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Free Radicals Are Involved in Methylmethacrylate-Induced Neurotoxicity in Human Primary Neocortical Cell Cultures

May Show Chen[1], Jian Nan Wu[2], San Nan Yang[2], Wen Yia Hsieh[2], Jiang Chuan Liu[3], Earl Fu[4] and Demeral David Liu[5]

[1]*School of Dentistry, Taipei Medical College, Taipei 110*
[2]*Departments of Physiology and* [3]*Anatomy,*
[4]*School of Dentistry and* [5]*Graduate Institute of Medical Sciences,*
National Defense Medical Center,
Taipei 100, Taiwan, ROC

Abstract

Methylmethacrylate monomer (MMA), a highly volatile material, has been extensively used for the construction of complete or partial dental prostheses. While previous studies have indicated a variety of complications and untoward side-effects associated with its use, the possible neurotoxicity induced by this monomer has not been addressed. In this study, we have investigated the MMA-produced neuronal injury in human neuron-enriched primary culture. Embryonic brain tissue (8-10 weeks postconception) was used for the primary neuron-enriched culture. Phase-contrast microscopy was used to evaluate morphological changes of cultured neurons. Extracellular concentrations of lactate dehydrogenase (LDH) and nitrite was measured from the culture medium to assess the magnitude of neuronal damage and nitric oxide formation, respectively. Neocortical neurons exposed to the monomer (1/200, $V_{monomer}/V_{glycerol}$) for two days resulted in a significant increase in the LDH level but monomer (1/20000, 1/2000, 1/1000, or 1/200; $V_{monomer}/V_{glycerol}$) failed to increase the nitrite level. Morphologically, the neurons subjected to monomer treatment exhibited irregular shrunken cell bodies with dystrophic and/or fragmented neurities, or even cell lysis. Moreover, superoxide dismutase plus catalase or vitamin C pretreatment protected against monomer-induced neurotoxicity. Our results suggest that this neurotoxicity can not likely be attributed to the cytotoxic effects of nitric oxide but may be mediated through the toxicity of superoxide and other free radicals. This is the first time, to our knowledge, that neurotoxicity induced by MMA has been demonstrated in human cortical neurons

Key Words: cytotoxicity, free radicals, methylmethacrylate, monomer, neurotoxicity, nitric oxide

Introduction

Methylmethacrylate monomer (MMA), a highly volatile material, has been extensively used not only in dentistry for the construction of complete or partial dental prostheses but also in orthopedic surgery as a cement for insertion of joint prostheses. In addition, monomer has also been widely used in other medical fields (20). Unlike the crystalline polymer, the liquid MMA is volatile at room temperature, has an irritating aromatic odor, and exhibits systemic effects through ingestion or inhalation. While previous studies described a variety of complications and untoward side-effects associated with its use, such as headache and dizziness as well as abnormal cardiovascular and respiratory functions (2, 10, 21, 30), the possible neurotoxicity induced by this monomer has not been addressed. It is also possible that MMA may primarily produce neuronal injury in central nervous system and lead to the secondary abnormalities in cardiovascular and pulmonary function.

The results from the previous survey suggested that MMA may cause acute and chronic damage of the central nervous system (16, 21, 23, 28). The chronic administration of MMA in rats resulted in a markedly impaired locomotor activity and learning, with

Corresponding author: Dr. Demeral David Liu, Graduate Institute of Medical Sciences, National Defense Medical Center, P. O. BOX 90048-503, Taipei, Taiwan, ROC. Tel: 886-2-2367-3441, Fax: 886-2-23656860.

aggressive behavior significantly increasing (16). Furthermore, local neurotoxicity has been reported through the absorbed MMA (3, 9, 22, 26). Although the distribution of MMA in the brain tissue following inhalation has been documented (21), the experimental evidence of MMA-induced neurotoxicity remains to be established.

Within the central nervous system, nitric oxide (NO) plays an important role in the pathogenesis involved in neuronal death (5, 6, 7, 31). The generation of NO by the calcium-dependent stimulation of NO synthase (NOS) has been reported to be associated with neurotoxic effects of many neurotoxins (5, 13, 31). Accumulated data suggest that excessive free radicals formation such as nitric oxide and superoxide are responsible for the excitotoxicity in the central nervous system (5, 29, 31). To our knowledge, there is no clear evidence to demonstrate that free radicals are involved in this neuronal damage induced by MMA. Previously, we have established a human primary neuron-enriched culture and have reported that these neocortical neurons can generate nitric oxide in response to NMDA challenge (19). We have also demonstrated that the nitric oxide is involved in the β amyloid peptide- and glutamate- induced neurotoxicity (31). Thus, the major purpose of the present study was to investigate the involvement of free radicals in MMA-induced neurotoxicity using primary human neocortical neurons. Furthermore, selective inhibitors of superoxide or free radical scavengers were also used to determine whether these pharmacological agents demonstrated therapeutic potential.

Materials and Methods

Brain tissue was obtained from the cortex of six human embryos. These embryos obtained for cell culture were from 8-10 weeks postconception following elective abortion. These abortions were carried out under regulations provided by the Abortion Act of the Republic of China. The use of human embryos was approved by the ethical committee of our institute and followed the guidelines proposed by Human Embryo Research Panel of National Institutes of Health (NIH, USA). The primary neuron enriched neocortical culture was performed as previous described (19, 27). Basically, the cerebral cortex was dissected and chopped into 2 mm pieces under light microscopy. The cortical chunks were suspended in 10 ml of 0.25% (w/v) trypsin solution (pH 7.4) and placed in a shaking water bath for 5 minutes at 37°C. The dissociated cells were diluted with serum supplemented medium (SSM: Dulbecco's modified Eagles medium with Ham's nutrient mixture F-12 and 10% fetal bovine serum) and were centrifuged at 600 × g for 10 min. The resultant pellet was resuspended in 10 ml SSM and was further dissociated by trituration through a fire-polished glass pipette. With this procedures, the viability of the dissociated cells (>95%) was examined by the trypan blue exclusion method. The cell suspensions were plated at 2 million cells/well in 1.5 ml of SSM using 6-well dishes (Nunc, 35 mm in diameter), precoated with poly-L-lysine and were incubated at 37°C in a humidified 95% air 5% CO_2 for 3 days. Thereafter, two replacements of SSM were made on the 3rd (with 5 μM cytosine arabinoside) and 5th day (without cytosine arabinoside) of incubation. The cultured cells were selected for MMA-induced neurotoxic study on the 9th day of incubation. Under this circumstance, the purity of the neurons were greater than 90% (19, 31). Methylmethacrylate monomer was purchased from Hygenic Corporation (Akron, Ohio, USA). Superoxide dismutase (SOD), catalase, and vitamin C were purchased from Sigma Chemical Co. (St. Louis, MO, USA). One hour prior to any experimentation, MMA was dissolved in glycerol and the other drugs were dissolved in sterile, distilled water. The neuron-enriched neocortical cultures (DIV=9) were routinely exposed to experimental drugs for 48 hours prior to the assessment of NO production and neuronal damage.

The levels of NO, lactate dehydrogenase (LDH), and neuronal morphology were evaluated from the same culture. In brief, NO was monitored by measuring nitrite, a stable oxidation breakdown product of NO (12). Nitrite levels were analyzed by mixing 100 μl of culture medium with 100 μl of Greiss reagent (1 part 1% sulfanilamide in 60% acetic acid plus 1 part 0.1% naphthylenediamine dihydrochloride in distilled water) for 10 min, and read on a spectrophotometer at 546 nm. Neuronal damage was first evaluated by the examination of neuronal morphology under phase-contrast microscopy followed by quantification of LDH released from damaged or destroyed cells into the culture medium, as previously described (6, 31). LDH was measured using a LDH colorimetric assay kit (Cat. No. 500, Sigma) with an absorbance peak of 450 nm. Unless otherwise stated, data are expressed as mean ± standard error of mean. Statistical evaluation was performed using one-way ANOVA followed by Dunnett F test for *post hoc* comparisons.

Results

To determine if MMA-induced neuronal damage is associated with an elevation in NO production, experiments were conducted using six dishes of primary neocortical neuron-enriched cultures from

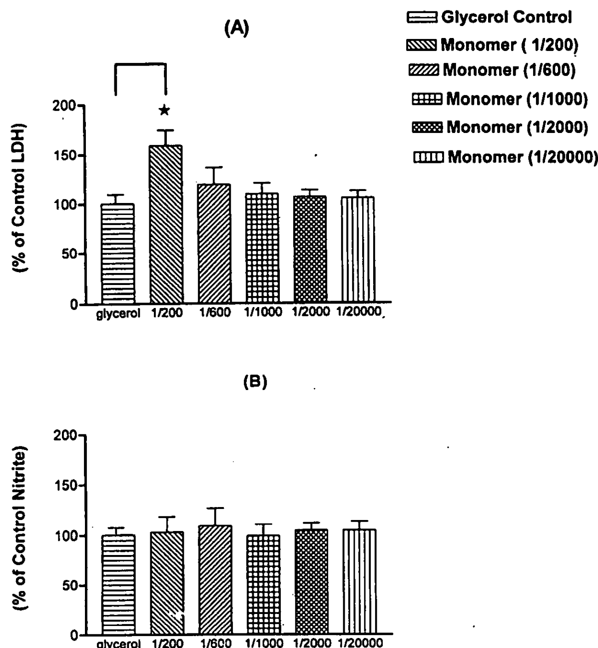

Fig. 1. Neurotoxicity and NO production induced by methylmethacrylate (MMA). (A). MMA-induced cell death in human neuron-enriched primary cultures as measured by LDH kit. A summary of six separate experiments. *p<0.01, as compared to the vehicle control, one-way ANOVA followed by Dunnett F test for post hoc comparisons. The average of the glycerol control LDH per well (10^6 cells) was 693.5±78.4 units/ml. (B). MMA-induced neurotoxicity was not associated with increasing the nitric oxide production as determined by Greiss reagent method. No significant change was found as compared to glycerol control (p>0.05, one way ANOVA, n=6). The average of the vehicle control nitrite level per well (10^6 cells) was 2.85± 0.19 nmole.

human embryos. MMA was dissolved in glycerol serving as a vehicle. Previous (31) and present studies revealed that the glycerol alone did not produce discernible neurotoxic effect. Monomer induced neuronal damage in a concentration-dependent manner (Fig.1A). However, the significant increase in LDH (158.5±15.2%, p<0.01, n=6) was generated at relatively higher concentration (1/200 $V_{monomer}/V_{glycerol}$) of monomer treatment. This MMA-induced neurotoxicty was clearly seen with phase-contrast microscopy (Fig. 2). Morphologically, the neurons subjected to monomer treatment revealed irregular shrunken cell bodies with dystrophic and/or fragmented neurities, and even cell lysis. Moreover, the increased LDH release in response to monomer treatment was not associated with an elevation of nitrite formation (Fig. 1B).

To study whether superoxide was involved in the MMA-induced neurotoxicity in human neocortical neurons, in another six separate experiments of neuron-enriched cultures, we examined if SOD/catalase can attenuate monomer-induced neuronal damage as observed in Fig. 1A. SOD (100 IU/ml) plus catalase (100 IU/ml) and monomer were applied to neocortical cultures simultaneously. Application with SOD and catalase significantly attenuated the magnitudes of MMA-induced neuronal cell death (145.9±17.8% vs. 120.2±7.6%, p<0.01, n=6; Fig. 3). We also noted a significant reduction (110.8±17.1 vs. 82.4±7.6%, p< 0.01, n=6) of the nitrite formation in this paradigm, while the monomer did not increase the nitrite formation. SOD/catalse alone control did not alter the LDH but significantly reduced the nitrite level as compared to MMA alone. In addition to the attenuation of LDH release, the neuroprotective effect of SOD plus catalase was also observed at the morphological level. As demonstrated in the Fig. 4C, the damaged neurons as indicated by irregular shrunken cell bodies with dystrophic or fragmented neurites were noted in the monomer treated dish but a relatively healthy neuron having smooth and round soma with clear neurites and axons were noted in the monomer with SOD plus catalase treated dish.

To further examine if a free radical scavenger can attenuate the monomer-induced neurotoxicity, vitamin C and monomer were applied to the culture medium in another six separate experiments of neuron-enriched cultures. The neuroprotective effect of vitamin C was shown in the Figure 4D and 5. The monomer-induced elevation of LDH was significantly attenuated by the treatment of relatively high dose of vitamin C (142.9±17.3 vs. 106.2±11.4%, p<0.01, n=6). Nitrite formation was not altered following this treatment. Neither LDH nor nitrite level was significantly changed in the vitamin C alone control experiment (data not shown)

Discussion

In this study, we found that MMA can produce neurotoxocity as revealed by phase contrast microscopic observation in the human neocortical neuron/glial cultures. This MMA-elicited neurotoxicity was accompanied by an elevation of LDH but not nitrite level. The neurotoxicity of MMA was attenuated by pretreatment with either SOD/catalase or vitamin C. Our data suggest that human cortical neurons are vulnerable to MMA and that this MMA-elicited neurotoxicity is unlikely to be attributed to the cytotoxic effects of nitric oxide, but instead may be mediated by superoxide and other free radicals.

MMA, a monomer extensively used in the manufacture of acrylic polymers, has been reported to cause neurological deficits in industrial workers (9, 17, 25). The distribution of MMA in the brain tissue following inhalation exposure to MMA has been documented (21). Furthermore, the effects upon central neuronal activity associated with acute

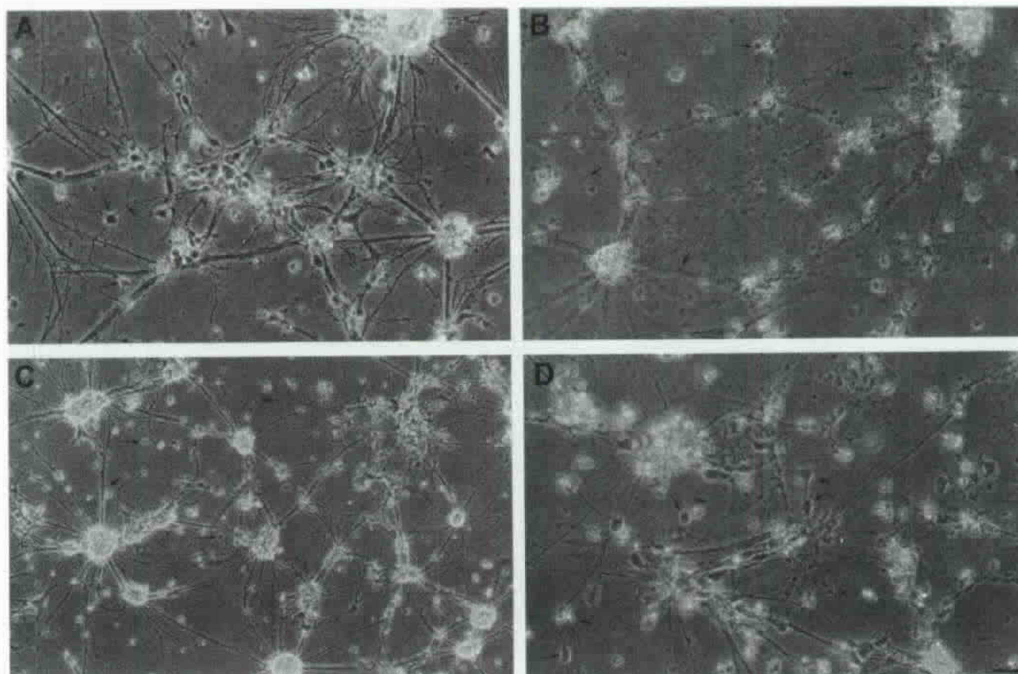

Fig. 2. Phase-contrast photomicrographs of primary cortical neurons taken 48 hours after the following treatments: (A) Control neurons (large arrow) showing extensive neurities with a relatively uniform diameter and a smooth appearance (small arrow); (B) Neurons treated with monomer (1/200, $V_{monomer}/V_{glycerol}$) revealing extensive neurite fragmentation (small arrow), and cell lysis or shrunken cell body (large arrow); (C) Vehicle control neurons treated with glycerol indicating a relatively normal morphological appearance as compared with control; (D) Neurons treated with monomer (1/600, $V_{monomer}/V_{glycerol}$) demonstrating a relatively normal morphological appearance as compared with (C). Bar = 50 μm.

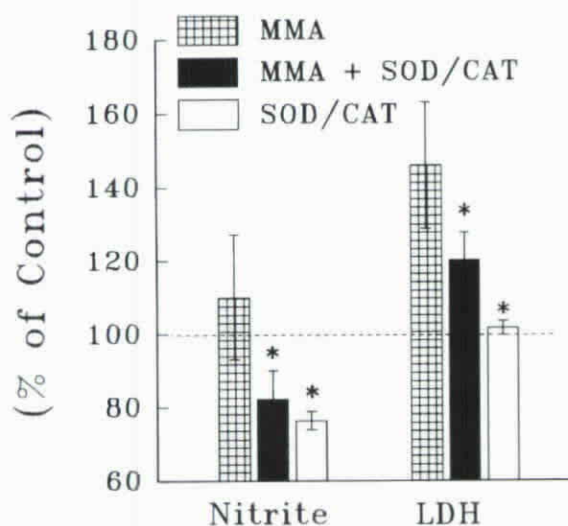

Fig. 3. Effects of superoxide dismutase (SOD) plus catalase (CAT) on neurotoxicity and nitrite level induced by the methylmethacrylate (MMA). A summary of six separate experiments. MMA (1/200)-induced neurotoxicity was significantly attenuated by the pre-treatment with SOD (100 IU/ml) plus CAT (100 IU/ml). The average of the control LDH per well (10^6 cells) was 576.7±35.7 units/ml. *$p<0.01$ as compared with MMA (1/200) alone. While nitrite level was not significantly increased following 48 hours of MMA (1/200) treatment, the SOD plus CAT pretreatment significantly attenuated the nitrite formation in this MMA-induced nuerotoxicity. The average of the control nitrite level per well (10^6 cells) was 3.62±0.67 nmole. A summary of six separate experiments. *$p<0.01$, as compared with MMA (1/200) alone.

exposure to monomer vapor have been examined in anesthetized rats (17). The most remarkable finding of this study was the depression of multiple-unit electrical activity in the lateral hypothalamus and ventral hippocampus as these animals were exposed to 400 ppm of MMA in air for 60 min. Moreover, the chronic administration of MMA in rats resulted in a markedly impaired locomotor activity and learning, while aggressive behavior significantly increased (16). Taken together, these results suggested that MMA may cause acute and chronic damage the central nervous system (16, 23, 28).

The toxic effects of MMA have been reported on the cellular integrity of monocytes, granulocytes, endothelial cells, and osteoblasts *in vitro* (8, 14, 20). These results are consistent with the ability of MMA to block an electron transport and to uncouple oxidative phosphorylation in rat liver mitochondria (1). Recently, free radicals induced by MMA have been suggested to be responsible for the cytotoxic effects *in vitro* (15, 20, 24, 25). Peripheral neuropathy associated with MMA exposure has been reported in dental technicians (3, 9, 22, 26). Results from a dose-response analysis in dental technicians and opticians has suggested that the symptoms of the organic dementia may have a connection to MMA exposure (23). In a case study, it was found that insertion of MMA appeared to produce profound hypotension in a

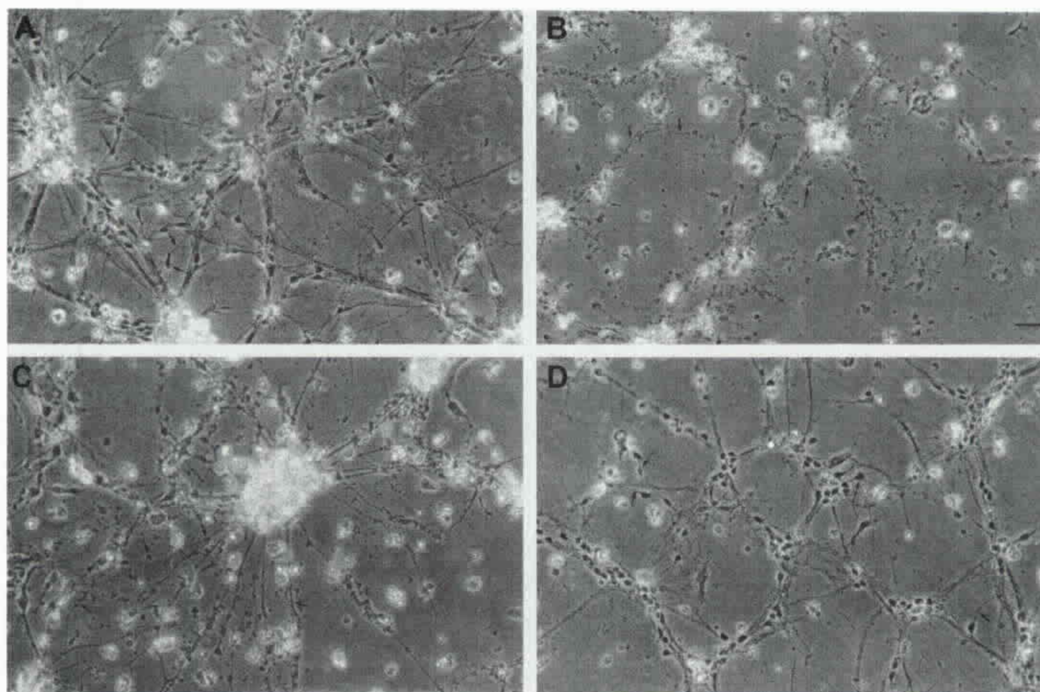

Fig. 4. Phase-contrast photomicrographs of primary cortical neurons taken 48 hours after the following treatments: (A) Control neurons (large arrow) showing extensive neurities with a relatively uniform diameter and a smooth appearance (small arrow); (B) Neurons treated with monomer (1/200, $V_{monomer}/V_{glycerol}$) revealing extensive neurite fragmentation (small arrow), and cell lysis or shrunken cell body (large arrow); (C) Neurons treated with monomer (1/200, $V_{monomer}/V_{glycerol}$) plus superoxide dismutase (100 unit/ml) and catalase (100 unit/ml) revealing shrunken cell bodies (large arrow) with dystrophic or fragmented neurites (small arrow); (D) Neurons treated with monomer (1/200 $V_{monomer}/V_{glycerol}$ plus vitamin C (2.5×10^{-3} M) indicating a relatively normal morphological appearance as compared with control. Bar = 50 μm.

patient with long-term levodopa-treated paralyis agitans in spite of normovolumia and proper anesthetic management (18). Consistent with previous reports, cytotoxic effects induced by MMA was also observed in the present study. Dawson et al. (6) reported that SOD (100 IU/ml) plus catalase (100 IU/ml) is capable of attenuating the superoxide-induced neurotoxicity *in vitro*. Our data indicate that superoxide and/or other free radicals was, at least partially, responsible for this MMA-induced neuronal damage in primary cortical neurons.

Accumulated data indicate that components of resin composites have effects on biological membranes. Terakado et al. (24) had shown that benzoyl peroxide, a catalyst for resin composites, was capable of converting polyunsaturated fatty acids and phospholipids to peroxides. This finding suggested a mechanism of action by MMA on lipid layers of cell membranes. Moreover, Zofia (33) found that the prosthetic monomer containing additional substances has stronger cytotoxicity than the pure monomer. Additionally, Fujisawa et al. (11) reported that the actions of this monomer are primarily on the cell membrane leading to increased permeability and exposure of internal plasma membranes to even higher concentrations of this monomer. Furthermore, Moreau et al. (20) found that free radicals released during MMA polymerization are cytotoxic for osteblasts. Vitamin C is widely distributed in the body as ascorbate monoanion. Ascorbate is a reducing agent that is capable of readily donating electrons to repair highly reactive free radicals, such as superoxide, hydroxyl radical, and lipid peroxyl radicals (4). Our data suggest that vitamin C, a potent antioxidant, is capable of attenuating the MMA-induced neurotoxicity *in vitro*. Further investigation is warranted to determine if vitamin C is able to detoxify the MMA-induced toxicity *in vivo*.

In this study, we found that the neurotoxicity-induced by MMA was not associated with increasing the nitrite formation. NO plays an important role in the pathogenesis involved in neuronal death (5, 6). Accumulated data suggest that excessive free radicals such as nitric oxide formation is an important pathogenesis underlying excitotoxicity in the central nervous system (5, 13, 32). Our data suggest that MMA-induced neurotoxicity was presumably not linked to the nitric oxide-dependent mechanism. Alternatively, the measurement nitrite production via Greiss reagent method is relatively insensitive as compared to others such as electrochemical method (19). It is still possible that the amount of increasing nitric oxide production is beyond the detective limitation (around micromolar range) of Greiss reagent

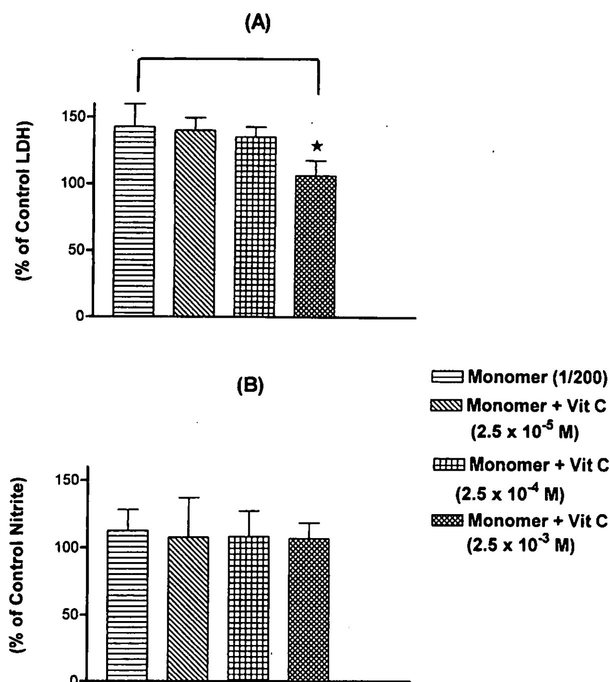

Fig. 5. Neuroprotective effects of vitamin C (Vit C) on the methylmethacrylate monomer (MMA)-induced neurotoxicity. Monomer (1/200)-induced neurotoxicity was significantly attenuated by the pretreatment with vitamin C (2.5×10^{-3} M). The average of the control LDH per well (10^6 cells) was 483.8±42.8 units/ml. A summary of six separate experiments. *$p<0.01$ as compared with MMA (1/200) alone. (B) Neither MMA nor vitamin C pretreatment altered the nitrite formation in this MMA-induced neurotoxicity. The average of the control nitrite level per well (10^6 cells) was 2.79±0.65 nmole. No significant change was found as compared with MMA (1/200) alone, $p>0.05$, $n=6$.

method (31) especially for the constitutive form NO release (19).

In conclusion, neurotoxicity induced by MMA was observed in the present study. Furthermore, we demonstrated that superoxide and/or other free radicals was, at least partially, responsible for this neuronal damage in primary cortical neurons. Thus, an awareness of the potential neurotoxicity of MMA is important in developing safety guidelines for the occupational environment of dental technicians and surgical staff.

Acknowledgments

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