A Possible Biomarker for Methadone Related Deaths.
Short Running Head: The tractus solitaries neurons are directly vulnerable to MTH

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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 is that marker were known to disrupt effective ventilation, As the brainstem houses the regulatory centers for cardio-respiratorycontrol, it would seem to bethe most likely anatomical site to find abnormalities in that relation.

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 humansis more common in deaths due directly to Methadone MTH toxicity than Within the TSof the brainstemin control subjects.

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. A complete autopsyand complete toxicology testing were performedon 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

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volume, by definition, would have included the area of the Rostral Ventrolateral Medulla (RVLM), the location of the RT.Immunohistochemistry studies utilizing caspase-9 reaction (a protease enzyme involved in the process of preprogrammed death) were performed in order to estimatethe degree and proportion of neuronal apoptosis, and also classical necrososis 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, the central site regulating cardiovascular and breath reflexes.

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 immunoistochemical stain.

Conclusions and Relevance: Our study shows that neurons, primarily along the TS, but occasionally in other cell nuclei (even controls) are vulnerable, both to MTH directly (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 ofcardio respiratory arrest.

Key words: neurotoxicity, brainstem, methadone, apoptosis, caspase-9, biomarker.

Introduction: MTH(6-dimethylamino-4-4-diphenylheptanorn-3) is a synthetic full μ-receptor agonist with inhibition at the N-methyl-D-aspartate receptor, which might make it a better analgesic than morphine[1].Mainly metabolised by cytochrome P450 3A4 (CYP3A4), and to a lesser extent by CYP2B6, CYP2C19 and CYP2D6 [2].It is a secure and effective pharmaceutical drug when used for opioid substitution treatment(OST) [3]. It is also increasingly 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, nonmedically, and diverted into a “gray market, where those legitimately 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, 6, 7,8], especially in relation to illicit use of MTH inyoung adults[3], but often 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, 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 death, no matter whether death is arrhythmic, or respiratory in nature, are non-specific: pulmonary oedema 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 substantianigra 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 [22, 11]. The triggering of opioid receptor agonists can activate inhibitory G proteins, block adenylylcyclase activity reducing cyclic amp production and promoting apoptosis.

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

Materials and Methods: This was a retrospective studyof 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

Commento [SK1]: **It would be best to insert two new references here, as either I or Chris Milroy may very well be sent the paper to review: (1)** Milroy **CM,** Forrest **AR.** Methadone **deaths: a toxicological analysis. J ClinPathol. 2000 Apr;53(4):277-81. and Karch, SB. Toxicology and pathology of deaths related to methadone: retrospective review. West J Med. 2000 Jan;172(1):11-4.**

Commento [SK2]: There are lots of different G proteins but they are all designated by greek letters – I don't know what you mean by Giprotiein. It might be best just to leave it at "G proteins" and not specific which one.

obvious cause of death (macroscopically and microscopically), or other drugs of abuseand alcohol had already been eliminated. All deaths were classified as *unnatural*,or *accidental* in regard to the manner of death. The results of field investigatorssuggested that a majority of the decedents had acquired their fatal drug illegally, either through diversion from maintenance programs, or, purchase from users oflegal prescriptions (the ultimate reason for investigation by the public prosecutor).
There were a total of eleven decedents. Controlswere comprised of five drug-free, violent/ trauma-related deaths, who died in same period. The average age of the controls was 31 years, and one of the controls was a woman. The average PMI for the controls was 29.6 hours. There was no attemptedin either the subjects or controls.

Toxicology study

Peripheral (femoral) blood was collected for toxicological testing**.** Brains were removed and fixed in formal for 10 days prior to dissection. The entire medulla was removed, sectioned, and stain 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. MTH and its major metabolites measurement were made in the same manner, but chiral separations was notcarried out[5, 22-24].

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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.

STA [23]

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Generic investigation for the detection of basic compounds

All the available biological fluids are analyzed by GC/MS from a qualitative point of view, i.e. identified molecules thanks to known Retention Time (R.T.) and mass spectra. 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 purposes. Sample preparation (e.g. initial volume of matrix) and instrument settings are targeted to the compounds to quantify, and 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), are analysed together with the unknown specimens. The quantification is usually applied to methadone, and its main metabolites EMDP (2-Ethyl-5-Methyl-3,3-Diphenyl-Pyrroline) and EDDP (2-Ethyl-1,5-Dimethyl-3,3-Diphenyl-Pyrrolidine), using proadifen as Internal Standard (IS); amphetamine, methamphetamine, 3,4-Methylendioxymethamphetamine (MDMA), using 2-phenyl-ethyl amine as IS; cocaine and benzoylecgonine, using scopolamine as IS; codeine, morphine, 6-MAM and acetylcodeine using nalorphine as IS.
Blood, urine or bile (an aliquot between 250 µL and 2 mL depending on the type and availability of the sample) are added with 50 µL of IS at a concentration of 20 µg/mL in methanol, water until a volume of 4 mL and 2 mL of pH 9 buffer. The mixture is centrifuged at 3500 rpm for 10 min and then loaded (1-2 mL/min) on a Bond Elut Certify (Agilent) cartridge (a mixed-mode cartridge packed with non-polar C8 and strong anion exchange SAX sorbent), previously conditioned with methanol (2 mL) and water (2mL). The cartridge is then washed with water (2 mL) and 1M acetic acid (3 mL), desiccated for 5 min and then washed with 0.5 mL methanol. The column is left under vacuum for 1 min and the analytes are eluted twice with 1 mL of a freshly prepared mixture of dichloromethane, isopropanol and 30% ammonium hydroxide (8/2/0.2 v/v/v). The solvent is evaporated and the residue analysed in GC/MS dissolved in methanol (50 µL) and then, after drying, derivatized adding 50 µL of BSTFA-1% TMCS and heating at 70 °C for 30 min.

Generic investigation for the detection of acidic compounds

Blood, urine or bile (an aliquot between 250 µL and 2 mL depending on the type and availability of the sample) are added with 50 µL of IS (hepta-barbital 50 µg/mL in methanol) water until a volume of 4 mL and 2 mL of pH 9 buffer. The mixture undergoes SPE extraction as described in the previuos case but the analytes are eluted with 4 mL of a freshly prepared mixture of acetone/chloroform (1:1). The solvent is evaporated and the residue analysed in GC/MS dissolved in methanol (50 µL) and then derivatized adding 20 µL MethElut Reagent in methanol (Pierce Companies).

GC/MS for generic investigation

The analyses were performed on a HP6890 Series II GC system, with a split-splitless injection system and an MSD HP5973 MS detector (Agilent Technologies, Santa Clara, CA, USA) operated in electron ionization (EI) mode (70 eV). The GC was equipped with a Rxi®-5Sil MS (5% diphenyl/95%

dimethyl polysiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm) capillary column (Restek, Bellefonte, PA, USA).
The GC/MS conditions: splitless; solvent delay, 3.5 min; injector temperature, 280°C; interface transfer line, 280°C; ion source, 280°C; oven temperature program, initial 70°C, 40°C/min up to 110°C, then 15°C/min up to 300°C (3 min). Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The MS detector was operated in the scan mode, acquiring ions from m/z 50 to 550. The total analysis time was 21 min.

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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. The following protocol was realized using 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 ten 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 (2x 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 incubating sections with antiserum saturated with homologous antigen.

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Image Analysis.

The immunohistochemical specimens were examined by using a Leica DMLB Microscope (Leica Microsystem GmbH Wetzlar, Germany) with a Nikon DSFi1 digital photographic system. Each sample was analyzed by two different operators who were unaware of the category of slide being evaluated.The results were also compared to an image analysis obtained from TIF files. Adobe Photoshop CS6 extended (Adobe System Inc, San Jose, CA) was used to

elaborate images and perform an image analysis (28, 29). After the conversion image color profile from RGB to CMYK the yellow channel was chosen because the literature indicates that it has the best linear response to color intensity and thus to protein presence. The quantification of colorimetric staining has been represented using a score with values from +1 to +5 (30, 31, 32) in all nuclei of solitary tract at 40x magnification.

Results: The controls were all drug free**.** Figure 1 shows fragmented, compacted nuclei, with condensed nuclear matter –so called "bodies of apoptosis"- along the nuclear membrane fragmented nuclei (Figure 1). Other neurons appeared to be undergoing early necrotic change, with homogenous shrunken cytoplasm and no nucleus visible. In several of the MTH cases, the neuropil was markedlyedematous. Necrotic neurons were also visible in several of the controls.Figure 2 showsCaspase 9 immunoreactivity on Nucleous of the solitary tract in MTH cases (Figure 2 Left, A) and in the controls (Figure 2,B). Table 1 showsa comparison of score Caspase 9 cleaved colorimetric quantification by image analysis in cases of MTH death and controls.

Discussion

The exact location of the neural control mechanisms controlling respiration vary from species to species [33]; in human[34], the tractus and nucleus solitaries extend throughout the length of the medulla(Figure 4)[25]. The tract is composed of general visceral afferents from the vagus and glossopharyngeal nerves. The nucleus and its central connection with the reticular formation subserve the reflex control of cardiovascular, respiratory and cardiac function [26]; the rostral portion of the nucleus solitarius (the gustatory nucleus) receives taste fibres from cranial nerves VII, IX and X. These same cranial nerves send general visceral sensation to the caudal portion of the nucleus solitaries (the cardiorespiratory nucleus). Still, there can be little doubt that the area of the RVLM certainly plays a central part in the autonomic control of breathing [35], even if other areas are involved as well. More recent studies illustrated that the NTS in some way integrates autonomic and voluntary respiratory efforts[36] and is also the first central site where cardiovascular reflexes regulating blood pressure and fluid balance are coordinated [37].

The NTS is the primary site of termination of afferent fibres arising from many cardiovascular receptors, including those in the aortic nerve, carotid sinuses and bodies, and is also a major site of termination for second-order neurons receiving inputs from many other visceral and somatic receptors [38]. Centrally, the NTS receives afferent inputs from many brain regions

including the cortex, hypothalamus, parabrachial complex nuclei of the pons and the area postrema. In turn, efferent outputs return to the same regions or various other ones. Projection from the NTS to the dorsal motor nucleus of the vagus (DMNV) are of particular interest since they innervate the preganglionic spinal neurons of the sympathetic system involved in blood pressure regulation [39].

Unlike other brainstem nuclei, which are relatively compact, collections of cells in the the solitary nucleus of humans is comprised of a loosely related groups of a neurons which loosely surround the tractus itself. When visualized three dimensionally, they can be seen running through the medulla, scattered around a bundle of myelinated nerve fibers of the tract itself (Figure 3). The ventral (V) subdivision is the nucleus of most interest as it extends to the subjacent reticular formation. The cells of this area are larger and sparsely packed.

The second messenger cyclic AMP (cAMP) controls cell proliferation, differentiation, and apoptosis. Down regulation of cAMP sensitizes tumor cells for anti-cancer treatment. Once triggered, opioid receptors can activate inhibitory Gi (?) proteins, which, in turn, block adenylyl cyclase activity reducing cAMP. When MTH receptors are activated in the presence of specific G-protein-coupled receptors, adenylyl-cyclases are inhibited and adenylcyclase is blocked. The result is that opioid receptor activation triggers downregulation of cAMP and improves effectiveness of anti-cancer drugs in treatment of glioblastoma and other tumors [22]. MTH also inhibits proliferation in leukemia cells and induces cell death by activated apoptosis pathways through the activation of caspase-9 and caspase-3, down-regulation of Bcl-x(L) X chromosome-linked inhibitor of apoptosis, and cleavage of poly(ADP-ribose) polymerase [40].

Limitations of Study: We realize that the number of patients studied was relatively small, however published studies allowed us a reasonable accurate way of locating the nucleus solitarius in humans. Histo-immunochemical studies arenormally used to identify and quantitate the amount of apoptosis, in add to our morphologic observationswere sufficientto make verify our microscopic observations; in particular, we took into consideration previous observation of studiesshowing the release of caspase -9 from mitochondria during neuronal apoptosis and ischemia in vivo, both in rat and canine models [41, 42, 43, 44], making caspase-9 himmunoistochemistry reaction the most preferable markers to study apoptotic cascade in neuronal tissue [45].Finally, we realize that apoptosis is not uniquely a result of MTH toxicity, but suggested that ongoing apoptosis in one nucleus, adjoining a nucleus with

no histological changes, must be of significance[46].We discuss the implications of these findings and directions that this may lead in future research.

Conclusions. The presence of apoptotic neurons within various areas of the RVLM of a MTH user does not, of course, prove that MTH was the cause of death, any more than the detection of necrotic cells can be considered as a cause of death. Nonetheless, we believe that the observation is of forensic value. Even in the face of high MTH concentrations, if neuronal apoptosis is not detected in the RVLM of a MTH user, and other possible causes of death are apparent, it may well be that MTH was not the cause of death but, rather, and incidental finding.

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