significant differences between data are marked with * (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Results and Discussion

Smoking is one of the major global health problems, and the World Health Organization recognizes chronic tobacco use as a substance use disorder. Tobacco products contain nicotine, which is responsible for both rapid and intense addiction, as well as a wide range of pathologies, including those affecting the skin and the cardiovascular system [16]. Nicotine-containing products are being marketed as substitutes for smoking and to ease the process of quitting smoking. These include non-pharmacological products, such as electronic cigarettes, as well as pharmacological products, such as pills, sprays or chewing gum [35]. *In vitro* evaluations play an important role in the quality control of nicotine products. As part of these tests, nicotine content, purity, stability, degradation kinetics and the presence of impurities or contaminants may be assessed. *In vitro* evaluations can compare different formulations or variations of nicotine products to assess similarities or differences between them. To support product development or regulatory decisions,

this may include comparing release profiles, absorption kinetics or cytotoxicity of different formulations [22]. A major objective of the current study was to evaluate, *in vitro*, at the level of human keratinocytes and cardiomyocytes, the effects of e-liquid used in electronic cigarette and nicotine containing chewing gum - on cell viability, morphology, as well as on the structure of nuclei. To gain a deeper understanding of the toxicological profile, the *in ovo* method was used to assess the potential for irritants at the level of the vascular plexus of the chorioallantoic membrane. To evaluate the impact on cell viability, keratinocytes and cardiomyocytes were exposed to two different nicotine samples, labelled as e-liquid and NCG, at five distinct concentrations (0.1, 10, 25, 50 and 100 μg/mL) over a 24 hours period. Nicotine concentrations were determined based on the information provided by the manufacturer of electronic cigarettes, respectively, chewing gum. The liquid from the electronic cigarettes was diluted so that the final nicotine concentrations were those presented in the article, and the chewing gum was inserted into the artificial saliva to obtain the tested concentrations. The release of nicotine from chewing gum is not a constant process, being influenced by certain factors (mechanical force, retention time in the oral cavity, etc.). For this reason, the concentrations tested in the present study were determined based on the nicotine content mentioned on the label of the tested products.

In human keratinocytes, e-liquid exhibited a significant cytotoxic effect in a dose-dependent manner. At the lowest evaluated concentration of 0.1 μg/mL, there was no notable decrease in cell viability. However, at 10 μg/mL, cell viability began to decline, reaching approximately 87%, and at 25 μg/mL, it dropped to around 71%. Further, at 50 μg/mL, cell viability decreased to approximately 58%. The most substantial reduction in cell viability was observed at the 100 μg/mL concentration, where it plummeted to approximately 46% (Figure 1). In contrast, when nicotine containing chewing gum (NCG) was tested at the same concentrations, it did not induce significant reductions in cell viability. Although there was also a dose-dependent decrease in cell viability, this was not so dramatic. Thus, the lowest cell viability (approximately 80%) was recorded in the case of the concentration of 100 μg/mL (Figure 1).

Nicotine has been a significant subject in scientific research, being previously assessed *in vitro* across various cell lines. Keratinocytes, which are present in the outermost layer of the skin, hold a pivotal responsibility in upholding the skin's barrier function [14]. Prior research has demonstrated nicotine's potent cytotoxic properties when interacting with these specific cell types. Thus, Lee and colleagues evaluated the effect of nicotine on various types of skin cells - human oral keratinocytes (IHOK), primary oral cancer cells (HN4), metastatic oral cancer cells (HN12) and human skin keratinocytes (HaCaT). The results indicated that nicotine dose-dependently inhibited cell proliferation, similar to what was observed in the present study [24]. In a similar fashion, Pozuelos and colleagues assessed the effects of nicotine both at the level of reconstructed epidermal tissue and human keratinocytes. The results indicated that nicotine derived from electronic cigarettes exerts a pronounced cytotoxic effect by altering mitochondrial and peroxisomal functions. These alterations can lead to an increase in reactive oxygen species, contributing to the occurrence of oxidative damage and hindering wound healing [29]. Furthermore, a study conducted at the level of oral keratinocytes (OKC) indicated that nicotine induces transcriptional and translational changes, leading to disruptions in the cell cycle and cellular differentiation genes [2]. It is important to mention that in a previous study conducted by our research group, the effect of nicotine from conventional cigarettes on human keratinocytes has been evaluated, and it is concluded that nicotine from conventional cigarettes is significantly more cytotoxic than nicotine from electronic cigarettes [9]. As for nicotine contained within chewing gum, to the best of our knowledge, no *in vitro* assessments have been conducted on human keratinocytes to investigate its effects. This constitutes a novel aspect of the current research.

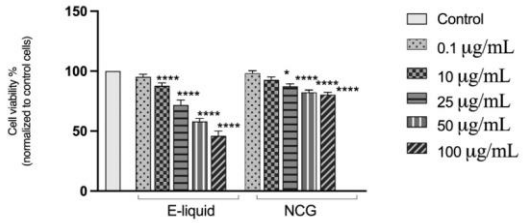

Figure 1.

In vitro evaluation of cytotoxic effects on keratinocytes - HaCaT after 24 hours of treatment with e-liquid and NCG (0.1, 10, 25, 50 and 100 μg/mL)

Results are expressed as a percentage based on the standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's post-test with multiple comparisons (*p < 0.05 and ****p < 0.00001)

The observed impact on cardiomyocytes closely paralleled the results previously documented in human keratinocytes. Specifically, nicotine from electronic cigarettes exhibited a potent cytotoxic effect that was concentration-dependent. While the lowest concentration did not lead to a substantial decrease in cell viability, mirroring the control cell viability, the highest concentration tested (100 μg/mL) resulted in a notable reduction of approximately 44% (Figure 2). In contrast, nicotine from chewing gum, a product available in pharmacies, did not manifest such a pronounced cytotoxic effect. In this context, the initial

two concentrations examined (0.1 and 10 μg/mL) did not induce significant decreases in cell viability compared to the control cells. The most significant decrease in cell viability in this case was approximately 77%, observed at the 100 μg/mL concentration (Figure 2).

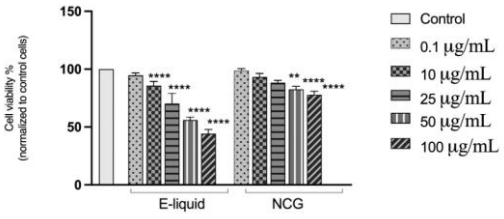

Figure 2.

In vitro evaluation of cytotoxic effects in cardiomyocytes - H9C2(2-1) after 24 hours of treatment with e-liquid and NCG (0.1, 10, 25, 50 and 100 μg/mL)

Results are expressed as a percentage based on the standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's post-test with multiple comparisons (**$p < 0.0001$ and ****$p < 0.00001$)

The cytotoxic effect of nicotine on cardiomyocytes *in vitro* has been a subject of study and concern, particularly in relation to the understanding of the potential effects of nicotine on cardiac cells. Cardiomyocytes are specialized muscle cells that contract the heart and maintain cardiac function [23]. The effects of nicotine on cardiomyocytes were studied by Xing *et al*. According to their findings, nicotine inhibits autophagy in cardiac myocytes at high doses, resulting in the death of the cells [37]. In addition, Zhou *et al.* evaluated nicotine's effects on cardiomyocytes *in vitro*. Results from the study were similar to those obtained in the present study, indicating that nicotine inhibits cell viability dose-dependently. Furthermore, it was noted that Nic induces cell apoptosis, resulting in a reduction in anti-apoptotic gene expression and an increase in pro-apoptotic gene expression [38]. According to the study conducted by Basma and colleagues regarding the effects of nicotine from electronic cigarettes, it is shown that it has a strong cytotoxic effect on cardiomyocytes, increasing the production of reactive oxygen species and altering genes involved in cell proliferation and apoptosis [3]. On the other hand, the study conducted by Chioran and colleagues revealed that nicotine from conventional cigarettes is more cytotoxic at the level of cardiomyocytes than nicotine from electronic cigarettes and chewing gum [9]. To our knowledge, no studies have been conducted on the effects of nicotine from chewing gum on cardiomyocytes.

Following the observation that the two types of nicotine reduced cell viability, the next step of the study was to determine how they affected the morphology of the cells. Cellular morphology is a crucial aspect of cytotoxic evaluations, which involve assessing the toxic effects of various substances on living cells. Cellular morphology refers to the physical structure and shape of cells, and it plays a significant role in understanding the impact of cytotoxic agents [30].

Nicotine derived from electronic cigarettes induced a profound and concentration-dependent alteration in the morphology of human keratinocytes. At the lowest concentrations tested, there were no notable changes in cell morphology when compared to the control cells. In this scenario, the most conspicuous change observed was a decrease in the density of the cells, notably affecting their confluence. Conversely, at the concentration of 100 μg/mL, the cellular morphology underwent significant disruption. This disturbance was evident in the appearance of cells assuming a rounded shape, detachment of cells from the culture dish, reduced confluence, and the loss of intercellular connections (as depicted in Figure 3).

In contrast, NCG did not induce substantial alterations in cell morphology at lower concentrations, with the cells maintaining a similar appearance to the control cells. However, at a concentration of 100 μg/mL, subtle morphological changes became discernible, including the emergence of rounded cells that had detached from the culture dish, as depicted in Figure 3.

In a previous study, nicotine from conventional cigarettes was evaluated in relation to human keratinocyte morphology. Based on the results of the study, it appeared that it has a significant effect on cellular confluence and shape [9]. In a study conducted by Pagano and colleagues, Heat-not-burn tobacco (IQOS) was evaluated at the level of fibroblasts and keratinocytes. This study indicates that they do not have as intense an effect on morphology as nicotine, which was studied in the present study [28].

Similar changes were observed in the case of cardiomyocytes. Thus, nicotine from electronic cigarettes induced dose-dependent alterations in cellular morphology. At the lowest tested concentration, 0.1 μg/mL, no significant modifications were observed, and the cells maintained a similar appearance to control cells. However, at a concentration of 100 μg/mL, pronounced alterations in cellular morphology were evident, including cell rounding, detachment from the culture substrate, reduced cellular confluence and the loss of intercellular connections. These changes collectively serve as indicators of the cytotoxic effect induced by e-liquid (Figure 4).

Conversely, nicotine derived from chewing gum did not induce substantial modifications in cellular morphology, except at the concentration of 100 μg/mL, where characteristic signs of cell death, such as cell rounding and decreased cellular confluence, were observed. Nonetheless, these changes were not as pronounced as those observed with electronic cigarette-derived nicotine (Figure 4).

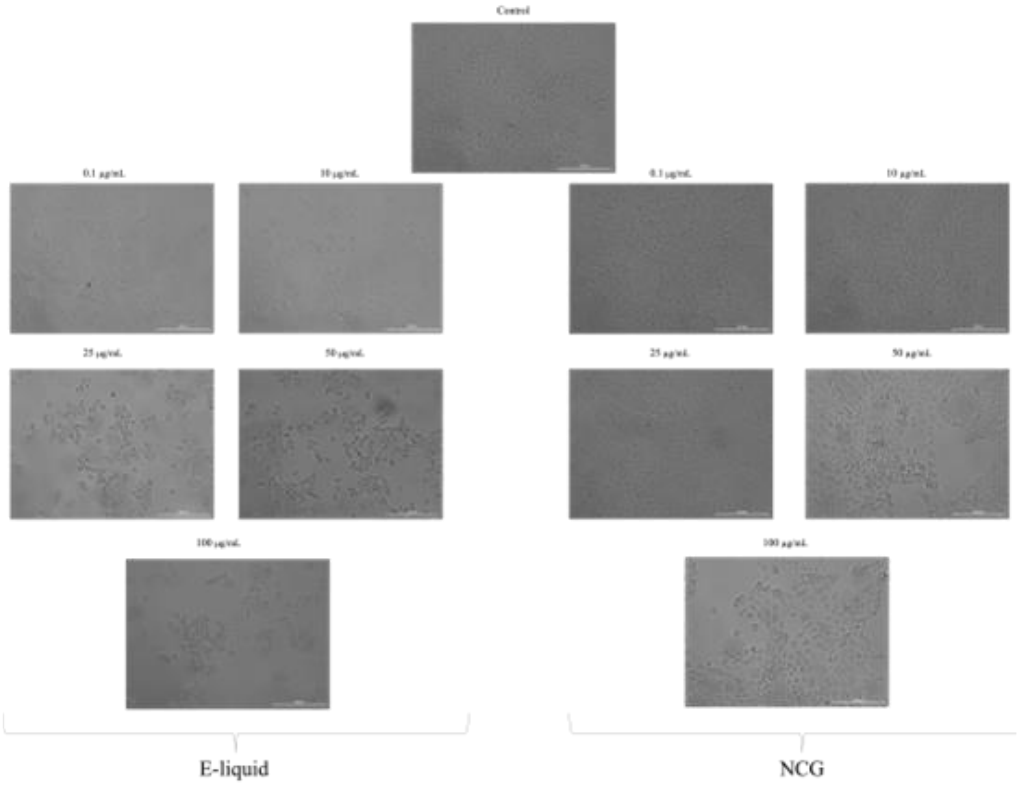

Figure 3.
Morphological appearance of HaCaT cells after stimulation with e-liquid and NCG in five concentrations (0.1, 10, 25, 50 and 100 μg/mL) for 24 hours
Scale bar indicates 200 μm

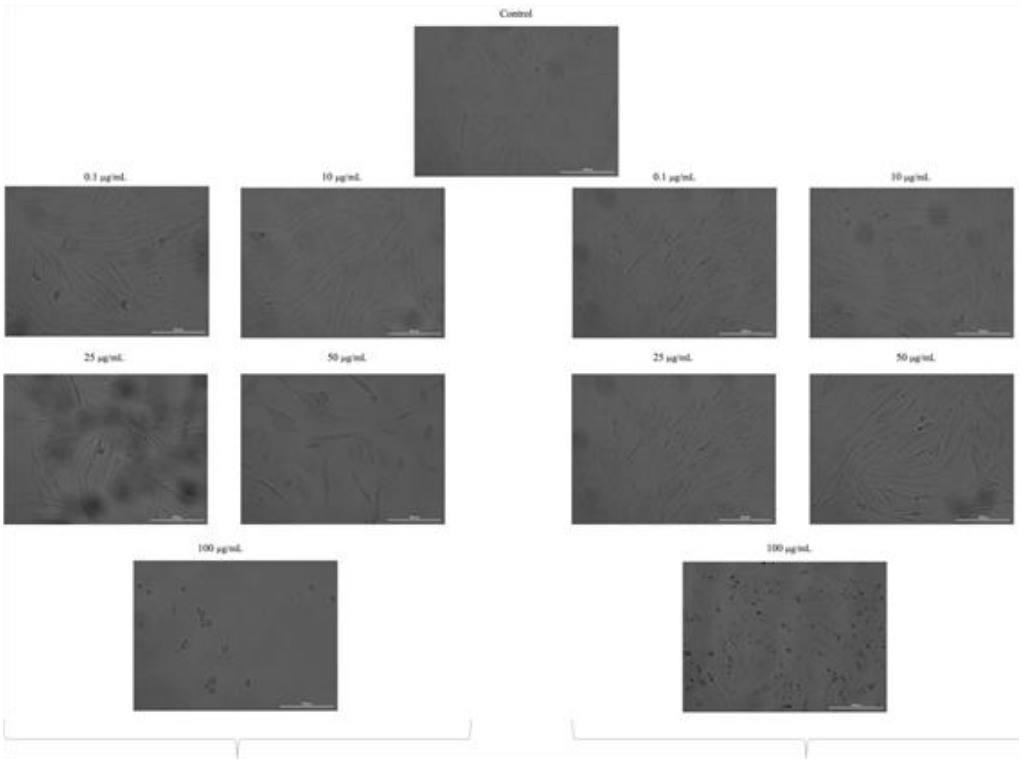

Figure 4.
Morphological appearance of H9C2(2-1) cells after stimulation with e-liquid and NCG in five concentrations (0.1, 10, 25, 50 and 100 μg/mL) for 24 hours
Scale bar indicates 200 μm

Zhou and colleagues evaluated the effects of nicotine on neonatal rat cardiomyocytes in a previous study. According to the results of the study, the cell morphology displayed changes similar to those observed in the present study, indicating that apoptosis is involved [38]. Another study evaluating the potential effects of nicotine on human embryonic stem cell (hESC) line H9 observed morphological changes that affected cardiogenic differentiation of hESCs [20]. Furthermore, nicotine's influence on cell morphology was also studied in the case of other tumour cell lines. Various types of cells were affected by nicotine, which induced epithelial-mesenchymal changes that contributed to invasion and metastasis of tumours [11]. Therefore, an evaluation of nicotine's effects on cellular morphology is crucial to the understanding of the mechanism of toxicity.

Staining nuclei in research on nicotine toxicity is a crucial tool for uncovering the hidden effects of nicotine on cellular health and genetic integrity, shedding light on the perils of tobacco use. The DAPI staining method is pivotal in molecular and cell biology, allowing researchers to understand cell nuclei, their structure and function, as well as applying this knowledge to a wide array of research, diagnostic and clinical applications. It is a versatile and indispensable tool for analysing nuclei in biological samples, contributing to advancements in scientific understanding and medical diagnosis [17].

In human keratinocytes, nicotine derived from electronic cigarettes induces nuclear alterations, exhibiting characteristic signs of cellular apoptosis. Thus, a concentration of 50 µg/mL resulted in a relatively strong condensation of nuclear chromatin, the appearance of apoptotic bodies and nuclear fragmentation. In contrast, nicotine from chewing gum did not induce very intense changes, with only slight condensation of nuclear chromatin and a decrease in the number of nuclei observed (Figure 5).

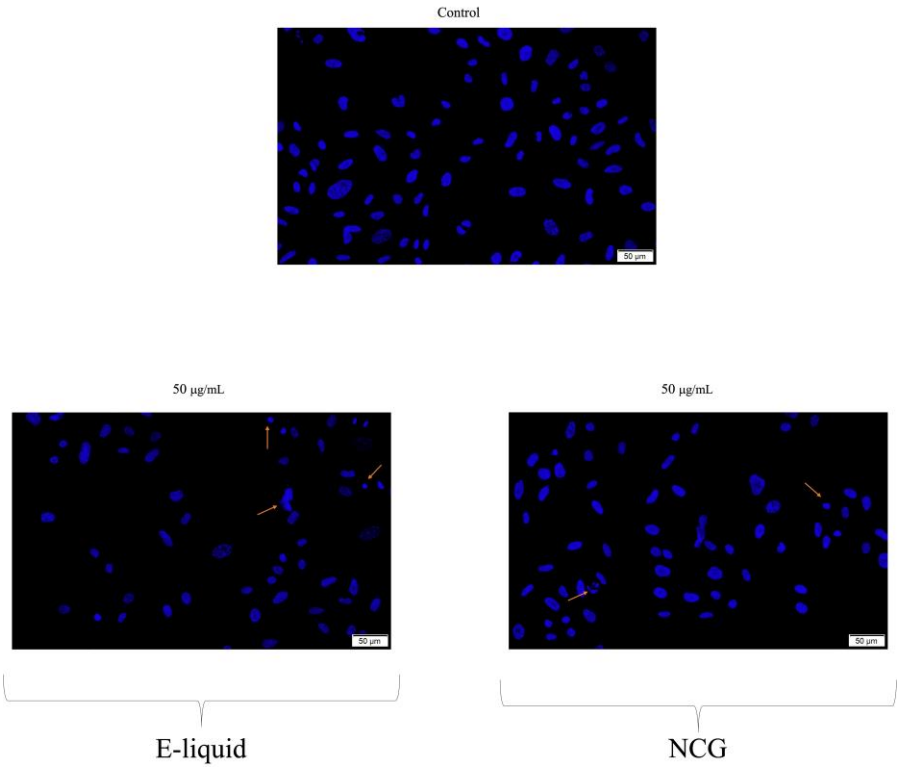

Figure 5.
Evaluation of the effects induced by the 24 hours treatment with e-liquid and NCG (50 µg/mL) at the level of keratinocyte nuclei - HaCaT
Photographs were taken with the 20x objective, and the scale bar indicates 50 µm

According to a previous study, nicotine from conventional cigarettes induces a strong condensation of chromatin, fragmentation of nuclei and the appearance of apoptotic bodies in the nuclei of human keratinocytes. All these effects are more intense than those observed in the present case with nicotine derived from electronic cigarettes and chewing gum [9]. Moreover, Ruiz and colleagues examined the effects of nicotine on the nuclei of human mesenchymal stem cells, which are fibroblast-like cells found in several tissues, including the skin. According to the study findings, cells exposed to nicotine had a more condensed and rigid nucleus as compared to those not exposed [33].

Simultaneously, e-liquid induced alterations like those previously observed in cardiomyocytes. At this level, a concentration of 50 µg/mL induced a series

of changes in nuclear structure compared to control cells, including: strong chromatin condensation, reduction in nuclear size, a decrease in the number of nuclei and the appearance of apoptotic bodies. However, at the same concentration, nicotine derived from pharmaceutical products, such as chewing gum, did not induce significant changes, with only slight chromatin condensation and a relatively small decrease in the number of nuclei observed (Figure 6).

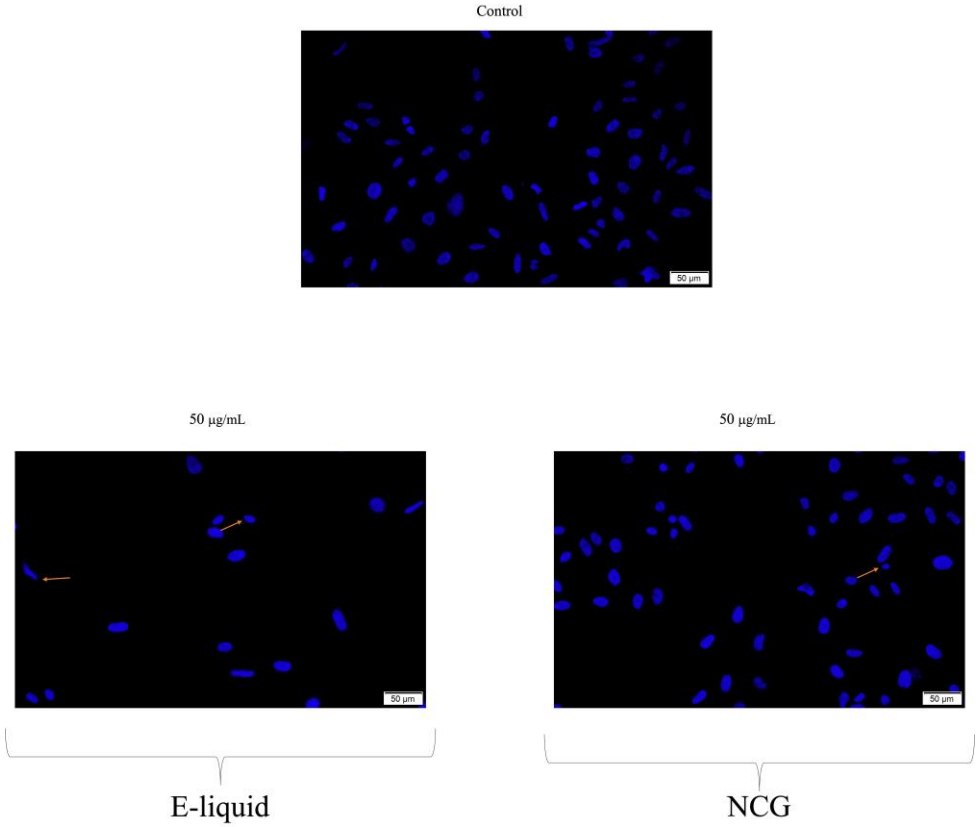

Figure 6.
Evaluation of the effects induced by the 24 hours treatment with e-liquid and NCG (50 µg/mL) at the level of cardiomyocytes nuclei - H9C2(2-1)
Photographs were taken with the 20x objective, and the scale bar indicates 50 µm

Electronic cigarettes have been controversially discussed in the past regarding their negative impact on the cardiovascular system [13]. The effects of nicotine on cardiomyocyte nuclei have been evaluated previously and it has been concluded that nicotine from conventional cigarettes is much more toxic at the cellular level than the nicotine studied in the present study [9].

The process of apoptosis is known to involve changes at the molecular and morphological levels. Thus, at the nuclear level, apoptosis is characterized by chromatin condensation, nuclear contraction and the appearance of apoptotic bodies [32]. These changes were also observed in the current study, in the case of nicotine from electronic cigarettes, which indicates that it induces an apoptotic-like effect on keratinocytes and cardiomyocytes. In contrast, nicotine from chewing gum exhibits less intense similarities at the morphological and nuclear levels, with relatively reduced changes even at high concentrations.

The HET-CAM assay, an invaluable tool in toxicity evaluation, plays a pivotal role in assessing the safety and potential irritative effects of substances, offering a humane and reliable alternative to animal testing in the field of toxicology [4].

To determine whether the two types of nicotine were irritants, they were applied at a concentration of 50 ug/mL to the chorioallantoic membrane, and the following effects were observed: haemorrhage, lysis and coagulation. In order to facilitate a more comprehensive assessment, water was used as a negative control and 1% sodium dodecylsulphate solution (SDS) was used as a positive control. The most intense irritation was observed with the 1% SDS solution, resulting in signs of massive haemorrhage, lysis and intravascular coagulation. In contrast, no significant changes were observed in the case of water. A mild form of microhaemorrhage as well as signs of coagulation was also induced by e-liquid. NCG did not induce such significant changes as e-liquid, in this case only slight signs of vascular lysis and coagulation were observed (Figure 7).

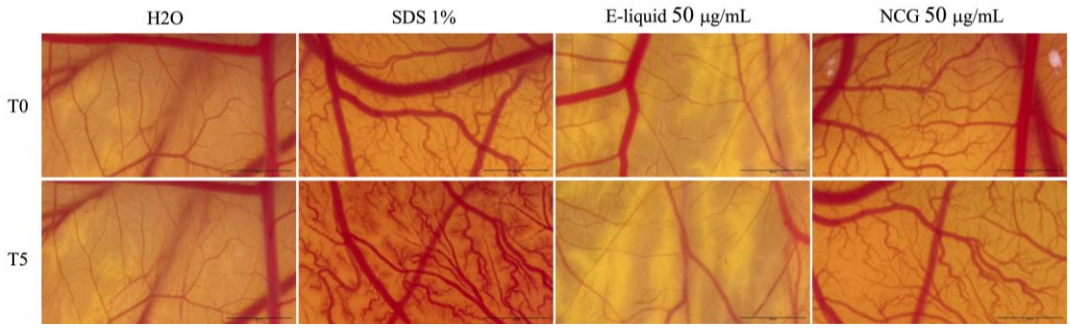

Figure 7.

Analysis of the irritant potential of e-liquid and NCG (50 µg/mL) by the HET-CAM method
Stereomicroscopic images of CAMs inoculated with negative control-H_2O, positive control-SDS and samples.

The irritation score was calculated for the positive and negative controls, as well as for the two samples, in order to quantify the irritant potential. In the case of the positive control (SDS 1%), the irritation score was highest (18.73), while the lowest irritation score (0.07) was recorded in the case of the negative control, water. In terms of nicotine from electronic cigarettes, the irritation score was 9.98, and for nicotine from chewing gum, the irritation score was 2.49 (Table I).

Table I

Irritation score (IS) for e-liquid and NCG and the occurrence time of haemorrhage (tH), lysis (tL) and coagulation (tC)

	H_2O	**SDS 1%**	**E-liquid 50 µg/mL**	**NCG 50 µg/mL**
IS	0.07	18.73	9.98	2.49
tH	300	47	170	290
tL	300	37	270	242
tC	300	23	65	270

Based on the assessment of the irritation score, substances are classified into the following categories: (I) non-irritating substances with an IS ranging from 0 to 0.9, (II) irritating substances with an IS between 1 and 8.9 and (III) highly irritating substances with an IS falling within the range of 9 to 21 [6]. According to these classifications, nicotine from electronic cigarettes is considered a strong irritant, while nicotine from chewing gum is considered an irritant. Based on the results of the previous study, nicotine from conventional cigarettes exerted a much more irritating effect on the CAM level than the results obtained in the current study [9].

Conclusions

The present study investigated the cytotoxic profile of nicotine in electronic cigarettes and chewing gum, both *in vitro*, at the level of keratinocytes and cardiomyocytes, and *in ovo*, at the level of the chorioallantoic membrane. According to the findings, the two types of nicotine possess different effects on the viability, morphology and structure of nuclei. In this manner, nicotine from electronic cigarettes was responsible for reducing cell viability significantly and at the same time resulting in changes in cell morphology and nuclei structure that were characteristic of apoptosis (rounding of cells, condensation of chromatin, appearance of apoptotic bodies). In contrast, nicotine found in chewing gum did not exhibit such a strong cytotoxic profile, with cell viability and changes in cell morphology and nucleus not being as critical as in the first case. At the level of the chorioallantoic membrane, similar results were also observed: e-liquid caused haemorrhages, lysis and intravascular coagulation, while NCG caused milder effects. In conclusion, e-liquid used in electronic cigarette has a highly pronounced cytotoxic profile in comparison with the nicotine in chewing gum, a pharmaceutical product. The results of this study can contribute to better understanding the toxicity mechanism associated with electronic cigarette smoking, highlighting the need for individuals to cease smoking and replace it with pharmaceutical alternatives.

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