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A possible biomarker for methadone related deaths

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ABSTRACT

Methadone (MTH) concentrations in those dying of MTH toxicity totally overlap concentrations where the presence of MTH is only an incidental finding, making it very difficult to make distinctions in actual cases. A biomarker, be it anatomical or biochemical for MTH toxicity is badly needed, particularly if that markers were known to disrupt effective ventilation. Because the brainstem houses the regulatory centers for cardiorespiratory-control enters, it would seem to be the most likely anatomical site to seek abnormalities in cardiorespiratory control.

Objective: To locate and describe the cells of nucleus of the solitary tract (TS)(NTS) in human brainstem and determine if neuronal cell death, either necrotic or apoptotic, within the TS of humans is more common in deaths due directly to MTH toxicity than with in the solitary tract itself.

Design, setting, participants: This was a single cohort study of MTH related decedents autopsied at a large university hospital. Each decedent had a recent history of non medical/illicit MTH use and had been pronounced dead in the field, prior to ever reaching the hospital. Complete autopsy and complete toxicology testing were performed on the formalin fixed brains of each individual. Multiple blocks were prepared of the area of interest, namely the tissue lying immediately between the inferior and the super colliculi. This volume, by definition, would have included the area of the Rostral Ventrolateral Medulla (RVLM), the location of the TS. Immunohistochemistry studies utilizing caspase-9 reaction (a protease enzyme involved in the process of preprogrammed death) were performed in order to estimate the degree and proportion of neuronal apoptosis, and also access the degree of classical necrosis within the NTS.

Main outcomes and measures: The primary outcome measure was the presence or absence of neuronal apoptosis and/or necrosis within the NTS.

Results: Cells displaying evidence of early apoptosis and advanced apoptosis, consisting primarily of nuclear fragmentation, admixed with other neurons displaying the features of classic necrosis were found. Evidence of classic necrosis was identifiable in most of the controls, though minor degrees of apoptosis were identifiable with Caspase staining and quantitative image analysis of immunohistochemical stains.

Conclusions: and Relevance: Our study shows that neurons, primarily along the TS, but occasionally in other cell nuclei (even controls) are vulnerable, both to direct MTH toxicity (via apoptosis) and indirectly (via hypoxia leading to classical cell necrosis). When MTH is found to be present in significant concentrations, but apoptotic lesions are absent, it would be reasonable to assume that MTH was not primarily the cause of cardiorespiratory arrest.

1. Introduction

MTH (6-dimethylamino-4-4-diphenylheptanorn-3) is a synthetic full μ-receptor agonist an inhibits the *N*-methyl-D-aspartate

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receptor. For that reason it could well make it better analgesic than morphine.[1] MTH is mainly metabolised by cytochrome P450 3A4 (CYP3A4), and to a lesser extent by CYP2B6, CYP2C19 and CYP2D6.[2] It's effectiveness when used opioid as an substitute treatment (OST) has repeatedly been demonstrated.[3] Increasingly, it is also being used in the treatment of chronic pain syndromes.

CDC surveys show that six times as many people died of MTH overdose in 2009 as died in 1999. Pharmacologic investigations indicate that MTH has the potential to be misused, non-medically, and diverted into a "gray market, where those who have legitimately been prescribed MTH sell their prescriptions for a profit.[4] The connection between the increasing number of deaths and the growing availability of this drug is clear,[5–8] especially in relation to illicit use of MTH in young adults.[3] Still, the actual mechanism leading to sudden death remains obscure.[9] Respiratory arrest is not the only mechanism that could account for MTH-related deaths. Even therapeutic doses of MTH can cause QT prolongation and QT dispersion, thereby providing the substrate for the torsades des pointes (TdP) and sudden cardiac death (SCD).[10] Postmortem blood concentrations in cases of MTH induced respiratory depression totally overlap concentrations where MTH is an unrelated isolated finding (such as in trauma victims) and even concentrations found in life.[11] Autopsy findings, in MTH-related deaths, no matter whether death is arrhythmic, or respiratory in nature are non-specific: pulmonary edema and visceral congestion.[12,13]

For more than 15 years is has been known that under some circumstances, opiates can cause neuronal death.[14] In experimental animals, chronic MTH treatment induces frank apoptosis.[15] In a like fashion, ultrastructural studies of melanized cells from the substanigra of Parkinson's victims show changes consistent with frank apoptosis after MTH exposure.[16] However, in tissue culture studies, MTH seems to activate both apoptotic and necrosis pathways.[17] MRI studies of humans have shown reductions in both the white[18] and the gray matter of heroin users undergoing MTH maintenance therapy (MMT).[19] Other imaging studies have shown changes in the rostral ventromedial medulla (RVLM) – the area of the brain containing the nucleus solitarius and solitary tract, the structures that control cardiovascular reflexes and respiration.[20,21] It has recently been shown that MTH cytotoxicity is a function of cyclic AMP (cAMP) concentrations that, in turn, control cell proliferation, differentiation, and apoptosis.[11,22] The triggering of opioid receptor agonists can activate inhibitory G proteins, block adenylcyclase activity thereby reducing cyclic amp production and promoting apoptosis.

Here we report the findings of systematic histological and histochemical studies of the rostral medulla to determine if failure of sympathetic vasomotor discharges emanating from the RVLM might lead to the respiratory failure. If a reliable biomarker could be found it would be of great value for forensic pathologists trying to determining the cause of death in human MTH users.

2. Materials and methods

This was a retrospective study of autopsy cases examined at the pathology department of a University Hospital during the years 2009–2012. Eleven of the decedents had been classified as MTH *abuse* fatalities, primarily on the basis of medical history, scene investigation, and known criminal background. The possibility of any other obvious cause of death (macroscopically and microscopically), or other drugs of abuse and alcohol had already been eliminated.

All deaths were classified as *unnatural*, or *accidental* in regard to the manner of death. The results of field investigators suggested that a majority of the decedents had acquired their fatal drug illegally, either through diversion from maintenance programs, or, by purchase from users of legal prescriptions (the ultimate reason for investigation by the public prosecutor). There were a total of eleven decedents. The average postmortem interval was 29,3 h. Controls were comprised of five drug-free, violent/trauma-related deaths, who died during the same period. The average age of the controls was 31 years, and one of the controls was a woman. The average post mortem interval for the controls was 29.6 h (see Table 1).

2.1. Toxicology study

Peripheral (femoral) blood was collected for toxicological testing. Brains were removed and fixed in formalin for 10 days prior to dissection. The entire medulla was removed, sectioned, and stained with hematoxylin/eosin. Subsequently, histochemical analysis of Caspase -9 was performed.

Immunological screening for MTH and other drugs of abuse was performed and the results confirmed by Systematic Toxicological Analysis (STA) with GC/MS measurement of Alkaline, Acid, and Neutral fractions. Volatile organic compounds were also measured.[23]

All the available biological fluids were analyzed by GC/MS. If a drug of abuse and/or its metabolites and/or other exogenous compounds are detected, a further GC/MS analysis is performed to quantitative them. Sample preparation (e.g. initial volume of matrix) and instrument settings are varied depending on the amount of drug thought to be present. Working Standard samples (WSs), i.e. "blank" biological fluid spiked with standard solutions of the most common drugs of abuse and their metabolites (total amount: 1 μg), were analyzed together with the unknown specimens.

Measurement of MTH and its major metabolites measurement were made in the same manner (Table 2), but chiral separations was not performed.[5,22–24] The anatomical sites of nucleus solitarius and tractus solitarius were located at brainstem level of medulla oblongata,[25,26] with transverse section of the medulla at the caudal end of the fourth ventricle. Histological sections of hematoxylin/eosin stained slides were read independently by three pathologists with formal neuropathology training. A simple grading system was used with 1 = rare evidence of apoptosis and 3 = frequent presence of apoptotic cells.

2.2. Immunohistochemistry study

Serial sections, 8 μm thick, were cut on Leica microtome RM2145, dried overnight at 37 °C and then stored at room temperature. The day after, the slides were dewaxed and rehydrated by sequential immersion in a graded series of alcohols and transferred into water for 5 min. To inhibit any endogenous peroxidase activity the slides were treated for 5 min with peroxidase quenching solution in hydrated incubation enclosure at room temperature. The slides were then transferred to PBS (Na_2HP0_4, KH_2P0_4, KCl, NaCl pH 7.4–7.6) at room temperature. We used Histostain®-Plus 3rd Gen IHC Detection Kit with DAB chromogen as substrate (Invitrogen).

After rinsing with PBS for 4 min, the sections were incubated with a blocking solution for 10 min and then incubated overnight at 4 °C with rabbit Caspase 9 cleaved (Cell Signaling Technology) diluted 1:100. After incubation, any excess antibody was removed by washing with PBS for 5 min and the sections were incubated with biotinylated secondary antibody for 20 min at room temperature. Unbound antibody was removed by washing (2× with PBS, 5 min each), followed by Streptavidin-Peroxidase conjugate for 10 min, and to reveal the reaction DAB chromogen in substrate buffer was added for 5 min and stopped in distilled water. The slides were removed from the water and mounted with one drop of aqueous mounting medium (DAKO Faramount). Negative controls were performed by omission of primary antibody, and by