Thesis

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Measurement of the gene expression rate of certain p53 family genes in experimental animals after metal oxide fume inhalation.

The p53 gene family activation after metal oxide fume inhalation in experimental animals and the investigation of the role of these genes in the pathophysiology of the disease called metal fume fever.

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Abstract:

The p53 protein family is pivotal in preventing tumoral cell transformations by aiding DNA repair or inducing apoptosis in cases of irreparable damage. This mechanism activates not only for tumor prevention but also for various DNA damages, such as those caused by oxidative stress from inhalation of Zn or Cu oxides. These oxides can induce metal fume fever (MFF), an occupational ailment primarily affecting welders and metal workers, potentially leading to chronic asthma.

Our study aimed to investigate the impact of ZnO fume inhalation on multiple genes from the p53-family associated with tumor suppression in mice, focusing on lung and the analysis of lymph node samples, to unravel their role in MFF's pathogenesis. During the experiments, animals were exposed to a sub-toxic amount of ZnO fume for 4 hours daily, consecutively for 3 days, with sample collection at 3- and 12-hours post-treatment. Significantly, our findings reveal unprecedentedly elevated levels of ATM gene expression at 12-hour sampling time points, along with heightened expression rates of various p53-family genes, including BRCA1&2, CHK1&2, CCNB1 & E2F1.

This pioneering animal experiment offers novel insights into the cellular processes underlying early metal fume fever development. Notably, all these genes play crucial roles in DNA repair, cell cycle regulations, and cell proliferation, encompassing both mitosis and apoptosis pathways. Our examinations revealed possible double & single stranded DNA damage caused by inhalation of zinc-oxide in mice which explain both the oxidative stress origin of the disease and the further immune reactions as well. Our results make a much deeper understanding of the MFF pathomechanism than any previous investigation before, however, further research is required for uncovering the complete process.

Összefoglalás:

A p53 géncsalád kulcsfontosságú szerepet tölt be a tumorok kialakulásának megelőzésében azáltal, hogy elősegíti a DNS-javító mechanizmusok működését vagy helyrehozhatatlan károsodás esetén apoptózist indukál. DNS-károsodás endogén- és exogén okból is létrejöhet, többek között különböző fém-oxidok belélegzésének következtében kialakuló oxidatív stressz miatt. Főként a cink-oxid hosszabbtávú, szubtoxikus koncentrációjú inhalációja előidézhet egy speciális tünetegyüttest, melyet fémfüst-láznak (MFF) vagy öntőláznak neveznek. A fémfüst- láz foglalkozási betegség, mely elsősorban a hegesztőket és a fémipari dolgozókat érinti és krónikus asztmához is vezethet.

Munkánk célja az volt, hogy egérmodellen szubtoxikus koncentrációjú cink-oxid belélegzésének hatására öntőlázat váltsunk ki, illetve vizsgáljuk a tünetegyüttes patomechanizmusát és a p53-as rendszernek az abban betöltött szerepét. A p53-géncsaládon belül több, tumorszuppresszióval összefüggő gén expresszióját mértük realtime PCR segítségével tüdő- és mediastinális nyirokcsomó mintákon.

Kísérleteinkben egyidőben 4 Balb-C egeret ZnO-füstnek tettünk ki 3 egymást követő napon keresztül napi 4 óra időtartamban. Az állatokból a szövetmintákat 3 és 12 órával a kezelést követően vettük.

Eredményeinkben a kezelés befejezése utáni 12 órás mintavételi időpontban több p53-családba tartozó gén, úgymint az Atm, Brca1&2, Chek1&2, Ccnb1 és E2f1 fokozott génexpressziós szintjét mutattuk ki. Kiemelendő, hogy az említett gének expressziója a 3 órás mintákban kontrolszinten mozgott.

Kísérletünk segítséget nyújthat a fémfüst-láz kialakulásához vezető korai sejtfolyamatok megértéséhez. Az említett gének döntő szerepet játszanak a DNS-javításban, a sejtciklus szabályozásában és a sejtproliferációban, beleértve a mitózis és az apoptózis folyamatát is. Vizsgálataink megerősítették a cink-oxid belélegzése által egerekben okozott lehetséges szimpla- és kettősszálú DNS-károsodást, ami bizonyítékot szolgáltathat a betegség kialakulását az oxidatív stresszel magyarázó elméletre és a további immunreakciókra is. Tudomásunk szerint elsőként vizsgáltuk a p53 géncsaládot az öntőlázzal összefüggésben. Az MFF patomechanizmusának tanulmányozása során eredményeink a korábbi vizsgálatokhoz képest átfogóbb megismerést tesznek lehetővé, azonban a teljes folyamat feltárásához további kutatásokra van szükség.

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Abbreviations:

ATM: Ataxia Telangiectasia Mutated	ATR: Ataxia Telangiectasia and Rad3-Related	BALF: Bronchoalveolar Lavage Fluid
BLM: Bloom Syndrome Protein	BRCA2: Breast Cancer Type 2 Susceptibility Protein	CAT: Catalase
CDKs: Cyclin-Dependent Kinases	CCNB1: Cyclin B1	CCNB2: Cyclin B2
CHEK1: Checkpoint Kinase 1	CHEK2: Checkpoint Kinase 2	CRP: C-Reactive Protein
DSB: Double-Strand Break	DDR: DNA Damage Response	DNA: Deoxyribonucleic Acid
DNA2: DNA Replication Helicase/Nuclease 2	E2F1: E2F Transcription Factor 1	EXO1: Exonuclease 1
γH2AX: Gamma-H2A Histone Family Member X	HRR: Homologous Recombination Repair	IL: Interleukin
MDC1: Mediator of DNA Damage Checkpoint 1	MFF: Metal Fume Fever	MK2: Mitogen-Activated Protein Kinase-Activated Protein Kinase 2
MRE11 (Meiotic Recombination 11 Homolog A)	MRN: MRE11-RAD50-NBS1 Complex	NBS1: Nijmegen Breakage Syndrome 1
NPs: Nanoparticles	PALB2: Partner And Localizer Of BRCA2	PMN: Polymorphonuclear Neutrophil
RAD50: DNA Repair Protein RAD50	RAD51: DNA Repair Protein RAD51	RAD54: DNA Repair Protein RAD54
RAD54B: DNA Repair Protein RAD54 Homolog B	RNF8: Ring Finger Protein 8	RNF168: Ring Finger Protein 168
ROS: Reactive Oxygen Species	RPA: Replication Protein A	SAA: Serum Amyloid A
SOD: Superoxide Dismutase	TNF-α: Tumor Necrosis Factor Alpha	ZnO: Zinc Oxide

Introduction:

Metal fume fever (MFF) is a well-documented occupational disease triggered by the inhalation of biogenic metals and metal oxides. While the oral uptake of these metals is strictly regulated, their inhalation can instigate inflammation in various organs. Zinc oxide, in particular, elicits specific symptoms in humans and has garnered various names, such as "foundry fever," "metal fume fever," "copper fever," "brass chills," "zinc chills," "brazier's disease," "spelter shakes," and "welder's ague" [1–3]. Additionally, the disease is informally referred to as "Monday morning fever" due to its tendency to manifest symptoms on Monday mornings after a period of non-exposure, typically correlated with weekend rest [4, 5].

Metal fume fever (MFF) can arise not only from zinc exposure but also from other metals like copper, other can have influence as manganese, cadmium, mercury, and magnesium in combination [1, 4]. Welders and metal workers, being highly exposed, bear the brunt of this widespread disease, impacting millions of individuals worldwide. Among welders, MFF prevalence can reach up to 35% due to repeated exposure, Wardhana & El-zein implied a potential genetic predisposition [6, 7]. Reports suggest cases of recurrent MFF, with some individuals experiencing more than 10 occurrences [4, 8, 9]. Although the U.S. Toxicology CenGenoter treated over 3300 cases of this occupational hazard between 2006 and 2012, the actual number might be higher due to general symptoms [1, 4]. Workers facing zinc chills also showed other serious symptoms. Examples of these adverse effects encompass respiratory dysfunction, pulmonary fibrosis, siderosis, accumulation of diverse metal particles within the pulmonary region, and in severe cases, the potential development of lung carcinoma. Contributing to the already existing pathomechanism of the disease are exposure to smoke and dust from smoking and other sources [10–13].

Manifestations of metal fume fever (MFF) typically become apparent within a few hours of inhaling the metal and involve experiencing a sweet, metallic taste in the mouth, irritation of the throat, and shortness of breath. Following this, flu-like respiratory symptoms may develop, including a dry cough, challenges in breathing, fever, muscular discomfort, chills, exhaustion, and a general sense of unease. In some cases, vomiting and headaches can also occur. The non-specific nature of these symptoms makes accurate diagnosis challenging, necessitating knowledge of welding work for proper assessment. Currently, treatment remains symptomatic, involving bed rest, pain relief, and fever

management. Hence, prevention should be the primary focus [1, 4].

In addition, beyond the customary symptoms, certain individuals may undergo allergic responses, manifesting as urticaria and angioedema, whereas others might endure the enduring ramifications of recurrent zinc oxide exposure in the form of persistent asthma [2, 14–16]. Overall, metal fume fever poses a significant occupational hazard with the potential to cause substantial harm to workers.

Various welding processes generate metal oxide particles of different sizes. Manual metal arc welding (MMAW) with a covered electrode, suitable for home use, and consumable electrode metal inert gas (MIG) and metal active gas (MAG) welding, widely employed in industrial settings, primarily produce larger particles ranging from 60 to 200 nm. In contrast, Tungsten inert gas (TIG) and resistance spot welding (RSW) mainly processes yield smaller particles, measuring less than 50 nm [17, 18].

Nanoparticles (NPs) with sizes below 100 nm exhibit a high mass/surface area ratio, rendering their biological activity more reliant on physical properties than chemical compositions. Consequently, the inhalation of diverse metals can result in comparable symptoms [19– 21]. Particles measuring less than 5 microns have the ability to permeate the alveolar barrier and are not susceptible to filtration by ciliary action within the airways. Metal oxide particles within the range of 20 to 3000 nm, produced from any welding process, pose a hazard to workers [22]. Consequently, many countries have established an occupational exposure limit for ZnO at 5 mg/m3 for an 8-hour workday [1, 4, 7, 23–25]. Within an extended investigative period, it was observed that 39.2% of the cohort consisting of 286 welders presented manifestations indicative of metal fume fever (MFF), while 13.8% experienced the onset of welding-induced asthma over an average 15-month monitoring duration. At the culmination of the study, a Prick test revealed a 11.8% positivity rate for metals associated with MFF, specifically Copper (Cu), Zinc (Zn), Chromium (Cr), and Manganese (Mn) [6].

Metal fume fever symptoms can develop even at sub-toxic concentrations of metal inhalation. In one experiment, inhaling 1 mg/m3 of zinc oxide resulted in increased blood C reactive protein (CRP) and serum amyloid A (SAA) levels, while inhalation of 2 mg/m3 of zinc oxide led to a fever of 39.5°C [26]. The acute immune response triggered by this exposure level resulted in a five-fold increase in SAA levels, associated with a three-fold higher risk of cardiovascular diseases like myocardial infarction and stroke in the long term

(24).
Consequently, several studies have recommended revising the existing health limits for occupational exposure to ZnO [24, 26, 27].

The exact pathophysiology of MFF remains uncertain, but several studies indicate an inflammatory process as the underlying cause of clinical symptoms. Exposure to sub-toxic concentrations of ZnO has been found to increase endogenous pyrogens, such as tumor necrosis factor-α (TNF-α), interleukin (IL) IL-1, IL-6, which contribute to fever development, as well as acute phase proteins SAA and CRP, detected in both serum and broncho-alveolar lavage fluid (BALF) [7, 24, 26, 28–30].

The major zinc-binding protein metallothionein plays a critical role in regulating cellular zinc levels and facilitating the translocation of zinc within the nucleus during various cellular processes, including the cell cycle and differentiation [31]. In the bloodstream, manganese (Mn) is predominantly present in its Mn^{2+} form, where it complexes with molecules such as citrate and proteins like albumin and α2-macroglobulin (α2M) [32]. Additionally, Mn can exist in the Mn^{3+} state, primarily bound to transferrin, which serves as the principal carrier protein for Mn in the plasma [32].
When it comes to iron (Fe), once it is absorbed by enterocytes in the intestinal lining, it traverses the cytosol until it reaches the basolateral membrane. At this stage, iron is transported into the bloodstream via ferroportin, the sole known protein responsible for iron export [33]. Within the bloodstream, Fe^{2+} is subsequently oxidized to Fe^{3+} and tightly binds to transferrin, which serves as the primary transport protein for iron throughout the plasma [33]. This orchestrated interplay of proteins and molecules is essential for maintaining the balance and proper distribution of these essential metals within the body.
Crucial defense mechanisms against toxic metal ions center around the action of molecules such as glutathione (GSH) and metallothionein (MT), both of which possess thiol groups capable of binding metal ions effectively. Experimental investigations have yielded evidence of heightened MT expression subsequent to exposure to ZnO, providing further substantiation of their pivotal role in mitigating the toxicity associated with metal exposure [34, 35].

Additionally, it is noteworthy that IL-27 exerts a promotive effect on MT synthesis, thereby contributing to the neutralization of free Zn^{+} ions and a consequent reduction in

oxidative stress. This observation aligns with the clinical observation that symptoms of metal fume fever often abate spontaneously within a span of several days [36]. In instances characterized by even more pronounced oxidative stress, cellular responses may culminate in apoptosis or necrosis [37].

Additionally, polymorpho-nuclear (PMN) cells are increased in the blood [16, 38]. Researchers are actively investigating the immune system's initial response after inhaling metals, but the exact triggers of disease development during this phase remains to be fully elucidated.

Currently, two hypotheses are accepted to explain the pathomechanisms.

The immunoallergic theory suggests that metal oxide can bind to proteins, forming complete antigens, leading to a type I hypersensitivity reaction. This reaction can cause symptoms such as urticaria and angioedema. McCord proposed this theory, explaining symptoms through histamine release from zinc protein complexes. [2, 39]

In type I hypersensitivity reactions, IgE-mediated interactions with mast cells and eosinophils cause symptoms. This process follows sensitization, where allergen exposure activates the humoral pathway, switching plasma cells to produce IgE. Eosinophilic granulocytes play a role. This reaction, involving histamine and enzymes, can lead to tissue damage and inflammation. [40]

Elevated MMP-9 levels have been found in metal fume fever (MFF). The NF-kB transcription factor can be activated via the MAPK pathway, leading to pro-inflammatory cytokine production and fever [26, 41]. Allergy, triggered by various allergens, can have systemic or localized symptoms. Late-type allergy may resemble MFF symptoms, but this link isn't fully confirmed, and results vary [42].

As per the oxidative stress theory, zinc oxide particles are transported into the lysosomes within immune cells, where they undergo ionization due to the acidic milieu. This ionization disrupts lysosomal integrity, resulting in the release of reactive oxygen species (ROS). These ROS can initiate both an inflammatory cascade and cellular apoptosis [28, 43]. Physiologically, cells naturally generate ROS, but cellular protection is afforded by the antioxidant enzyme system, which encompasses superoxide dismutase (SOD) and catalase (CAT) [28, 43].

Studies on golden mussels (Limnoperna fortunei) exposed to evaporated Zink Oxide for

24 hours revealed a significant increase in SOD and CAT activity, indicating a cellular response to oxidative stress [19]. Under higher levels of oxidative stress, cells may undergo apoptosis or necrosis [37]. In recent studies, it has been demonstrated that when vanadium salts are administered to mice, apoptosis is induced through the generation of hydrogen peroxide and other reactive oxygen species (ROS) [44].

Among the various reactive oxygen species (ROS) identified, the most prominent ones include superoxide radicals (•O– 2), hydrogen peroxide (H2O2), and the highly reactive hydroxyl radical (•OH) [[45]. Notably, the •OH radical is generated as a byproduct of the Fenton's reaction between H2O2 and Fe21, and it exhibits the highest reactivity, capable of causing damage to critical cellular components such as DNA, proteins, and lipids [46, 47].

The impact of •OH radicals on DNA bases involves several distinct mechanisms: (1) addition to double bonds, (2) extraction of hydrogen atoms from methyl groups, and (3) attacking the sugar residue in their proximity [48, 49]. As a result of this damage, specialized sensor proteins responsible for lesion recognition initiate a DNA damage response (DDR). The DDR comprises a sophisticated network of mechanisms designed to detect the presence of DNA damage, signal its occurrence, and subsequently facilitate repair processes [50]. The generation of ROS and following DDR activates the tumor suppressor gene-family p53, which is known to be involved in cell regulation, DNA repair, and apoptosis [44].

The gene-family p53 serves as a crucial tumor suppressor, playing a vital role in safeguarding cells from tumorigenic changes. Interestingly, most cancers tend to carry gene mutations within the p53 gene-family [51].

The stabilization of p53 in response to DNA damage is facilitated, at least in part, by the DNA damage signaling pathway (DDR). This pathway entails the participation of sensor kinases, including Ataxia-Telangiectasia Mutated (ATM) and Ataxia Telangiectasia Rad3-related protein (ATR), which are responsible for detecting the DNA damage, and effector kinases such as Checkpoint Kinase 1 & 2 (CHEK1/2) & Breast Cancer Type 1 gene (BRCA1), which play a role in transmitting the damage signal to downstream targets. Through this coordinated network of phosphorylation events & kinases, p53 is stabilized, leading to its activation and subsequent engagement in critical cellular processes, including cell cycle arrest, DNA repair, and apoptosis, all essential for the preservation of cellular

health and avoidance of deleterious outcomes such as cancer development [52] [55, 56] [53].

The phosphatidyl-inositol kinase-related protein ATM assumes a critical role as the foremost signal transducer, initiating cell cycle alterations in response to DNA damage [54]. Notably, multiple pathways are involved in safeguarding genetic integrity following exposure to ionizing radiation, with a substantial focus on cell cycle regulation [55].

In response to DNA-damaging agents, cells commonly activate cell cycle checkpoints, providing a controlled and temporary arrest at a specific stage of the cell cycle to facilitate the correction of potential defects [55, 56]. As master transducers of DNA signals, ATM and ATR orchestrate an extensive cellular network to ensure the maintenance of genomic integrity [53].

The initiation of Double stranded break (DSB) recognition and subsequent signaling cascade begins with chromatin modification, which serves as the primary event responsible for sensing the presence of DSBs. This process leads to the activation of ATM and targeted phosphorylation of Histone 2A family member (γH2AX), chromatin Poly(ADP- ribose)ylation, recruitment of mediator of DNA damage checkpoint protein 1 (MDC1), and, eventually, the recruitment of tumor suppressors TP53-Binding Protein 1 (53BP1) and BRCA1 [57–63].

Once ATM is activated, it phosphorylates H2AX and MDC1 at multiple sites, which, in turn, triggers the recruitment of RNF8 and RNF168. This recruitment initiates a series of intricate steps in DNA repair, involving proteins like Breast Cancer Type I (BRCA1), TP53-Binding Protein I (53BP1), MRN (A complex of three proteins - MRE11 (Meiotic Recombination 11 Homolog A), RAD50 (DNA Repair Protein RAD50), and NBS1 (Nijmegen Breakage Syndrome 1 Protein)), Exonuclease 1 (EXO1) or Bloom Syndrome Protein (BLM) with DNA Replication Helicase/Nuclease 2 (DNA2), Replication Protein A (RPA), DNA Repair Protein RAD51, Breast Cancer Type II (BRCA2), Partner and Localizer of BRCA 2 (PALB2), DNA Repair Protein RAD54 Homolog (RAD54), DNA Repair Protein RAD54 Homolog B (RAD54B), and various polymerases (d, j, and m). [64–78].

ATM and ATR, being key regulators of the DNA damage response, play pivotal roles in orchestrating multiple phosphorylation events. One aspect of their function involves activating CHEK1, CHEK2, and Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 (MK2), thereby initiating a second wave of phosphorylation [79–81].

Furthermore, ATM serves a dual role, as it is not only required for ATR-CHEK1 activation but also plays a critical part in initiating DNA repair via homologous recombination (HRR) [82, 83].

The recruitment and activation of ATM primarily occurs at DNA DSBs in conjunction with the MRE11:RAD50: NBS1 (MRN) sensor complex [84, 85]. On the other hand, ATR is activated through its recruitment to stretches of single-stranded DNA (ssDNA) in association with its partner protein, ATRIP [86–88].

Following phosphorylation by ATM, CHEK2 is hypothesized to disperse as a monomeric form across the nucleus, enabling its interaction with various substrates critical to fundamental cellular functions such as cell cycle advancement, programmed cell death (apoptosis), and gene transcription [89, 90]. Some of the known substrates of CHEK2 include the p53 gene-family, the tumor suppressor BRCA1, and E2F1 [91, 92] [93, 94]. Additionally, an increasing corpus of evidence indicates that ATM might possess substrates and exert functions within the cytoplasm [95].

Contrasting with ATM, ATR-CHEK1 signaling is most strongly activated when DNA replication faces impediments, such as nucleotide depletion or replication-blocking DNA damage lesions, as encountered in response to ultraviolet (UV) light exposure [82]. These intricate phosphorylation cascades regulated by ATM and ATR are essential for maintaining genome integrity and orchestrating effective DNA damage responses in cells.

Cells exposed to various genotoxic stresses, such as ionizing radiation and cytotoxic chemotherapy agents, frequently activate both the ATM–CHEK2 and ATR–CHEK1 pathways simultaneously. Unlike ATM, there is currently no evidence supporting autophosphorylation or other posttranslational modifications in ATR activation [82].

CHEK1, in addition to its role in the DNA damage response, is believed to influence recombination processes by phosphorylating Rad51 and BRCA2 [96] [97]. Moreover, CHEK1 is implicated in mediating the repression of gene transcription induced by DNA damage through the phosphorylation of histone H3 [98]. During unperturbed cell cycles, particularly in S-phase, ATR and CHEK1 are thought to be active at low levels, underscoring their importance in cellular homeostasis [99].

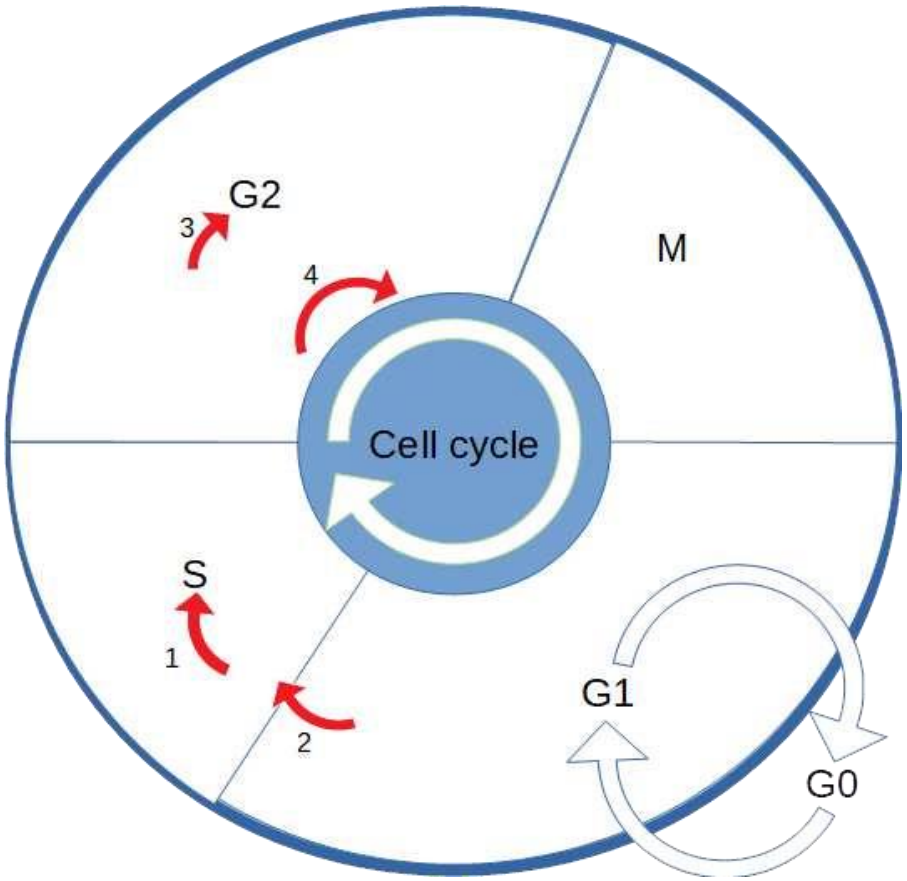

Figure 1: Cell cycle checkpoints of ATM-CHEK2 & ATR-CHEK1 pathways in case of DNA-damage 1: intra-S checkpoint – slows replication of damaged DNA
2: G1 checkpoint – delays entry into S-phase
3: G2 checkpoint – prevents entry into mitosis (M)
4: S-M checkpoint – delays onset of mitosis until DNA replication is complete

In response to DNA damage and DNA synthesis inhibition, vertebrate cells elicit multiple checkpoint responses, each governed by distinct mechanisms and controlled by the ATM–CHEK2 and ATR–CHEK1 pathways. These checkpoints serve critical functions in regulating cell cycle progression and maintaining genomic stability. When DNA damage is detected, cellular checkpoints are capable of postponing initiation of the S-phase (G1 checkpoint), retarding the replication of impaired DNA (intra-S checkpoint), or inhibiting commencement of mitosis until the damage is rectified (G2 checkpoint) [100, 101].

In the event of DNA synthesis inhibition, specific checkpoint responses are activated. These responses are targeted at stabilizing paused replication forks (fork stabilization), restraining the activation of dormant replication origins (origin suppression) [102–104], and deferring the initiation of mitosis until DNA replication achieves completion (S-M checkpoint) [100]. Within these checkpoint processes, ATR–CHEK1 emerges as the principal and direct effector, governing both DNA damage and replication responses, while ATM–CHEK2

assumes an auxiliary role, particularly in the response to DNA double-strand breaks (DSBs) [101, 105]. The intricate coordination of these pathways ensures efficient DNA damage repair and proper cell cycle control, safeguarding cellular integrity and minimizing the risk of genomic instability.

The proteins Brca1 and Brca2 form a complex with Rad51, which is essential for the repair of DNA double-strand breaks through homologous recombination [106–109]. During the S phase of the cell cycle, these three proteins localize to distinct nuclear foci, exhibit similar developmental expression patterns, and reach their highest expression levels at the G1-S transition [107, 110–112].

Upon exposure of cells to ionizing radiation or hydroxyurea, the BRCA1 foci disperse, relocating to sites of DNA synthesis where DNA repair processes may take place [112]. Through mass spectrometry analysis, ATM was identified as one of the proteins associated with Brca1, a finding further validated by reciprocal coimmunoprecipitation [110–114]. This association suggests that ATM might play a role in the phosphorylation of Brca1 in response to DNA damage induced by gamma irradiation. ATM appears to be particularly critical in the response to DNA double-strand breaks caused by ionizing radiation. Notably, the phosphorylation of Brca1 in response to other types of damage, such as ultraviolet light, methyl methanesulfonate, and hydroxyurea, seems to be independent of ATM [112].

Moreover, the optimal phosphorylation of other ATM substrates, like p53 and CHEK2, also appears to be specific to DNA double-strand breaks, as other types of damage can induce ATM-independent phosphorylation of these substrates [115, 116].These findings highlight the intricate interplay between Brca1, Brca2, Rad51, and ATM in the context of DNA damage response and repair, shedding light on the complex mechanisms involved in safeguarding genomic stability.

The interaction between E2F1 and its target genes is closely regulated by phosphorylation events catalyzed by cyclin-dependent kinases (Cdks), allowing it to function as an active transcription factor [117]. The E2F subunits collectively play a crucial role in the intricate control of the cell cycle and cellular proliferation, as well as being involved in numerous other essential functions such as differentiation, DNA damage checkpoints, and metabolism [118, 119].

This response exhibits an all-or-nothing nature, triggered by various mitogenic signals that effectively convert the cellular response into a binary switch. E2F1, in particular, plays a vital

role in this process by upregulating the transcription of genes required for cells to progress into late G1/S phase and advance through the cell cycle, thus acting as a mediator of this binary switch. However, prolonged and excessive expression of E2F1 can lead to the activation of the G1 checkpoint and initiation of apoptosis, serving as a protective mechanism to prevent inappropriate reinitiation of DNA synthesis [120].

These phosphorylation events serve as key regulatory steps in governing the precise timing and coordination of gene expression during the cell cycle, ensuring proper cell proliferation and avoiding potential aberrations that could lead to genomic instability.

CCNB1, a vital member of the cyclin family, serves a crucial role in the initiation and regulation of mitosis. For increased expression it needs to be phosphorylated by CHEK1 [121]. It exhibits a dynamic pattern of accumulation, peaking during the S phase and reaching its maximum level during mitosis, after which it undergoes rapid degradation as the cell cycle transitions from metaphase to anaphase. In the context of various cancers, the significance of CCNB1 has been extensively studied. For instance, in pancreatic cancer cells, the silencing of CCNB1 has been found to promote cell senescence, inhibiting cell proliferation and fostering cell apoptosis [122]

Moreover, CCNB1, along with its counterpart CCNB2, has been implicated in the regulation of mast cell activation and macrophage polarization [123]. The integration of CCNB1 with a cell cycle-dependent Ser/Thr kinase stimulates the production of the maturation-promoting factor, a process indispensable for mitosis [124]. Functionally, CCNB1 plays a crucial role as an important regulator of G2/M phase [125].

Inhibition of CCNB1 expression has been shown to effectively suppress cell proliferation, block cell cycle progression, and induce apoptosis [121]. These findings underscore the central role of CCNB1 in governing cellular processes related to proliferation and differentiation and demonstrate that its downregulation can lead to growth inhibition and enhanced apoptosis [126].

Overall, CCNB1 emerges as a key player in cell cycle regulation and its dysregulation has implications in various pathological conditions, making it an important target for cancer research and therapeutic interventions.

Aims:

Our study aimed to investigate the impact of ZnO fume inhalation on multiple genes from the p53-family associated with tumor suppression in mice, focusing on lung and the analysis of lymph node samples, to unravel their role in MFF's pathogenesis. This pioneering animal experiment offers novel insights into the cellular processes underlying early metal fume fever development.

Material and methods:

Ethical statement

The Animal Experiment Allowance (No. PE/EA/1335-8/2019) for our experiments was obtained from the Animal Protection Authority of the Hungarian Government Office. All procedures adhered to the relevant guidelines and regulations and are reported in accordance with the ARRIVE guidelines. Cervical dislocation was employed to euthanize the animals. No sedatives were administered before the extermination to prevent potential interactions with the inflammatory processes, ensuring the accuracy of our results (16).

Animal model

Male mice of the BALB/C strain (Mus musculus), aged 8-10 weeks, were procured from the National Institute of Oncology of Hungary (Budapest, Hungary) and maintained under specific pathogen-free (SPF) conditions. The mice were housed in standard boxes with chip trays, provided with uniform mouse food, and had unrestricted access to drinking water. The room temperature was maintained at 20-24°C, with a relative humidity of 60% and a 12-hour light/dark cycle. Visual observation of the animals was conducted at least once daily to ensure their well-being.

ZnO fume generation and fume concentration control

To replicate realistic conditions, welding fumes containing zinc oxide (ZnO) were generated using the Tungsten Inert Gas (TIG) welding technique. Analytic grade Zn shots (Merck Group, Darmstadt, Germany) were melted and evaporated during the process. The Rehm TIGER 180 AC/DC High welding machine (Rehm GmbH., Uhingen, Germany) was used with 99.99% pure argon (Linde Hungary Ltd., Répcelak, Hungary) as the shielding gas. The welding parameters were set according to professional guidelines, with a welding current of 80 A, using a WT40 type tungsten electrode with a diameter of 2.5 mm, and a shielding gas flow of 5 l/min. The resulting ZnO fumes were collected and filtered by a Kemper Smart

Master fume extraction and filtering device (Kemper GmbH., Vreden, Germany), after which the air was recycled back into the workshop.

To monitor ZnO concentration in the treatment cabinet, an Aeroqual Model 500 instrument (Aeroqual, Auckland, New Zealand) equipped with PM10 and PM2.5 sensors was used. The target ZnO concentration during the experiment was set to 2 mg/m3, which is below the work health limit concentration in workplaces in Hungary. In other countries, the limit value is 5 mg/m3, as listed in the 5/2020 (II. 6.) ITM legislation (52). Since the measurement device reports particle concentrations in ppm, a conversion was needed to calculate the concentration in mg/m3, which was done using the provided equation (53).

$$X\frac{mg}{m^3} = \frac{(Yppm) * (molecularweight)}{24{,}45}$$

The molar weight of ZnO (81.38 g/mol) was used to convert the measured concentration of ZnO in ppm to 0.6 mg/m3, as it exceeded the required concentration of 2 mg/m³. To achieve the desired time mean concentration of 0.6 ppm, intermittent ZnO fume generation was employed. In each ZnO fume generation cycle, fumes were generated until the PM10 concentration reached 1.2 ppm in the treatment chambers, and then allowed to decrease to 0.1 ppm over time. The planned concentration profile is depicted in Figure 2a.

Prior to conducting the animal treatments, the generated ZnO fume was sampled and analyzed for particle size and morphology using Scanning Electron Microscopy (SEM), as well as for chemical composition using Energy Dispersive X-ray Spectroscopy (EDS). These analyses were carried out at the laboratory of the Material Science and Technology Department, Faculty of Mechanical Engineering, Budapest University of Technology and Economics, Hungary.

Animal treatment

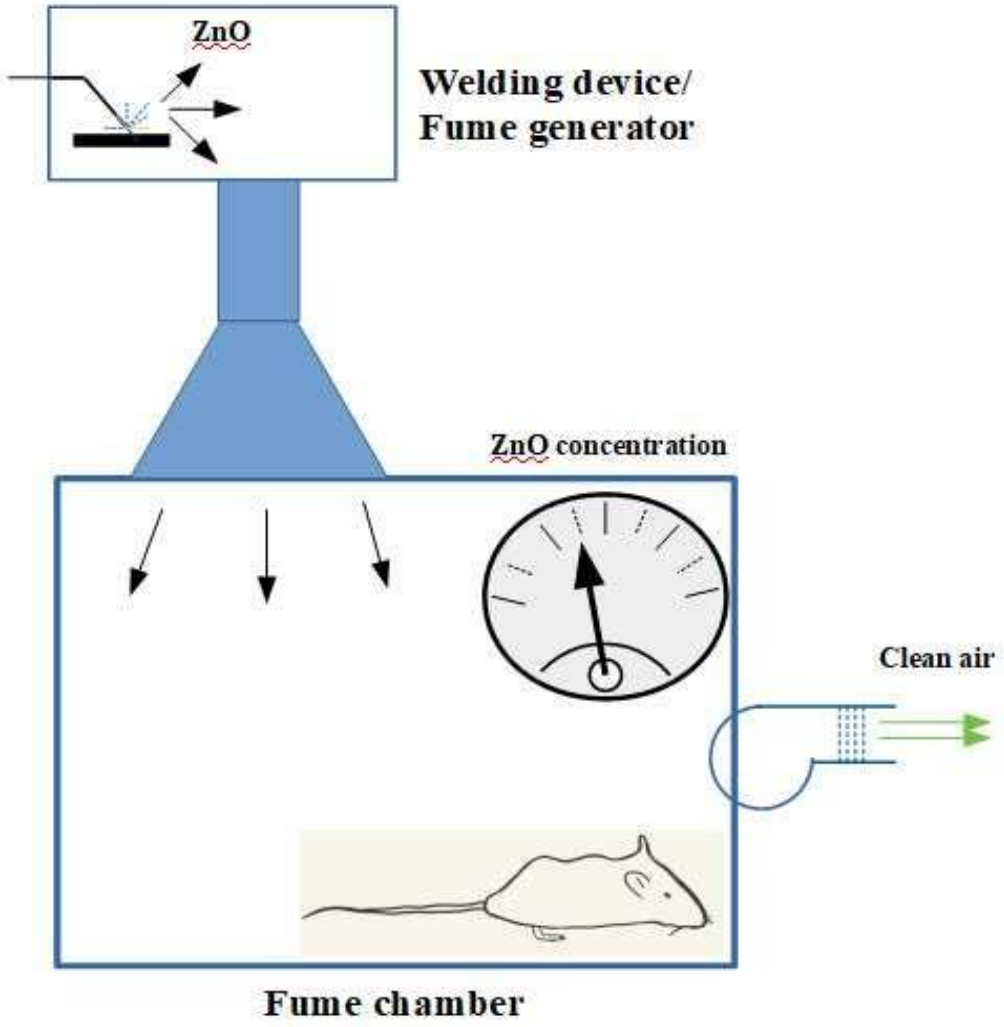

Figure 2: Schematic diagram of the laboratory construction and Fume treatment

In the welding cabinet, intermittent generation of zinc oxide (ZnO) fume took place. The fume-containing air was then directed to an animal treatment cabinet, where individual animals were housed in the treatment chambers of an EMKA Whole Body Plethysmograph system (EMKA Scientific, Paris, France). Throughout the fume exposure, the animals had access to food and drinking water ad libitum. The air flow rate through the chambers was set to 0.5 l/min. Each daily treatment lasted for 4 hours, preceded by an acclimatization period of approximately 10 minutes before fume exposure. During each treatment period, which spanned three consecutive days, four animals were exposed. Continuous monitoring of fume concentration occurred in the animal treatment cabinet, and the polluted air underwent filtration and recirculation as previously described.

Pretreatment and selection of susceptible individuals by measuring locomotor activity

In each experiment, four animals were exposed to ZnO fume-containing air as described earlier. To identify susceptible individuals, we monitored their locomotor activity using a thermal imaging system. After the last exposure, the animals were observed for 72 hours using the Teledyne FLIR Duo R thermal imaging camera (Teledyne-FLIR, Wilsonville, Oregon, USA). The camera recorded thermal images of the four animals, individually housed in adjacent cages, with a time interval of 6 minutes between images. R Studio statistical software was used to analyze the images, and the animals' positions were determined based on the hottest points inside the rectangles representing their respective cages.

By dividing the images into four horizontal rectangles representing the animal boxes, we obtained position data of the animals in 6-hour time intervals. This data was then used to create two-dimensional frequency charts, taking into account the animals' circadian rhythm. We identified animals with consistently low locomotor activity for at least two consecutive 6- hour intervals, indicating a febrile state and potential sensitivity to metal fume fever. These selected animals underwent further examination. To gather enough sensitive animals for the further experiment, these preliminary experiments were repeated three times.

Main experiment with susceptible animals

After the pretreatment phase, the selected animals underwent a 3-week exposure-free rest period to reduce the likelihood of developing tolerance. Following this rest period, the mice were included in the main experiment. They were exposed to ZnO fume-containing air for 4 hours daily on three consecutive days, using the same parameters as described earlier.

At two different time points after the last zinc exposure (3 hours and 12 hours), two mice were euthanized. Lung and mediastinal lymph node samples were collected from each euthanized mouse. To preserve the samples for further analysis, they were stored in RNAlater stabilization solution at room temperature according the suppliers specifications (ThermoFisher Scientific, Waltham, MA, USA). These stored samples will be used for subsequent investigations and analysis.

RNA isolation

Total RNA was extracted from the collected samples using the QIAGEN RNeasy Kit, following the manufacturer's instructions (Qiagen, Hilden, Germany). It should be noted that the samples taken at a specific sampling time (from both animals, including lung and lymph nodes) were combined to create one pooled sample before the RNA isolation process. The concentration of RNA in the pooled sample was quantified using a NanoDrop ND-1000 UV- Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, United States).

Transcription of RNA into copyDNA

Complementary DNA (cDNA) was generated from the isolated RNA using the Qiagen RT2 First Strand Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The thermal profile for cDNA synthesis involved incubation at 42°C for 15 minutes, followed by denaturation at 95°C for 5 minutes.

Gene expression measurement by quantitative real-time PCR

Real-time PCR was conducted to assess the expression of 84 genes associated with inflammatory mediators using the Qiagen RT2 Profiler PCR Array Mouse Inflammatory Cytokines and Receptors (PAMM-011ZA) kit (Qiagen, Hilden, Germany). The complete gene list can be found in Additional Information Table 1.

RT-PCR reactions were carried out with a Bio-Rad CFX96 Touch Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA), and cycle threshold (Ct) values were determined using Bio-Rad CFX Maestro software. Gene expression values were calculated using ΔΔCt analysis, and the assay was validated with internal, positive, and negative controls, as well as by measuring the expression of 5 housekeeping genes. The most suitable housekeeping gene for ΔΔCt analysis was selected using RefFinder freeware online software (http://blooge.cn/RefFinder/).

To evaluate the gene expression results of tissue samples from ZnO-exposed mice, they were compared to the gene expression results of lung and mediastinal lymph node samples from two untreated, healthy mice. Due to technical constraints, statistical analysis was not feasible as each gene was examined in a single well. Thus values for control animals were set as 1, and a 10-fold up- or down-regulation compared to control values was considered a significant change in gene expression. The choice of this 10-fold threshold was based on the manufacturer's recommendations and existing literature data 56.

Due to technical constraints, statistical analysis was not feasible as each gene was examined in a single well.

Results:

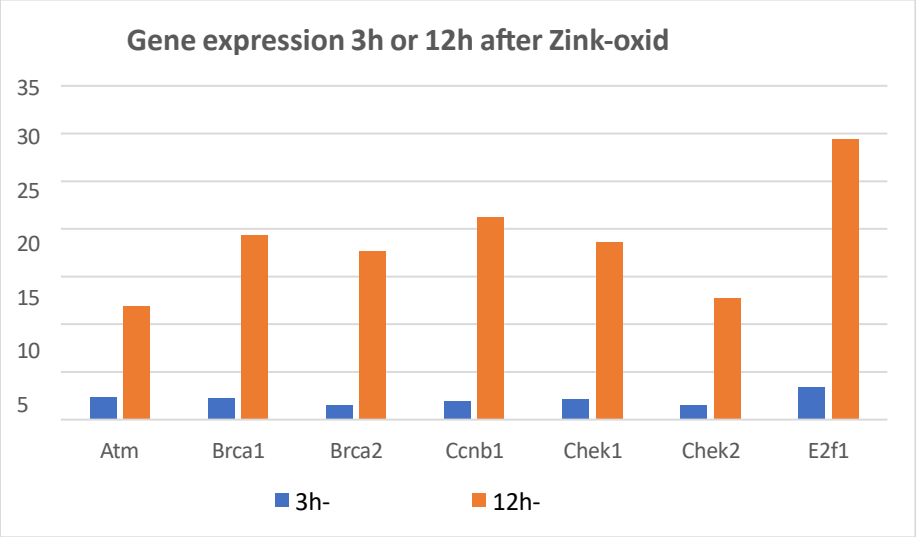

Figure 3: Gene expression 3- or 12h after Zink-oxide inhalation

In this experiment 84 gene expressions were measured, 7 genes showed notable changes compared to the baseline physiological expression (set at 1). After 3 hours, CHEK2 and BRCA2 increased to 1.4 times the baseline. BRCA1, CCNB1, and CHEK1 increased by 2.0 to 2.1 times, while ATM increased by 2.4 times, and the highest increase was observed in E2F1 with 3.4 times the baseline.

At the 12-hour mark, ATM expression escalated significantly to 11.9, almost equal to CHEK2 (12.7). BRCA1, BRCA2, and CHEK1 showed remarkable increments, rising by 17 to 19-fold. CCNB increased by 21 times, and once again, E2F1 exhibited the highest elevation with 29 times the baseline expression.

Discussion:

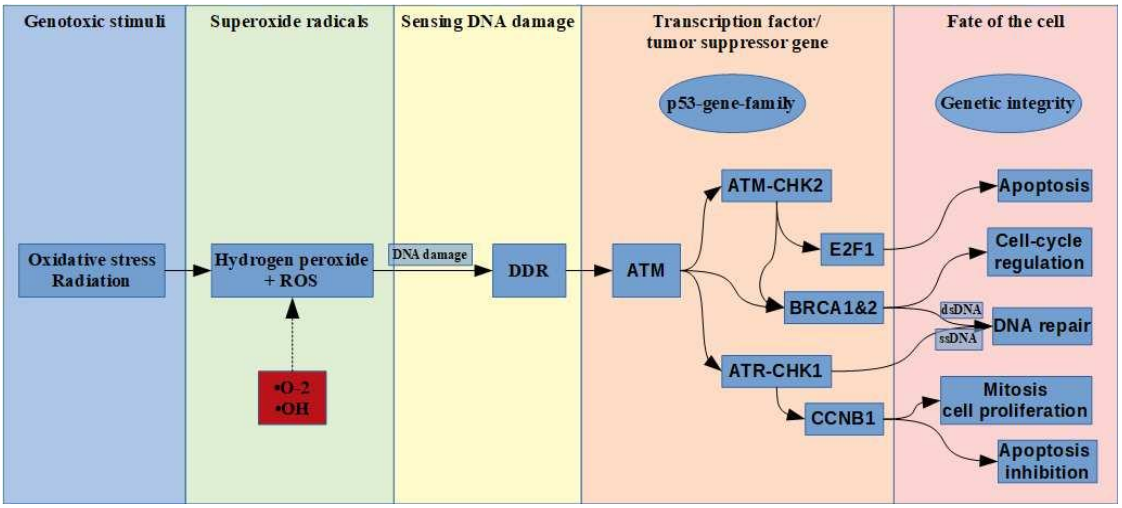

Figure 4: Genotoxic stimuli, such as oxidative stress and radiation, lead to the production of superoxide radicals, including hydrogen peroxide and other reactive oxygen species (ROS). This, in turn, triggers DNA damage and activates the DNA damage response (DDR). The transcription factor and tumor suppressor protein p53, as well as ATM through chromatin modification, are expressed in response to this DDR. ATM then phosphorylates CHEK2, inducing programmed cell death and activating E2F1. BRCA is responsible for maintaining genomic integrity and facilitating double-strand DNA repair and cell-cycle regulation. ATR-CHEK1, on the other hand, is involved in single-strand DNA repair. Overexpression of ATR leads to increased CCNB1, which promotes mitosis, cell proliferation, and differentiation while inhibiting apoptosis.
For additional information and references, please find the extended content.

Our report marks the first-ever exploration of MFF with an inclusion of mediastinal lymph node samples, derived from a comprehensive review of global scientific literature. Our findings propose an alternate avenue for the pathomechanism of MFF, suggesting that lung involvement might not be central, aligning with earlier conjectures that underscored the role of blood in the process [127, 128].

The pretreatment phase was devised to pinpoint animals with susceptibility to metal fume fever. Though this was a technical facet of our study, it is noteworthy that the occurrence rate of identified sensitive animals stood at 30%. This figure strikingly aligns with the sensitivity incidence observed in the broader human populace, which generally ranges from 30% to 35% [6, 7].

Due to their diminutive size, ZnONPs have the capability to reach the lung alveoli. Here, they are primarily phagocytized by alveolar macrophages. Inside the lysosomes of these cells, the high concentration of Zn+ ions from the particles instigates the formation of reactive oxygen species. Consequently, this situation escalates the levels of oxidative stress within the cells.

Furthermore, this surge in oxidative stress triggers the activation of the DNA Damage Response (DDR) pathway. This activation is attributed to the dissolution of ZnO particles

[129].

The transcription factor and tumor suppressor protein p53 play pivotal roles in orchestrating cellular responses to DNA damage [51]. Additionally, ATM, facilitated through chromatin modification, responds robustly within the DNA Damage Response (DDR) [50]. This response involves the activation of ATM, which then phosphorylates CHEK2 [57–63]. In our study, at the 12-hour mark, ATM expression displayed a remarkable surge, reaching a value of 11.9. Working in tandem, Chk-2 partners with ATM, CHK-2 exhibiting an expression nearly equivalent to ATM's (12.7). Eliciting a cascade that ultimately leads to programmed cell death and the activation of E2F1 [94]. E2F1, a transcription factor, and responsible for apoptosis, exhibited a substantial 29-fold increase [117].

Furthermore, in that cascade, the breast cancer susceptibility gene, BRCA 1&2, is a key player in maintaining genomic integrity. Its functions encompass the facilitation of double-strand DNA repair and cell-cycle regulation, essential processes for upholding the stability of the genetic material 91 [106–109], our experiment indicated an augmentation by 17 to 19- fold.

Conversely, the ATR-CHEK1 pathway primarily engages in single-strand DNA repair, contributing to the intricate DNA repair network [82, 83]. Interestingly, an upregulation or overexpression of ATR is associated with heightened levels of CCNB1, a critical protein linked to cell cycle regulation. Elevated CCNB1 levels further drive processes such as mitosis, cell proliferation, and cellular differentiation, while concurrently inhibiting apoptosis, marking the complex interplay of these factors in cell fate determination and genomic maintenance [122]. This cell cycle regulator demonstrated a notable elevation of 21.

From our results we can see elevated levels of genes which play a role in cell cycle checkpoints, cell cycle regulation, DNA repair and cell proliferation. The most important for tumor suppression will be the maintenance of genomic integrity. These cellular processes prove a single and double stranded DNA damage. Combined with the existing publications we can now better understand the pathogenesis of Metal Fume Fever. Inhalation of Zink- Oxide nanoparticles within the allowed EU-regulations under 5mg per m^3, causes DNA- damage in an extend which is not dangerous at first [62]. The cellular

processes involved and explained in this publication, normally work error-free. In case of a faulty DNA composition the risk of a faulty cell function is increased and the progression of tumorous cells is increased. This is mainly the case in mutations of the p53-gene family for example BRCA1&2, these mutations have an occurrence in the human population of 1 in 300 [52, 130]. There is no cross connection to be found between the p-53 gene family and the generally descripted symptoms of Metal Fume Fever. Through ROS many other cytokines get activated, e.g. IL-17, TNF-alpha, IL-1&6, which promotes inflammation and protect the body from further damage [131]. These are the symptoms normally descripted with MFF [10–13], it's important to note that severe damages to DNA don't need to have further consequences to the body after they are error-free repaired.

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Vincent Michelberger

Appendix 4. Supervisor counter-signature form

I hereby confirm that I am familiar with the content of the thesis entitled *Measurement of the gene expression rate of certain p53 family genes in experimental animals after metal oxide fume inhalation*

written by *Vincent Michaelberger* (student name)

which I deem suitable for submission and defence.

Date: Budapest, *28* day *11* month *2024* year

DR. SLÜCS-SOMLYÓ EVA

Supervisor name and signature

Microbiology and Infectious Diseases

Department

Appendix 5. Declaration regarding TDK research paper-thesis equivalence

DECLARATION

I hereby declare that the thesis entitled ..Measurement of the gene expression rate of certain p53 family genes in experimental animals after metal oxide fume inhalation.....

.....

is identical in terms of content and formal requirements to the TDK research paper submitted in 2023 (year).

Date: Budapest, 29 day..November.....month..2024.....year

.....
Vincent Michelberger
Student name and signature

Thesis progress report for veterinary students

Name of student: Vincent Felix Michelberger

Neptun code of the student: ZMB1K5

Name and title of the supervisor: Éva Szücs-Somlyó

Department: of Microbiology and Infectious Diseases

Thesis title: The p53 gene family activation after metal oxid fume inhalation in experimental animals and the investigation of the role of these genes in the pathophysiology of the disease called metal fume fever

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	05	18	Introduction into Topic theoretical	
2.	2023	06	09	Visiting lab & going through research/results	
3.	2023	06	20	Table of Contents	
4.	2023	08	01	Checking literature Review/first diagrams	
5.	2023	08	20	Checking discussion and further diagrams	

Grade achieved at the end of the first semester: ...jeles (5).....

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	09	26	Abstract finalization	
2.	2023	10	01	Plagiarism check	
3.	2023	10	20	Formal/Final check	

4.	2023	11	15	Presentation Check	
5.	2023	11	20	Rehearsal TDK	

Grade achieved at the end of the second semester: ...jeles (5).....

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....
signature of the supervisor

Signature of the student: .....

Signature of the secretary of the department: ...Bársúné Kolonics Mária

Date of handing the thesis in...nov. 17....2023.