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EXPERIMENTAL STUDY

Late effects of cutaneous 3-methylcholanthrene exposure on DNA damage-related pleiotropic growth factors and oxidative stress markers in mice

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ABSTRACT
BACKGROUND: Skin is the body's first defence against direct exposure to variety of chemicals. Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (3-MC) are common in polluted urban air and have a potential of producing harmful effects. Moreover, their late effects can occur months or years after exposure.
OBJECTIVES: We aimed to investigate the long-term effects of 3-MC induced dermal toxicity on the expression of markers of apoptosis, pleiotropic cytokines, and oxidative stress and to determine the protective effect of cisplatin.
METHODS: Groups were designed as control (group 1), 3-MC applied (group 2) and 3-MC+cisplatin applied mice (group 3). Cutaneous expressions of TGFβ, PDGFA, PDGFC, bFGF, PDGFRα, USP28, and Ki67 were evaluated with qPCR. Total oxidant (TOS), total antioxidant (TAS) and oxidative stress index (OSI) values were determined in liver and kidney tissues.
RESULTS: The expression levels of TGFβ, PDGFRα, USP-28, Ki67, and PDGFA were decreased significantly in MC applied groups. Renal TAS levels were significantly lower in group-3. Liver and kidney OSI values were increased in both groups 2 and 3.
CONCLUSION: The results indicated that low dose 3-MC caused oxidative stress and downregulated apoptotic and cytokine markers in the long term and cisplatin had no ameliorative effects on this degeneration processes *(Tab. 3, Fig. 3, Ref. 32)*. Text in PDF *www.elis.sk.*
KEY WORDS: 3-methylcholanthrene, late effect, oxidative stress, polycyclic aromatic hydrocarbons, pleiotropic cytokines.

Introduction

Reactive oxygen species are produced by living beings as an outcome of normal cellular metabolism and environmental agents, such as air pollutants or cigarette smoke (1). Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (3-MC) are common in polluted urban air and cause untoward effects including toxicity and carcinogenesis (2, 3). 3-MC induced applications have been used conventionally for determining immunosurveillance of tumorigenesis (4). The skin is the main natural cover for the body and, as the result of its direct exposure to variety of chemicals, it is at a considerable risk of developing harmful effects to the organs and late effects can occur months or years after exposure (5).

Pleiotropic growth factors have wide tissue dispersion and play crucial roles during embryonic development, normal tissue homeostasis, and carcinogenesis. These factors have cytostatic activities on normal epithelial cells, however, through a tumour suppressor pathway, they are proposed as powerful pro-tumorigenic agents, acting to coordinate peri-tumoral angiogenesis, together with tumour cell migration, immune escape, and dissemination to metastatic sites (6).

Cisplatin is commonly used in cancer chemotherapy (7) and is suggested as a new therapeutic intervention of precancerous stages before the invasive stage begins (8). Here we aimed to evaluate the alterations of pleiotropic growth factors in the skin tissues and oxidative stress levels in the kidney and liver samples of single dose 3-MC induced dermal toxicity in mice after a long- term period. We also investigated the effect of cisplatin on the late effects of single and low dose 3-MC application.

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Tab. 1. Oligo-primer sequences used in the qPCR analyses of the present study.

Gene	Primers	Reference
Transforming growth factor beta (TGFβ)	F:ACTGCCGTACAACTCCAGTGAC R: CAACGCCATCTATGAGAAAACC	Nan et al (12)
Platelet-derived growth factor A (PDGFA)	F:CCTGTGCCCATCCGCAGGAAGAGA R: TTGGCCACCTTGACGCTGCGGTG	Jin et al (13)
Platelet-derived growth factor C (PDGFC)	F: ACCACGAGTCCTTCGGTGTT R: GCATTGTTGAGCAGGTCCAA	di Tomaso et al (14)
Basic fibroblast growth factor (bFGF)	F: GAAACACTCTTCTGTAACACACTT R: GTCAAACTACAACTCCAAGCAG	Du et al (15)
Platelet-derived growth factor receptor α (PDGFR α)	F: CGACTCCAGATGGGAGTTCCC R: TGCCATCCACTTCACAGGCA	Gonzalez et al (16)
Ubiquitin specific peptidase 28 (USP28)	F:ACTCAGACTATTGAACAGATGTACTGC R: CTG CATGCAAGCGATAAGG	Saei et al (17)
Ki67	F: AATCCAACTCAAGTAAACGGGG R: TTGGCTTGCTTCCATCCTCA	Sobecki et al (18)
GAPDH	F: CTGGGATGGAAATTGTGAGG R: TGGCCTCCAAGGAGTAAGAA	Sonobe et al (19)

Materials and methods

Animals and husbandry

The study design was approved by the Animal Experiments Local Ethics Committee (Decision no. 2018/47). A total of 30 male, 10 weeks old BALB/c mice (Kobay Inc, TR) weighing 30 ± 3.59 g were randomly divided into three groups, each consisting of 10 mice. The conditions of the cage environment, care, and feeding of the animals were designed as previously reported (9).

Experimental design and treatment of animals

The study groups were designed as the healthy control group (group 1), 3-methylcholanthrene (3-MC) applied group (group 2) and the 3-MC and cisplatin applied group (group 3). At the beginning of the study, a single intradermal (i.d.) injection of 15 mg/kg 3-MC (213942, Sigma-Aldrich, Germany) dissolved in 0.1 ml sesame oil was performed to the interscapular skin of group-2 and group-3 mice. After a six months period, 10 mg/kg intraperitoneal cisplatin (Sigma-Aldrich, Germany) was injected in the group-3 mice. Group-1 injections were performed with saline in the same way. The mice were euthanized by an exposure to gradually increasing concentrations of carbon dioxide (CO_2) two weeks after the cisplatin injection.

Histopathological evaluation of skin tissues

Tissues were collected from mice at necropsy and fixed in formalin, followed by embedding in paraffin wax. After that, sections of about 5mm thick were stained with haematoxylin and eosin (H&E). The H&E stained slides of the skin samples were evaluated histopathologically.

Biochemical analysis

Kidney and liver tissues were washed with saline at +4 °C, dried on blotting paper and stored in 1 ml storage vials and stored in deep freezer (–80 °C) until analyses were performed. Before the analyses, renal and liver tissues were homogenized using a homogenizer (Stuart, SHM1/EURO, UK) by adding (10 %, w/v) 140 mM KCl solution at +4 °C (10). The obtained homogenates were centrifuged at 3000 rpm for 5 minutes at +4 °C. Oxidative stress parameters were measured in supernatants formed after a centrifugation (9). The levels of Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) in liver and kidney tissue homogenates were measured spectrophotometrically (MultiskanGO, Thermo, USA) using the methods described in the kits (Rel Assay Kit Diagnostics, TR). Results were expressed in mmol Trolox equiv./lt for TAS and μmol H_2O_2 equiv./lt for TOS (9). In addition, the oxidative stress index (OSI), expressed as a percentage of the ratio of TOS levels to TAS levels, was also calculated and the results were expressed in the arbitrary unit (AU) (10).

Quantitative Real-Time PCR

Total RNA was isolated from sampled skin tissues using the total RNA isolation kit (74106, QIAGEN, Venlo, The Netherlands). The concentration and purity values of obtained total RNA samples were evaluated by determining the absorbance at 260 and 280 nm using a nanodrop spectrophotometer (MultiskanGO, Thermo Scientific, Vantaa, Uusimaa, Finland) and calculating A260/A280 ratio. cDNA samples were synthesized using a commercial kit (K1671, Vantaa, Uusimaa, Finland) and each of them was quantified to 200 ng and used for amplifying the investigated genes using a quantitative real-time PCR (qPCR) reactions.

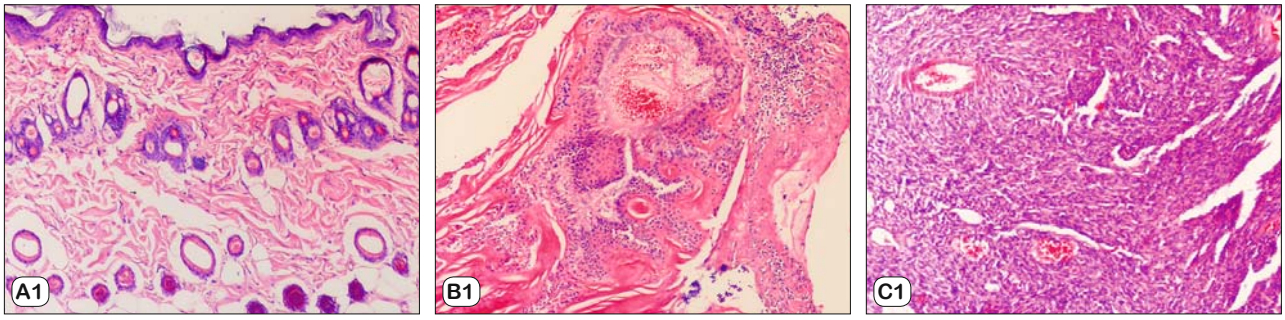

Fig. 1. Hematoxylin and Eosin Stained sections. A1: Normal skin tissue (x100), B1: Squamous cell carcinoma (x100), and C1: Fibrosarcoma (x100).

Tab. 2. Levels of oxidative stress markers in kidney and liver tissues.

		Groups			p
		Control	3-MC	3-MC+Cisplatin	
Kidney	TAS (mmol Trolox equiv./l)	0.92±0.078	0.73±0.03	0.68±0.02	0.03
	TOS (μmol H_2O_2 equiv./l)	7.10±0.89	8.85±0.72	9.56±1.14	>0.05
	OSI (AU)	0.78±0.08	1.22±0.10	1.42±0.18	<0.01
Liver	TAS (mmol Trolox equiv./l)	1.52±0.06	1.49±0.05	1.44±0.03	>0.05
	TOS (μmol H_2O_2 equiv./l)	8.07±0.68	10.38±0.55	9.87±0.49	>0.05
	OSI (AU)	0.53±0.03	0.71±0.05	0.68±0.03	0.01

3-MC: 3-methylcholanthrene, TAS: Total Antioxidant Status, TOS: Total Oxidant Status, OSI: Oxidative Stress Index. $p < 0.05$ was considered significant (Kruskal–Wallis). Data are presented as the mean ± standard error (SE).

Amplifications were performed by a qPCR instrument (LightCycler® 480 II, Roche, Mannheim, Baden-Württemberg, Germany) and its supply (SYBR Green I Master Kit, Roche). This way, the expressions of TGFβ, PDGFA, PDGFC, bFGF, PDGFRα, USP28, and Ki67 genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene (Tab. 1). The obtained crossing point (Cp) values were utilized to determine the relative expressions using the equation of $2^{-\Delta\Delta Ct}$ (11).

Statistical analyses

Statistical analyses were performed by the Statistical Package for the Social Sciences (IBM, New York, USA). Differences of the expression values were evaluated by the Kruskal–Wallis test and Mann–Whitney U test. Data are presented as the mean ± standard error (SE). p values less than 0.05 were considered significant.

Results

The skin tissues, with intradermally applied low dose 3-MC, were evaluated for tumour development. Fibrosarcoma (FSA) and squamous cell carcinoma (SCC) cases were detected at the appli-

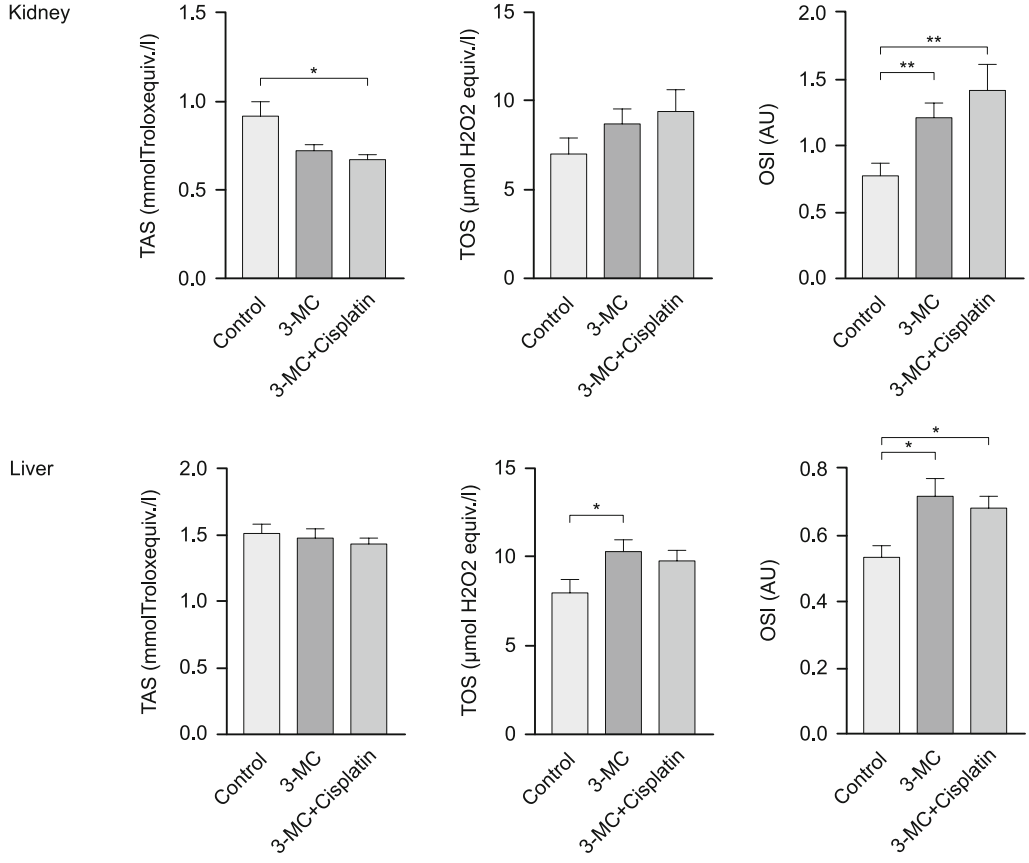

Fig. 2. Results of the oxidative stress markers in kidney and liver tissues in control, 3-MC applied and 3-MC+cisplatin applied study groups. Asterisks indicate significant (*: p<0.05; **: p<0.01) differences between the groups (Mann-Whitney U test). Data are presented as the mean±standard error (SE).

cation site of 3-MC in four mice. Tumours >2 mm in diameter, and demonstrating progressive growth were recorded as positive (20). FSA and SCC cases were represented in Figure 1.

The levels of the oxidative stress markers in kidney and liver tissues are indicated in Table 2. Renal TAS levels were significantly lower in group-3 than in the controls ($p < 0.05$). Liver and kidney OSI values were increased in both groups 2 and 3, compared to the controls ($p < 0.05$). No significant alterations were observed between the values of the groups 2 and 3. The levels of renal OSI ($p < 0.01$), liver TOS ($p < 0.05$), and liver OSI ($p = 0.01$) were increased significantly in the group 2 compared to the controls. Renal ($p < 0.01$) and liver OSI ($p < 0.05$) values were increased significantly in the group 3 compared to the controls. However, the levels of renal TAS ($p = 0.01$) were decreased in the group-3 compared to the controls (Fig. 2).

qPCR results

TGFβ, PDGFRα, USP-28, and Ki67 genes were decreased significantly (Kruskal–Wallis test, p all < 0.05) in the groups 2 and 3, compared to the controls (Tab. 3). Further, the difference between the groups was evaluated by the Mann–Whitney U test. The expression levels of Ki67 ($p = 0.01$), USP28 ($p = 0.02$), TGFβ ($p < 0.05$), and PDGFRα ($p < 0.01$) were decreased significantly in the group 2 compared to the controls. Also, the expression levels of Ki67 ($p < 0.05$), USP28 ($p < 0.05$), TGFβ ($p = 0.02$), PDGFA ($p < 0.05$) and PDGFRα ($p < 0.01$) were diminished significantly in the group-3 compared to the controls (Fig. 3).

Tab. 3. Levels of relative gene expressions in skin tissues.

Genes	Groups			p
	Control	3-MC	3-MC+Cisplatin	
TGFβ	1.46±0.36	1.03±0.34	0.66±0.18	0.03
PDGFA	1.14±0.18	1.23±0.47	1.05±0.47	>0.05
PDGFC	56.87±15.07	53.83±18.62	21.55±4.67	>0.05
bFGF	10.58±3.82	14.45±4.61	6.24±2.36	>0.05
PDGFRα	2.30±0.79	0.62±0.33	0.10±0.05	0.001
USP-28	1.15±0.21	0.25±0.11	0.35±0.20	0.04
Ki67	1.38±0.28	0.71±0.28	0.89±0.27	0.03

3-MC: 3-methylcholanthrene, $p < 0.05$ was considered significant (Kruskal–Wallis test). Data are presented as the mean ± standard error (SE)

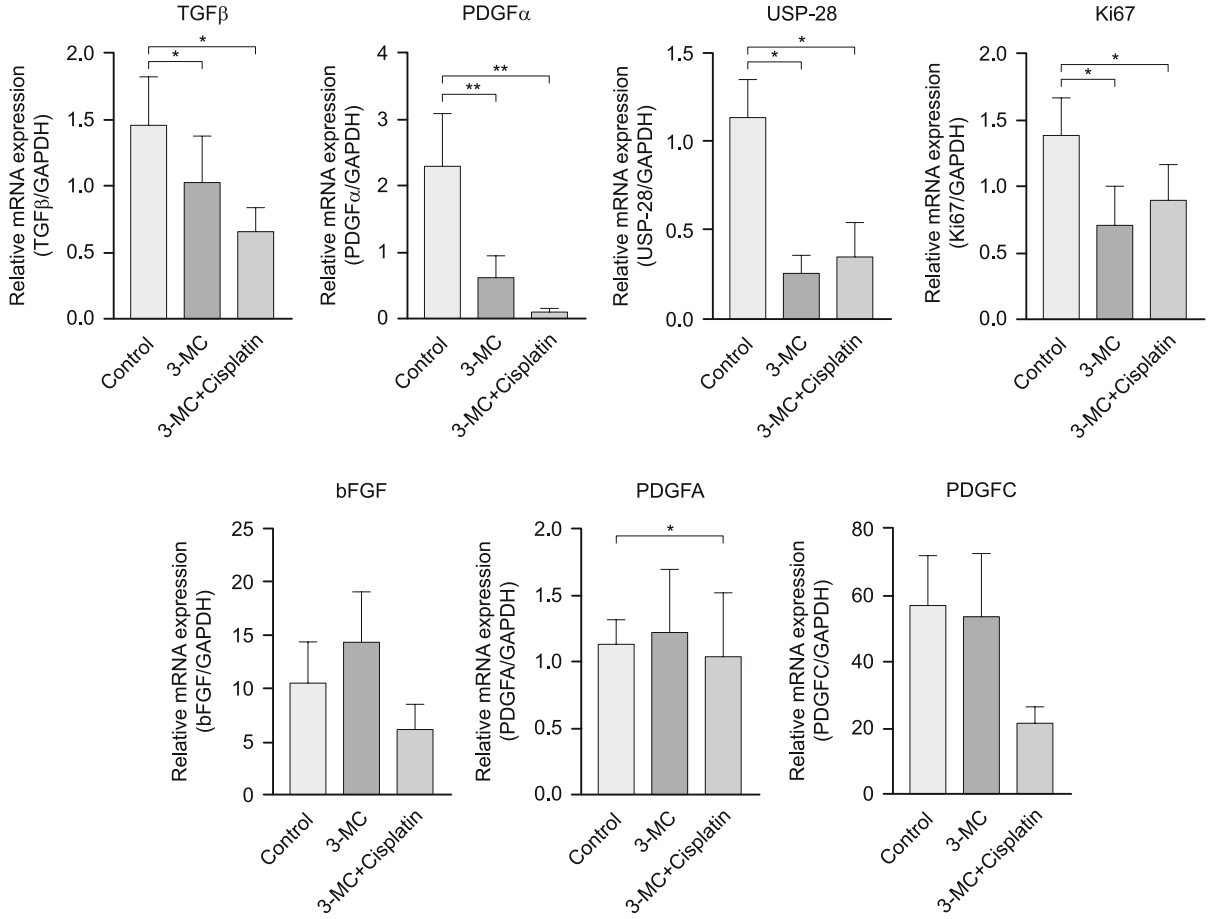

Fig. 3. Quantitative Real-Time PCR (qPCR) analysis results of the control, 3-MC applied and 3-MC+cisplatin applied study groups. Asterisks indicate significant (*: p<0.05; **: p<0.01) differences between the groups determined with Mann-Whitney U test. Data are presented as the mean±standard error (SE).

Discussion

Here we investigated the late effects of a single and low dose (21) i.d. 3-MC application, a polycyclic aromatic hydrocarbon displaying carcinogenic activity, and cisplatin, a well-known chemotherapeutic drug, on the levels of pleiotropic cytokines as well as apoptosis and oxidative stress markers in mice. We found cytokinal and anti-apoptotic alterations among the experimental groups, as well as the formation of FSA and SCC in four 3-MC applied mice. We determined that applying a low dose and evaluating a long term 3-MC induction gave rise to a disruption in the levels of DNA damage-related pleiotropic growth factors and oxidative stress markers. To the best of our knowledge, the present study is the first in literature in terms of investigating the late effects of low dose 3-MC on the skin tissue.

Among DNA damage-related pleiotropic growth factors, TGF-β plays an important role in maintaining tissue homeostasis (22). With its pro-apoptotic effect, it acts as a tumour suppressor, also suppressing epithelial cell proliferation and inflammatory response (23). A disruption of the TGF-β signalling pathway is of great importance in cancer initiation and progression (24). In our study, we found a statistically significant decrease in TGF-β expression levels in both 3-MC and 3-MC+cisplatin groups ($p < 0.05$). In the study applying the same dose and administration of 3-MC, authors induce both SCC and FSA by a single i.d. injection of 3-MC (25). We also detected SCC and FSA in four cases of our study. Authors reported lower levels of TGF-β in the mice with FSA than in the mice having SCC (25). In parallel with this finding, we found a 2-fold decrease in our FSA cases compared to the SCCs.

Platelet-derived growth factors (PDGFs) and the PDGF receptors have significant roles in improving connective tissue cells. PDGFRα signalling audits gastrulation and the improvement of varied organs like skin, lung, intestine, kidney, bones, and testis tissues (26). PDGFRα is frequently stated in the pathological conditions e.g. atherosclerosis, fibrosis, and cancer, probably reflective of a complicated process of the mesenchymal stem cell activity during pathogenesis (27). We found a significant downregulation in the expression of PDGFRα in the group-2 and 3 by qPCR ($P < 0.05$). Similar to our results, D'Arcangelo et al. (28) reported significantly reduced PDGFRα levels in melanoma biopsies. Authors suggested that PDGFRα strongly inhibits carcinogenesis and endothelium proliferation, and melanoma progression eliminates cells expressing PDGFRα (28). We consider the same perspective for 3-MC application and think that observed downregulations in the PDGFRα levels might have been due to its carcinogenic activity.

Ubiquitination is one of the most significant post-translational modifications and it enacts variable functions in cancer-related mechanisms. It also has functions in cell-cycle progression, apoptosis, and transcription (29). Adaptable reactions adjusting deubiquitinating enzyme (DUBs) activity has been shown to control signalling in a number of carcinogenic pathways (30). The Ubiquitin Specific Peptidase 28 (USP28) is a deubiquitinase playing important roles in the DNA damage pathway (31). USP28 fights against the activity of E3 ligases and antagonizes FBW7, an F-box protein and an important component of the E3 ubiquitin ligase aiming transcriptional agents to ubiquitin-directed proteasome degradation (29). We found significantly decreased USP28 gene expression levels in the group-2 and 3 of the present study. Saei and Eichhorn (30) reported that skin showed subdued levels of USP28 and the enzymatic activity of USP28 was limited by FBW7 leading to the corruption of oncogenes. We think that suppressed USP28 levels in the skin tissues we examined might be based on the mechanism mentioned by the authors.

Comba et al (32) reported elevated levels of TOS and OSI by 3-MC administration. The authors performed their study in blood sera of intraperitoneally 25 mg/kg 3-MC applied rats. The levels of the oxidative stress markers of the present study determined that intradermal 3-MC application caused oxidative stress in liver and kidney in the long term. Also, it was determined that cisplatin had no remedial effects on the oxidative tissue degeneration processes induced by 3-MC application in mice. Consequently, the present study proposed that hepatotoxicity and nephrotoxicity were important drawbacks of 3-MC as a late effect.

Conclusion

In conclusion, this paper discussed the assessment of the pathobiochemical effect of exposure to low doses of a carcinogen. Human skin is often exposed to chemicals at a low dose and presents their effects after a long period of time. This issue is of great significance both in the areas of occupational and environmental hazards. The present study proposed that late effects of 3-MC-induced toxigenicity could lead to oxidative stress in liver and kidney and the disruption of the immunoprotective mechanisms mediated by cytokines in the skin tissue.

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