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Chemical toxicity and radioactivity of depleted uranium: The evidence from *in vivo* and *in vitro* studies

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ABSTRACT

The main aim of this review is to summarize and discuss the current state of knowledge on chemical toxicity and radioactivity of depleted uranium (DU) and their effect on living systems and cell lines. This was done by presenting a summary of previous investigations conducted on different mammalian body systems and cell cultures in terms of potential changes caused by either chemical toxicity or radioactivity of DU. In addition, the authors aimed to point out the limitations of those studies and possible future directions. The majority of both *in vitro* and *in vivo* studies performed using animal models regarding possible effects caused by acute or chronic DU exposure has been reviewed. Furthermore, exposure time and dose, DU particle solubility, and uranium isotopes as factors affecting the extent of DU effects have been discussed. Special attention has been dedicated to chromosomal aberrations, DNA damage and DNA breaks, as well as micronuclei formation and epigenetic changes, as DU has recently been considered a possible causative factor of all these processes. Therefore, this approach might represent a novel area of study of DU-related irradiation effects on health. Since different studies offer contradictory results, the main aim of this review is to summarize and briefly discuss previously obtained results in order to identify the current opinion on DU toxicity and radioactivity effects in relation to exposure type and duration, as well as DU properties.

1. What is depleted uranium (DU)?

Uranium is a heavy metal from the actinide series that is both chemically toxic and radioactive (Craft et al., 2004; Dewar et al., 2013; Domingo, 2001). It is a naturally abundant element, found in air, soil, water and rocks (Albina et al., 2005; Danesi et al., 2003; HPS 2010; Linares et al., 2007) with a half-life of 4.5 billion years, such that the level of radiation does not significantly decrease over time (UNEP 2003). Uranium is considered radioactive because it emits α-particles and is, in addition, capable of emitting β-particles and γ-rays. If insoluble, these particles are too big to pass through human skin (two protons and two neutrons). Therefore, they are mostly inhaled (Dewar et al., 2013). The element itself was first isolated and scientifically described by the German pharmacist Martin Heinrich Klaproth in 1789 and named after the then recently discovered planet Uranus, but its radioactivity was not considered important until Marie Curie characterized the element radium (Briner, 2010; Craft et al., 2004).

Natural uranium consists of three isotopes, namely ^{238}U (99.27%), ^{235}U (0.72%) and ^{234}U (0.0054%). Enriched uranium which is necessary for the production of nuclear energy (Bellés et al., 2005) contains higher amounts of the ^{235}U isotope (in the range from 1.5% to 4.6%). After the major part of ^{235}U isotope is removed from the natural uranium, the remaining residue is referred to as depleted uranium (DU), which is around 40% less radioactive and less stable than naturally occurring uranium and contains the abovementioned isotopes in the following ratio: 99.8% ^{238}U, 0.2–0.3% ^{235}U and 0.001% ^{234}U (Hao et al., 2013). DU is mainly used for the production of armor-penetrating bullets. It can also be used for civil purposes, for example, for the production of shields for protection from irradiation in hospitals and containers for the transport of radioactive materials. Finally, highly

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Table 1
An overview of in vivo studies on DU effects performed on mammalian models, instead of: An overview of in vivo studies on DU effects on health performed on mammalian models.

Cell line	Major findings	Reference
Chinese hamster ovary (CHO) cells	The study demonstrated that uranyl nitrate decreased the viability of CHO cells in a dose-dependent manner. It also inhibited cell cycle kinetics, increased the incidence of micronuclei, sister chromatid exchanges and chromosomal aberrations. In this way, chemical toxicity of uranyl nitrate on CHO cells was proven.	Lin et al. (1993)
Human osteoblast cells (HOS TE85 cell line)	It was shown that DU-uranyl chloride caused a 9.6-fold elevation in cell transformation frequency compared to untreated cells. The transformants exhibited anchorage-independent growth, produced tumors when injected into nude mice, expressed high levels of k-*ras* oncogene, altered the phosphorylation of the pRb tumor-suppressor protein and increased the levels of sister chromatid exchanges. DU was proven to chemically induce neoplastic changes in these cells.	Miller et al. (1998a)
Mouse macrophage cell line J774	The study indicated that the uptake of uranyl chloride by macrophages increased in a time dependent manner, reaching a peak after 24 h of exposure. Uranyl chloride was also shown to decrease cell viability, cause translocation of phosphatidylserine, followed by morphological changes, such as cell shrinkage, chromatin condensation, cell blebbing and formation of apoptotic bodies and DNA fragmentation. DU exposure to macrophage cells resulted in cell apoptosis due to the accumulation of damage.	Kalinich et al. (2002)
Macrophages and primary CD4+ T cells	Macrophages and primary CD4+ T cells were exposed to uranyl nitrate to find it cytotoxic in both cell types in a concentration-dependent manner. DU was also shown to alter gene expression patterns in both cell types, with signal transduction genes being most differentially expressed, indicating the potential involvement of DU in carcinogenesis and autoimmune diseases.	Wan et al. (2006)
Human colon cells (primary colon cells, preneoplastic LT97 adenoma cells, highly transformed tumor cells HT29 clone 19A)	Uranyl nitrilotriacetate (U-NTA) genotoxicity in three types of human colon cells was studied. U-NTA has shown to be cytotoxic in HT29 clone 19A cells, and genotoxic in the other two cell lines. It also elevated the incidence of chromosomal aberrations in chromosomes 5, 12 and 17. The results could not prove its radiological toxicity, but confirmed the chemical genotoxicity.	Knöbel et al. (2006)
Human bronchial epithelial cells (BEP2D)	The results implied that DU induces loss of contact inhibition in addition to anchorage independent growth after 24 h of exposure, as well as that 53% of transformants exhibited a hypodiploid phenotype (chromosome numbers ranging between 7 and 43). DU is, therefore, capable of inducing neoplastic transformation and chromosome instabilities.	Xie et al. (2009)
Human osteoblast cells (HOS TE85 cell line)	Uranyl nitrate increased the incidence of dicentrics after 24 h of exposure. DU compounds of the same concentration and chemical effects, but different uranium isotopic concentrations, exhibiting different specific activities (^{238}U-uranyl nitrate, DU-uranyl nitrate and ^{235}U-uranyl nitrate) were also investigated. It was shown that the neoplastic transformation incidence increases in an activity-dependent manner, indicating the role of radiation.	Miller et al. (2002)
Chinese hamster lung fibroblast V79 cells	Mutagenicity was proven by a dose-dependent increase in mutagenic response after uranyl nitrate exposure. To prove radiation-related effects of DU, two uranyl nitrate isotopes of different specific activities were used (^{238}U-uranyl nitrate and DU-uranyl nitrate) and it was recorded that the increased specific activity corroborates with the increased mutation incidence.	Miller et al. (2007)
Rat lung epithelial cells	The results indicated that the mechanism by which DU induces oxidative stress in a time- and dose-dependent manner was the ability of DU to decrease the antioxidant potential of the cells. Also, it was found to decrease cell proliferation after 72 h of exposure.	Periyakaruppan et al. (2007)
Rat lung epithelial cells	The study demonstrated that uranyl acetate-induced oxidative stress may lead to apoptotic signaling pathways by increasing the activity of caspases − 3 and − 8 and cytochrome-c oxidase concentrations, leading to the conclusion that DU induces apoptotic cell death.	Periyakaruppan et al. (2009)
A1–5 rat embryo fibroblast cell line	The results have shown that neither uranyl acetate nor uranyl nitrate induce cell cycle arrest or increased apoptosis, that the levels of total p53 and active p53 did not increase after treatment with uranyl acetate, and that the level of active p53 did not increase in the presence of uranyl acetate or uranyl nitrate. The exposure to depleted uranium compounds does not trigger a p53-mediated pathway.	Heintze et al. (2011)

enriched uranium is used for making explosives and it contains more than 20% ^{235}U (Craft et al., 2004; HPS 2010; Islamović and Selimović, 2008; UNEP 2003). It is noteworthy that all phases of uranium processing (mining, fuel production, reactors and re-processing) produce high amounts of nuclear waste (Islamović and Selimović, 2008; Paternain et al., 1989).

There are several methods used to detect uranium and measure its concentration, including gravimetric, fluorimetric and polarographic methods. Specific analyses include fission track, phosphorescence kinetics, atomic emission, instrumental neutron activation, delayed neutron emission and α-particle counting (Craft et al., 2004; Hillson et al., 2007). A novel approach to uranium detection is the development of a biosensor for micromolar concentration of uranium, which is made from an engineered bacterium *Caulobacter crescentus,* that produces green fluorescence in the presence of uranium when exposed to a UV lamp. It is responsive to uranium but not to other heavy metals and has shown little non-specific activity towards NO_3^-, Pb, Cd and Cr (Hillson et al., 2007).

Human exposure to DU can be a consequence of different types of contacts, including dust inhalation, skin contact, embedded DU particles, entry through wounds and through water and food (Briner, 2010; Domingo et al., 1987). Once it enters the human body, uranium can undergo a series of chemical reactions resulting in formation of oxides, hydroxides and carbonates (Craft et al., 2004). LD_{50} value (median lethal dose, which is the dose of a substance that could kill half of individuals from an examined population after a specified time period; EHSC 2007) of uranium for humans is 14 mg/kg (Briner, 2010), although this value is currently being re-assessed (see below). Con-

sidering the fact that the consequences of DU exposure have been extensively investigated *in vitro,* on different mammal models and through human epidemiological studies in the last two decades in different laboratories, producing somewhat opposing results, the main aim of this review is to summarize and discuss the current state of knowledge about the DU exposure and its influence *in vivo* and *in vitro*. The authors of this review have tried to accomplish this by presenting a summary of previous investigations conducted on different mammalian body systems and cell cultures in terms of potential changes induced by either chemical toxicity or radioactivity of DU. The goal of such an approach is to re-assess the opinion that kidneys and bones are the primary targets of DU toxicity, that is, to examine whether, how and to what extent could other body systems also be affected by exposure to DU. Chromosomal aberrations, DNA damage, micronuclei formation and epigenetic changes are especially highlighted in this review, since these changes are speculated to be signature changes induced by DU radioactivity and not by its chemical toxicity. Furthermore, the authors aimed to differentiate between the changes caused by U chemotoxicity that has been a common concern related to heavy metal usage throughout human history, and DU radiation effects that might have carcinogenic abilities in living systems, as well as in cell cultures.

This review was written based on original research papers that were published in peer-reviewed journals and were collected through the search of relevant databases. Keywords consisting the terms “depleted uranium”, “*in vivo*”, “*in vitro*”, “micronuclei”, “epigenetics”, “carcinogenesis”, “malignancy”, “transformation”, “kidney”, “liver”, “nervous system”, “lungs”, “intestines”, “immune system”, “reproductive system” were searched through PubMed, PMC, ScienceDirect and ResearchGate.

Studies were selected based on the following parameters: cell lines and/or organ systems tested regarding exposure to DU were clearly stated; DU exposure levels and duration were clearly defined and results of the studies were unambiguous in terms of adequate statistical analyses and isotopic ratio of used DU source. According to these criteria, 19 papers investigating DU exposure on cell lines were selected, and 29 papers investigating DU exposure *in vivo*.

2. *In vitro* studies on the effects of DU

Since cell culture models are extremely important in the assessment of cellular and molecular mechanisms of potential or known carcinogens (Miller et al., 1998a), a variety of *in vitro* studies that demonstrate the carcinogenetic effects of depleted uranium have been conducted in the last 20 years (summarized in Table 1). The general conclusion that can be drawn from these studies is that the majority of changes induced in cell lines following DU exposure can actually be assigned to its chemical cytotoxicity resulting from uranium being a heavy metal rather than its relatively weak radioactive properties. Only two studies (Miller et al., 2002, 2007) actually used uranium sources of different isotopic content, thus managing to prove that cell damage resulted from radioactivity, and have both shown that DU is less harmful to cell cultures when compared to natural uranium containing higher amounts of the ^{235}U isotope. The remaining studies, however, managed to prove the induction of apoptotic pathways and even *de novo* neoplastic transformation in both cancer cells (Miller et al., 1998a, 2002; Xie et al., 2009) and non-mutated models (Wan et al., 2006).

3. *In vivo* studies on DU-related chemical toxicity and radioactivity

Evidence of DU chemical toxicity and irradiation effects following either acute or chronic exposure have been a focus of research interest during the past two decades. Numerous *in vivo* studies have been conducted on animal models with a focus on different systems within the body (Table 2).

One of the most comprehensive models of the main targets of uranium toxicity in living organisms came from a previous study in which rats were implanted with low, medium or high dose DU pellets and exposed for 1 day, or 1, 6, 12 or 18 months (Pellmar et al., 1999a). Negative controls were tantalum-implanted animals. Tantalum (Ta) is a biologically inert material, usually used in prosthetics that serves as a negative control in DU implantation studies. The results implied that the primary targets of DU exposure are kidney and bone, since they showed the most dramatic increase in DU concentration at all time points and DU doses. Other sites of increased DU concentration were liver, spleen, muscle, brain, lymph nodes, testicles, teeth, heart and lung. A minor increase was recorded in serum, while DU excreted in urine was higher than in controls a month after exposure and at all time points afterwards. Generally, DU concentration in target organs was neither time- nor dose-dependent (Pellmar et al., 1999a). Another general *in vivo* research on uranium exposure set the goal of retesting the limiting value of U concentration in drinking water, previously set by WHO (30 μg/l for human use; Dublineau et al., 2014). In the study, five rat organs were tested (small intestine, kidneys, hematopoietic cells, liver and brain) following exposure of 9 months to different concentrations of DU in drinking water. An extensive analysis of physiological parameters, organ size and uranium accumulation in the organs failed to prove serious harm to the rat body, thus indicating that the WHO threshold should be reconsidered, as authors argue that U content up to 1350 μg/l is safe for human use, and that kidney damage will not occur at a concentration below 300 μg/l. When taken together, previous *in vivo* studies investigating the effect of DU on animal models failed to determine long-term exposure to low DU doses, which is the most common scenario arising as a consequence of the remaining ammunition residues in the areas afflicted by war activities. Instead, the majority of the studies investigated DU effects on rats or mice, which has the major drawback of making it impossible to compare human and rat lifespan. One of the studies (Pellmar et al., 1999b) has shown that the effects of animal aging overcame the effects of DU exposure in rats as rapidly as in 18 months, therefore making it impossible to study prolonged chronic exposure. Additionally, the studies generated inconclusive results, meaning that chemical toxicity of DU can be confirmed by frequent alterations in the immune system response to exposure (Dublineau et al., 2006b; Hao et al., 2013), but transformation efficiency caused by irradiation was not successfully determined in the investigated models. The only studies that managed to prove an increased incidence of sarcomas (Hahn et al., 2002) and leukemias (Miller et al., 2005) were performed on rats and mice, respectively, therefore suffering from the abovementioned drawbacks. A study investigating the genomic instability transmission to unexposed offspring done on a rat model is particularly interesting as it succeeded in proving that the litter from DU-implanted male parents tends to be smaller and more prone to mutations. At the same time, a statistically significant increase in the formation of solid tumors has not been observed (Miller et al., 2010). However, the authors did not mention the possibility of blood tumor formation, which would be an expected consequence of an instable genome early in the childhood.

4. Chromosome aberrations and DNA strand breaks caused by DU

Depleted uranium is a suspected clastogen (causing breaks in chromosomes; LaCerte et al., 2010; Wise et al., 2007) and aneugen (an agent causing a cell to have an abnormal number of chromosomes; Miller, 2006; Nriagu et al., 2012). Therefore, it is capable of causing DNA damage and different types of chromosome aberrations through double strand DNA breaks (DSB) and incorrect rejoining (Capocaccia et al., 2015; Holmes et al., 2014; Ibrulj et al., 2007; Jovičić et al., 2004; Milačić and Simić, 2009; Wise et al., 2007). Depending on where the breaks and rejoining occurred, these aberrations can be divided into three major categories: inter-chromosomal, intra-chromosomal inter-arm and intra-chromosomal intra-arm (Brenner et al., 2001). Numerous *in vitro* and *in vivo* studies were performed on animal models and with human samples in order to assess the relationship between chromosome

Table 2
An overview of *in vivo* studies on DU effects performed on mammalian models.

Investigated body system/metabolic process	Major findings	Reference
Kidney	Rats were exposed to 40 mg/l DU in drinking water for 9 months. RBC count was reduced by 20%, the number of apoptotic cells was increased and change in expression of mRNAs corresponding to renoprotective genes was recorded. Renoprotective genes with anti-apoptotic function were downregulated by 90%, while those with antioxidant activity were 12-fold upregulated.	Berradi et al. (2008)
Kidney	Wistar rats were injected with uranyl acetate in the concentration of 0–2 mg/kg body weight and kidney mitochondria were isolated 24 h after the injection to check potential nephrotoxicity. An increase in blood urea nitrogen and creatinine levels was recorded at all UA concentrations, while electron transfer chain was disrupted. *In vitro* studies revealed the damage to mitochondrial complexes II and III, which causes oxidative stress, as well as decreased ATP concentration, mitochondrial swelling and cytochrome c release.	Shaki et al. (2012)
Kidney	Rats were implanted with 0, 0.1, 0.2 or 0.3 g DU fragments and euthanized 3, 6 or 12 months after implantation surgery. DU concentration in kidney tissue was dose-dependent and reached a peak after 3 months of exposure. Kidney size decreased in a dose-dependent manner with the signs of inflammation and changes in nucleus and mitochondrion shape. Many other morphological changes were detected when compared to negative and Ta-implanted controls. Moreover, renal dysfunction was detected by studying urinary and serum markers.	Zhu et al. (2009)
Liver	Hypercholesterolemic apolipoprotein E-deficient mice were exposed to 20 mg/l DU in drinking water for 3 months in order to study the effects of DU ingestion on liver cholesterol metabolism. Since no important differences between the control and test groups were observed, the conclusion of the study is that low DU concentrations do not cause alterations in liver metabolism, even if studied in hypersensitive animal models.	Souidi et al. (2012)
Intestines	Male rats exposed to drinking water contaminated with 40 mg/l DU for 3, 6 or 9 months were proven to have elevated neutrophil and reduced macrophage levels. The levels of certain cytokines and chemokines were changed during the exposure period, and NO (nitric oxide) pathway was inhibited by DU contamination. These effects correspond to those caused by heavy metal chemical toxicity and not radioactivity.	Dublineau et al. (2007)
Intestines	Male Sprague-Dawley rats were exposed to 40 mg/l DU in drinking water for 3 or 9 months in order to check for DU effects on Peyer's patches in intestines. Although Peyer's patches were proven to accumulate DU, the number of apoptotic cells and cytokine levels were not elevated in these structures, suggesting no DU-induced damage.	Dublineau et al. (2006a)
Intestines	Rats were acutely exposed to a sublethal dose of DU (204 mg/kg in water) and tested after 1 or 3 days. Proliferation, differentiation and apoptosis analyses revealed no DU toxicity in intestines. However, a slight modulation in gene expression and production of cytokines and chemokines in intestines was noted, suggesting possible immunological reaction.	Dublineau et al. (2006b)
Immune system	Kunming mice were daily fed with DU concentrations of 0, 3, 30 and 300 mg/kg in food for 4 months. The group that received 300 mg/kg DU in feeding doses showed a decline in innate immunity functions, as well as abnormal cellular and humoral immunity, while the groups that received 3 and 30 mg/kg doses did not differ significantly from the control unexposed group. However, changes in body or organ size and blood parameters were not observed in any of the tested groups.	Hao et al. (2013)
Nervous system	Male rats implanted with DU pellets for 6, 12 or 18 months were tested for potential neurotoxicity through four exposure groups (0, 4, 10 and 20 DU pellets). After 6 and 12 mo of exposure, electrophysiological reactions to external stimuli were shifted. After 18 months of exposure electrophysiological potential did not differ from the control group, although DU was detected in the brain tissue. Such an observation is explained by the fact that the aging of the animals overcame the effects of DU exposure.	Pellmar et al. (1999b)
Nervous system	Sprague-Dawley rats injected with uranyl nitrate of three concentrations (0, 70 and 144 μg/kg) were analyzed after three days. DU was detectable in a group that received a high dose, which was not the case with the unexposed control and with the group injected with low dose DU. It was also observed that sleeping processes were affected.	Lestaevel et al. (2005)
Reproductive system	Researchers investigated the effect of a different number of implanted DU alloys (0, 12 or 20 for male rats, and 4, 8, 12 or 20 for female rats) on reproduction in Sprague-Dawley rats. No differences were found between DU-implanted and control groups regarding sperm motility, caudal sperm concentration or mating success in any of test groups.	Arfsten et al. (2006)
Reproductive system	Sprague-Dawley rat F0 generation was implanted with different numbers of DU pellets (0, 12 or 20 for male rats, and 0, 4, 8, 12 or 20 for female rats) and mated 120 days following implantation. F0, F1 and F2 generations were investigated for mating success, litter number and average size, and histopathology of bodily systems. A consistent absence of DU effects on the study parameters was observed, indicating no important influence of DU exposure on the reproductive success and offspring generation.	Arfsen et al. (2009)
Damage transmission to offspring	Transgenic Big Blue mice were studied in order to test the probability of DNA damage transmission and the increase in mutagenicity level in unexposed offspring from male parents exposed to low, medium and high doses of internalized DU for up to 7 months. There were no significant differences in the frequency of visible mutations in litter, such as baldness and anatomical abnormalities. However, the size of the litter was lower when the male parents were DU-exposed. Mutation frequency analysis of bone marrow samples showed that male parents exposed for four and seven months produced litter with an increased mutation frequency since male parent mice had mutations in testes that were both time- and dose-dependent. Although solid tumors were not detected in the offspring, obvious genomic instability transferred from the male parent and sperm damage in an exposed male parent can make an offspring more prone to cancer development early in the lifetime.	Miller et al. (2010)
Serum and urine mutagenicity	Male Sprague-Dawley rats were implanted with different doses of DU (0, 4, 10 or 20 pellets), exposed for different time periods (0, 6, 12 or 18 months) and evaluated using Ames reversion test. Hydrophobic and hydrophilic urine fractions showed an increase in mutagenicity when compared to unexposed animals in time- and dose-dependent manner. However, serum mutagenicity was not increased in any experimental group in comparison to the control group.	Miller et al. (1998b)
Sarcoma development	Male Wistar rats were implanted with DU pellets or DU fragments into soft muscle tissue and observed regarding their life span. Negative controls either received a sham surgery or tantalum implants. After a year, a change in life span was not detected, but 11 out of 50 implanted animals developed sarcomas, while no disease was detected in non-implanted animals. Tantalum-implanted animals developed 2 sarcomas. Tumor	Hahn et al. (2002)

(continued on next page)

Table 2 *(continued)*

Investigated body system/metabolic process	Major findings	Reference
	incidence increased with the size of implants.	
Leukemia development	DBA/2 mice implanted with 2, 6 or 8 DU pellets and injected with murine hematopoietic cell line FDC-P1 60 days after implantation surgery were tested for leukemia development. The difference in the number of detected cancers between test and control group was significant (76% *vs.* 12%, respectively).	Miller et al. (2005)

and DNA breakage and DU exposure.

The effect of low linear energy transfer (LET) radiation on spatial clustering of 22 pairs of human autosomes in lymphocytes during metaphase was studied *in vitro* using multicolor FISH (fluorescence *in situ* hybridization). Authors have identified two important chromosome clusters, namely chromosomes 13, 14, 15, 21, and 22, which were found to be close to each other in the nucleolus, while chromosomes 1, 16, 17, 19, and 22 were more likely to be located in the center of the interphase nucleus. The non-random proximity of these chromosome clusters implies an increased possibility of inter-chromosomal aberrations (Arsuaga et al., 2004). The results of the study apply to DU irradiation because DU, as an alpha- and beta-particle emitter (Dewar et al., 2013; McDiarmid et al., 2011) produces both low- and high-LET. The studies discussed in the present review provide evidence of an increased frequency of dicentric chromosomes *in vitro* (Miller et al., 2002) and *in vivo* (Ibrulj et al., 2007; Jovičić et al., 2004), thus proving that DU really influences spatial orientation of chromosomes in the cell in a way that enables easier interchromosomal fusions.

4.1. DU exposure can cause different types of chromosomal aberrations

A study of dependence between chromosomal aberrations in peripheral blood lymphocytes and DU exposure, either through inhalation or embedded DU-containing particles, was performed on 35 Gulf War veterans in Baltimore, MD using FISH. The authors did not detect any statistically significant relation between any type of chromosomal aberration and uranium concentration in urine since the aberration frequency was consistent with that of the general population. However, it was concluded that the incidence of translocations, dicentrics, color junctions and abnormal cells depended on the age of the examined individuals (Bakhmutsky et al., 2013).

On the other hand, a study done by Jovičić and colleagues (2004) has produced somewhat opposing results. In this study, a control non-exposed group had a frequency of chromosomal aberrations of 0.2%, while the study group (individuals from uranium-contaminated areas in Serbia) had an incidence of 20.6%, with dicentrics, acentrics and chromosome rings. The frequency of structural chromosomal aberrations was 0.3 per subject in the control group and 1.3 per subject in the study group (Jovičić et al., 2004). In another study performed in Serbia, four different groups exposed to radiation were compared: the first group were subjects chronically exposed to DU, second group were subjects who were temporarily exposed to DU at some point in life, and the third and fourth group were control groups consisting of subjects exposed to non-DU chronic or acute radiation, respectively (Milačić and Simić, 2009). This study found that individuals with chronic exposure to DU irradiation had an increased number of chromosomal aberrations, lesions and damaged cells when compared to the reference groups. A group consisting of individuals who were temporarily exposed to DU irradiation was not statistically different from the control groups (Milačić and Simić, 2009). A study of the Bosnian-Herzegovinian population from three locations (Sarajevo, Posušje and Hadžići, with the last one being the DU-exposed study group) confirmed significant differences between the study group and the controls. In Hadžići, 95% of all aberrations were chromosome-type, mainly dicentrics, and the remaining 5% were chromatid-type (Ibrulj et al., 2007).

Fišter and Jović (2014) performed a study on sheep inhabiting DU-contaminated areas in Serbia in order to detect chromosome abnormalities and karyotype changes. Sheep inhabiting the area that was affected by the NATO air strikes in 1999 in southern Serbia (Bujanovac) were analyzed and compared to two control groups from the northern part of the country (Zemun and Ovča). Sheep inhabiting the Bujanovac region showed a significantly higher incidence of chromosome and chromatid breaks and gaps, while the number of polyploid and aneuploid cells did not differ significantly between the groups. The results of the study could not confirm that chromosomal aberrations were a consequence of DU exposure alone, since such changes could also happen due to breeding and feeding practices and heavy metal pollution in the study area (Fišter and Jović, 2014).

An *in vitro* study by Wise and colleagues (2007) investigated the effect of uranium trioxide (UO_3, a source of particulate DU) and uranium acetate (UA, a source of soluble DU) on normal human bronchial fibroblasts. UO_3 caused an increase in the number of damaged metaphase cells and the number of chromosomal aberrations, indicating that it might act as a clastogen, with chromatid lesions being the most common type of aberration. On the other hand, UA did not increase cell damage and chromosomal aberrations (Wise et al., 2007). *In vitro* effects of UO_3 were also studied on Chinese hamster ovary cell line (CHO) by Holmes and colleagues in 2014. The results showed that UO_3 caused an increase in the number of damaged metaphases and chromosome damage after 24 h exposure. The most common aberrations were break-type chromatid lesions and fragmented chromosomes (Holmes et al., 2014).

4.2. DNA strand breaks

As depleted uranium has been suspected to cause different types of cancers, it is interesting to assess its potential involvement in DNA strand breaks (Capocaccia et al., 2015). DNA breaks induced by DU exposure were studied on both ssDNA and dsDNA on several occasions.

Single-stranded DNA breaks were analyzed *in vitro* on pBlue-script SK^+ plasmid DNA incubated in the presence of uranyl-acetate (UA) and ascorbate (Asc, vitamin C), which caused 80% plasmid relaxation (Yazzie et al., 2003). Single strand breaks were 6–8 times more likely to occur in the case of coupled UA + Asc incubation than when DNA was incubated with UA or Asc alone. In addition, DNA relaxation was 5 times higher than in the case of DNA incubation with Cr(VI) + Asc, which served as a positive control, since hexavalent chromium is a known DNA mutagen that causes ssDNA breaks in the presence of ascorbate. It was proposed by the authors that breaks occur at least in part due to H_2O_2 production, which is an indirect mechanism for uranium-caused DNA breaks. The direct mechanism, which was also suggested, involves the reaction of the UA + Asc complex with the negatively charged phosphate backbone of DNA (Yazzie et al., 2003).

An *in vitro* study was conducted on two CHO cell lines, parental CHO AA8 line and CHO EM9 line (Stearns et al., 2005). The CHO EM9 cell line is deficient in DNA-repair mechanism and has reduced levels of XRCC1-DNA ligase III complex. This complex plays a role in rejoining the phosphodiester backbone of DNA and therefore in DNA strand break repair. DNA strand breaks were detected in both cell lines following 40 min and 24 h of exposure to UA, but the response was not dose-dependent and there was no significant difference between the

two cell lines. The analysis of DNA-uranium adducts showed that both cell lines experienced adduct formation on the scale of a few uranium atoms per 1000 nucleotides that was both dose- and time-dependent and without significant differences between the two cell lines (Stearns et al., 2005). This was the first study to show that uranium can directly interact with and bind to the DNA.

Monleau et al. (2006b) did an extensive work on DNA breaks caused by soluble and insoluble depleted uranium particles. An *in vivo* study was performed on pathogen-free adult male rats using UO_2 (uranium dioxide, insoluble DU) and UO_4 (uranium peroxide, soluble form). Epithelial nasal cells did not show changes in the number of DNA double strand breaks following the exposure to any DU form or dose. BAL (broncho-alveolar lavage) cells experienced DNA breaks in the case of UO_2 exposure and the consequences lasted longer in the case of repeated than in the case of acute exposure. Kidney cells had detectable DNA breaks only in the case of repeated exposure to UO_2, but with consequences detectable for a shorter time period (Monleau et al., 2006b). Another *in vivo* study of similar design used the same model organisms and DU sources. The first test group was acutely exposed to UO_4 only for 30 min ($AcuUO_4$), the second one was acutely exposed to UO_2 for 3 h followed by 30 min exposure to UO_4 ($AcuUO_2 + UO_4$), while the third group was repeatedly exposed to UO_2 for 3 weeks, followed by UO_4 exposure for 30 min ($RepUO_2 + UO_4$). The results of the previous two studies together imply that the extent of DNA damage caused by inhaled DU particles depends on the solubility of those particles. The insoluble form causes more DNA damage, while the soluble form would cause DNA breaks only in the case of pre-exposure to UO_2. In addition, it was shown that the frequency of DNA breaks increases with repeated exposure to DU and not in the case of acute exposure (Monleau et al., 2006a).

Finally, an *in vitro* study done on normal rat kidney proximal cells ($NRK\text{-}52^{E}$) investigated DNA damage and chromosomal breaks since kidneys, along with bones, are known to be the primary targets of DU toxicity (Craft, 2004). DNA damage was proportional to the duration of exposure and concentration of DU to which the cells were exposed. Also, an increased incidence of DNA ds breaks was noticed after DU exposure, with a peak reached at 500 μM DU, while the cells were treated with DU in the range from 200 to 600 μM (Thiébault et al., 2007).

4.3. Micronuclei formation in DU-exposed subjects and samples

Exposure to depleted uranium is suspected to cause micronuclei (MN) formation (Al-Muqdadi and Al-Ansari, 2011; Briner, 2010), which is a direct consequence of mutagenetic stress and plays an important role in the genomic plasticity of tumor cells (Huang et al., 2011; Utani et al., 2010). These structures are formed from whole chromosomes or their fragments which did not reach mitotic spindle poles during cell division. Micronuclei were the subject of several studies. Ibrulj and colleagues (2006) investigated 30 individuals who lived in Sarajevo, B & H, during the war activities and afterwards. Out of 1000 binuclear cells, the number of cells with micronuclei ranged from 3 to 31 with a mean value of 10.97. This value was higher than in individuals who were not exposed to war activities, but significantly lower than in individuals who were confirmed to be DU-irradiated (Ibrulj et al., 2006). Another study investigating MN frequency in B & H was done by Krunić et al. in 2005. The frequency of micronuclei in binucleated cells was examined in Hadžići, close to Sarajevo (DU-exposed group) and West Herzegovina inhabitants (control group). In the exposed group, 60% individuals had an increased MN frequency, while the value was 37% in the control group, which is a statistically significant difference (Krunić et al., 2005).

A study was conducted on Gulf War veterans in 2011. Study participants were divided into two groups, low and high, depending on urine uranium concentration. There was no control non-exposed group. Statistical analysis did not show important differences in the number of cells with MN or in the frequency of micronucleated binuclear cells between these two groups (Bakhmutsky et al., 2011). A similar study designed by McDiarmid and colleagues (2011) compared the number of MN in the peripheral blood cells and chromosome breaks in 35 Gulf War I veterans divided into high urine U (uU; 13 participants) and low uU (22 participants) groups. Again, no significant differences between the two groups were found in terms of number of micronuclei formed, as well as related to the overall number of aberrant chromosomes detected by FISH (McDiarmid et al., 2011).

An *in vitro* study was performed by Miller et al. (2003) on immortalized human osteosarcoma cells (HOS). The number of micronuclei-producing cells in the DU-exposed group was compared to non-exposed controls and to gamma radiation- and Ni-exposed cells. The incidence of MN in the DU-exposed group was higher than in all three control groups. Micronuclei formation was increased even 36 days after the end of exposure, which was not the case with the two other study groups, indicating prolonged effects of DU exposure and *de novo* formation of micronucleated cells even after the direct exposure was over. While the frequency of micronuclei in untreated cells was below 1.8%, the value ranged between 2.2% and 4.5% in DU-exposed cells (Miller et al., 2003).

A general conclusion that can be drawn from previously performed studies regarding chromosome aberrations and DNA strand breaks induced by DU is that, although less radioactive than natural and enriched uranium, DU was on few occasions pointed out as a cytotoxic and clastogenic pollutant. Also, different research groups managed to prove statistically significant changes in the number of chromosome aberrations, DNA breaks and micronucleated cells in DU-exposed individuals and cell lines when compared to control populations. These results, however, are not definite confirmation of DU influence on chromosome aberrations as another important group of studies failed to find any statistically significant differences between exposed and unexposed individuals, and especially not between individuals with low vs. high uU content. While *in vitro* studies seem to be capable of proving an increased frequency of cell deaths in DU-exposed cultures, *in vivo* studies on animal models and humans failed to detect the trend of DU genotoxicity and clastogenic effects on many occasions. A reasoning offered by McDiarmid and colleagues in their work published in 2011 is a very interesting insight into this area, as it suggests that certain study groups might have had an increased cancer incidence due to exposure to radon, a byproduct of uranium radioactive decay, rather than due to uranium itself. Furthermore, DU which is even less radioactive than natural U, would be even less expected to exert carcinogenic and/or mutagenic effects in living organisms (McDiarmid et al., 2011). An important consideration related to these studies is the follow-up period. Since DU release events in B & H happened during 1995, that is, 20 years ago, there is a definite possibility that the follow-up period is relatively short and that prolonged DU effects are still to be detected. This was suggested by an *in vitro* study done by Miller et al. (2003) where the authors revealed a possibility of the appearance of micronuclei formation even after DU exposure. The same conclusion can be drawn from the studies performed on DNA breaks and consequential chromosomal aberrations, either numerical or structural, as longer follow-up periods would definitely be necessary to fully assess the long-term effects of either acute or chronic exposure to DU particles on genome instability. Finally, it is worth noting that DU exposure seems to leave a specific fingerprint when it comes to the type of chromosome aberrations. As discussed above, DU exposure is capable of inducing different types of these changes. There appears to be a pattern in a way that *in vivo* studies mainly prove chromosome-type, while *in vitro* studies tend to prove an increase in chromatid-type chromosome changes (Holmes et al., 2014; Ibrulj et al., 2007; Jovičić et al., 2004; Wise et al., 2007). Additionally, the studies are consistent in proving that chronic exposure to DU affects health more than acute exposure, as well as that insoluble DU particles tend to cause more frequent DNA breaks when compared to soluble DU (Milačić and Simić, 2009;

Monleau et al., 2006b, 2006a).

5. Epigenetic changes due to DU exposure: DNA methylation status

Epigenetic changes, a novel focal point in molecular biology research, are also suspected to be related to many types of hematopoietic cancers (Costa, 2010; De et al., 2013; Jelinek et al., 2012; Thathia et al., 2011), which is why such changes deserve attention during the investigation of DU effects on human health. Studies investigating epigenetic changes induced by DU exposure are rather scarce, with only two relevant original research papers available in the literature, in which, however, a strong relationship between DU exposure and aberrant DNA methylation patterns has been detected.

In vivo leukemia development and the methylation status of spleen cells were studied by Miller's team (2009) in DU-implanted DBA/2 mice that were exposed to DU irradiation for two months. DNA 5-^{Me}C level of mice from the un-implanted control group was taken to be 1.0, while exposed mice that developed acute myeloid leukemia (AML) had a normalized methylation content of 0.75, which indicated hypomethylation. Transcriptional activation of endogenous retrotransposable repeat elements was studied by Northern blot, since these elements are normally silenced through methylation. The results of this study indicated that the presence of endogenous retrotransposable repeat elements in the control samples (spleen and bone marrow) was negligible. On the other hand, their levels were significant in all eight AML samples tested. This Led to the conclusion that hypomethylation can cause an increased expression of retrotransposable elements and insertional mutagenesis, which can further lead to cancer development (Miller et al., 2009).

Although not performed on a mammal model, a recent *in vivo* study performed by Gombeau and colleagues (2016) on zebrafish (*Danio rerio* AB strain) is worth mentioning. Zebrafish brain, eyes and gonads were analyzed following exposure to water-borne DU in concentrations of either 2 or 20 μg/l for 7 or 24 days to check for sex- and tissue-specific epigenetic changes. Methylation at CpG sites was investigated by restriction digestion with *Hpa*II (5′-CCGG-3′). Changes in brain tissues in females were not detected, but were significant in males after 24 days of exposure with 32% and 54% reduction in methylation for groups exposed to 2 and 20 μg/l DU, respectively. In gonads, hypomethylation was observed in both sexes, but was more pronounced in males. In eyes, no significant effects in females were observed. In males, 42% and 71% reduction in methylation was observed after 7 and 24 days of exposure to 20 μg/l DU, respectively.

In the same study (Gombeau et al., 2016), HPLC-MS/MS was used to investigate whole-genome methylation patterns on cytosine residues. Hypermethylation was observed in female brain tissue at both concentrations after 7 days of exposure, but this trend was not detected after 24 days. In the case of males, 34% hypermethylation was detected after 24 days of exposure to 20 μg/l DU. In the case of gonads, females did not show significant changes, while males had 11% lower methylation than controls 24 days after exposure to 20 μg/l DU. In eyes, males showed 11% higher methylation frequency after 24 days of exposure to 20 μg/l DU when compared to unexposed controls, while females did not show any epigenetic changes. The authors proposed three possible pathways through which DU might affect epigenetics: (1) interaction with epigenetic machinery, e.g., affecting methyltransferase enzyme, (2) binding to nuclear receptors to induce or silence epigenetic factors and (3) activation of membrane receptor signaling cascades (Gombeau et al., 2016). The third pathway is presented as the most likely to occur. Namely, it is known that calcium gets substituted by uranyl cations in proteins. Furthermore, in the form of Ca^{2+}, it is the second messenger in a number of signaling cascades, which respond to exposure to extracellular signaling molecules.

The literature synopsis above clearly indicates the absence of investigation regarding the effect of DU on epigenetic changes, *i.e.* aberrant DNA methylation patterns. Current literature provides only a single study concerning such changes in mammals, and a single study done on zebrafish as a study species. However, both studies successfully proved the impact of DU on such changes, which is the reason they are both included. While exclusion criteria for the preparation of the present review states that the studies on non-mammalian models were excluded, the only exception is the study by Gombeau and colleagues (2016) since the evidence of DU-caused epigenetic changes is almost absent from the literature and any evidence should be used for the topic elaboration. Additionally, the study on zebrafish is giving results that are partially in compliance with the results obtained previously on a mouse model (Miller et al., 2009), thus implying that the research should be continued in both directions in order to discover a stronger relationship between DU irradiation and epigenetic changes in different animal species.

6. Concluding remarks and future perspectives

Previous research on the consequences of either acute or prolonged depleted uranium exposure managed to prove that, as a heavy metal, this element is capable of inducing chemical toxicity and cell damage in the form of cell morphology alteration, immune response and/or apoptotic death, which is evident in the *in vitro* studies reviewed in this paper. Furthermore, *in vivo* studies confirmed chemical toxicity of DU by frequent alterations in the immune system response to exposure (Dublineau et al., 2006b; Hao et al., 2013). Radiation-related effects are still, however, in the domain of speculation. Studies done *in vitro* investigating cell damage as a result of radioactivity showed that DU is less damaging to cell cultures when compared to natural uranium containing higher amounts of the ^{235}U isotope. *In vivo* studies failed to determine a significant increase in transformation efficiency as a consequence of either acute or chronic irradiation of the investigated models. In order to either confirm or disband the hypothesis that DU irradiation is capable of inducing neoplastic changes *in vivo* and *in vitro,* it would be necessary to conduct experiments using uranium isotopes of different radiation potential, different DU concentrations, as well as to conduct studies with long-term follow-up periods that would truly reveal the consequences of prolonged exposure to low-dose DU, which is the most common type of exposure encountered in everyday practice.

Some studies regarding chromosome aberrations and DNA strand breaks as a result of DU indicated that DU can be considered as cytotoxic and clastogenic, as DU exposure induced cell death in several studies. Respective studies confirmed statistically significant changes in the number of chromosome aberrations, DNA breaks and micronucleated cells in DU-exposed individuals and cell lines when compared to control populations. In addition, it appears that DU exposure leaves a specific signature regarding chromosome aberrations, and while *in vivo* investigations showed chromosome-type changes, *in vitro* studies demonstrated chromatid-type chromosomal changes (Holmes et al., 2014; Ibrulj et al., 2007; Jovičić et al., 2004; Wise et al., 2007). Literature also demonstrated that chronic exposure to DU affects health more than acute exposure, as well as that insoluble DU particles tend to cause more frequent DNA breaks when compared to soluble DU (Milačić and Simić, 2009; Monleau et al., 2006b, 2006a). Longer follow-up periods would definitely be necessary to fully assess the long-term effects of either acute or chronic exposure to DU particles on genome instability. In addition, one must also take into consideration that continuous follow-up on soldiers and inhabitants of DU-contaminated areas and better healthcare management of such individuals would surely improve our current understanding of the potential damage caused by this exposure and whether it arose due to chemical toxicity of DU, which is characteristic for heavy metals, or is primarily related to its irradiation effects. Moreover, the closer follow-up of mine workers would help us understand if any increase in cancer incidence is due to DU or radon exposure, as radon is known to be an important source of radiation that is capable of cell transformation. The properties

of DU when it comes to radiation are, however, opposing the statements that it is causing neoplastic transformation simply due to its low radioactivity. Finally, some studies presented in this review consider age an important variable (Bakhmutsky et al., 2013; Ibrulj et al., 2006, 2007), while other studies reported that there seems to be no correlation between age and any of the possible changes induced by DU (Milačić and Simić, 2009). This leads to the conclusion that an increased frequency of changes that correlated positively with older participants might simply be a result of mutation and damage accumulation over lifetime and not solely due to exposure to DU.

The literature synopsis regarding the effect of DU on epigenetic changes, i.e., aberrant DNA methylation patterns clearly indicates the absence of such investigation. Current literature provides only a single study concerning such changes in mammals, and a single study done on zebrafish as a study species. However, both studies successfully proved the impact of DU on such changes. The differential methylation status of DU-exposed cell lines and individuals is to be taken into consideration and to be studied as a possible signature of DU radiation and not its chemical toxicity. Identifying specific changes caused by DU irradiation would help in a more specific approach of identifying malignant diseases that arose solely due to such exposure, and not due to genetics, lifestyle, or any other environmental influence, which was the main problem in the abovementioned studies that keep proving the chemical toxicity but not the carcinogenicity of DU.

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