Preliminary Study to Investigate the Distribution and Effects of Certain Metals After Inhalation of Welding Fumes in Mice

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Abstract

The most important welding processes used are the Gas Metal Arc (GMA) welding, the Tungsten Inert Gas (TIG) welding, and the Manual Metal Arc (MMA) welding processes. The goal of our investigation was to monitor the distribution of iron (Fe), manganese (Mn), calcium (Ca) and magnesium (Mg) in the lung, spleen, liver, and kidney of mice after inhalation exposure of different welding methods using different steel base materials. The treatment groups were the following: MMA-mild steel, MMA-molybdenum-manganese (MoMn) alloy, TIG-mild steel, and TIG-stainless steel. The samples were taken 24- and 96 hours after the treatments.

Most importantly, it was found that the Mn concentration in the lung' samples of the MMA-mild steel and the MMA-MoMn groups was increased extremely at both sampling times and in the spleen' samples also. In the TIG groups, the rise of the Mn concentration was only considerable in the lungs and spleens at 24h, and emerged concentration was found in the liver in 96h samples.

Histopathology demonstrated emerged siderin content in the spleens of the treated animals and in siderin filled macrophages in the lungs mostly in all treated groups. Traces of high-level glycogen retention was found in the MMA groups at both sampling times. Similar glycogen retention in TIG-Ms and TIG stainless group's liver samples and emerged number of vacuoles, especially in the hepatocytes of the TIG-stainless steel 96h group were also found.

The mentioned results raise the consequence that there is a considerable difference in the kinetics of the Mn distribution between the MMA- and the TIG-fume treated groups. Hence, the result suggests that manganese has a particle-size dependent toxico-kinetics property. The anomaly of the glycogen metabolism indicates the systemic effect of the welding fumes. Also, the numerous vacuoles mentioned above show a possible liver-specific adverse effect of some components of the TIG-stainless steel welding fumes.

Introduction

Welding is the most often used joining method of metals (especially steel) in the industrial manufacturing worldwide. Different technologies are developed for joining ferrous and non-ferrous metal parts, which can differ considerably from each other. A large part of these processes utilizes the energy and heat of the electric arc for melting and joining the workpieces. The most widely used joining process for steel constructions is the Gas Metal Arc (GMA) welding, the Tungsten Inert Gas (TIG) welding and the Manual Metal Arc (MMA) welding processes. Recently, however, the automatization of the welding methods in manufacturing has undergone a remarkable development, the welding work is still performed mostly by human welders all over the world (IARC 2018). Thus, many hundred-thousand workers are involved in full- or part time job in the world, and suffer from the chemical, thermal, radiation and other exposures of the welding. Since welding requires high skills, these workers are precious human resources of the manufacturers and often there are shortages from skilled enough personnel on this field for example in our country and whole Europe as well. In this aspect, it can be concluded that welding has a high economic

and healthcare impact. Workers affected by the fume exposure of welding are often suffering from moderate to high-level respiratory function changes, pulmonary fibrosis, siderosis and deposition of other metal particles in the lungs, and even from lung cancer. It should be mentioned that these problems are usually originated from multiple causes besides of welding. Smoking, fume- and dust exposure from other sources also play a role in the pathomechanism (Al-Otaibi 2014, Flynn & Susi 2010, Kazi et al. 2021, Mur et al. 1989, Sferlazza & Beckett 1991).

To understand and eliminate the possible risks and dangers generated by the welding fumes, several *in vivo* and *in vitro* experiments have been performed and epidemiological cohort analyses were made. The majority of them were concentrating on the GMA and the MMA welding processes, since obviously they are the most fume-generating methods that are widely used in manufacturing. These experiments underlined the importance of different metal content in the fume (especially welding high-alloy steels) causing irritation, decreased pulmonary function, increased airway hyperresponsiveness, bronchitis, even lung cancer, like Cr, Al, Ni, Mn, Mg. Some of the metals can be absorbed from the lungs and have systemic effects on the nervous system or in the kidneys (Akbarkhanzadeh 1980, Antonini et al. 2003, Pandeh et al. 2017, Rossignol et al. 1990). TIG welding process is highly underrepresented in these kinds of investigations, probably because it is less widely used and produces significantly less amount of visible fume. This method can be used to join several kinds of metals, like mild to high alloy steels, aluminium-, manganese-, titan-, and copper- alloys (Khan 2007). However, some investigations are showing that this welding procedure can also generate fine and mostly ultrafine metal-containing particles that are available to inhale (Berlinger et al. 2011, Berlinger et al. 2008, Floros 2018).

The goal of the investigation was to monitor the distribution of iron (Fe), manganese (Mn), calcium (Ca), magnesium (Mg) in the lung, spleen, liver and kidney of mice after inhalation exposure to different welding methods using different steel alloys.

Materials And Methods

Experimental design

Thirty-eight BALB-C mice originated from the National Institute of Oncology of Hungary, (Budapest, Hungary), with Specific Pathogen Free (SPF) status, were used in the study. They were grouped randomly into five groups (Table 1).

Table 1
Experimental design

Group name	Treatment	No. of animals
C	Untreated control	8
MMA-Ms	MMA-mild steel	8
MMA MoMn	MMA MoMno alloyed high strength steel	6
TIG-Ms	TIG-mild steel	8
TIG-X	TIG stainless steel	8
Mo=Molibdenum, Mn=Manganese		

The exposure of the animals was applied for a four-hour time interval in the case of each group.

The experiment was performed with the permission of Animal Experiment Allowance No. PE/EA/289-7/2018 given by the Animal Protection Authority of the Hungarian Government Office.

Sampling

Samples were taken twice from each group at 24- and 96 hours after exposure. During the sampling, the animals (4 mice but 3 ones from MMA MoMn treated group) were euthanized, and tissue samples (lung, spleen, liver, kidney) were dissected for chemical and histopathology examinations. Since these samples were to be used in chemical- and histopathological analysis, the organs were separated from one animal as follows (the individual sample masses were not measured):

- One lung, two-third of the spleen, the half of the liver and one kidney was collected for the chemical analysis.
- One lung, one-third of the spleen, the half of the liver and one kidney was collected for the histopathology.

Welding setup

In order to perform the fume inhalation treatments, actual welding work has been done. The welding was performed as a manual overlay weld with multiple layers on the indicated base metal plate according to the general technical requirements, and it was done by a qualified welder. The technical parameters for each treatment group are shown in the second Table 2. The base metal types are designated according to EN 10027-2:2015 standard, the filler metal types are designated according to the manufacturer's designation system. Data are declared by the manufacturers or by international standards. The chemical composition of the used base- and filler metals are shown in the Table 3.

Table 2
Technical parameters of welding methods used in the different experimental groups.

Method	Base metal plate	Filler metal rod	Electrode polarity	Electrode diameter	Welding current
MMA-Ms	EN 1.0038	OK46.00	DC -	2 mm	80 A
MMA MoMn	EN 1.0038	OK74.78	DC +	5 mm	180 A
TIG-Ms	EN 1.0038	TigRod 12.64	DC -	2 mm	80 A
TIG-X	EN 1.4301	TigRod 16.12	DC -	2 mm	80 A
DC: direct current					

Table 3
Chemical composition of the base- and filler metals declared either by EN 100272:2015 standard or by the manufacturer

Base metal	Chemical composition (maximum, m/m%) of the metal	Coating type
EN 1.0038	C: 0.2, Mn: 1.4	N.a.
EN 1.4301	C: 0.07, Mn: 2, Si: 1, Cr: 19.5, Ni: 10.5	N.a.
Filler material		
OK 46.00	C: 0.08, Mn: 0.4, Si: 0.3	rutile
OK 74.78	C: 0.06; Mn: 1.5, Si: 0.35, Mo: 0.40	basic
TigRod 12.64	C: 0.12, Mn: 1.85, Si:1.15	N.a.
TigRod 16.12	C: 0.03, Mn: 2.1, Si: 1, Cr: 21, Ni: 11	N.a.
C=Carbon, Mn=Manganese, Si=Silicium, Mo=Molibdenum, N.a.: not applicable		

In the case of TIG welding, the shielding gas was 99.996% Ar (Linde Hungary Limited, Répcelak, Hungary) and WT20 0.2% thoriated tungsten electrode with a diameter of 2 mm was used.

The fume was eliminated from the welding chamber using a Kemper SmartMaster (Kemper GmbH, Vreden, Germany) local fume excreting and filtering device. The animal treatment chamber was 800 × 540 × 330 mm, in size, and it was placed between the smoke source and the filtering unit, so the excreted welding fume containing air was forced to go through the treatment chamber. The SmartMaster unit has an air excretion rate of 950 m³/h, according to the manufacturer's technical specification. The test animals were put into the treatment chamber with their own housing boxes with feedstuff and water *ad libitum* supplied. The animals were free to move around their boxes, and no forced exercise was applied during the treatment. During one treatment, four animals were placed into the treatment chamber. Since this experiment was intended to be a model study, we did not had the chance to measure the exact composition of the polluted air, however we made some measurements with a first version Kemper

AirWatch (Kemper GmbH, Vreden, Germany) air monitor both in the breathing zone of the welder and in the airflow of the treatment chamber, which showed that the air pollution is about 10 times higher in the treatment chamber than in the breathing zone during the welding process (data not shown). Unfortunately, this device is designed to perform a real-time air analysis in an industrial site rather than to be used as a research instrument. This setup was used in the entire experiment.

Chemical analysis of biological samples

The detection of metals such as Fe, Mn, Ca, Mg was performed using a PE Optima 8300 Inductively Coupled Plasma – Optical Emission Spectroscopy device (ICP-OES) (PerkinElmer Inc., Waltham, Massachusetts, USA). The specific organs of the animals in the specific experimental groups indicated above were pooled and homogenized to provide enough sample mass for the examination. The amount of 0.5 g from each tissue sample was digested using 5 ml concentrated HNO_3 and 5 ml H_2O_2 in a CEM MARS 6 microwave digesting system (CEM Corporation Matthews, NC, USA) at 200°C at 1700 W power for 50 minutes. After the digestion process, the samples were filled up to 25 ml with ultraclean water and were 2-fold diluted for the examination. Standard solution with a concentration of 1 mg/l was used for each measured element, the used chemicals were ultrapure quality (Sigma Aldrich, St. Louis, Missouri, USA), the ultrapure water was originated from a Zeneer Power I water purification system (Human Corporation, Seoul, Republic of Korea). Since the experiment was a model study with no previous data, we arbitrary set the limit of 10% difference from the control values as a threshold that we consider as deviation caused by the treatment in our setup. This 10% value is used to set the error bar values in the graphical representation of our results. Since the sample size is small, we were not able to use statistical methods to evaluate our data further.

Histopathology examinations

Tissue samples for histopathology examination were preserved in 10% buffered formaldehyde solution (Molar Chemicals Kft., Halásztelek, Hungary). After 48 hours, the samples were placed into embedding cassettes and were embedded into paraffine using a Thermo Shandon Excelsior automatic embedding machine (Thermo Electron Corporation, Waltham, Massachusetts, USA). The slides were stained with Haematoxylin-Eosin (HE) staining and Perls's Prussian blue staining described elsewhere (Lillie et al. 1976, Parmley et al. 1978).

Digital microscopy

The histopathology slides were digitalized by a 3DHitech Panoramic 1000 slide scanner (3DHISTECH Ltd, Budapest, Hungary) utilizing a 40-times magnification objective lens for the acquisition. The micrographs have been made by the 3DHitech Panoramic Viewer 1.15.4 software (3DHISTECH Ltd, Budapest, Hungary).

Material examinations

Scanning electron microscopy (SEM)

The SEM examinations were performed by a Carl Zeiss Leo EVO 50 instrument (Carl Zeiss AG, Oberkochen, Germany) at the Department of Material Science and Technology, Budapest University of Technology and Economics. Pictures were taken from the samples, and the chemical composition was also identified by the attached energy dispersive X-Ray spectroscopy (EDS) device. In the case of MMA covered stick electrodes, both the wire and the covering material were examined separately.

Spectroscopy

Spectroscopy analysis was performed on the suitable samples using a PMI-MASTER Sort (Worldwide Analytical Systems AG, Uedem, Germany) instrument at the Department of Material Science and Technology, Budapest University of Technology and Economics.

Results

Material examinations

The measured chemical composition of the welding materials and the final welds are summarised in Table 4. The most important finding in this examination that should be emphasized is that the Mn concentration in the covering material of the OK 46.00 and the OK 74.78 welding sticks which were measured as 18.05 m/m% and 3.15 m/m% respectively. These findings are important since manufacturers only declare the chemical composition of the metal core wire, but the composition of the coating material is kept confidential. During the welding, a large part of the fumes is originated from this covering material (Hedberg et al. 2021, Kirichenko et al. 2018). The difference of the Mn content between the raw welding materials and the final weld seam is caused by the different coating material (rutile or basic) because these have different capabilities to save the alloying metals in the weld seam. This is resulting the shown higher Mn content in the weld seam of the MoMn alloyed OK 74.78 electrode despite of the lower overall Mn content of it compared to the OK 46.00 electrode.

Table 4

Concentrations of specific metals in the different samples (m/m%, average $\pm$ SD) measured by EDS

Metal	MMA- 46.00 weld	MMA 46.00 wire	MMA 46.00 coating	MMA- 74.78 weld	MMA 74.78 wire	MMA 74.78 coating	TIG- 12.64 weld	TIG 12.64 rod	TIG- 16.12 weld	TIG 16.12 rod
Fe	99.07 ± 0.1	98.02 ± 1.7	4.38 ± 0.4	97.90 ± 0.2	98.62 ± 1.6	1.86 ± 0.3	98.30 ± 0.2	95.84 ± 1.6	66.80 ± 1.0	63.8 ± 1.4
Mn	0.48 ± 0.0	1.17 ± 0.2	18.05 ± 0.62	1.14 ± 0.0	0.96 ± 0.2	3.15 ± 0.2	0.52 ± 0.0	1.59 ± 0.2	1.51 ± 0.0	1.88 ± 0.2
Mo	ND	ND	ND	0.25 ± 0.0	ND	ND	ND	ND	0.08 ± 0.0	ND
Cr	0.08 ± 0.0	ND	ND	0.05 ± 0.0	ND	ND	0.11 ± 0.0	ND	19.33 ± 0.8	20.92 ± 0.5
Ni	0.03 ± 0.0	ND	ND	0.06 ± 0.0	ND	ND	0.11 ± 0.0	ND	11.30 ± 0.2	9.34 ± 0.5
Al	0.04 ± 0.0	0.58 ± 0.1	9.49 ± 0.6	0.01 ± 0.0	0.25 ± 0.1	1.57 ± 0.2	0.01 ± 0.00	0.67 ± 0.1	0.01 ± 0.0	1.3 ± 0.2
Cu	0.05 ± 0.0	ND	ND	0.15 ± 0.1	ND	ND	0.44 ± 0.0	ND	0.08 ± 0.0	ND
Co	0.01 ± 0.0	ND	ND	0.01 ± 0.0	ND	ND	0.01 ± 0.0	ND	0.06 ± 0.0	ND
Ti	0.01 ± 0.0	ND	1.76 ± 0.3	0.01 ± 0.0	ND	6.29 ± 0.3	0.01 ± 0.0	ND	0.01 ± 0.0	ND
Ca	ND	ND	4.45 ± 0.3	ND	ND	50.01 ± 1.0	ND	ND	ND	ND
Mg	ND	ND	ND	ND	ND	1.18 ± 0.1	ND	ND	ND	ND

ND=Not Detected, concentration of the specific metal in the sample was under detection limit, "weld" means the weld seems made during the process excluding the base metal plate

Chemical analysis of the biological samples

During both the exposition and the incubation period neither any kind of physical lesions nor pathological changes in the behaviour of the experimental animals were observed. In this section, the measurement

results are shown both in absolute concentrations (mg/kg, wet) in Table 5.

Table 5

Metal concentrations measured in different organs of the mice in different treatment groups and sampling times (mg/kg)

	MMA Ms			MMA MoMn		TIG Ms		TIG X	
Fe (mg/kg)	Control	24h	96h	24h	96h	24h	96h	24h	96h
lung	201.92	186.09	179.21	192.53	153.55	187.32	162.40	197.29	197.57
spleen	794.99	1013.34	866.20	956.19	927.83	951.87	881.45	929.35	942.40
liver	203.89	184.68	172.07	164.94	146.46	206.39	193.56	200.46	190.84
kidney	139.44	110.93	91.89	89.82	89.11	115.27	102.13	98.08	111.48
Mn (mg/kg)	Control	24h	96h	24h	96h	24h	96h	24h	96h
lung	0.22	2.08	1.24	1.49	0.53	0.24	0.20	0.24	0.19
spleen	0.24	0.71	0.18	0.46	0.27	0.26	0.16	0.39	0.19
liver	1.00	1.01	1.00	0.95	0.87	1.10	1.33	1.08	1.08
kidney	1.36	1.42	1.30	1.32	1.19	1.29	1.30	1.28	1.33
Mg (mg/kg)	Control	24h	96h	24h	96h	24h	96h	24h	96h
lung	123.35	115.86	126.50	122.16	117.89	120.44	127.17	122.44	120.26
spleen	239.32	225.45	217.74	214.20	214.57	227.00	224.52	234.90	216.60
liver	216.43	196.49	205.56	212.71	190.13	224.89	220.28	216.11	221.90
kidney	185.61	177.24	160.29	182.80	175.73	177.97	171.61	172.53	186.73
Ca (mg/kg)	Control	24h	96h	24h	96h	24h	96h	24h	96h
lung	257.57	99.77	212.69	173.18	153.81	177.49	196.92	142.58	147.61
spleen	137.24	102.10	63.82	97.70	96.35	84.29	139.95	62.44	74.54
liver	47.87	50.77	35.58	39.10	36.30	48.95	42.23	32.76	34.58
kidney	80.35	52.48	70.78	55.66	61.08	51.84	60.55	46.05	64.28

As it is shown by the data, in the case of iron, there can not be found any considerable difference in the concentrations in the lungs of the treated animal compared to the control. In the spleen samples the iron concentration emerged with at least 10% compared to the control independently of the incubation time. In

the liver, the Fe concentration dropped in the groups treated with MMA fumes but has not changed considerably in the TIG-fume treated groups. In the case of kidney samples it was found that the iron concentration decreased in every sample of every treatment group.

It was found that the manganese concentrations in the lungs showed extreme differences between the treatment groups. Whereas in the lungs of the TIG-fume treated groups there were no considerable differences compared to the control, extremely high Mn concentrations have been measured in the 24h samples MMA-fume treated groups which decreased in the 96h samples, but still remained higher than the control value. The concentrations of the same metal in the spleen have been detected to be increased in the 24h samples (except in the case of TIG-Ms 24h sample), which decreased to control-level in the 96h samples, except in the case of MMA-MoMn 96h sample, in which the manganese concentration was detected higher than in the control. In the liver samples larger Mn concentration was only measured in the case of TIG-Ms 96h sample. Otherwise the concentrations were found in the +/- 10% range compared to the control data. As for the kidney samples, no considerable divergence of Mn concentration was found.

The magnesium concentrations in all samples were found in the +/- 10% range of the control results, except in the case of kidney sample of the MMA-Ms 96h group, in that case the concentration decreased a little amount compared to the control.

It was found that the calcium levels remained at the control concentrations only in the case of MMA-Ms 24h liver, TIG-Ms 24h liver and TIG-Ms 96h kidney samples. In all other samples the Ca concentrations were measured to be considerably lower than in the controls. The most extreme results were detected in the MMA-Ms 24h lung, 99.77 mg/kg, in the MMA-Ms 96h spleen, 62.82 mg/kg and in the TIG-X 96h spleen sample, 62.44 mg/kg. The concentration of the Ca in the control lung and spleen were found 257.57 mg/kg and 137.24 mg/kg, respectively.

The mentioned data are shown also in Figure 1, depicted as a percentage of the untreated control results. The control level is also shown with a green line.

Histopathological evaluation

During our investigations, we performed HE and Perls's Prussian blue staining on the microscopic slides originated from the experimental groups.

In the case of Perls's Prussian blue staining, generally, the ferric state iron-containing siderine and ferritine bodies are stained in deep blue, whereas other organelles of the cells and heme-connected iron are showing up in purple. This staining method is intended to find the trace of the inhaled iron-containing fume particles in the organs. In the lung of the MMA fume treated animals 24h after the treatment cells were able to be detected with Perls's positive bodies in their cytoplasm in the alveoli and around the larger airways as well (Figure 2). There was no visible difference in the morphology, and the numbers of these bodies between the MMA St and MMA MoMn treated animals. The Perls's positive cells contained mostly corpuscular blue bodies in their cytoplasm, but sometimes the positivity showed up as a cloudy blue reaction. In the samples of the later sampling time the Perls's positive cells concentrated more to the region

of the larger airways and the blue bodies showed up as condensed, corpuscular particles, localised perinuclearly in the macrophages (Figure 3).

In the case of TIG-treated 24h samples, the Perls's positivity was less prominent than in the case of MMA samples. Mostly cloudy bluish Prussian blue reaction has been detected in the samples. However, there were cells with fine particle-like blue bodies (Figure 4). The samples gathered 96h after the TIG fume treatment showed a rather similar picture. However, at this time a lesser amount of Perls's positive cells were found, and these cells localized more often in the larger airways, similarly to the findings of the MMA treated 96h samples (Figure 5).

In the spleen it is normal to see Perls's positive bodies in the cells, since the haemoglobin content of the dead erythrocytes are metabolised here. As it is shown in Figure 6A, the blue bodies are localized mostly in the red pulp of the control spleen. In the white pulp area, there are just a few of them. It is rather visible that we can see more siderine content in any welding fume-treated samples at any time compared to the control (Figure 6. B-C). In the case of MMA treated samples (independent of the welding metal) in the 24h samples the Perls's positive bodies are much more numerous and larger in size than normal. Also, coarse blue bodies can be seen not just in the red-, but also in the white pulp as well (Figure 6. B). In the 96h samples, still more than the normal amount of blue bodies can be detected in the spleen, but the amount is reduced compared to the MMA 24h samples (Figure 6. C).

In the case of TIG samples, 24 hours after the treatment more Perls's positive bodies were found in the cells than in the control. Similar to the MMA results, coarser and larger blue bodies showed up in the white pulp region as well (Figure 7. B). In our view, the Prussian blue positive bodies in the TIG samples showed up more dark-stained (sometimes even black or blackish-blue), hence more dense than in the case of MMA treated samples. The TIG fume treated spleen samples gathered 96h after the treatment showed a reduced amount of the Perls's positive particles, but still more compared to the control, and the density of these bodies are showing still high (Figure 7. C).

Evaluation of the HE stained slides showed no obvious pathologic signs in any organs in any groups. However, in the case of liver, big differences were able to be detected between the treated and the control animals (control liver slide is shown in Figure 8. A). In the case of MMA treated animals, big perinuclear cavities were observable (Figure 8. B) which are most likely remnants of glycogen bodies that were dissolved away during the staining. The biggest size cavities were observed in the MMA-Ms 24h group, but the picture of the MMA-MoMn 24h group was also comparable. The size of the cavities in the 96h samples were smaller but still larger than any cavities that were observed in the control samples. In the case of TIG samples, in the 24h samples, we were able to detect similar cavities, but in smaller size than the MMA samples. The 96h TIG liver samples showed up very similar to the control samples, but in the case of TIG X samples, round empty vesicles in higher numbers were detected compared to the control (Figure 8., C). These features most likely were lipid vesicles, but the content was dissolved during the preparation of the slides. In other organs (lung, spleen, and kidney) there were no differences compared to the control organs.

Discussion

In our study, the main goal was to test our experimental setup and to test our concept about the possible toxico-kinetic differences between the fume particles originating from different welding methods. According to our results, we can conclude that the basic setup of our experiment is usable for the purpose it was intended for. As the data show, metal content originating from the welding fume is measurable in different organs of the experimental animals.

Iron levels were measured to serve as internal control, since according to the literature, the iron ion concentration and –metabolism is strictly regulated in mammalian organisms. However, this regulation takes the oral iron intake as default, so in the intestinal path, the uptake of the iron can be stopped if the metal content in the body is optimal to prevent excess iron to circulate in the body. Excess iron can cause oxidative stress, and it can give an opportunity to pathogenic bacteria to multiply since these microbes usually need iron for development and multiplication. One important protein in the regulation process is the hepcidin (Siah et al. 2006). This protein can inhibit the ferroportin from binding the iron, also can enhance the internalization and degradation of the same protein. Since the ferroportin is presented on the macrophages outer surface and on the basolateral side of the enterocytes, high hepcidin levels inhibit or at least slow down the transport of the iron to other cells. Hence, in the case of physiological iron concentration in the liver, the production of the hepcidin will prevent further iron uptake from the intestines and will also inhibit the transfer of the iron content of the macrophages to the liver cells. (De Domenico et al. 2005, Nemeth et al. 2004). As for the inhalation uptake of iron-containing particles, such regulation can not be applied. In this case, immune cells collect the inhaled particles in the lung and carry them to the possible depo organs. In the case of iron, this depo is the liver (Celada 1977), but, as it can be seen the iron concentration in the liver does not increase, but even decrease a little in every groups. The animals were fed with complete rodent feedstuff, which contained the necessary amount of minerals, including iron, for the animals. So, we hypothesise that the iron depo was on physiological level, and this resulted in an elevated hepcidin level in the blood. In a situation like this, the extra amount of iron collected by the macrophages in the lungs will trigger even higher hepcidin production and the downregulation of the liver iron uptake. Since the macrophages filled with iron-containing particles could not pass the iron to transport proteins, these cells were migrating into the spleen, causing the iron concentrations in the spleen to be increased compared to the control, which was well measurable. This iron-homeostasis regulation is well documented in the literature and applies to all mammalian species, including humans as well (Collins et al. 2008). It is known that lung siderosis is rather often diagnosed in welders, which seems to be combined with total body iron overload. In the literature, however, the spleen status is not often investigated since it is less invasive to gather blood and lung biopsy samples, but our results seem to comply with the literature data (Casjens et al. 2014).

Manganese concentration in the organs showed alterations in accordance with the used welding technology. It is important to emphasize that these alterations were selective to the manganese and seemed to be independent of the iron concentration alternations. As these changes do not follow the iron concentrations, currently, we cannot explain the process as the result of the hepcidin regulation, and according to our results, the process seems to depend on the welding technology used in the treatment as follows. In case of MMA welding, Mn concentrations in the lungs were multiple magnitudes higher than in

the control animals at 24 hours after the treatment and were still elevated at the 96 hours sampling time. The MMA-mild steel groups showed the most extreme data, in the case of MMA-MoMn groups the measured values were smaller. The spleen samples also showed over the normal Mn concentrations in these groups, but no noticeable changes were observed in the liver and in the kidney samples. However, as for the TIG-treated groups, there were slight increases of Mn concentration in the lungs, also in the spleen 24 hours after the treatment and in the liver as well 96 hours after treatment. To explain this phenomenon, several things should be taken into consideration. The covered welding electrodes, used for the MMA-Ms treatment, and the base metal contained low amount of manganese according to the documentation and to the material examination as well. The coating material of these electrodes, however, contained about 20 m/m% of Mn according to our measurements. Since a large part of the covering material is evaporated during the welding process, and the manganese content of the cover is used to protect the weld bath as antioxidant material, it can be understood that the welding fume will contain a high amount of oxidic Mn particles. It is also established in the literature that the MMA welding generates larger, usually 100 nm in diameter particles (Brand et al. 2013). These particles can penetrate through the upper and lower airways and can reach the alveoli of the lungs. According to our results, the oxidic Mn compounds originated from MMA welding are trapped in the lungs for a considerable time. This is true not just for the mild steel experiments, but for the MoMn experiments as well. In that case, the Mn content of the electrode cover is less than in the mild steel electrodes, and still, the Mn content in the lungs is also considerably high both 24 and 96 hour post-treatment. In the spleen, both the MMA-Ms and MMA-MoMn treatments, the Mn concentration is boosted in the 24 h post exposition samples, but in the liver and kidneys, we were not able to detect noticeable differences. According to this, it can be concluded that the oxidic Mn particles were collected in the lungs by macrophages, then transported to the spleen, where the manganese afterward was accumulated, and there was no further large-scale transport to the liver or to the kidneys. Unfortunately, in this experiment we were not able to measure the metal content in the brain. However, it can be assumed that some part of the excess Mn probably was deposited in the central nervous system, as it can also accumulate this metal according to previous investigations (Antonini et al. 2006, Chen et al. 2018, Graczyk et al. 2016, Taube 2013).

In the case of TIG treatments, manganese distribution showed different results. In both TIG-Ms and TIG-X treatment groups, there was a slight increase of Mn concentration in the lungs, spleen and in the liver 24 hours after the treatment, but after 96 hours, the metal excess cleared from the lungs and from the spleen and appeared in the liver. The Mn levels of the kidney samples again showed no important changes at any time. There was no high manganese content covering material in the system, so the lower level of excess Mn in the organs is easily understandable. As previous experiments showed that the particle size of the TIG welding fume measures about 20-70 nm in diameter (Brand et al. 2013, Mei et al. 2018), it can be concluded that these tiny particles were able to penetrate through the alveoli into the bloodstream, so they did not accumulate in the lungs and the liver. Mn content was also able to reach the liver despite the regulation systems. Taking into consideration that there is a huge difference in the Mn distribution between the MMA and the TIG fume-treated groups, and the fact, that the one of the main differences between these fumes is the particle size, the final conclusion is that in the case of manganese, a particle size-

dependent pharmaco-kinetics pattern has been demonstrated. Similar differences in the kinetic properties of other nanoparticles were found earlier (Hirn et al. 2011, Nabeshi et al. 2009).

The emergence of Mn concentration in the liver after the TIG-X treatment is strange since other investigations reported that the Mn influx to the liver is saturable, and the organ is capable of high clearance of excess manganese. These properties may cause that the Mn content in the liver is very stable and only application of high doses (like 3 g/m³ dose intratracheally as reported by Salehi et al. 2003) resulted in some rise of the Mn concentration in the liver. Our data complies with these results, except the results of the TIG-X treated group. Since the exposure level in the whole experiment was equal in all groups, in our opinion, in this case, the Mn-oxide nanoparticles evaded the liver's normal Mn-transport regulatory mechanisms and cause the higher Mn concentration of the liver samples in this group in the 96h sample (Roels et al. 1997, Salehi et al. 2003).

It should be mentioned that many experiments were done previously to investigate the kinetics of the Mn in a mammalian organism. However, many of these investigations were done using soluble manganese salts, like MnCl₂ or MnSO₄ and in some cases, insoluble manganese oxides were used (Roels et al. 1997, Salehi et al. 2003, Tjalve et al. 1996). The results suggest that oral uptake of the Mn is strictly regulated, and the bioavailability of the metal through this way is about 5%, whereas the intratracheal absorption rate is near to 100% for soluble salts. However, the solubility of the used Mn compound heavily influences the speed of the absorption. The water-insoluble oxides showed almost no adsorption at the oral application, but well measurable absorption in the case of material administered intratracheally. As many researchers suggest, we also can support the theory that in this exposure pathway, the uptake of the particles is done mostly by the reticuloendothelial cells and by the macrophages based on our results (Roels et al. 1997). Our opinion is supported by the high manganese content of the spleen after the MMA treatments. Similarly, Salehi et al. (2003) reported high Mn concentrations in the lungs after inhalation treatment of rodents with moderate and high doses of MnSO₄. This result complies with our measured Mn concentration in the lungs after MMA-fume exposure. It is very interesting that this result was obtained with insoluble Mn-oxide particles. These data suggest that the accumulation of the Mn in the lungs seems to be less dependent on the solubility of the inhaled manganese compound (Salehi et al. 2003).

As we mentioned, the most probable transient depo organ for the Mn is the central nervous system. Any habitual or locomotor activity changes were not experienced in the treated animals in our experiment, which can be central nervous system sign of manganese intoxication. Since the Mn concentrations were not measured in the different parts of the brain, no data are available to support or decline the theory about the direct absorption of the manganese via the *bulbus olfactorius* to the brain (Roels et al. 1997, Salehi et al. 2003, Tjalve et al. 1996).

Ca and Mg levels have also been examined in the mentioned organs after the treatments. Calcium and magnesium are important ions for ensuring the ion balance and buffer capabilities of the electrolytes in the body. Calcium is an essential secondary messenger in the signal transduction processes. During our experiments, heavy Ca depletion was experienced after every treatment in each group, nearly in every sample. As it can be seen in Figure 1., it is hard to find a general trend for the Ca depletion, neither in

correlation with the used welding method nor in connection with the base and the filler metal. In the lungs, the largest Ca depletion happened in the MMA-Ms group, 24 hours after the treatment, which was seemingly recovering for the 96 hours sample. However, the Ca level still has not reached the control concentration. In the case of spleen and liver, usually, the 96h measurements showed lower values, although not in every case. In contrast, Mg levels showed no extraordinary change in any groups in any sampling time in comparison with the control.

Currently the explanation of this behavior of calcium is unknown. Since the Ca is an important part of the signal transduction in the regulation of many metabolic pathways, the depletion of it may be in connection with the glycogen accumulation that we identified in the liver. Still, we will need more experimental data to understand this phenomenon.

There were no major signs of damage in the treated animals' organs. Perls's Prussian blue staining is available to make the iron-containing hemosiderin bodies in the histological slides visible. Hemosiderin is the form of the iron that the macrophages are holding and transporting the iron originated from dissolved red blood cells mainly under normal circumstances. Using this staining we were able to identify the amount and localization of the iron-containing particles in the organs. Similar to the results of the chemical analysis, we found higher amount of Perls's positive bodies in the lungs of both MMA fume treated groups in both sampling time however, the overall Fe concentrations in the lungs showed no considerable differences from the control. These particles were located mostly intracellular, in the septa of the alveoli, or in the lumen of the lungs. This fact indicates that the part of the inhaled fume-particles was captured by the macrophages in the lungs during the elimination process. In case of spleen, we saw a considerably higher amount so Perls's positive bodies than in the control organs. It is important to emphasize that the presence of this kind of hemosiderin formation in the spleen is normal since the hemoglobin content of the removed red blood cells is processed in the spleen. In our case, however, the amount and the roughness of the particles differed from the control. Also, we found slight change in the distribution of these particles because in the control, the hemosiderin is located mostly in the red pulp area, and little amount of it was found in the white pulp. In the treated animals, considerable amount of coarse particles was found in the white pulp areas as well. Taking the time into consideration, we found no real difference in the amount or in the distribution of the Perls's positive particles in the 24h and 96h spleen samples of the MMA-fume treated animals. As for the TIG experiments, the amount of detected hemosiderin particles in the lungs was nearly equal to the control in any sampling time. The spleens, however, showed a higher than normal amount of Perls's positive bodies than in the control organ, but it was less than we found in the MMA treated samples. Also, the grains were not coarser than in the control. These results show that there is a method-based difference between the experimental groups, but between the groups treated using the same welding method, there is no real difference despite the different welding materials or base metals. Taking the fact into consideration, that the TIG welding emits more ultrafine particles than MMA welding, we can explain the mentioned difference with the different particle size in the fumes inhaled by the animals. In case of MMA, the fume consists of coarser particles, which can not penetrate the wall of the alveoli, so the inhaled particles are collected by the macrophages of the lungs, which we were able to demonstrate. In the case of TIG welding, the ultrafine bodies (size between 10 and

50 nm) can either penetrate to the capillary of the lungs, or can be dissolved fast, so they can not be detected with Perls's Prussian blue staining, but chemically we can measure elevated iron content in the lungs.

The elevated amount of iron-containing particles in the spleens suggests that the iron-containing dust collected by the macrophages was deposited in these organs. This can be explained by the hepcidin iron metabolism regulation pathway as stated above. Since the animals were fed with complete feedstuff, the iron needs of them were completely fulfilled. Hence, these results also support our theory about the hepcidin regulation, so the excess amount of iron could not get transported into the liver but deposited into the spleen. This theory is also supported by the fact that we were not able to find seriously elevated iron contents in the liver of the treated animals.

As for the HE slides, in most of the organs, we were not able to detect any abnormalities. However, in the liver, multiple differences were able to be found compared to the control animals. One of these was the extensive polymorphic cavity formation, mostly in the MMA fume treated groups, but in some amount in the TIG-fume treated groups as well. According to the morphology of the cavities, we assume that these were locations of glycogen bodies in the hepatocytes which were dissolved during the histological processing. Since the extermination of the animals happened in the afternoon at the same time for all the groups respectively, including the control as well, this glycogen accumulation is assumed to be the result of the treatment, especially the MMA fumes, rather than the circadian rhythm of the glycogen in mice (Halberg et al. 1960, Nelson et al. 1975). It was the most severe in the samples taken 24 hours after the treatment and changed only a bit in the 96h samples, independently of the base- or filler material. Boyce et al. (2020) investigated the deposition of the lipids in the liver and similar polymorphic cavities were detected in the hepatic cells based on the histological images as we found in our experiment. The biological cause of this finding is still unclear. Taking into account that in human cases, it was reported that longer time exposure of welding fumes could cause hepatic iron overload and hepatic fibrosis (Mariani et al. 2021), the mentioned liver symptoms surely have consequences in a longer period of time on the function of the liver both in rodents and humans as well. In our experiment, however, the applied dose of fumes and the relatively short incubation period was not proper to identify these long-term effects.

Another finding in the TIG-X fume treated groups was the fact that emerged number of round vacuoles were found in the hepatocytes in comparison with the control sample. This phenomenon is the most visible only in the TIG-X 96h samples. According to the morphology, these vacuoles most probably contained lipids. An earlier experiment demonstrated that fume exposition of stainless steel welding combined with a high-fat diet could accelerate the lipid deposition in the liver of rats, so our result may show the very early phase of this process (Boyce et al. 2020). Our result can confirm that the TIG-X fume is causing disturbance of the lipid metabolism in treated samples. Since the stainless steel material has a high concentration (~20 m/m%) of chromium, there can be some relationship between the Cr content and the biological effect (Levina et al. 2003, Snitynskyi et al. 1999, Vincent 2000), but the proper mechanism of action needs to be evaluated in the future.

Conclusion

During this pilot study, we wanted to prove our concept of investigating the kinetics of certain metals in the body after inhaling fresh welding fumes in an experimental animal model. According to our results, first and foremost, we can declare that our experimental setup is basically capable of performing the targeted investigation, although fine-tuning of some parts of the experimental process is required in further examinations.

According to the indicated differences of the manganese concentrations between the MMA-fume and TIG fume treated groups, we conclude a possible difference in the manganese distribution kinetics depending on the size of the inhaled particles. Since other studies also found differences in the kinetics of the same materials (gold, silver, or silica) depending on the size, namely nanoparticles versus corpuscular particles, the same in case of manganese is considered possible. Many of our results are in compliance with the earlier investigation, but some show differences, so further, and larger scale examination is required to prove our theory and to confirm our data.

Declarations

Ethics approval and consent to participate

All applicable international, national and /or institutional guidelines for the care and use of animals were followed. The experiment (and the experimental protocol of the study) was performed with the permission of Animal Experiment Allowance No. PE/EA/289-7/2018 given by the Animal Protection Authority of the Hungarian Government Office.

Consent to Publish

Not applicable

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Competing interests

The authors declare no competing interests.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Concentrations of Fe (Fig. 1A), Mn (Fig. 1B), Mg (Fig. 1C) and Ca (Fig. 1D) measured in different organs in different treatment groups and sampling times. Results are represented the percentage of the untreated controls (control value = 100%, represented with the green line), error bars are representing +/-10% difference.

Figure 2

Microscopic image of the lung from an animal from MMA-Ms 24h group stained with Perls's Prussian blue. Green arrows are pointing to the cells filled with blue, Perls's – positive bodies. The insert shows the Perls's positive cells in higher magnification.

Figure 3

Microscopic image of the lung from an animal from MMA-Ms 96h group stained with Perls's Prussian blue. Green arrows are pointing to the cells filled with blue, Perl's – positive bodies. These cells are cumulating mainly next to the larger airways. Blue arrows show macrophages with perinuclear Perl's body arrangement.

Figure 4

Microscopic image of the lung from an animal from TIG-Ms 24h group stained with Perls's Prussian blue. Arrows are pointing to the cells filled with blue, Perls's – positive bodies, green arrows indicate the very fine, cloudy positivity whereas blue arrow indicates the more condensed, particle-like bodies.

Figure 5

Microscopic image of the lung of an animal from TIG-Ms 96h group stained with Perls's Prussian blue. Arrows are pointing to the cells filled with blue, Perls's – positive bodies, green arrows indicate the very fine, cloudy positivity whereas blue arrow indicates the more condensed, particle-like bodies.

Figure 6

Microscopic image of the spleen stained with Perls's Prussian blue. The blue particles represent the iron-containing hemosiderin bodies. A: control sample; B: MMA-Ms 24h group sample. The siderin bodies are larger, coarser, and darker than in the control organ. C: MMA-Ms 96h group sample. The amount of the blue bodies decreased compared to the MMA St 24h sample, but there are still more and larger bodies than in the control organ.

Figure 7

Microscopic image of the spleen stained with Perls's Prussian blue. The blue particles represent the iron containing hemosiderin bodies. A: control sample; B: TIG-Ms 24h group sample. The size and amount of the Perls's positive particles are a bit larger than we found in the control, but the density of the particles is much greater than that, many of them looking nearly black. C: TIG-Ms 96h group sample. The size of the blue bodies is still bigger than those in the control organ, and their density is still high.

Figure 8

Microscopic images of the liver (HE) A: control sample; B: MMA Ms – 24h group sample, Empty spaces around the nuclei are well visible. Most likely, these are remnants of the glycogen solved away during the staining process. C: TIG X – 96h group sample,

Every image has the same magnification, the arrows are pointing to the round vesicles, most likely containing lipids in the hepatocytes.