In vitro Cytotoxicity of Mild Steel and Stainless Steel Welding Fumes using Human-derived cells

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ABSTRACT: The potential toxicity of generated welding fumes was investigated using standardise welding rig and testing of welding fumes in *in vitro* toxicity assays. Toxicity were influenced by welding length (6.0 ± 1.0 m and 9.0 ± 2.0 m), duration (30 and 60 minutes), wire feed speed (2 m/min and 7 m/min) and types of material being welded (mild steel and stainless steel). The fumes were channelled to an *in vitro* dynamic exposure setting consisting of A549 pulmonary type II-like epithelial cell lines which were cultured on Snapwell membranes. The cells were exposed directly at the air/liquid interface and cytotoxicity were investigated using MTS3-(4,5-dimethylthiozol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 tetrazolium) assay. Chemical analysis using ICP-AES showed that stainless steel welding fumes contain Cr and Ni while mild steel consumables produce more Mn and Fe. Results showed that wire fed speed influenced fume generation and cell viability reduction as the welding duration was prolonged.

Key words: welding, automated platform, dynamic exposure, MTS, A549, wire feed speed

Introduction

Inhalation to occupational hazardous contaminants is an important route of exposure at many workplaces. Welders are exposed to several groups of hazards: physical, radiation, ergonomic and chemical. Primarily, they are exposed to multiple chemical contaminants which can gradually affect their health and well being. In typical industrialized countries, especially in important industries, welders constitute 0.2 to 2% of the total workforce (Taylor *et al*., 2003).

One of the most significant risks associated with the welding operation is exposure to generated metal particulates and noxious gases. A complex of metal oxide was generated by welding activities like iron oxide, manganese, chromium, nickel, silicon and copper (Uemitsu *et al*., 1984; NOHSC, 1990). The major sources of byproducts in the generated welding fumes are from the consumable electrode wire and the base metal being welded. Fumes generated from stainless steel electrodes usually contain 20% chromium with 10% nickel, whereas, fumes from mild steel consumables encompass of iron (more than 80%) with some manganese but no chromium or nickel present (Frankel & Lippold, 2004). The electrode used will partially volatilize due to the extreme temperature during the welding process. The vaporized material will react with air to form metal-enriched fumes. These fumes are a complex mixture of gases and small particulates of metal oxides coming from the vaporization and oxidation of oxidized metal particles. Moreover, dependence on the type of welding processes the fumes could form respirable-sized particles with different composition. It is also suggested that the aerosol formation mechanism during welding process comprise of nucleation of primary particles followed by growth through coalescence and clumping (Zimmer & Biswas, 2001).

A range of health effects from welding activity have been published and categorized from acute poisoning to the chronic toxic effect including bronchitis, asthma, dermatological and hypersensitivity, central nervous system and respiratory effect (Antonini *et al*., 2003). More recently, the finding of epidemiological studies showed a high likelihood of respiratory diseases among welders. The most frequent described respiratory illness among welders consisted of influenza-like symptoms known as metal fume fever (MFF) (Antonini *et al*., 2003). The inhalation of welding fumes that mainly comprised of manganese oxide could cause the MFF

(Pascal & Tessier, 2004). The symptoms included metallic taste in the mouth, fever and sweating, thorax irritation, chest tightness, dry cough, and general malaise (McNeilly *et al*., 2004).

An essential element in welding fumes is chromium which is generated after stainless steel material is welded. Cr (VI) is known to be a Group I proven human carcinogen (IARC, 1997). Thus, welding fume is declared as possibly carcinogenic to humans (Group 2B) presumably lung is the cancer site (Antonini, 2003). Pascal and Tessier (2003) demonstrate that manganese and the hexavalent form of chromium, but not nickel or elemental chromium, are cytotoxic to small airways epithelium within the concentration range of 0.2–200μM (Tessier & Pascal, 2006).

Furthermore, some exposure to Mn-containing fumes would affect nervous system which is described as manganism or also known as Manganese-induced Parkinsonism (MIP) (Flynn & Susi, 2009). Individuals with manganism exhibit an enhanced accumulation of Mn within the striatum and eventually exhibit symptoms, some of which were similar to those observed in Parkinson's disease (PD) (Kenangil et al.,2006; Santamaria, 2008). The symptoms of manganism that resembles PD include hallucinations, tremors, an awkward gait, abnormal balance, memory loss, impairment of motor skills, slurred speech, lack of facial expression and sleep disorders. It has been reported that manganese-induced neurotoxicity is a post chronic exposure to high levels of manganese, usually above the permissible exposure limit ceiling at 5 mg/m^3 set by the US Occupational Safety and Health Administration (Dale Marcy & Drake, 2007).

The toxicity of welding fume was investigated using a standardized welding apparatus. Welding fumes were exposed to cultured human cells in an *in vitro* cytotoxicity assay that allowed dynamic exposure of fume to cells at the air-cell interface (Bakand, 2006). Human pulmonary type II-like A549 epithelial cells lines were used in this assay. The A549 was frequently used as an *in vitro* model for studying lung toxicity of environmental toxicants to replicate human inhalation systems. Cytotoxicity of the generated fumes were investigated using the colorimetric MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) - 2-(4-sulfophenyl)-2H-tetrazolium, inner salt). Hence this study aims to assess the *in vitro* toxicity effect of welding

fumes using the dynamic exposure technique to replicate the real exposure that occurs among welders.

Materials and Methods

Chemical, materials and apparatus used

In vitro assay reagents were purchased from Promega (USA) and Sigma (USA). Chemicals and reagents for acid digestion were obtained from UNIVAR (distilled conc. nitric acid, HNO_3 70% w/w and distilled conc. hydrochloric acid, HCl 37% w/w).

Two types of steel, mild steel (MS) (Grade 350) and stainless steel (SS) (T304) were purchased from Mascot Steel and Midway Metals respectively. Shielding gas for Gas Metal Arc Welding (Argonshield, BOC) which contain CO_2 (7%), oxygen (1.5%) and argon (91.5%) was used. 5 µm polyvinyl chloride (PVC) membrane (GLA 5000, SKC) was used for the entrapment of welding fumes generated during exposure experiment.

The 6-well plate of porous membrane Snapwell^{TM} Inserts (3801 Corning) was used as cell growing membrane. The 96-wells plate purchased from Greiner Bio-one was utilized during the absorbance reading. A welder machine (model MIG 200C) was used for fume generation during all exposure experiments located in Mechanical Engineering workshop, UNSW (Australia). The analysis of digestion solution was performed on Inductively Coupled Plasma Atomic Emission Spectrophotometer (ICP-AES) (Optima 3000 DV, Perkin Elmer). Deionised water (MQ system, > 18 MΩ: Ultra Clear Basic UV plus-2004-B) was used throughout the metal elements analysis.

Automated welding platform design and construction

The automatic welding platform was developed and constructed in this study to replicate real welding scenario (Figure 1). The platform consisted of two plates that move in x and y direction namely Plate I (300 mm x 600 mm) as sample holder and Plate II (300 mm x 500 mm) as torch holder. A system of water circulation was inserted for cooling purpose. This welding unit

chamber had a total volume of 0.43 m^3 with 1.48 m (length) x 0.68 m (depth) x 0.42 m (height). The welder platform was enclosed to ensure reproducible fume generation and collection.

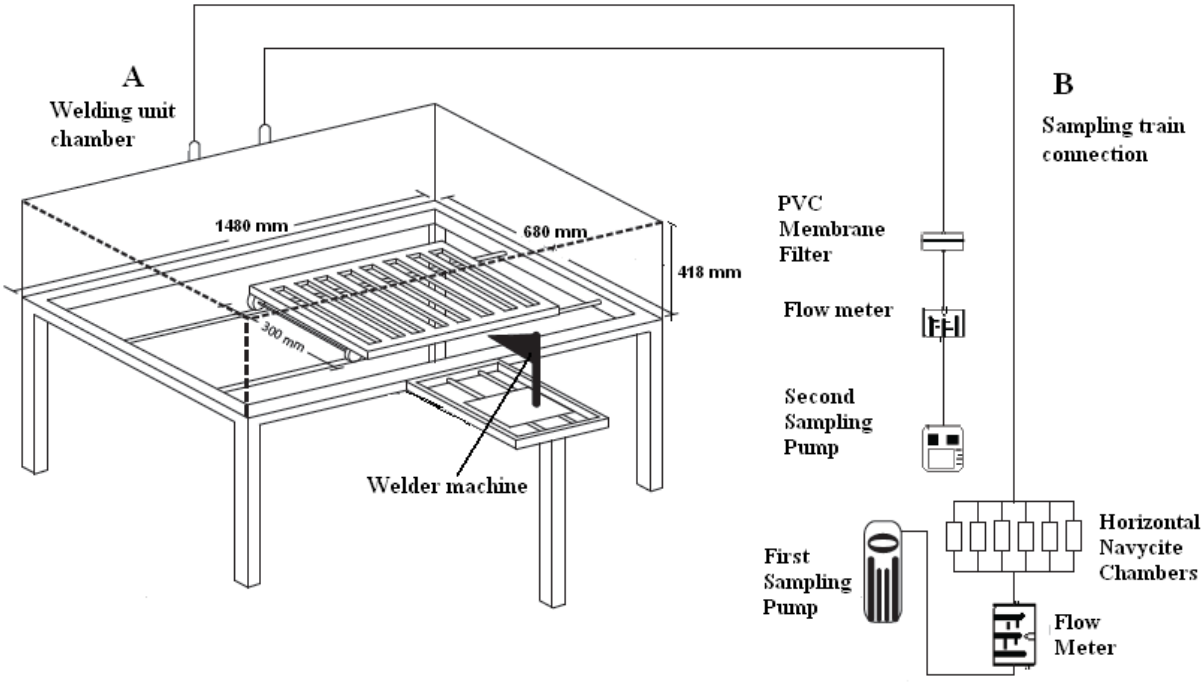

Figure 1: Automated welding unit chamber

A549 culture and preparation

Human pulmonary type II-like epithelial cell lines (A549, ATCC No. CCL-185) were cultured in sterile, vented 75-cm^2 cell culture flasks with Dulbecco's modified eagle medium: Ham's F12 nutrient mixture (DMEM/F12; Gibco, USA) supplemented with 5% (v/v) fetal calf serum (FCS); JS Bioscience, Australia), and 1% (v/v) of an antibiotic solution (Sigma, USA) containing: L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cultured cells were kept at 37^oC in a humidified 5% CO_2 incubator.

For cytotoxicity experiments, newly confluent cell layers were enzymatically removed, resuspended in culture medium and viable cell number determined (Bakand *et al*., 2005). Human cells were grown on porous membranes (0.4 ml) in Snapwell inserts (Bakan *et al*., 2007). The

Snapwell insert is a modified Transwell culture insert with a 12 mm diameter providing a growth area of 1.12 cm2 (clear polyester Snapwell^{TM} insert, 3801, Corning), supported by a detachable ring that was placed in a six well culture plate. Culture media supplemented with HEPES buffer (0.01 M) was added and the Snapwell inserts were incubated at 37^0C for 1 h as an initial equilibrium time to improve cell attachment. Culture media from the top was replaced with fresh media (0.5 ml) containing 25 x 10^4 cells, supplemented with HEPES buffer (0.01 M). Cell cultures were incubated at 37^0C in a humidified incubator for 24 h. Before exposure, cell confluence (75–80%) and attachment was observed using the light microscope, the medium was removed from newly confluent cells and membranes washed with Hank's balanced salt solution (HBSS; Gibco, USA). Cells were exposed to airborne concentrations of test gases on their apical side while being nourished from their basolateral side, using the dynamic direct exposure method (Bakand, 2006).

Direct dynamic exposure of A549 to welding fumes

Prior to the exposure, the sampling pump was calibrated. The flow rate for shielding gas supply was 15 L/min with pressure being used maintained at 5400 kPa while the test wire was fed to the arc-welding gun at speed ranging from 2 to 7 m/min for both MS and SS plate samples. The sampling train (Figure 1B) represents horizontal Navycite chamber (Harvard apparatus) which connected to SKC field flow meter (320-2A05) and calibrated SKC sampling pump (224-PCXR4) at established flow rate 25 – 50 mL per minute (21). The horizontal Navycite chambers were placed on the heat blocks to maintain the temperature at 37^oC. The tygon tubes (OD = 1/8 in, ID = 1/16 in, with length = 89 cm) were used to channel the fumes from the top outlet of welding unit chamber (Figure 1A) to the sampling train. The PVC filter was placed in line with the sampling train for fume collection for further composition analysis.

The intermittent welding process (2 minutes of welding followed by 2 minutes of pause) was performed during the collection of fumes. The overall exposure was up to 60 minutes without opening the welding unit chamber. Two types of exposure experiments were undertaken in this study. The first exposure experiment condition was for '30 minutes'. This investigation was conducted on a surface area of 300 mm (length) x 270 mm (width) on the welding plate. There

were two wire speeds supplied to the welding torch of 2 m/min and 7 m/min. The second exposure experiment investigated was '60 minutes'. A surface area of 300 – 600 mm (length) x 270 mm (width) of welding plate was used in this section. Similarly to the half hour exposure time, two different types of wire speed were studied during one hour exposure time, which were between 2 m/min and 7 m/min.

Cytotoxicity Assessment

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl) -2-H-tetrazolium) cytotoxicity assay was used to assess the toxic impact of welding fumes in cultured human lung cells. The MTS assay is based on the ability of viable cell to convert a soluble tetrazolium salt into a coloured formazan product.

After the grown cells were exposed to the generated welding fumes, the fresh culture medium was added to the bottom (2 mL) and 0.4 mL for the top of the Snapwell membranes. The MTS assay was performed either after 0 h or 24 h post incubation. Then an amount of 100 µL of final solution was transferred into 96-well plate for absorbance reading (Multiskan Ascent Thermo Labsystems) at wavelength 492 nm. The absorbance results were analysed to calculate the percentage of cell viability after welding fume exposure using mean absorbance of exposed cell over mean absorbance of unexposed control cells. Experimental results were expressed as mean ± standard deviation (m ± S.D.) for at least three different replicates (n = 3) at each experiment condition. After testing the homogeneity of variances using the F-test, the Student's t-test was used to compare the average cell viability of exposed cell after exposure. Differences were considered as statistically significant at $p < 0.05$.

In this study a number of controls were set up with identical cell preparation conditions. The controls are IC_0 (0% inhibitory concentration, without cells and test chemical); IC_{100} (100% inhibitory concentration, with cells but no test chemical) and exposure to ambient air control only (with cells and air instead of test chemical).

Welding fumes analysis

The generated particulate trapped on the PVC filter was analysed to determine the composition using ICP-AES. The composition of welding fumes and the plate samples have been analysed with modification of the NIOSH Manual of Analytical Methods (Method 7300).

Results

Effect of MS-welding length on fumes generation and toxicity

The average of MS-welding length operated were 6.0 ± 1.0 m and 9.0 ± 2.0 m respectively. Figure 2 showed no significance different of percentage cell viability ($p > 0.05$) between 0 and 24 hours post exposure incubation.

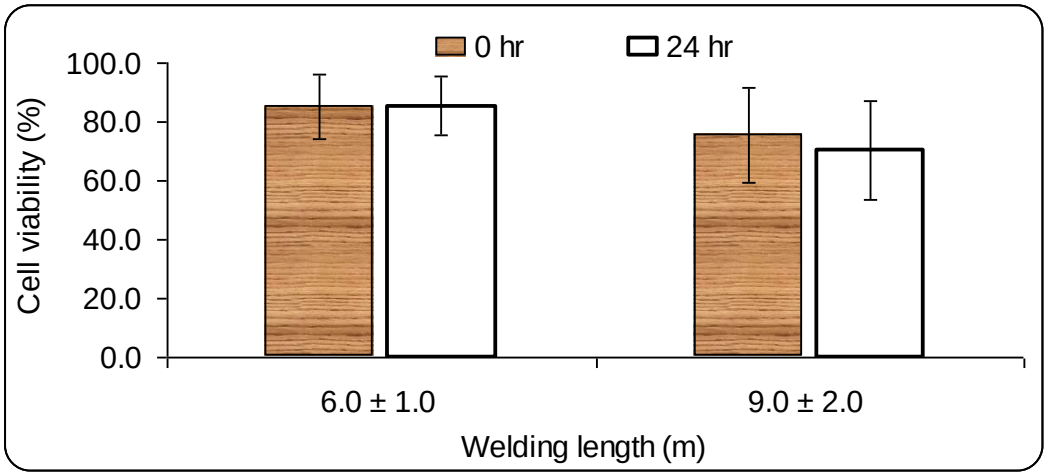

Figure 2: Cytotoxic effect of MS-welding length on A549, assayed 0 and 24 hours post exposure incubation

Further investigations were carried out to clarify the exact cytotoxicity that was observed in Figure 2, however, the welding was set at a specific welding length 7m (Figure 3) with two different time of experiment; 30 and 60 minutes. The data suggested that no significant difference ($p > 0.05$) was observed between 0 and 24 hour post exposure incubation either in 30

minutes exposure or in 60 minutes exposure. However, there was a significant differences between 30 and 60 minutes exposure for 0 h post exposure incubation ($p < 0.05$).

Figure 3: Cytotoxic effect of 7 m length of MS-welding in 30 and 60 minutes experiment

Data in Table 1 indicated mass of five metal elements that were grouped based on the two length-based MS-welding operations. Each value represents the mean ± SD of at least six separate experiments. The results depicted that longer length of welding operation (9 m length) would doubled the mass of Fe and Mn. The data also indicated that Fe was higher than Mn in both length-based experiments. Furthermore, it was also observed that increased productions of metals did not significantly alter cell death either 0 or 24 hrs post exposure incubations ($p > 0.05$).

Table 1: Mass of metals present in the generated welding fume as determined by ICP-AES

Welding length (m)	**Exposure mass (mg)**				
	Fe	**Mn**	**Cr**	**Ni**	**Cd**
6.0 ± 1.0	0.50 ± 0.05	0.08 ± 0.02	n.d	n.d	n.d
9.0 ± 2.0	0.85 ± 0.17	0.12 ± 0.03	n.d	n.d	n.d

n.d = not detected

Effects of MS-wire feed speed on fumes generation and toxicity

Figure 4A reflected the 0 hour post exposure incubation while the 24 hour post exposure incubation was depicted in Figure 4B. No significant toxic deviations ($p > 0.05$) were observed at low and high speed in 30 minutes exposure time (Figure 4A). However, the high wire speed (7 m/min) during one hour exposure significantly affected the cell viability as compared to the low wire speed ($p < 0.05$).

In Figure 4B, data suggested that no significant effects on cell viability between low and high speeds either in 30 minutes experiment or one hour experiment ($p > 0.05$). The comparison of cell viability between half hour and one hour exposure time was not significantly different ($p > 0.05$) for both low and high speed of welding operation after the assay was performed 24 h post exposure incubation.

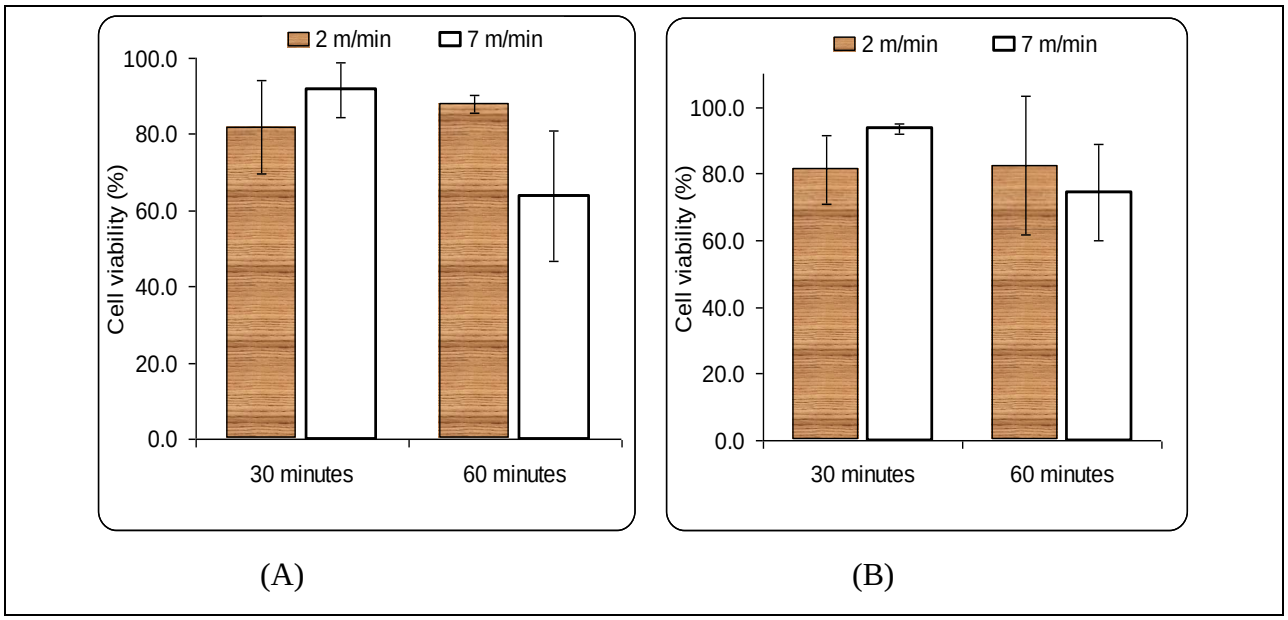

Figure 4: Cytotoxic effects of MS-welding fume generated at different wire feed speed in 30 and 60 minutes exposure experiment and was assayed: (A) 0 hour post exposure incubation and (B) 24 hour post exposure incubation

The metal composition of generated welding fume was presented in Table 2 where the metals were assorted according to the wire feed speed. In Table 2, increasing the wire feed speed had proportionally increased the amount of metals (Fe and Mn). Furthermore, the increased amount of Fe and Mn have not significantly ($p > 0.05$) affected the cell viability whether the MTS assay was performed after 0 or 24 hours post exposure incubation.

Table 2: Metal mass of different wire feed speed in 30 and 60 minutes of exposure time

Metal analysed	**Exposure mass (mg)**			
	2 m/min		**7 m/min**	
	30 minutes exposure	60 minutes exposure	30 minutes exposure	60 minutes exposure
Fe	0.48 ± 0.06	0.77 ± 0.22	0.54 ± 0.03	0.93 ± 0.14
Mn	0.08 ± 0.02	0.12 ± 0.03	0.09 ± 0.01	0.15 ± 0.05
Cr	n.d	n.d	n.d	n.d
Ni	n.d	n.d	n.d	n.d
Cd	n.d	n.d	n.d	n.d

n.d = not detecetd

Effect of welding consumables: Mild Steel (MS) and Stainless Steel (SS) on Cell Viability

A comparison between cytotoxicity effects of types of consumables, SS and MS was performed to assess the effects of materials used, and assayed at 0 h post exposure incubation (Figure 5). The parameters in these experiments consisted of 7 m of welding length with total exposure time of one hour. No significant cell viability reduction could be established ($p > 0.05$) between MS and SS whether at low or high speed experiment (Figure 5).

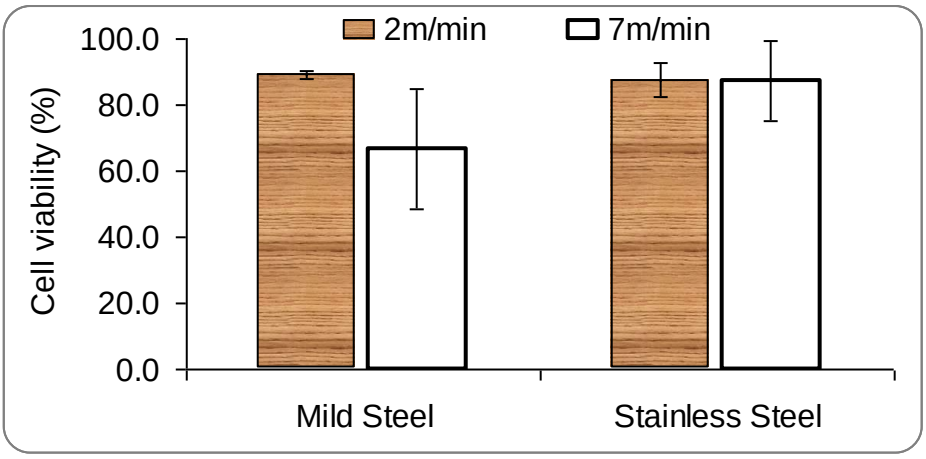

Figure 5: Cytotoxic effects of welding fume generated using MS and SS consumables within 60 minutes welding duration, assayed at 0 h post exposure

Table 3 shows Mn and Fe were detected from both types of materials (MS and SS) while Cr and Ni could only be detected from SS generated welding fumes. The results also indicated that Fe was higher in MS welding fumes as compared to SS welding fume. However, there was no significant differences of Mn detected either in MS and SS welding at low and high speeds ($p > 0.05$). The elemental anaysis data suggested that wire speed did not significantly affect the exposure mass.

Table 3: Metal mass of different wire feed speed and different types of consumables used collected from one hour exposure time

Metal Analysed	**Exposure mass (mg)**			
	Wire speed 2 m/min		**Wire speed 7 m/min**	
	MS	SS	MS	SS
Fe	0.82 ± 0.20	0.41 ± 0.10	0.86 ± 0.15	0.42 ± 0.10
Mn	0.15 ± 0.02	0.13 ± 0.04	0.13 ± 0.05	0.13 ± 0.03
Cr	n.d	0.13 ± 0.04	n.d	0.13 ± 0.03
Ni	n.d	0.06 ± 0.01	n.d	0.06 ± 0.02
Cd	n.d	n.d	n.d	n.d

n.d = not detected

Discussion

The cell viability of human cells exposed to welding fumes (at 25-50 ml/min flow rates) was investigated in this study. The generation of fumes is reproducible for up to 60 minutes of welding operation intermittently. However in the initial experiments, difficulties were encountered in attempting to weld continuously for 60 minutes without opening the chamber. The problem occurred due to melting of the wire blockage at the tip of nozzle, causing the whole welding process to stop. The problem managed to be solved by developing the intermittent welding process.

The literature clearly indicates that the risk of welding fumes exposure was dependent on the duration of exposure, composition and mass of generated fumes (Gerhardsson, 1994). However, the results indicated that the MS-welding length (6 and 9 m) did not have a signifcant impact on the cytotoxicity of exposed cells (Figure 2). The observed results could be attributed to the insufficient welding duration which led to an inadequate generation of welding fumes. In addition to the exposure time, types of consumables used during welding operation could influenced the quantity and composition of generated fumes, therefore interfere with the toxicity effect. The data in Table 1 clearly indicated that the generated MS-welding fumes consisted primarily of Fe and Mn. Welding fumes were generated using mild steel (MS) contained iron (80 – 95%) and manganese (1-15%) (Antonini et al., 2004). The manganese profile of the generated MS particles had mimicked the MS welding wire consumed during the welding process. Welding fumes contain all chemical elements present in the consumable, however the proportion and toxic nature change as it is dependent on the physical and chemical processes that occurred during welding operation (Gerhardsson, 1994).

The second effect that could contribute to toxicity effect was the speed of the MS-wire fed. The results indicated a toxic effect on cell viabiliy resulting from increased MS-wire speed combined with longer exposure duration. This agreed with published literature where it was reported that increased welding rates triggered a higher rate of fume generation (Spear, 2009). The toxicological potential of airborne manganese-compound-containing particles in welder's

workplaces depends largely on dose received (exposure concentration x time) and their chemical composition, size structure and resulting bioavailability (Spiegel-Ciobanu & Millan, 2007).

Our data indicated that small accelerated metals production did not significantly decrease cell viability. Furthermore, the generated metals at different wire speeds appeared to not cause reduction of cell viability even though the result was not significant. The insignificant cell viability reduction in the Figures 4 could be attributed to low metal mass generation. Furthermore, in previous literature had reported that iron and manganese at low levels were essential metals to most of living system (Piscator, 1979). Iron is essential to life because of its unique ability to serve as both an electron donor and acceptor.

The ICP-AES data on chemical composition of SS-welding particles was mainly characterized by amounts of Fe, Mn, Cr and Ni (Table 3). Although, other studies in the literature reported that Cr^{6+} and Ni^{2+} have a potential toxic effect in exposed welders, the toxicity data generated by our assay systems clearly indicated no cytotoxic effects (Figure 5). This might be explained by the amount of generated fumes from the automated welders, whereby the SS-fume mass was not enough to trigger observable effects in the cytotoxicity assays used. The SS-fumes metal profile consisted predominantly of iron (most abundant metal) as shown in Table 3, followed by Mn, Cr and Ni and this agreed with published literature (Antonini et al., 2007). Among the elements, Ni, Cr and Mn are metals with toxic potential whereas the toxic potential of Fe is much less so.

Conclusion

The toxicity of fumes created by welding can be evaluated using this system. The estimation of toxic effect of fumes through replication the exposure using direct dynamic method could possibly draw some answers to the variation of result in determining the health effect of welding fumes among welders. Variables such as duration of welding and type of materials being welded impact on the observed toxicity, however the estimation of toxicity could be used to identify toxicity of welding fumes and could determine the health effects among welder.

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