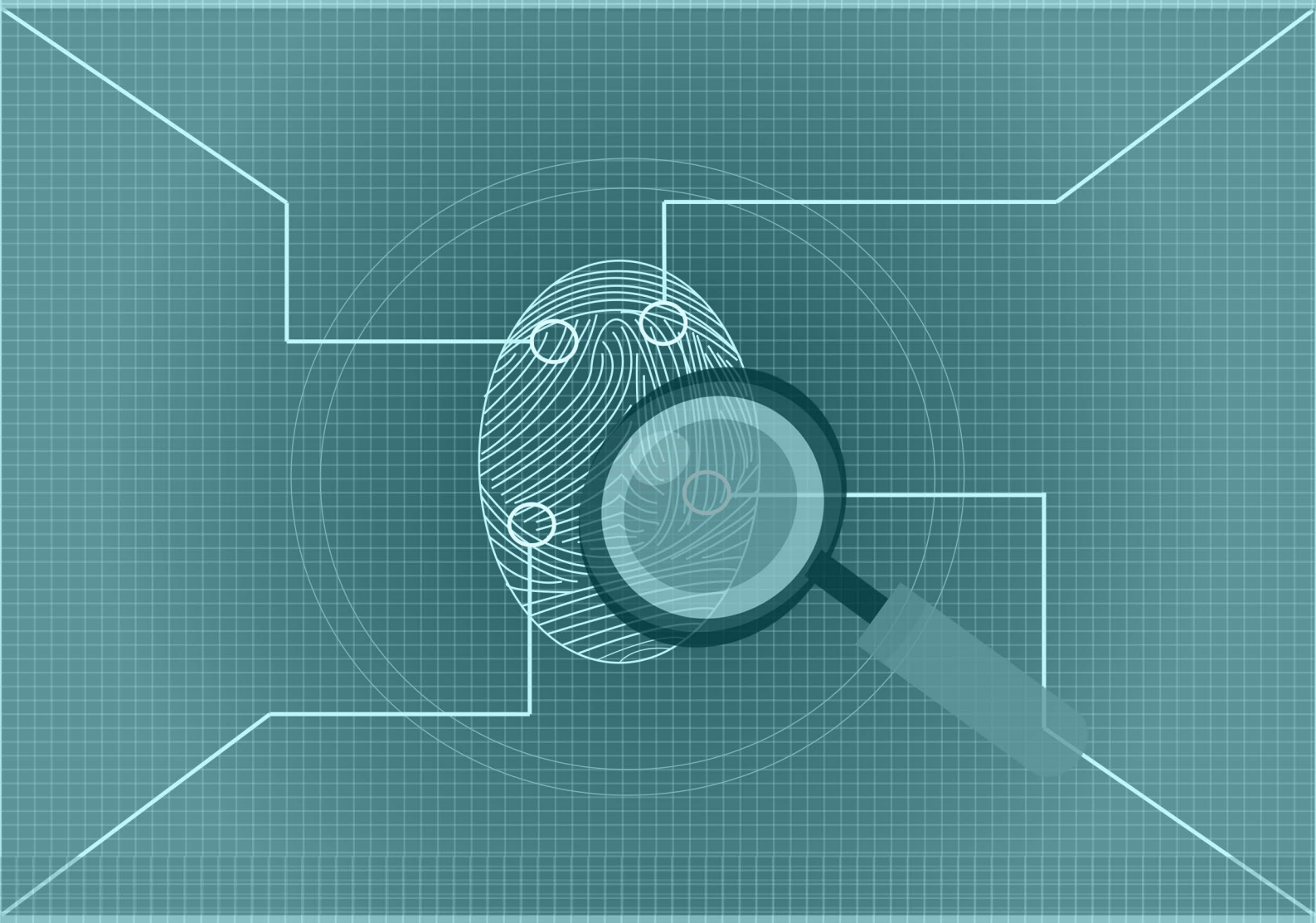
Openventio PUBLISHERS

ISSN 2474-8978

TOXICOLOGY AND FORENSIC MEDICINE

Open Journal

| December 2017 | Volume 2 | Issue 2 |



www.openventio.org

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978

Open Journal

CONTENTS

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-116

Rubric

*Corresponding author
Georg A. Petroianu, MD, PhD, FCP
Professor and Founding Chair
Department of Cellular Biology and Pharmacology
Herbert Wertheim College of Medicine
Florida International University
Miami, FL 33199, USA
E-mail: georg.petroianu@fiu.edu

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2116

Article History
Received: July 8th, 2017
Accepted: August 9th, 2017
Published: August 10th, 2017

Citation
Petroianu GA. A case of star fruit Diet. *Toxicol Forensic Med Open J*. 2017; 2(2): 37-38. doi: 10.17140/TFMOJ-2-116

A Case of Star Fruit Diet

Georg A. Petroianu, MD, PhD, FCP*

Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, FL 33199, USA

A 47-year-old obese woman is brought to the emergency room of the local hospital with generalized seizures, impaired consciousness and hiccupping; according to her relatives she never experienced such symptoms in the past. Her past medical history is non-contributory except for a moderate reduction in renal function. She regularly uses histamine receptor blockers (ranitidine) for acidity and nonsteroidal anti-inflammatory drugs (NSAIDs) (ibuprofen) for headaches as needed.

Her relatives report that despite numerous attempts at diet, exercise, and enrollment in a support group she never really managed to lose weight. However, she recently started a new aggressive diet based on almost exclusive consumption of organic star fruit based products (star fruit juice and dried or fresh star fruits).

Which of the foods/drinks listed below has the highest content of oxalic acid per serving?

1. Spinach
2. Black tea
3. Cashew nuts
4. Cucumbers
5. Soy beans

Which of the substances listed below is most likely responsible for the presenting symptoms in the case described?

1. Organophosphate pesticides
2. Endogenous benzodiazepines (endozepines)
3. Endogenous opioids (endorphins)
4. Caramboxin
5. Carbolines

Oxalic acid derived from food combines with divalent cations such as calcium to form oxalates which are then excreted in the urine. When oxalate concentrations are high and solubility limits are exceeded kidney stones can form and damage the kidney tubules. High oxalic acid foods are thus potentially nephrotoxic and should be avoided in patients with impaired kidney function. Spinach and rhubarb top the list of oxalate content but oxalate nephropathy has also been reported with many other foods such as cashew nuts,[1] iced-tea,[2] cucumber fruit (*Averrhoa bilimbi*)[3] and star fruit (*Averrhoa carambola*).[4] An aggressive diet based on almost exclusive consumption of star fruit based products (organic or not) has the potential to inflict kidney injury.

While oxalates can induce or exacerbate nephropathy they do not explain the neurotoxicity associated with star fruit (carambola) and the closely related cucumber fruit (bilimbi) ingestion.[5,6]

The mechanism of neurotoxicity was recently elucidated by Garcia-Cairasco et al[7], who identified in the star fruit (carambola) a phenylalanine-like molecule they named caramboxin. Caramboxin is excitotoxic and activates N-methyl-D-aspartic acid (NMDA) and alpha-

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-116

amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Metabolic inactivation and excretion of caramboxin is impaired in renal failure. The vast majority of patients experienced hiccups and seizures.

Endozepines and endorphins are not excitotoxic; high levels are likely to be associated with sedation.

β-Carboline alkaloids are widespread in plants and animals, and frequently act as endogenous ligand is γ-aminobutyric acid ($GABA_{A)}$ receptor inverse agonists and can therefore have convulsive and anxiogenic effects. Some β-carbolines may be formed naturally in the human body and possibly function as endogenous endozepine antagonists.

Star fruits are not known to contain β-carbolines; Syrian rue (Peganum harmala) is high in β-carboline content.[8] The plant's seeds have been used for centuries in the rites of many cultures.

The symptoms of organophosphate exposure are due to inhibition of the esterase responsible for acetylcholine metabolism: they correspond to over-activity of the parasympathetic component of the autonomic nervous system.

REFERENCES

1. Bernardino M, Parmar MS. Oxalate nephropathy from cashew nut intake. *CMAJ*. 2017; 189(10): E405-E408. doi: 10.1503/cmaj.151327

2. Syed F, Mena-Gutierrez A, Ghaffar U. A case of iced-tea nephropathy. *N Engl J Med*. 372: 1377-1378. doi: 10.1056/NEJMc1414481

3. Bakul G, Unni VN, Seethaleksmy NV, et al. Acute oxalate nephropathy due to 'Averrhoa bilimbi' fruit juice ingestion. *Indian J Nephrol*. 2013; 23(4): 297-300. doi: 10.4103/0971-4065.114481

4. Chen CL, Fang HC, Chou KJ, Wang JS, Chung HM. Acute oxalate nephropathy after ingestion of star fruit. *Am J Kidney Dis*. 2001; 37: 418-422. doi: 10.1053/ajkd.2001.21333

5. Neto MM, Robl F, Netto JC. Intoxication by star fruit (*Averrhoa carambola*) in six dialysis patients? *Nephrol Dial Transplant*. 1998; 13(3): 570-572. doi: 10.1093/ndt/13.3.570

6. Neto MM, da Costa JA, Garcia-Cairasco N, Netto JC, Nakagawa B, Dantas M. Intoxication by star fruit (*Averrhoa carambola*) in 32 uraemic patients: Treatment and outcome. *Nephrol Dial Transplant*. 2003; 18(1): 120-125. doi: 10.1093/ndt/18.1.120

7. Garcia-Cairasco N, Moyses-Neto M, Del Vecchio F, et al. Elucidating the neurotoxicity of the star fruit. *Angew Chem Int Ed Engl*. 2013; 52(49): 13067-13070. doi: 10.1002/anie.201305382

8. Herraiz T, González D, Ancín-Azpilicueta C, Arán VJ, Guillén H. β-Carboline alkaloids in Peganumharmala and inhibition of human monoamine oxidase (MAO). *Food Chem Toxicol*. 2010; 48(3): 839-845. doi: 10.1016/j.fct.2009.12.019

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-117

Letter to the Editor

Corresponding author
Soria Sanchez Mª. Luisa, PhD
Quality Manager
Seville Department
National Institute of Toxicology and Forensic Science
Avda. Doctor Fedriani, s/n 41015 Sevilla, Spain
Tel. 954389872
Fax: 954370262
E-mail: luisa.soria@justicia.es

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2117

Article History
Received: May 17th, 2017
Accepted: May 23rd, 2017
Published: May 25th, 2017

Citation
Soria ML. New psychoactive substances: Risks and challenges. *Toxicol Forensic Med Open J*. 2017; 2(2): 39-40. doi: 10.17140/TFMOJ-2-117

New Psychoactive Substances: Risks and Challenges

Soria Sanchez Mª. Luisa, PhD*

Seville Department, National Institute of Toxicology and Forensic Science, Avda. Doctor Fedriani, s/n 41015 Sevilla, Spain

New psychoactive substances (NPS) have emerged in the last few years in response to market trends and legislative control. Only in the EU, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported in its European Drug Report 2016 the monitoring of more than 560 novel substances, of which 380 (70%) were detected in the past 5 years.[1]

NPS are usually drawn as analogues or derivatives of controlled substances, easy to obtain, inexpensive, not detected by standard toxicological screens and produced to circumvent regulations and imitate the effects of controlled drugs. Synthetic cannabinoids are among the most commonly seized NPS in Europe responsible for causing about 8 to 10 seizures per year, followed by cathinones. NPS are mainly manufactured in China and India, and purchased online *via* "darknets" (anonymous networks) without any age restriction. Other than phenethylamine, piperazine, tryptamine or aminoindan derivatives, compounds are also included in chemical classes of NPS.

The consumption or usage of a substance which "demands control", is usually characterised by the following attributes: (a) the substance should be psychoactive (b) the usage or consumption of the substance should be associated with a motive of abuse or intoxication; and (c) the substance should possess certain characteristics which is capable of causing harm or threat to the human health. Traditional response to the discovery of a new 'drug' at a time when the discovery of such chemical compounds was a relatively rare event; necessitated the evaluation and assessment of the risks it poses to public health and accordingly include them in the national list of controlled substances. The current situation, marked by the discovery of many new substances and very limited evidence of related health risks, potentially stretches the credibility of the control systems.

Helander and Bäckberg[2] indicated that when launched, most NPS have not been tested on humans; increasing the risks of causing harmful and adverse conditions on the human health. Another potential threat to human health is due to the substances that are typically produced in the clandestine laboratories. The consequences of poor quality control in the production of these substances are attributed to unknown chemical structure, dose, and the presence of contaminants. Hence, dealing with medical complications associated with NPS use and overdose has become a growing problem at the emergency departments and intensive care units worldwide.

Previously, Baumann and Volkow[3] said that, although NPS can elicit subjective effects that resemble their progenitors, potential off-target sites of action are unknown and adverse medical consequences are common. Also, many of the case reports of intoxication are due to a combination of drugs and the lack of information concerning tolerance, routes of administration, dosage and sudden drug withdrawal syndrome meaning that reliable and accurate interpretation of NPS concentrations is not possible. A study published in the Australian Journal of Forensic Sciences in 2016 concerning the prevalence of new psychoactive substances in the Victorian fatally-injured drivers, reported that consumption of NPS can lead to a variety of psychological and physiological effects and these effects may last for a few to several hours

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-117

post dosage.[4] Repeated use of NPS can also lead to a more intense response and a longer duration of effect. The commonly reported responses towards synthetic cathinones include aggression, anxiety, euphoria, empathy, enhancement of mood and hallucinations. Synthetic cannabinoids have been shown to result in a dreamy state, confusion, depression, paranoia and psychosis. Different studies have indicated that, though the degree of impairment of driving skills caused due to NPS have not been reported, the impairment of cognitive and psychomotor functions due to NPS can affect motor skills required to keep a vehicle safe and on track on the roads, therefore possibly being considered as a public health risk.

In comparison to the classical controlled drugs, there are a lesser number of fatal cases reported and a limited amount of data available relevant in terms of examining the metabolism and toxicity caused due to NPS. However, there have been fatal cases of NPS reported continuously in USA, Europe, Japan and other countries.

The novel substances are typically not detectable with the usual drug of abuse immunoassays. It is therefore possible that they contribute towards acute toxicities and medical complications, or even deaths, by escaping detection. A major challenge is the lack of analytical research information available on these substances and the lack of reference standards since they are new to the market and consequently have not yet been characterized.

As reported by Lobo et al[5] sometimes the combination of different analytical techniques as nuclear magnetic resonance (NMR) spectroscopy, gas-chromatography–mass-spectrometry (GC-MS), fourier transform infrared spectroscopy (FTIR), high-resolution tandem mass-spectrometry (HR-MS/MS) and chemoinformatic tools, is necessary in order to confirm the true identity of these substances.

In relation to the interpretation of NPS concentration in biological fluids, from a forensic point of view and according to Gerostamoulos et al[6] the major concern presently would be to establish minimum concentrations for the screening of NPS, to determine if the detection of the parent drug is adequate or whether metabolite screening is required. Overall, the qualitative detection of these drugs would enable the identification of NPS in casework much more readily than is possible under the existing situations, thereby improving the availability of prevalent related information. Unless the knowledge of the toxicity of these substances improves significantly using pharmacological studies, the toxicologists should be careful when determining the concentration of an NPS regardless of the matrix in which it is measured.

REFERENCES

1. European Drug Report 2016: Trends and Developments. (EMCDDA). 2016.

2. Helander A, Bäckberg M. New psychoactive substances (NPS) – the Hydra monster of recreational drugs. *Clin Toxicol (Phila)*. 2017; 55:1: 1-3. doi: 10.1080/15563650.2016.1217003

3. Baumann MH, Volkow ND. Neuropsychopharmacology. 2016; 41: 663-665. doi: 10.1038/npp.2015.260

4. Yapa S, Drummer OH. Prevalence of new psychoactive substances in Victorian fatally-injured drivers. *Aust J Forensic Sci*. 2016; 48(2): 230-243. doi: 10.1080/00450618.2015.1050066

5. Lobo J, Chassaigne H, Holland M, et al. Systematic analytical characterization of new psychoactive substances: A case study. *Forensic Sci Int*. 2016; 265: 107-115. doi: 10.1016/j.forsciint.2016.01.024

6. Gerostamoulos D, Elliott S, Walls HC. To measure or not to measure? That is the NPS question. *J Anal Toxicol*. 2016; 40: 318-320. doi: 10.1093/jat/bkw013

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-118

Retrospective Study

Corresponding author
Ismail M. Sebetan, MD, PhD
Forensic Sciences Program
National University
11255 North Torrey Pines Road
La Jolla, CA 92037, USA
Tel. (858) 642 8419
E-mail: isebetan@nu.edu

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2118

Article History
Received: May 9th, 2017
Accepted: June 6th, 2017
Published: June 7th, 2017

Citation
Admani S, Burritt B, Sebetan IM. The effect of dna evidence on incidence of property crime arrests. *Toxicol Forensic Med Open J*. 2017; 2(2): 41-45. doi: 10.17140/TFMOJ-2-118

The Effect of DNA Evidence on Incidence of Property Crime Arrests

Shabana Admani, MFS[1,2]; Brian Burritt, BS[2]; Ismail M. Sebetan, MD, PhD[1*]

[1]Forensic Sciences Program, National University, La Jolla, California 92037, USA
[2]Forensic Biology Unit, San Diego Police Department, San Diego, California, 92101, USA

ABSTRACT

Background: With a high volume of incoming cases and ever-present backlogs, forensic laboratories are often limited with the funding and resources necessary to test every evidence item waiting to be processed.[1] Properly allocating resources can be a persistent challenge.
Aim: The purpose of this study was to determine which types of physical evidence collected from property crime scenes, upon the completion of deoxyribonucleic acid (DNA) analysis, would most likely lead to cold hits in the Combined DNA Index System database (CODIS) and ultimately result in arrests. The goal here is to provide law enforcement agencies and forensic laboratories with useful information that could improve evidence processing methods and allow them to better utilize limited resources.
Materials and Methods: All of San Diego Police Department's DNA cases from property crimes during a one-year period were reviewed to determine which evidence types yielded the highest number of arrests. Bloodstains, drinking containers, cigarette butts, clothing, rocks, and various types of tools were the most common types of evidence submitted for DNA testing in property crime investigations.
Results: Bloodstains resulted in the highest amount of cold hits (73%) and yielded nearly 250% more arrests than non-blood evidence.
Conclusion: When allocating resources in forensic laboratories, property crime cases with blood evidence should be given the highest priority. Further prioritization based on evidence type, however, is not definitively supported by the results of this study.

KEY WORDS: Deoxyribonucleic acid (DNA); combined DNA index system (CODIS); Cold hit; Arrest; Evidence; Backlog; Property crime.

INTRODUCTION

Allocation of resources in most crime laboratories is a constant struggle. Ever-present backlogs, partly due to an increase in the use of forensic deoxyribonucleic acid (DNA) testing amongst law enforcement investigators, cause regular evaluations of the best uses of the limited resources available.[2,3] The classic model of allocation of resources in the modern forensic DNA laboratory is to provide for the investigation of all homicide and sexual assault cases and then for as many lesser crime cases, such as robberies and burglaries, as possible.[4] For simplicity, these lesser crimes will be referred to as property crimes throughout the study. As the number of property crime cases usually exceeds the number that the crime laboratory can process, an effective method of prioritizing cases would be of significant value.[5] This study attempts to measure the value of testing different types of evidence commonly found during property crime investigations. Common items collected at property crime scenes include bloodstains, drinking containers, cigarette butts, clothing, rocks, and various types of tools. There is limited data currently available in the literature specifically focusing on property crimes to determine which type of evidence yields the best results in terms of obtaining usable DNA samples, identifying potential suspects, and leading to arrests.

TOXICOLOGY AND FORENSIC MEDICINE
Open Journal
Openventio PUBLISHERS
ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-118

MATERIALS AND METHODS

All of San Diego Police Department's DNA cases from the year 2010 were reviewed. Cases from which data was collected focused specifically on suspect-less property crimes with single evidence items. Crime and evidence type were documented for each case, and whether each item resulted in usable DNA results, a mixture of DNA profiles, a cold hit in the Combined DNA Index System database (CODIS), and an arrest of the subject of the cold hit. Focusing on single-item cases was done to remove as many variables as possible, as it was more difficult to judge the value of an individual evidence item when multiple items were tested. While useable results, mixtures of DNA profiles, and cold hits were easy to assess from the available laboratory reports, accurate arrest data was dependent on the appropriate recording of information by officers in the San Diego Police Department records system.

RESULTS

Of the 430 total assessed cases, 330 involved evidence items that were included in the study criteria (Table 1). The remaining 100 cases consisted of a variety of additional evidence items (e.g., steering wheels, cell phones, hair samples) which were not included in the study analysis due to small sample size.

Of all the items included in the analysis, bloodstains (n=63) gave the highest incidence of usable results (97%) (Figure 1), cold hits (73%) (Figure 2), and arrests (51%) (Figure 3), while having the lowest number of mixed profiles (1.6%) (Figure 4). These findings are in agreement with previous studies which concluded that blood evidence yields more successful DNA results than any other biological evidence or items that have been handled or touched.[6] Tools (n=89), on the other hand, were the most numerous type of evidence tested, yet gave the lowest arrest rate (11%) (Figure 3).

DISCUSSION

Every case is different, and there are many factors that determine the value of a DNA cold hit in a given case. One factor that is of particular interest to the laboratory is the type of evidence from

Table 1: Data Summary.

Data Summary by Tier and Evidence Type						
Tier	**Types of Evidence**	**No. of Samples**	**No. of Usable Results**	**No. of Mixed Profiles**	**No. of Cold Hits**	**No. of Arrests**
1	Bloodstains	63	61 (97%)	1 (1.6%)	46 (73%)	32 (51%)
	Tier 1 Totals	63	61 (97%)	1 (1.6%)	46 (73%)	32 (51%)
2	Drinking Containers	36	30 (83%)	9 (25%)	20 (56%)	8 (22%)
	Cigarette Butts	25	20 (80%)	5 (20%)	9 (36%)	4 (16%)
	Tier 2 Totals	61	50 (82%)	14 (23%)	29 (48%)	12 (20%)
3	Clothing	82	65 (79%)	53 (65%)	50 (61%)	24 (29%)
	Rocks	35	16 (46%)	18 (51%)	10 (29%)	5 (14%)
	Tools	89	38 (43%)	44 (50%)	23 (26%)	10 (11%)
	Tier 3 Totals	206	119 (58%)	115 (56%)	83 (40%)	39 (19%)

Figure 1: Incidence of Usable Results by Evidence Type.

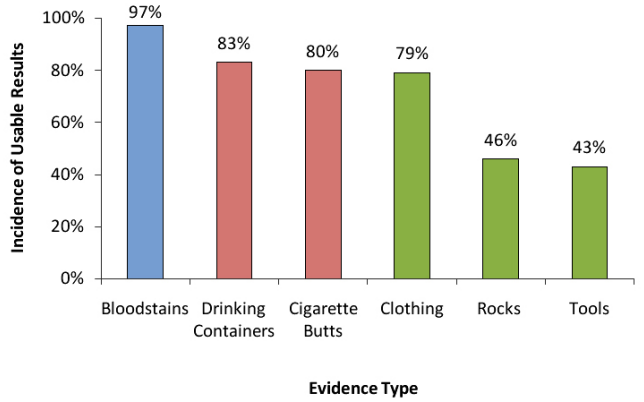
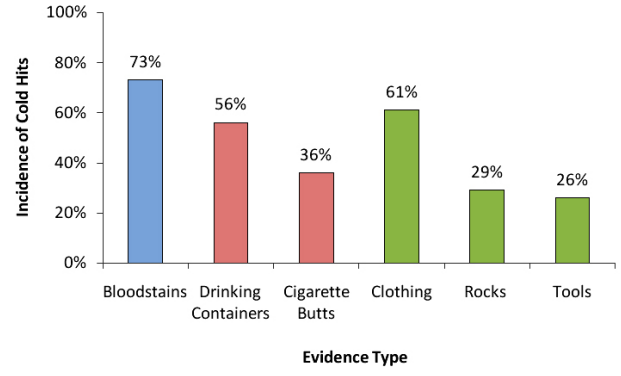


Figure 2: Incidence of Cold Hits by Evidence Type.



ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-118

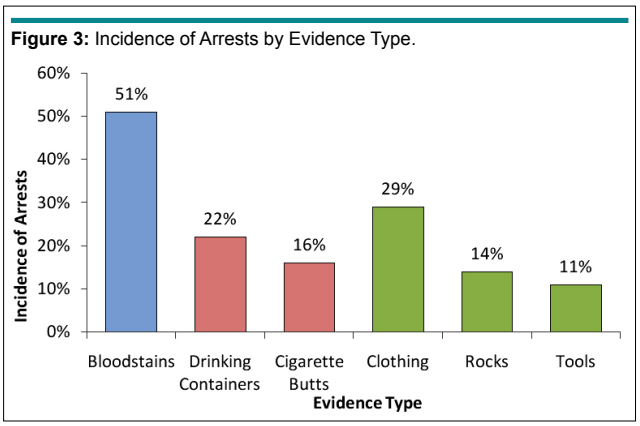


Figure 3: Incidence of Arrests by Evidence Type.

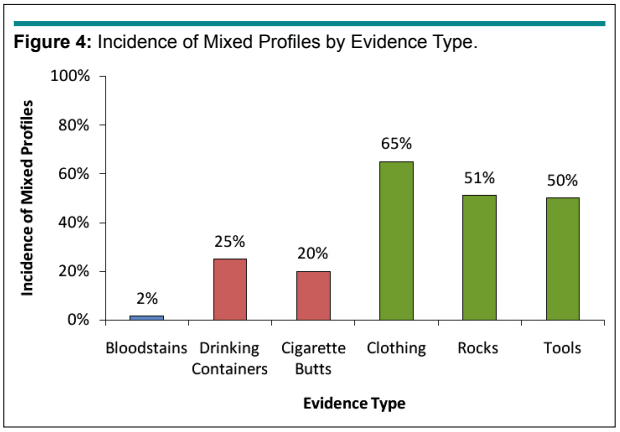


Figure 4: Incidence of Mixed Profiles by Evidence Type.

which the DNA was collected. While there are other important factors, evidence type is one of the easiest factors by which laboratory managers can base some of their decisions.

Although there is also a large variation in the types of evidence that can be submitted to the laboratory for DNA testing, the bulk of the items tested fall into a handful of types, which can be further grouped into three main categories, or tiers. We have proposed a three-tier model that will be used as a conceptual tool for categorizing and prioritizing evidence. These tiers consist of evidence that tends to link someone to a crime (Tier 1), evidence that tends to link someone to a crime scene (Tier 2), and evidence that tends to link someone to an item (Tier 3) (Figure 5).

These descriptions are simplifications; alternate explanations for the presence of someone's DNA on an item or at a crime scene are limited only by one's imagination. To facilitate a reasonable discussion, it will be assumed that evidence was not planted at the crime scene nor the result of contamination, and that items such as a cigarette butts are not normally moved after being used and discarded. In court, the implication of finding someone's DNA at a crime scene or on an item is often a major source of contention. Does the presence of someone's DNA on an item truly link them to the crime, or is there an innocent explanation for the presence of their DNA on an item or at a crime scene? The generalizations made in this discussion do not take away from the natural debate or disagreement that can occur on this matter.

DNA testing of evidence from homicide and sexual assault cases is typically given the highest priority, and often easily fits within the laboratories' resources.[4] Generally, forensic laboratories process all requested homicide and sexual assault evidence, and as much property crime evidence as they can. The testing of DNA evidence from these high volume property crimes often causes laboratory management to find itself with more potential work than can be completed, and thus will have the need to make informed decisions concerning the optimum utilization of resources. As such, the following discussion will focus primarily on evidence that is most often tested in property crime investigations.

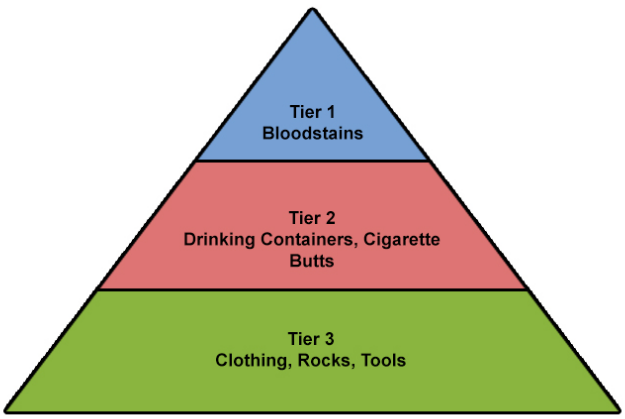


Figure 5: Proposed 3-Tier Model.

Tier 1: Evidence that links a person to a crime

Tier 2: Evidence that links a person to a crime scene

Tier 3: Evidence that links a person to an object

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-118

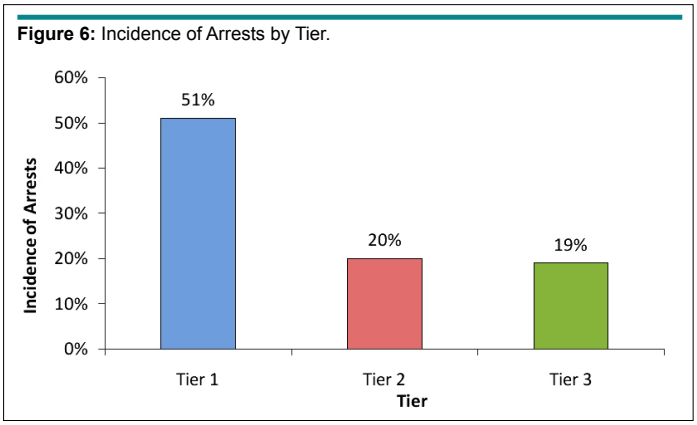


Figure 6: Incidence of Arrests by Tier.

Tier 1 evidence is that which tends to link someone to an actual crime and is naturally the most valuable evidence to examine during a criminal investigation. This type of evidence however, usually makes up a fairly small portion of the DNA testing that is performed in the crime laboratory. At property crime scenes, the most common situation of finding Tier 1 evidence is when a bloodstain is left at a burglary scene where a window was broken.[4] A bloodstain from a burglary is very likely to be from the perpetrator and will presumably yield a large amount of DNA from a single person. Evidence of this type is sometimes referred to as *prima facie* evidence, in that the evidence that links the person to a crime is so strong that it becomes the burden of the person considered as the source of the DNA to provide a non-criminal reason for the presence of their DNA at the crime scene.

Tier 2 evidence is that which tends to link someone to a location. While this evidence may link a person directly to a crime scene, it does not necessarily link that person to the crime. Common examples of this type of Tier 2 evidence are cigarette butts and drinking containers. These types of items are generally used once, discarded after use, and in most cases not immediately moved from where they were discarded. Biological samples found on cigarette butts and drinking containers are usually saliva, which is a strong source of DNA. Because these items are typically only used by one or two people, they tend to provide robust single-source results or simple mixtures.

Tier 3 evidence is that which tends to link a person to an item. This item may have been found at a crime scene, but it does not necessarily mean that the person was ever at that location. It simply means that the person likely made contact with that item at some point in time. Common examples of this type of evidence are clothing and tools. Based on their nature, these items can prove to be more challenging to analyze than Tier 1 and 2 items. Clothing and tools are items that are designed to be used repeatedly over a long period of time. They are often used by multiple people, which can lead to complex mixtures of varying levels of DNA.

Tier 2 and 3 evidence does not prove guilt; this type of evidence merely provides an investigative lead. It is logical to believe that sometimes people who are the subject of a DNA cold hit to this type of evidence are not the perpetrator of the crime being investigated. An investigator typically would compare information about the subject of the cold hit to any available information about the perpetrator of the crime. In the event that a detective continues to suspect a person's potential involvement in a crime, further investigation may lead to the discovery of additional evidence implicating the person. Common examples of corroborating evidence include witness identification, video recordings, and the finding of stolen property. Linking a person to less than ideal evidence found at numerous crime scenes can also be an effective method to implicate a person in a series of crimes.

The descriptions of the various types of evidence and their relative values can help explain why the arrest rate for cold hits to bloodstains found at property crime scenes is over twice that of other types of evidence (Figure 3). These descriptions and the observed arrest rates (Figure 6) support giving Tier 1 evidence higher priority than Tier 2 or 3 evidence by a crime laboratory or law enforcement agency experiencing a limitation of resources.

CONCLUSION

The primary goal of this study was to determine which types of DNA evidence from property crimes would most likely lead to arrests. The arrest rate for cases with blood evidence were approximately 250% greater than for those with non-blood evidence (Figure 6). These results suggest that evidence that tends to link a person to a crime (Tier 1), such as a bloodstain left at a burglary scene, should be given the highest priority in criminal investigations. Although, it could be argued that evidence that links a person to a location (Tier 2) would be more valuable than evidence that merely links a person to an item (Tier 3), both categories of evidence resulted in nearly identical rates of arrest (Figure 6). While individual evidence types gave differing arrest rates, the limited amount of data present suggests treating this information with an appropriate amount of caution. Our findings suggest that while bloodstains from property crimes should be given the highest priority, further prioritization based on evidence type is not definitively supported by the results of

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-118

this study. Considering other factors such as the intensity of violence and the severity of the crime could potentially be more effective in maximizing the public safety impact of testing DNA evidence.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Spagnoli L. *Beyond CODIS: The changing face of forensic DNA analysis*. Law Enforcement Technology. Website. http://www.officer.com/aricle/10249651/beyond-codis.2007. Accessed March 25, 2017.

2. Makin MA. *DNA and Property Crime Scene Investigation: Forensic Evidence and Law Enforcement.* Waltham, MA, USA: Anderson Publishing; 2014.

3. National Institute of Justice. *DNA evidence:* What law enforcement officers should know? *National Institute of Justice Journal*. 2003; 249: 10-15. Web site. http://www.policemag.com/channel/patrol/articles/2011/02/dna-evidence-what-patrol-officers-need-to-know/page/2.aspx. Accessed May 8, 2017.

4. National Institute of Justice. *DNA in "Minor" Crimes Yields Major Benefits in Public Safety.* Washington, DC, USA: U.S. Department of Justice; 2004.

5. Gabriel M, Boland C, Holt C. Beyond the cold hit: Measuring the impact of the national DNA data bank on public safety at the city and county level. *Journal of Law, Medicine & Ethics* 2010; 38(2): 396-411. doi: 10.1111/j.1748-720X.2010.00498.x

6. Roman JK, Reid S, Reid J, Chalfin A, Adams W, Knight C. *The DNA Field Experiment: Cost-Effectiveness Analysis of the use of DNA in the Investigation of High-Volume Crime*. Washington, DC, USA: U.S. Department of Justice; 2008.

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-119

Brief Research Report

Corresponding author
Héctor Rangel-Villalobos, PhD
Instituto de Investigación en Genética Molecular, Universidad de Guadalajara
Centro Universitario de la Ciénega (CUCiénega-UdeG)
Av. Universidad #1115, Col. Paso Blanco, CP 47810, Ocotlán
Jalisco, México
Tel. 52+ (392) 92 59400, ext. 8363
Fax: +52 (392) 92 57112
E-mail: hrangel13@hotmail.com

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2119

Article History
Received: May 4th, 2017
Accepted: June 12th, 2017
Published: June 14th, 2017

Citation
Locia-Aguilar GJ, López-Saucedo B, Deheza-Bautista S, Salado-Beltrán OV, Martínez-Sevilla VM, Rangel-Villalobos H. Statistical parameters of forensic importance for 15 autosomal STRs in Mestizo population from the State of Guerrero (South Mexico). *Toxicol Forensic Med Open J.* 2017; 2(2): 46-50. doi: 10.17140/TFMOJ-2-119

Statistical Parameters of Forensic Importance for 15 Autosomal STRs in Mestizo Population from the State of Guerrero (South Mexico)

Gorge Locia-Aguilar, MSc[1]; Briselda López-Saucedo, QFB[1]; Susana Deheza-Bautista, QFB[1]; Oscar Venturita Salado-Beltrán, QFB[1]; Victor Manuel Martínez-Sevilla, MSc[2]; Héctor Rangel-Villalobos, PhD[2*]

[1]Dirección General de Servicios Periciales de la Fiscalía, General del Estado de Guerrero (FGE-G), México

[2]Instituto de Investigación en Genética Molecular, Universidad de Guadalajara (CUCiénega-UdeG), Ocotlán, Jalisco, México

ABSTRACT

Background: Human identification requires the maintenance of population databases of short tandem repeat (STR) loci that allow for their correct interpretation during paternity testing and forensic cases.
Material and Methods: We analyzed 15 STR loci with the AmpF*l*STR® Identifiler kit in a Mestizo (admixed) population sample from the state of Guerrero (South Mexico).
Results: We estimated the allele distribution and different forensic parameters in this population. Genotype distribution was in agreement with the Hardy-Weinberg expectations for all 15 STRs. Similarly, linkage disequilibrium test demonstrated no association between the pair of loci. The power of exclusion and power of discrimination values were 99.9994% and >99.99999%, respectively. Interpopulation analysis suggested that the Mestizo populations from the Central and the Southern regions conform one population cluster, separated from the Southeastern and Northwestern regions.
Conclusions: We describe the genetic structure of Mexican Mestizos based on 15 STRs reporting for the first time a population representing the Southern region.

KEY WORDS: Short tandem repeats (STRs); Mestizos; Guerrero; Mexico; Population; Structure.

ABBREVIATIONS: STRs: Short Tandem Repeats; CODIS: Combined DNA Index System; HWE: Hardy-Weinberg Equilibrium; MDS: Multidimensional scaling.

INTRODUCTION

In Mexico, most of the population (>90%) is the consequence of an admixture on account of European contact principally with the Spaniards, Amerindians, and African slaves. This part of the population is commonly referred to as the Mestizos, who speak Spanish and live in both urban and rural regions throughout the country. Many studies have been conducted in Mexican-Mestizos to validate the application of autosomal short tandem repeats (STRs) for human identification purposes.[1,2] However, in Mexico, this forensic validation requires the study of different populations, given the genetic structure demonstrated among Mestizos from different regions.[3] Based on the 13 STRs of the Combined DNA Index System (CODIS), it has been claimed that the European ancestry increases gradually towards the Northwestern region and diminishes towards the Central and Southeastern regions; conversely, the Amerindian ancestry displays an opposite pattern increasing towards the Central and Southeastern region, and decreasing towards the Northwestern region.[1,2] Although, several Mexican-Mestizo populations have been studied, some geographical areas have been poorly analyzed for STR

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-119

markers from a forensic perspective; particularly the Southern region characterized by the presence of numerous Mexican indigenous communities, which could potentially increase the Amerindian ancestry of the neighboring southern Mestizo populations.[4] Therefore, the two defined objectives of this study were:

1. To report the statistical parameters of forensic efficiency for 15 STRs of the Identifiler® kit in the state of Guerrero, a representative of the southern Mestizo population;

2. To compare the collected data with the populations from the main geographical regions of Mexico.

MATERIALS AND METHODS

DNA Samples

We analyzed the DNA samples of 251 unrelated Mexican-Mestizo individuals from the following regions of the southern state of Guerrero: Costa Chica (n=14), Acapulco (n=80), Centro (n=53), Montaña (n=23), Norte (n=56), and Tierra Caliente (n=25) (Figure 1). All individuals participating in the study, signed a written informed consent according to the ethical guidelines of the Helsinki Declaration.

STR Genotyping

DNA was extracted from a dried blood specimen spotted on the FTA paper or buccal swabs using Chelex® 100 or the standard phenol-chloroform method. Genotypes for 15 STRs were obtained with the AmpF*l*STR Identifiler® kit followed by capillary electrophoresis according to the supplier´s instructions using the ABI-Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Allele ladder provided in the kit and the software Genemapper® 3.2 were used for allele calling.

Quality Control

Positive and negative controls were used as specified in the Identifiler® kit user's manual. The genotype data was analyzed and verified independently by two different statistical methods.

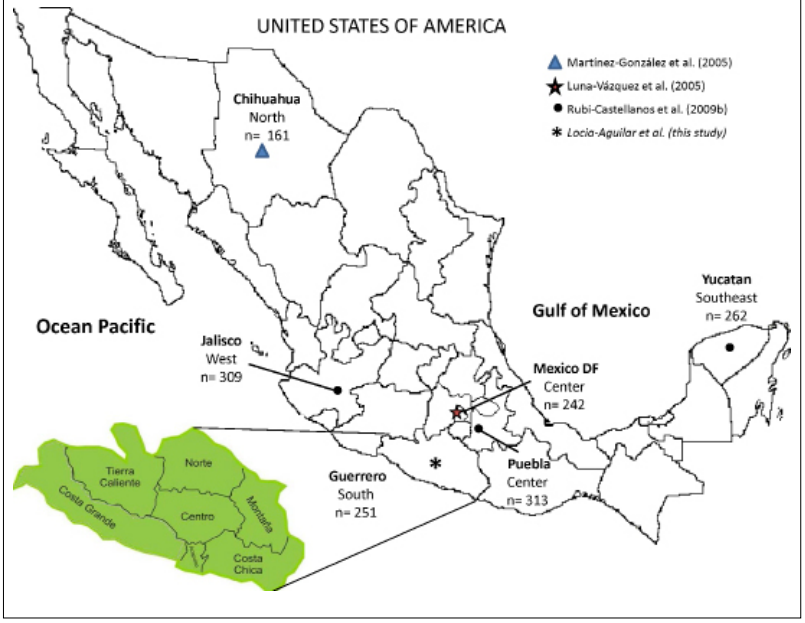
Data Analysis

Allele frequencies and statistical parameters of forensic efficiency were estimated with the spreadsheet PowerStats.[5] Hardy-Weinberg equilibrium (HWE) for each and the combined loci were computed by performing the exact tests using the software Genetic Data Analysis (GDA) 1.1.[6] Population differentiation was evaluated by performing pairwise comparisons (exact test *p*-value) and normalized F_{ST} genetic distances represented in a Multidimensional scaling (MDS) plot using the software Arlequin 3.0[7] and Statistical Package for the Social Sciences (SPSS) version 20.0, respectively. For this purpose, four previously reported Mexican-Mestizo populations from different geographic regions were included in the interpopulational analysis: Chihuahua (North),[8] Jalisco (West),[9] Valley of Mexico,[10] and state of Puebla (Center),[9] and Yucatan (Southeast)[9] (Figure 1).

RESULTS AND DISCUSSIONS

Allele frequencies and statistical parameters of forensic efficiency of the 15 STRs for the Identifiler® kit have been summarized in Table 1. In addition, complete genotype STR dataset is available as electronic supplementary material in Supplementary Table 1. This information will be helpful to the Mexican forensic

Figure 1: Geographical Location, Sample Size (n) and Reference of the Mexican-Mestizo Populations Used for Interpopulation Analysis in this Study.



ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-119

Table 1. Allele Frequency Distribution and Statistical Parameters of Forensic Efficiency of 15 STRs (Identifiler kit) in 251 Mexican-Mestizos from the State of Guerrero (South, Mexico).

Allele STR	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	THO1	D13S317	D16S539	D2S1338	D19S433	VWA	TPOX	D18S51	D5S818	FGA
2				0.0020								0.0020			
4						0.0020									
6				0.0020		0.3406						0.0020			
7			0.0060	0.0040		0.3845								0.0797	
8			0.0697	0.0080		0.0378	0.0600	0.0060				0.4741		0.0080	
9			0.0299	0.0259		0.0598	0.2520	0.1195				0.0359		0.0618	
9.3						0.1713									
10	0.0996		0.2550	0.2390		0.0040	0.1240	0.1932		0.0020		0.0378	0.0020	0.0538	
11	0.0518		0.3446	0.2610			0.1560	0.2928				0.2769	0.0120	0.4801	
12	0.1315		0.2351	0.3884			0.2120	0.3028		0.0378		0.1614	0.0777	0.2371	
12.2										0.0139					
13	0.3805		0.0478	0.0637	0.0080		0.1140	0.0777		0.1793	0.0020	0.0100	0.1633	0.0737	
13.2										0.1255					
14	0.2311		0.0120	0.0040	0.0498		0.0820	0.0080		0.2530	0.0598		0.1693	0.0060	
14.2										0.0717					
15	0.0837			0.0020	0.5060					0.1096	0.0837		0.1394		
15.2										0.1295					
16	0.0159				0.2649				0.0179	0.0458	0.3805		0.0976		
16.2										0.0239					
17	0.0040				0.1016				0.1295	0.0020	0.2590		0.1773		
17.2										0.0020					
18	0.0020				0.0657				0.0598	0.0040	0.1494		0.0717		0.0060
18.2															0.0020
19					0.0020				0.2331		0.0458		0.0359		0.0797
20		0.0020			0.0020				0.1773		0.0179		0.0239		0.0378
21									0.0159		0.0020		0.0139		0.1215
22									0.0618				0.0080		0.0936
23									0.2151				0.0040		0.1414
23.2															0.0020
24									0.0657				0.0040		0.1514
24.2		0.0020													
25									0.0239						0.1972
25.2		0.0020													
26															0.1056
27		0.0100													0.0458
28		0.0876													0.0120
29		0.1912													0.0020
30		0.2629													
30.2		0.0100													
31		0.1096													
31.2		0.1155													
32		0.0199													
32.2		0.1215													
33.2		0.0578													
34.2		0.0040													
35.2		0.0020													
36		0.0020													
43.2															0.0020
MAF	0.0114	0.0120	0.0112	0.0114	0.0106	0.0107	0.0126	0.0116	0.0127	0.0119	0.0111	0.0108	0.0123	0.0108	0.0124
PD	0.9113	0.9537	0.8994	0.8615	0.8389	0.8540	0.9460	0.9021	0.9500	0.9582	0.9039	0.8344	0.9687	0.8572	0.9687
PE	0.5218	0.6380	0.4681	0.5218	0.3490	0.3654	0.7306	0.5498	0.7557	0.6229	0.4553	0.3882	0.6921	0.3999	0.7079
Het	0.7570	0.8207	0.7251	0.7570	0.6454	0.6574	0.8680	0.7729	0.8805	0.8127	0.7171	0.6733	0.8486	0.6813	0.8566

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-119

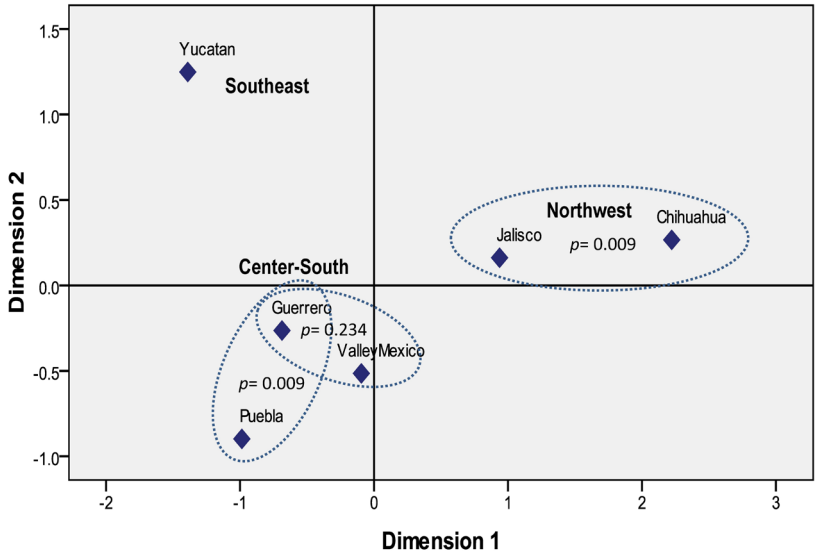
PIC	0.7336	0.8244	0.7130	0.6708	0.6093	0.6490	0.8062	0.7269	0.8186	0.8335	0.7178	0.6177	0.8571	0.6579	0.8605
TPI	2.0574	2.7889	1.8188	2.0574	1.4101	1.4593	3.7879	2.2018	4.1833	2.6702	1.7676	1.5305	3.3026	1.5688	3.4861
HWE	0.2509	0.0041	0.3088	0.4884	0.5088	0.0131	0.2281	0.7966	0.4156	0.6506	0.4413	0.0369	0.8978	0.1591	0.2472

MAF: Minimum allele frequency; HWE: *p*-value of exact tests for Hardy-Weinberg equilibrium expectations; PD: Power of Discrimination; PE. Power of Exclusion; Het. Heterozygosity; PIC. Polymorphism Informativity Content; TPI: Typical Paternity Index.

geneticists to evaluate paternity and forensic cases solved in the Guerrero state. According to these forensic parameters, the most informative loci were D18S51, D2S1338 and FGA; conversely, the STRs with the lowest forensic informativity were TPOX and D3S1358. The combined power of exclusion for the STR genetic system was 99.999634444%, whereas the combined power of discrimination was greater than >99.99999999%, confirming the potential of this kit for human identification purposes. Although, HWE test showed a low *p*-value for D21S11 (p=0.041), the application of the Bonferroni correction (p<0.0033) suggested that the genotype distribution was in agreement with the Hardy-Weinberg expectations for all the 15 STR loci in the population of Guerrero. Similarly, the exact linkage equilibrium was established between all pairs of loci (p>0.0038) (Data not shown). In brief, these results suggested that the binomial square and product rule can be confidently used in forensic caseworks to estimate DNA profiles obtained with the Identifiler® kit in the population of Guerrero.

The knowledge of the genetic relationships among populations is important because it guides the work of forensic geneticists to analyze alternative STR population data during the statistical interpretation, given that most of the Mexican-Mestizo populations do not have their own STR database. Until now, this interpopulation analyses of Mestizo populations including the main geographic regions of the Mexican territory have been carried out only with the 13 CODIS-STRs.[1,2] Therefore, we represented the genetic distances among six Mexican-Mestizo populations in an multidimensional scaling (MDS) plot with the complete set of 15 STRs constituting the Identifiler kit (Figure 2). Although, recently, three Mexican Native populations from Guerrero were analyzed with these 15 STRs,[11] the complete genotype dataset was not available and thus, could not be included in this report. The close similarity observed between the southern state of Guerrero and the central populations is partially in agreement with the geographical parameters. However, the greater extent of similarity between the population residing in the Valley of Mexico with Guerrero (p=0.2342) than with the nearby population of Puebla (p=0.0000), disregards the idea that geography could possibly explain such genetic associations (Table 2). This observation is in agreement with the first global study conducted on Mexican-Mestizos based on CODIS-STRs that discarded the Isolation by Distance (IBD) hypothesis[1] by means of the autocorrelation indices for DNA analysis (AIDA). These contradictory results suggest that Mexican population relationships have been explained by factors such as socioeconomic conditions and educational status associated with the higher European ancestry.[12] Similarly, the IBD hypothesis has not been followed for the study of Native American populations from Mexico when geographic distances are lesser than 1500 km,[13] which is in agreement with our results. A different quadrant of the MDS plot represents the populations of Chihuahua (North) and Jalisco (West), whereas the Yucatan population is isolated in a third different section of the plot (Figure 2). Al-

Figure 2: MDS Plot Representing the Genetic Relationships Among Six Mexican-Mestizo Populations Based upon the Powerplex 16 System.



MDS plot that represents F_{ST} distances between six Mexican-Mestizo populations from different regions of the country. Dotted ovals indicate population clusters based on Fst *p*-values.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-119

Table 2: Pairwise FST *p*-values (above diagonal) and FST Genetic Distances (below diagonal) between Five Mexican-Mestizo Populations from Different Geographic Regions based on 15 STR Loci.

	Chihuahua North	Jalisco West	Valley Mexico Center	Puebla Center	Guerrero South	Yucatan Southeast
Chihuahua	****	0.00901	0.00000	0.00000	0.00000	0.00000
Jalisco	0.00118	****	0.00000	0.00000	0.00000	0.00000
Valley Mexico	0.00484	0.00125	****	0.00000	0.23423	0.00000
Puebla	0.00928	0.00368	0.00108	****	0.00901	0.00000
Guerrero	0.00777	0.00264	0.00028	0.00104	****	0.00000
Yucatan	0.01029	0.00559	0.00409	0.00419	0.0027	****

though, this result is partially in agreement with a previous description that roughly classifies the distribution of the Mexican-Mestizo population into two principal regions (Northwest and Center-Southeast).[1,2] The observed distribution suggests that the Southeastern region is different and should be considered as independent from the Central and Southern regions.

CONCLUSION

We estimated for the first time the forensic parameters for 15 STRs in Mestizos from Guerrero (South, Mexico). We describe the genetic structure of Mexican-Mestizos based on 15 STRs, where the Southeastern region showed differences with the Central and Southern regions.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Rubi-Castellanos R, Martínez-Cortes G, Muñoz-Valle JF, et al. Pre-Hispanic Mesoamerican demography approximates the present-day ancestry of Mestizos throughout the territory of Mexico. *Am J Phys Anthropol*. 2009; 139: 284-294. doi: 10.1002/ajpa.20980

2. Salazar-Flores J, Zuñiga-Chiquete F, Martínez-Sevilla VM, et al. Admixture and genetic relationships of Mexican Mestizos regarding Latin American and Caribbean populations based on 13 CODIS-STRs. *J Comparative Hum Biol HOMO*. 2015; 66: 44-59. doi: 10.1016/j.jchb.2014.08.005

3. Moreno-Estrada A, Gignoux CR, Fernández-López JC, et al, The genetics of Mexico recapitulates Native American substructure and impacts biomedical traits. *Science*. 2014; 344: 1280-1285. doi: 10.1126/science.1251688

4. Martínez-Cortés G, Nuño-Arana I, Rubi-Castellanos R, et al. Origin and genetic differentiation of three native Mexican groups (Purépechas, Triquis, and Mayas): Contribution of CODIS-STRs to the history of human populations of mesoamerica. *Ann Hum Biol*. 2010; 37: 801-819. doi: 10.3109/03014461003743801

5. Tereba A. Tools for analysis of population statistics. In: *Profiles in DNA*: Madison, WI, USA: Promega Corp; 2001.

6. Lewis PO, Zaykin D (GDA). Genetic Data Analysis: Computer program for the analysis of allelic data version 1.0 2001. 2001. Web site. http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php. Accessed May 3, 2017.

7. Excoffier L, Laval G, Scheneider S, Arlequin ver. 3.0: An integrated software package for population genetic analysis. *Evol Bioinform Online*. 2005; 1: 47-50.

8. Martínez-González LJ, Martínez-Espín EM, Fernández-Rosado F, et al. Mexican population data on fifteen STR loci (Identifiler kit) in a Chihuahua (North Central Mexico) sample. *J Forensic Sci*. 2005; 50: 236-238.

9. Rubi-Castellanos R, Anaya-Palafox M, Mena-Rojas E, Bautista-España D, Muñoz-Valle JF, Rangel-Villalobos H. Genetic data of 15 autosomal STRs (Identifiler kit) of Three Mexican Mestizo Population samples from the States of Yucatan (Southeast), Jalisco (West), and Puebla (Center). *Forensic Sci Int Genet*. 2009; 3: e71-e76. doi: 10.1016/j.fsigen.2008.07.006

10. Luna-Vázquez A, Vilchis-Dorantes G, Aguilar-Ruiz MO, et al. Population data for 15 Loci (Identifiler® Kit) in a sample from the Valley of México. *Legal Med*. 2005; 7: 331-333. doi: 10.1016/j.legalmed.2005.06.002

11. López-Parra AM, Chiñas PI, Huang Q, Arroyo-Pardo E, Mesa MS. Genetic polymorphism of 15 STR loci in 3 ethnics groups of Guerrero State, Mexico. *Forensic Sci Int Genet*. 2016; 25: e8-e9. doi: 10.1016/j.fsigen.2016.06.018

12. Martínez-Marignac VL, Valladares A, Cameron E, et al. Admixture in Mexico City: Implications for admixture mapping of type diabetes genetic risk factors. *Hum Genet*. 2007; 120: 807-819. doi: 10.1007/s00439-006-0273-3

13. Rangel-Villalobos H, Martinez-Sevilla VM, Martinez-Cortes G, et al. Importance of the geographic barriers to promote gene drift and avoid (pre and post-Columbian) gene flow in Mexican Native groups: Evidence from forensic STR loci. *Am J Phys Anthropol*. 2016; 160(2): 298-316. doi: 10.1002/ajpa.22969

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-120

Research

*Corresponding author
David R. Wallace, PhD
Professor
Department of Pharmacology and Physiology
Oklahoma State University Center for Health Sciences
1111 West 17th Street
Tulsa, Oklahoma 74107-1898, USA
Tel. +1-918-561-1407
Fax: +1-918-561-5729
E-mail: david.wallace@okstate.edu

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2120

Article History
Received: August 2nd, 2017
Accepted: August 17th, 2017
Published: August 17th, 2017

Citation
Wallace DR, Crosswy KK. Interactions between over-the-counter and illicit drugs utilizing cytochrome p450 metabolism: Potential for exacerbation of pharmacological response. *Toxicol Forensic Med Open J*. 2017; 2(2): 51-62. doi: 10.17140/TFMOJ-2-120

Interactions between Over-the-Counter and Illicit Drugs Utilizing Cytochrome P450 Metabolism: Potential for Exacerbation of Pharmacological Response

David R. Wallace, PhD[1,2*]; Kara K. Crosswy, MS[2]

[1]Department of Pharmacology and Physiology, Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma 74107-1898, USA

[2]Department of Forensic Sciences, Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma 74107-1898, USA

ABSTRACT

Aim: To determine the interaction of over-the-counter (OTC) and illicit psychostimulants at the cytochrome P450 enzyme, CYP2D6. CYP2D6 is responsible for 20% of hepatic Phase I metabolism and is a site of drug-drug interactions, leading to increased drug toxicity.
Materials and Methods: We examined the effects the OTC drugs; 1) the prototype H_2-antagonist cimetidine (CMT) and 2) the opioid agonist cough suppressant dextromethorphan (DEX); as well as two scheduled drugs, methamphetamine (MA) and 3,4-methylenedioxymethamphetamine (MDMA) for their ability to interfere with CYP2D6 activity. Assays with human CYP2D6 determined the inhibitory potential (IC_{50}) of each drug. Kinetic analysis (V_{max} and K_m) was accomplished using rodent hepatic microsomes.
Results: Maximum inhibition of CYP2D6 activity following exposure to CMT+MDMA was significantly reduced 75-85% compared to quinidine (control) values. These data showed inhibitory effects in CYP2D6 activity in each compound tested. Alterations in CYP2D6 activity may result in complex drug-drug interactions leading to elevated plasma levels of drugs and increased risk for toxicity. Assays using rat CYP2D2 demonstrated V_{max} elevations in the CMT group (493%) compared to control (naïve, no treatment) values (19.9±5.1 pmol/mg protein/min). The K_m was increased 218% in CMT compared to controls (3.1±0.5 μM). Collectively, all MA challenged groups exhibited increases in total enzyme [V_{max}; 280-490%] and affinity [K_m; 165-220%] values compared to the control group. The increase in both V_{max} and K_m suggests that the low affinity/high capacity CYP2D2 isoform is upregulated.
Conclusion: Our findings suggest that *in vivo*, MA acts as a CYP2D2-inducer, which will lead to altered secondary drug metabolism, increasing the risk of drug-related toxicity. Coupled with the ability of CMT and DEX to interfere with MA metabolism, a complex drug-drug interaction is possible, leading to increased toxicity. Our findings substantiate the hypothesis that the combination of illicit and OTC drugs could result in complex drug-drug interactions increasing the risk for severe drug-related toxicity.

KEY WORDS: Cimetidine; Methamphetamine; Dextromethorphan; Ecstasy; CYP2D6; Hepatosomes; Methylenedioxymethamphetamine.

ABBREVIATIONS: MA: Methamphetamine; AM: Amphetamine; OTC: Over-the-counter; MDMA: 3,4-Methylenedioxymethamphetamine; CMT: Cimetidine; DEX: Dextromethorphan; CYP: Cytochrome P450; NE: Norepinephrine; DA: Dopamine; 5-HT: Serotonin; MDA: 3,4-Methylenedioxymethamphetamine; AMMC: 3-[2-(*N,N*-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin; HHMA: 3,4-Dihydroxymethamphetamine; AHMC: 3-[2-(*N,N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin; PCP: Phencyclidine; DXO: Dextrorphan.

TOXICOLOGY AND FORENSIC MEDICINE
Open Journal
Openventio PUBLISHERS
ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

INTRODUCTION

The cytochrome P450 (CYP) enzyme system is a superfamily of hemoproteins that catalyze the Phase I metabolism of numerous xenobiotics and is usually associated with hepatic metabolism.[1,2] CYP enzymes are also found in the small intestine, kidney, lung, and brain.[3] CYP2D6 is the second largest drug metabolizer (20%) and is an important part of the phase I drug metabolism, which transforms a functional group or adds a functional group to the drug to introduce or unmask polar bodies.[4,5] The active catalytic site of CYP2D6 contains acidic amino acid residues (Asp301 and Glu216) and as such will bind substrates which contain basic nitrogen and planar aromatic ring groups.[6] CYP2D6 has been studied extensively due to its genetic polymorphisms and its large number of substrates.[1] Based on genetic profiling, CYP2D6 polymorphisms are separated into the following classifications: 1) poor, 2) normal, and 3) extensive metabolizer. Allelic variants that are prevalent in ethnic populations that are known to be poor metabolizers are the CYP2D6*10 and CYP2D6*17 variants.[7] Ethnic populations which express these alleles, would have reduced function and be 'poor' metabolizers, whereas individuals with the 'normal' alleles will be able to adequately metabolize, and the last group, the 'extensive' metabolizers, will either express more of the active alleles, or will have been 'induced' by a secondary drug leading to a greater number of enzyme molecules.[2,8,9] Most drugs or chemicals exhibit reversible inhibition where there is competition for the catalytic site.[9] Inhibition magnitude is a function of the inhibiting agent concentration and the affinity of the agent for CYP2D6.[2] It is clear that the combination of ethnic/genetic effects coupled with the induction/inhibition ability of various drugs, the potential for drug-drug interactions resulting in an increased incidence of toxicity is significant. Improved understanding of CYP2D6 activity in the presence of illicit drugs will lead to better interpretation and predictions of drug-drug interactions.

Methamphetamine (MA) and 3,4-methylenedioxymethamphetamine (MDMA) are abused psychostimulant drugs often ingested as part of polydrug use. Drug-drug interactions at the level of the hepatic CYP microsomal system (Phase I reactions) can result in unexpected and dangerous side effects. Interactions between drugs may also affect forensic/medical toxicological analysis and interpretation. OTC-related inhibition of MA or MDMA metabolism will result in the accumulation of the illicit drug leading to increased toxicity. Cimetidine (CMT) and MDMA[10-12] are inhibitors of CYP2D6 whereas dextromethorphan (DEX) and MA are substrates for the CYP2D6 isozyme.[2,13,14] Comparing the actions of CMT and DEX at CYP2D6, CMT exhibits 20-fold lower potency at inhibiting compared to DEX (200 μM *vs*. 10 μM).[15] Inhibitors of CYP activity lead to increased drug adverse effects associated with the increase in the drug concentration.[1,2,9] Clemens et al confirmed the increase in adverse effects when MDMA and MA are administered concurrently.[16] The ability of X-ray crystallography to determine the CYP2D6 three-dimensional structure has aided significantly in understanding the mechanism of substrate activity at the CYP2D6 catalytic site.[17] A sample schematic for the metabolism of MA, MDMA, CMT, and DEX (Figure 1) includes the primary metabolite for each of the parent compounds.

Amphetamine (AMP) was first synthesized as a nasal decongestant to replace ephedrine.[18] Later, AMP was used to treat

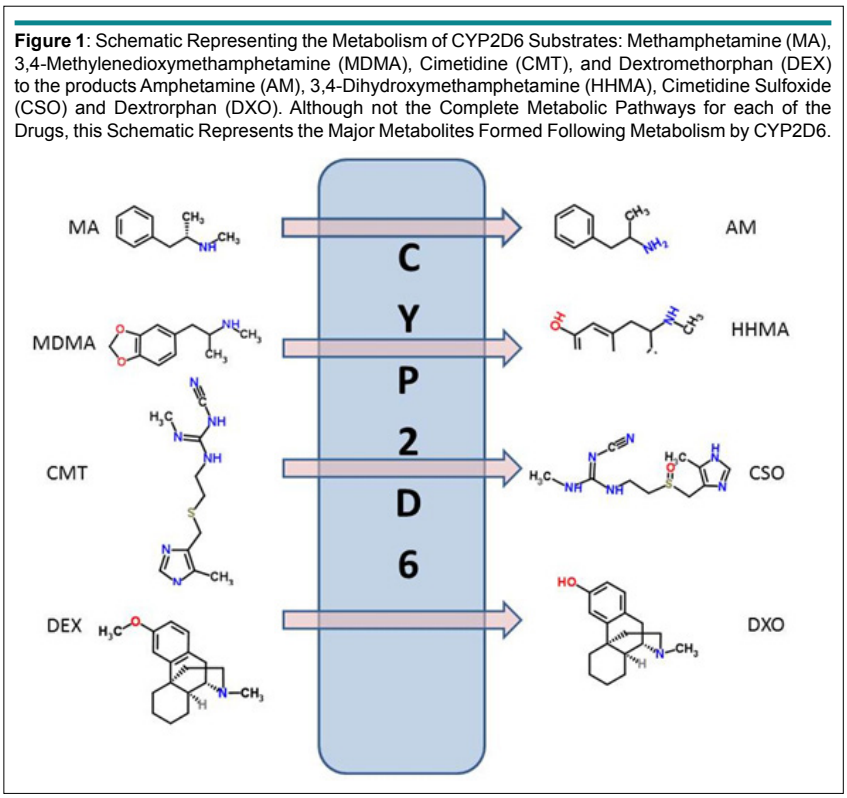


Figure 1: Schematic Representing the Metabolism of CYP2D6 Substrates: Methamphetamine (MA), 3,4-Methylenedioxymethamphetamine (MDMA), Cimetidine (CMT), and Dextromethorphan (DEX) to the products Amphetamine (AM), 3,4-Dihydroxymethamphetamine (HHMA), Cimetidine Sulfoxide (CSO) and Dextrorphan (DXO). Although not the Complete Metabolic Pathways for each of the Drugs, this Schematic Represents the Major Metabolites Formed Following Metabolism by CYP2D6.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

narcolepsy and hyperactivity, but its abuse skyrocketed.[19] After World War II, AMP and related drugs like methamphetamine (MA) were available without a prescription thus increasing use and abuse. It was not until the Controlled Substances Act (1986) that the regulation of AMP/MA increased and these agents were classified as "C-II" which means that there are medical uses, but the agents are highly addictive.[19] MA is a derivative of AMP and belongs to the phenethylamine class of psychostimulants.[20] MA is commonly accepted as more addictive and favored by drug addicts due to its increased ability to enter the central nervous system (CNS).[19,21] Of the two racemic forms of MA, the *d*-isomer exhibits greater stimulatory potency.[22] The mechanism associated with AMP and MA addiction involves increased activity of the dopaminergic system where dopamine (DA) release is increased, or DA is displaced from its storage vesicles elevating synaptic DA concentration.[19,20] At higher doses, MA reduced both DA and serotonin (5-HT)synthesis.[19,20] MA itself has low potency for stimulating DA release, but is metabolized to p-hydroxymethamphetamine (~15%) and the active metabolite AMP (4-7%) *via* CYP2D6. AMP is subsequently metabolized to p-hydroxyamphetamine and other inactive metabolites. Currently, MA is an easily obtainable drug, and the ease of production, increased potency, and availability has led to increased MA abuse.

Methylenedioxymethamphetamine (MDMA) was first synthesized as an appetite suppressant, yet never received approval for medical use. It was abused in the 1960s and 1970s for its psychoactive and hallucinogenic properties, and it was classified as "C-I" (no medicinal use, highly addictive).[19] Currently, MDMA is an illicit recreational drug that is very popular at all-night parties or raves. MDMA is an analog of MA formed by methylenedioxy substitution[22] and is structurally similar to mescaline and methylenedioxyamphetamine, MDA.[23] MDMA affects several neurotransmitter systems including norepinephrine (NE), DA, 5-HT, and the neurotransmitter γ-amino butyric acid, GABA.[22] MDMA is a strong and selective 5-HT neurotoxin in numerous animal species, including non-human primates.[24] In rats, MDMA stimulates 5-HT release which is believed to be *via* the reversal of the 5-HT transporter.[22] There are two forms of MDMA: the R-(-)-isomer and the more potent neurotoxin, the S-(+)-isomer. MDMA is O-demethylated to 3,4-dihydroxymethamphetamine [HHMA] *via* CYP2D6[23,25] followed by N-demethylation to the active metabolite MDA *via* CYP1A2 and CYP2D6.[22] MDA potently inhibits the activity of CYP2D6 through irreversible, covalent, binding to the CYP2D6 catalytic site.[10] When taken orally, MDMA is well absorbed with a half-life of 7 hours in humans, with MDA reaching peak plasma concentrations in 4-6 hours.[26] Nearly two-thirds of MDMA is excreted in the urine unchanged.[22,26] There is evidence to support long-term electrophysical abnormalities in MDMA users and suggest that typical recreational doses of MDMA are enough to cause long-term altered cortical activity in humans.[24] MDMA and MA, when taken concurrently, can produce greater adverse effects, dependent on the order of administration. The sequence of administration of MA and MDMA appears to have pharmacological relevance.[16] Collectively, these reports suggest that there could be a synergistic or additive effect of MDMA and MA that may be due to interactions at a common metabolic point, such as CYP2D6.

Cimetidine (CMT) is a commonly used over-the-counter medication for the treatment of acid reflux disease and heartburn. CMT was one of first in the class of histamine H_2 blockers/antagonists that prevent H^+ secretion from the parietal cells into the stomach lumen.[27] CMT can bind to the cytochrome P450 heme iron reactive site, inhibiting all cytochrome-dependent phase I enzyme activity.[28] Due to this interaction at P450 sites, CMT has been associated with many drug-drug interactions involving the inhibition of CYP2D6 and other P450 isozymes.[29-31] When CMT is combined with MA, levels of both MA and AMP were significantly higher in the rat CNS compared to rats that did not receive CMT.[32] CMT is metabolized by P450 enzymes to its major metabolite, an S-oxide.[27,29,33] The half-life of CMT is relatively short (2-4 hours) and is quickly cleared from the body by urinary excretion (70% of unchanged CMT) or the S-oxide form (20%).[34,35] The use of CMT as an acid-reducer is widespread, but the exact mechanism of P450 inhibition is not entirely understood. More work is needed to enhance our understanding of the potential of drug-drug interactions associated with CMT use.

Dextromethorphan (DEX) is an antitussive used in cold and cough medications to relieve non-productive coughs.[36] The abuse of DEX has been recognized since 1975, yet DEX has not been placed on the Controlled Substances Act.[37] DEX does not bind to opioid receptors like classical opioids and it has no analgesic activities; however, it does bind to a site associated with sigma-site ligands and also to the phencyclidine (PCP) N-methyl-D-aspartate (NMDA) glutamate receptor channel site.[38,39] At higher concentrations, recreational users reach dissociative effects similar to PCP or ketamine.[38,40] DEX is a substrate for CYP2D6 and may also interfere with the metabolism of many other drugs like CMT. DEX had been used to determine if a person is a rapid, normal or slow CYP2D6 metabolizer by measuring the rate which the active metabolite dextrorphan (DXO) is formed.[41] Both DEX and DXO have been shown to reduce MA self-administration at doses <30 mg/kg in rats,[42] suggesting that co-administration of DEX and MA may reduce the reinforcing properties associated with MA administration. Quinidine, a prototype substrate for CYP2D6, inhibits the DEXO-demethylation to DXO, thus increasing the DEX/DXO ratio.[36,43-45] After oral administration, DEX undergoes hepatic metabolism (85%) to increase elimination in the urine, but almost 15% of DEX is excreted unchanged.[44,46]

This study examines the interactions between two OTC drugs and two illicit drugs at CYP2D6 and asks: *can OTC drugs interfere with the metabolism of the illicit drugs leading to increased toxicity?* Drugs and Human Performance Fact sheets from the National Highway Traffic Safety Administration[47] also report "potential inhibitors of the CYP2D6 isozyme could decrease the rate of methamphetamine elimination if administered concurrently, while potential inducers could increase the rate of

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

elimination." Collectively, this work will improve our understanding of potential drug-drug interactions between OTC and illicit drugs and the potential hazards associated with polydrug use/abuse. This work is significant since unforeseen drug interactions may lead to the misinterpretation of toxicology results.

EXPERIMENTAL DESIGN

Chemicals and Drugs

Methamphetamine HCl, 3, 4-methylenedioxymethamphetamine HCl, cimetidine, dextromethorphan-HBr, acetonitrile, dimethyl sulfoxide (DMSO), 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin (AMMC)[48] were purchased from Sigma Chemical Company (St. Louis, MO, USA). The inhibitor screening kit CYP2D6/AMMC was purchased from BD Biosciences (Woburn, MA). The P450 HTS kit include: CYP2D6 (CYP2D6*1 + P450 reductase), non-fluorescent substrate (AMMC), fluorescent metabolite (AMHC), cofactors (1.3 mM NAPD+, 66 mM $MgCl_2$, and 66 mM glucose 6-phosphate), glucose 6-phosphate dehydrogenase (40 Units/ml in 5 mM sodium citrate buffer, pH 7.5), reaction buffer solution (0.5 M potassium phosphate, pH 7.4, filter sterilized), stop solution reagent (0.5 M Tris base reagent, filter sterilized), CYP2D6 positive control inhibitor (quinidine),[28] and NADPH regenerating system. The ability of this kit to perform *in vitro* screening of CYP2D6 substrates and inhibitors and its comparison to other screening modalities have been previously described.[49,50]

Animals

Male Sprague-Dawley rats (6-months old/375-425 grams, Harlan Sprague-Dawley Laboratories, Indianapolis, IN) were randomly assigned to treatment groups; group housed, and allowed access to food (Pro-lab Rat, Mouse, and Hamster Chow #3000) and water ad libitum in a temperature controlled room (23±2°C) and 12-hour light cycle. All animals experienced the same environmental conditions over the course of the experiment. Twenty rats (N=4 for each group) were placed in quarantine upon arrival for one week and then moved to the animal colony and allowed to habituate in their environment for at least one week prior to use. Animals were maintained according to NIH guidelines in our USDA-certified facilities. The protocol for the use of animals in this research was approved by the IACUC of Oklahoma State University Center for Health Sciences.

Inhibition Studies Purified CYP2D6

AMMC, 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin, is a non-fluorescent substrate that is demethylated to the fluorescent metabolite AHMC, 3-[2-(N, N-diethylamino) ethyl]-7-hydroxy-4-methylcoumarin hydrochloride, which was used to screen for interactions with CYP2D6.[51,52] Quinidine, a potent inhibitor of CYP2D6, was used as the control compound to determine intra- and inter- assay variability. Stock solutions (1 mM) of CMT, DEX, MA, and MDMA were each prepared in HPLC-grade acetonitrile before each CYP2D6 assay. Groups were divided by treatment: CMT, DEX, MA, MDMA, CMT+MA, CMT+MDMA, DEX+MA, DEX+MDMA, CMT+DEX+MA, and CMT+DEX+MDMA. Each drug was serially diluted (1:3) from highest concentrations (20 µM) to lowest concentrations (3 nM) and pre-incubated at 37 °C for 10 min in a black/clear bottom 96-well plates. Separate vehicle assays were performed (maximum 2% acetonitrile) and there was no effect on CYP2D6 activity (data not shown), which is similar to reports by the manufacturer. The enzyme-substrate mix [CYP2D6*1-P450 reductase + 10 mM AMMC] was added and plates were incubated at 37 °C for 30 min. The conversion of AMMC to AHMC was terminated by the addition of stop reagent and the amount of fluorescent product, AHMC, was determined using a fluorescent plate reader (Synergy HT with KC4 software; Bio-TEK Instruments, Inc., Winooski, VT, USA). Measurement parameters for determining AHMC fluorescence utilized an excitation/emission filter of 360 nm/460 nm. Background values were subtracted from the treatment wells before statistical analysis.

Inhibition Studies: Rat CYP2D2

Animals and treatment: Rats were randomly assigned to one of the 5 treatment groups: The first group was control (naïve); treatment groups included: vehicle control (VC; 0.9% saline and DMSO, 4%); CMT (10 mg/kg); DEX (10 mg/kg); and the combination of CMT+DEX (10 mg/kg each). Rats received a single daily injection from day 1 till day 7 at the same time each day. All compounds were administered *via* intraperitoneal (IP) injections to minimize first-pass effects. On Day 8, the drug-treated rats (not naïve rats) were challenged with a 5 mg/kg IP injection of MA. Seven hours after MA injection, rats were lightly anesthetized using carbon dioxide gas, and sacrificed by decapitation. The median and the left lateral hepatic lobes were harvested and frozen in liquid nitrogen. Samples were stored at -80 °C until use.

Hepatic microsome preparation: Hepatic microsomal fractions were prepared with slight modifications as previously described.[53] Briefly, frozen (-80 °C) rat livers were thawed and minced in 2-4 mL of homogenizing buffer (0.1 M potassium phosphate, pH 7.4 and 0.25 M sucrose), then brought to 30 mL with additional homogenizing buffer. Homogenization was completed with 10 strokes at 900 rpm using a Teflon pestle/glass homogenizer (Wheaton, USA). Nuclei and mitochondria were removed by centrifugation at 9,000 x*g* for 20 min at 4 °C. The resulting supernatant (S1) was centrifuged at 100,000 x*g* for 60 min. The pellet (P2; containing microsomes) was resuspended in 20 mL of incubation buffer (0.1 M potassium phosphate, pH 7.4, 0.25M sucrose, 1 mM EDTA, and 5% glycerol) and used immediately or stored frozen (-80 °C) until use. Florence et al showed washed microsomes could be stored at -80ºC for up to 30 days without loss of activity.[54] All stored microsomes in the present studies were used before the end of the 30 day period.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

Protein Analysis: Commercially available Coomassie-blue-based protein assay (Bio-Rad, Richmond, CA, USA) based on the Bradford method was used to determine total protein concentration.[55] Based on specific CYP2D2 activity, the calculations for pmol/mgprotein/min was determined.

***In vivo* Kinetic Studies:** The *in vivo* kinetic studies used the HTS kit from the *in vitro* studies with modifications. The assay was used to quantify the CYP2D2 (rat) enzyme activity for each of the drug treatment groups following MA challenge by measuring the production of fluorescent AHMC. The activity of CYP2D2 was determined following seven-day exposure to CMT, DEX, and CMT/ DEX, or saline using AMMC as a probe. This probe has been shown to be highly selective for rat CYP2D2, as well as the human CYP2D6 isoform.[48,56] Rat hepatic microsomes were used in place of the purified human CYP2D6 utilized in the *in vitro* assay.

Assays were performed as described above. The enzyme mix for the treatment groups was prepared for each microsomal fraction by adding H_2O, buffer (0.5 M potassium phosphate, pH 7.4, filter sterilized), and enzyme (microsomal fraction), a 79:20:0.75 mix. For the treatment groups, enzyme mix and AMMC (0.5 µM to 3.29 µM) were mixed in the well for a total volume of 100 µL. Varying AMMC concentrations produced a concentration response curve that determined enzyme kinetics. The plate was incubated for 30 min at 37 °C, and the reaction was terminated by the addition of stop reagent. The fluorescence generated by AMMC was quantified as described previously at excitation/emission wavelengths of 360 nm/460 nm. Assays were performed as four assays (N=4) in duplicate.

Statistical Analysis

For the *in vitro* CYP2D6 inhibition assays, the inhibitory potency of quinidine and each test compound was determined by measuring the IC_{50} value for each compound. This data was curve fit using an iterative nonlinear curve fitting program in PRISM 7.01 (GraphPad Software Inc., San Diego, CA, USA). IC_{50} values and the maximum inhibition percentage were then analyzed using the Kruskal-Wallis nonparametric test followed by the Dunn's comparison (using quinidine as the comparison group). A significance level was set at α=0.05.

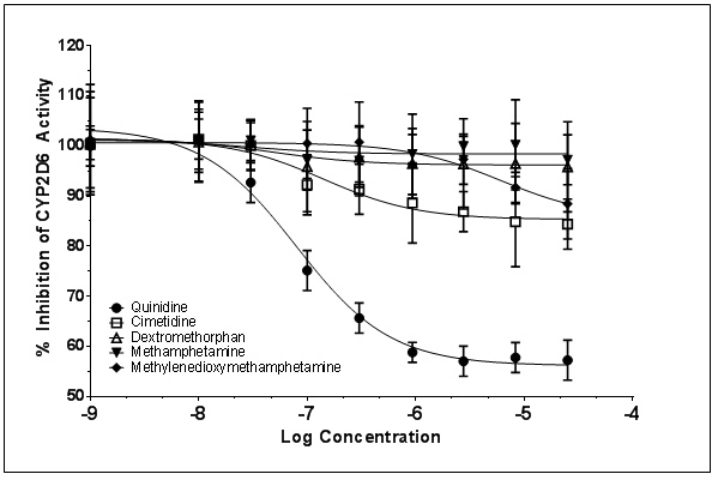
For the *in vivo* enzyme kinetic assay, data was collected similar to above and calculated using a nonlinear fit (rectangular hyperbola) of the data, yielding V_{max} and K_m values. Kinetic data were then analyzed using one-way ANOVA followed by Dunnett's posthoc test to compare each of the treatment groups to the K_m (µM) and V_{max} (pmol of AMHC formed/mg protein/minute) values obtained from naïve rats. The significance level again was set at α=0.05. All data are expressed as the mean±standard error of the mean (SEM).

RESULTS AND DISCUSSION

Inhibition Studies Purified CYP2D6

To determine inhibition of CYP2D6 by quinidine and test compounds the IC_{50} values were calculated for each inhibition curve (Figure 2). Then, the mean log IC_{50} values for each were group were obtained and compared (Figure 3A and 4A). The IC_{50} values for each test compound were then compared to the

Figure 2: Inhibition of CYP2D6 Activity by OTC and Illicit Drugs. Purified Human CYP2D6 was Incubated in the Presence of the Non-Fluorescent 3-[2-(N, N-Diethyl-N-Methylamino) Ethyl]-7-Methoxy-4-Methylcoumarin (AMMC, 10 mM) which was then Demethylated to the Fluorescent Metabolite 3-[2-(N, N-Diethylamino) Ethyl]-7-Hydroxy-4-Methylcoumarin Hydrochloride (AHMC). Enzyme-Substrate Mixtures were incubated with 8 Concentrations of CMT, DEX, MA or MDMA (0.2 nM – 100 µM) for 10 min at 37°C. The Quantity of Fluorescent Product (AHMC) was then Measured with a Fluorescence Excitation/Emission Filter of 360 nm/460 nm. Data was Fit using Nonlinear Regression Analysis and both 1-site and 2-site Models were Compared. Curves were best Fit to the Simpler, 1-site Model and from this Analysis, the IC_{50} and Maximum Inhibition Values could be Determined (expressed in Figures 3 and 4). Each of the Data Points Represent 4 Assays (N=4) Performed in Duplicate, Except for the DEX Group which was an N=3. Data then Expressed as the Mean±SEM.



ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

quinidine IC_{50} value (3.8 nM) which was used as the 'control.' Our values are similar to the values reported elsewhere and in the manufacturer literature (3-11 nM).[50] Figure 2 represents the inhibition curve from each test compound (and quinidine) and the data are expressed as the mean±SEM of 4 assays performed in duplicate for each test compound except for the DEX and CMT+MA groups which are three assays performed in duplicate. Another indicator of effect at CYP2D6 was the measurement of the maximum inhibition elicited by each of the test compounds. The maximum inhibition (35-40%) of quinidine was set as 100% inhibition, and each group was then calculated as a percentage of the quinidine maximum (Figure 3B and 4B). When examining single compound, there was no effect on the log IC_{50} values compared to quinidine ($H_{5,20}$=5.66; p=0.2263, Figure 3A). Comparing the maximum inhibition of the single test compounds to quinidine revealed a significant effect of treatment ($H_{5,20}$=13.69; p=0.0084, Figure 3B) with both the MA and MDMA groups exhibiting significantly less inhibition compared to the quinidine group (p<0.05). When examining the effects of multiple drug exposures, to determine if there may be synergistic, additive or potentiating effects, there was a generalized lowering of log IC_{50} values ($H_{7,21}$=14.42; p=0.0252, Figure 4A). Comparison to quinidine values revealed that only the CMT+DEX+METH log IC_{50} value was significantly (p<0.05) reduced compared to quinidine values. In the combination treatment groups, all groups exhibited significantly reduced inhibition compared to quinidine, yet there were no differences between any of the combination treatment groups (Figure 4B). Collectively these data suggest none of the drugs resulted in the inhibition of CYP2D6 to the same extent as quinidine, the prototype CYP2D6 inhibitor. Although, not potent inhibitors, each of the test compounds did elicit some level of inhibition of CYP2D6 activity. Each test compound (single or combination) displayed a lower affinity for CYP2D6 compared to quinidine (usually 10- to 100-fold lower IC_{50} values compared to the 3-11nM IC_{50} value reported for quinidine). In general, the *in vitro* assays suggest that each of the OTC and illicit drugs can inhibit CYP2D6 to a relatively small extent and that the weak

Figure 3: Effects of Single Drug Exposure on CYP2D6 Activity. The IC_{50} (A) and Maximum Inhibition (B) Values were Determined from the Nonlinear Analysis (Figure 2). The Values for Quinidine (IC_{50} and Maximum Inhibition) were used as the Positive Control to which the other Drug Exposure Effects were Compared. When Examining Single Compound Comparisons (A), there was no Effect ($H_{5,20}$=5.66; p=0.2263) on the log IC_{50} Values Compared to Quinidine Values. Comparing the Maximum Inhibition (B) of the Single Test Compounds to the Maximum CYP2D6 Inhibition by Quinidine, there was a Significant Effect of Treatment ($H_{5,20}$=13.69; p=0.0084) with both the MA and MDMA Groups Exhibiting Significantly Less Inhibition Compared to the Quinidine Group (p<0.05). Data are Expressed as mean±SEM of 4 Assays (3 for DEX) Run in Duplicate. *p<0.05 Compared to Quinidine Value.

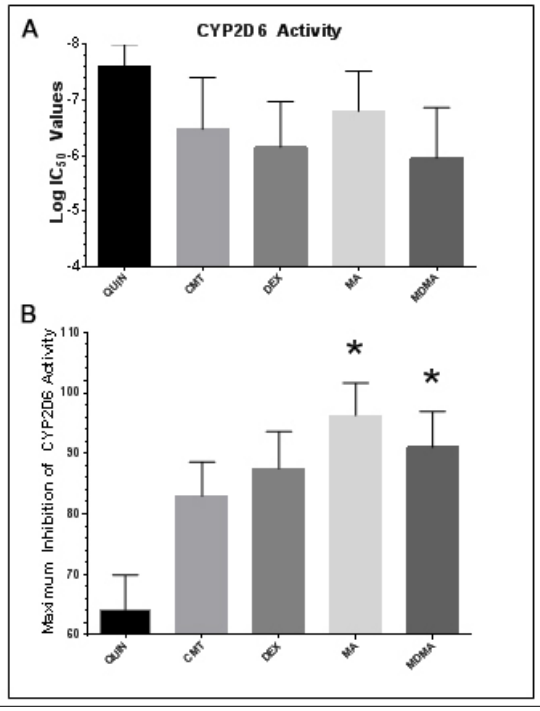
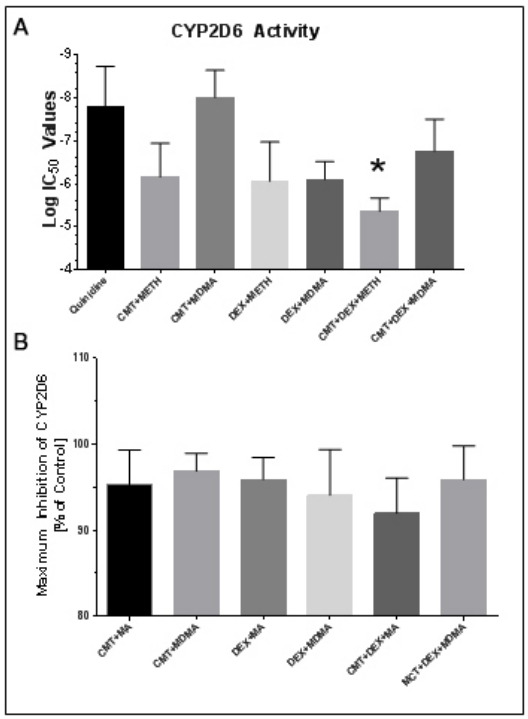


Figure 4: Effects of Combination Drug Exposure on CYP2D6 Activity. The IC_{50} (A) and Maximum Inhibition (B) Values were Determined from the Nonlinear Analysis (Figure 2). The Values for Quinidine (IC_{50} and Maximum Inhibition) were used as the Positive Control to which the other Drug Exposure Effects were Compared. Examining the IC_{50} Values (A), the Combination Drug Exposure had Generally Lower IC_{50} Values Compared to the Quinidine Groups ($H_{7,21}$=14.42; p=0.0252). Comparison to Quinidine Values Revealed that only the CMT+DEX+METH Combination Group had Significantly (p<0.05) Lower log IC_{50} Values Compared to the Quinidine Group. In the Combination Treatment Groups, all Groups Exhibited Significantly Reduced Inhibition Compared to Quinidine (Approximately 35% Inhibition to 5-10%), yet there were no differences between any of the Combination Treatment Groups (B). Data are Expressed as mean±SEM of 4 Assays (3 for CMT+MA) Performed in Duplicate. *p<0.05 Compared to Quinidine Value.



ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

inhibition observed does not exhibit any other characteristics in the combination groups such as synergism, potentiation or additivity.

Each of the drugs tested have reported action at CYP2D6 either as an inhibitor, an inducer, or a substrate. CMT has been reported to have inhibitory effects on CYP2D6 in both humans and rodents.[33,57-59] DEX interacts with a greater number of P450 isozymes, with CYP2D6 being a major contributor to DEX metabolism by O-demethylation.[60] It is clear that OTC drugs such as CMT and DEX can play a significant role in the development of drug-drug interactions leading to increased toxicity by interference with CYP2D6 metabolism. There is an extensive body of work regarding the metabolism of MA and MDMA. CYP2D6 is involved in multiple steps in the metabolism of both MA and MDMA.[61] Earlier reports suggest that the parent compound for MA and its parent compound, amphetamine, act at CYP2D6 with low micromolar affinity.[62] Our findings demonstrate that a potential interaction between OTC and illicit drugs does exist[63] and that the interaction may lead to increased risk of toxicity. Overall, our findings *in vitro* show the calculated IC_{50} values for the test compounds were reduced by a 75-85% decrease compared to IC_{50} values associated with quinidine. The IC_{50} value for quinidine of 3.8 nM was consistent with the value reported by the manufacturer and other researchers.[50,64,65] The efficacy of inhibition or maximum inhibition was lower than the inhibition that was observed with quinidine, suggesting that the test compound only weakly interacted with CYP2D6 compared to quinidine.

Studies using MA and MDMA employed a variety of different methodologies to measure kinetic parameters.[10-12,62,64] Taavitsainen et al reported an IC_{50} value for MA of 414 µM, but this study used DEX as the substrate probe, not AMMC.[64] De la Torre et al reported MDMA's rate of activation is decreased when quinidine is added, suggesting MDMA is metabolized in part by CYP2D6.[66] Studies that include CMT used different methodology such as Western blotting with serum containing anti-CYP2D6[67] and use different factors to determine the kinetic parameters for CMT.[29,68] Early studies examining the effects of CMT on CYP2D6 as well as other P450 isozymes describe the binding actions of CMT to CYP2D6 and that this interaction can reduce the metabolism of a benzodiazepine by up to 45%.[69,70] A report by Madeira et al. showed that CMT and DEX could reciprocally interfere with CYP2D6-mediated metabolism.[29] Literature describing the actions of DEX on CYP2D6 are the most prevalent and use DEX as a probe to determine enzyme activity or phenotyping.[29,41,68] Also DEX has been used to categorize the metabolizer-typing of the patient.[45] Studies on DEX-mediated CYP2D6 inhibition report IC_{50} values for DEX of 1.89 µM to 2.0 µM dependent on drug concentration.[48,65] Based on the DEX studies, it is clear that DEX exerts a robust effect at CYP2D6 and could be an important OTC drug when considering potential drug-drug interactions.

Inhibition Studies with rodent isozyme: CYP2D2

Rats were treated as described above for 7 days (day 1 to day 7) and then challenged on Day 8 with MA. Following challenge (7 hours), rats were sacrificed and hepatic lobes removed to determine the conversion of AMMC to AMHC. The K_m(µM) and V_{max}(pmol of AMHC/mg protein/minute) values are presented in Table 1. Comparison of K_m values across treatments revealed a significant effect of treatment ($F_{4,15}$=4.067; p=0.0199). Although reductions in K_m ranged from 65-121% compared to naïve values, the actual range was from 3-6.8 µM. The saline, CMT, and DEX groups were significantly different from naïve control values (p<0.05). The only group that was not statistically different from naïve was the combination of CMT+DEX. It is unclear as to whether these relatively small changes would result in significant changes in metabolism *via* CYP2D2 (or CYP2D6 in humans). Comparing V_{max} values revealed the more robust changes. Across all treatment groups, it appeared that the CYP2D2 activity had shifted to a higher capacity (280-490%). V_{max} values were significantly ($F_{4,15}$=4.342; p=0.0157) increased compared to naïve values by 2.8- to 4.9-fold. Dunnett's posthoc analysis showed that the only group that was significantly different from naïve was the CMT group (p<0.05).

We further confirmed and extended the *in vitro* findings with *in vitro* studies utilizing adult male Sprague-Dawley rats. Rodent CYP2D2 activity following daily drug administration was measured using a modified assay based on the *in vitro* studies. Our *in vivo* results suggest that both V_{max} and K_m values were elevated in the treatment groups. V_{max} values increased 280-490% and K_m values increased 165-220% in treatment groups compared to V_{max} and K_m values from the control (naïve) group. Collectively, these results suggest that following treatment with CMT, DEX or a combination, the CYP2D2 enzyme in the rat shifts towards a low affinity/high capacity CYP2D2 isoform.

Table 1: Mean K_m & V_{max} Values for each Treatment Groups.

	Naive	Saline	CMT	DEX	CMT/DEX
K_m (µM) Mean±SEM	3.08±0.46	6.81*±0.73	6.72*±1.34	6.40*±0.47	5.09±0.55
V_{max} (pmol/mg protein/min) Mean±SEM	19.9±5.1	65.9±12.0	98.3*±22.1	70.5±15.1	56.4±6.1

*p<0.05 compared to corresponding naïve values.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

The rightward shift direction of the kinetic curves would lead to a slowing of metabolism over time, and as a result, increasing the concentration of other drugs/compounds which require CYP2D6 for their metabolism. All test compounds reduced the activity of CYP2D6, i.e., weak inhibition; therefore further studies are relevant.

In vivo studies showed that pre-treatment with CMT, DEX or CMT+DEX for 7 days followed by an MA challenge on day 8 resulted in CYP2D2 activity that exhibited a lower affinity and higher capacity. This data implies that MA may be an inducer *via* CYP2D2 if elevations in V_{max} represent an increase in the quantity of CYP2D2 protein. Conversely, V_{max} elevation may be due to changes in the catalytic activity of the existing enzymes. Dostalek et al suggest that MA may be an inducer of DEX metabolism *via* CYP2D2; therefore the co-administration of MA with DEX may result in decreased drug plasma levels thus a decrease in drug effects.[71,72] Conversely, reports have shown that co-administration of DEX and MA reduces the reinforcing properties of MA, *via* inhibition of DA neurons in the brain.[73] This effect could lead to increased MA administration to achieve the same "high," which will bring plasma levels closer to toxicity. Similar results were reported by Glick et al and these findings also extended DEX and DXO effects to include reductions in both morphine and nicotine self-administration as well as MA.[42] Studies on MDMA metabolism are more recent, but tend to support previous findings with other MA analogs. Although CYP2D6 is the major route of metabolism, other P450 isozymes contribute to the overall metabolism of MDMA.[11,12,74,75] A recent study examined the potential drug-drug interactions between MDMA and caffeine.[76] Although, the authors report that there are no drug-drug interactions which significantly alter MDMA metabolism, the authors acknowledge the importance of the investigation into drug-drug interactions. DEX effects can be extended to the central nervous system where reductions in self-administration and neuroprotection are believed to be through non-NMDA glutamate receptor-mediated functions.[42,77,78] The K_m value in the control (naïve) group was consistent with the suggested K_m value reported by the manufacturer (BD Biosciences). This suggests that the results from both assays are relevant to each other and can be compared. Most studies that measure enzyme activity of CYP2D6 or CYP2D2 use DEX as the substrate,[12] but this study uses the reduction of AMMC to AHMC to determine the enzyme activity. A few studies report the kinetic parameters for MA, CMT, and DEX, but most use different methods or *in vitro* instead of *in vivo*.[29,61] Lin et al report V_{max} and K_m values for MA, but the values are for both isomers of MA and both types of reactions (4-hydroxylation and N-demethylation) whereas this study did not differentiate between the two isomers or the two types of reactions.[61] Madeira et al report V_{max} and K_m values for CMT but the study is done *in vitro* instead of *in vivo* and with DEX as the probe.[29]

CONCLUSION

Poly-drug use is a growing concern due to the potential for drug-drug interactions and increased risk for severe drug-related toxicity. The current study addressed this question by using the combination of the two scheduled drugs, MA (C-II) and MDMA (C-I), and two common and inexpensive OTC drugs, CMT and DEX. CYP2D6 is an important area of research due to the large role it plays in metabolism, its genetic polymorphisms among humans, and its large number of substrates. There has been some concern regarding the impact of hepatic CYP2D6 interactions since there are other sites that are involved in the metabolism of drugs, such as the intestine, kidney, and brain. The clinically relevant drug, Selegiline, has been used in the treatment of Parkinson's disease and is metabolized to MA by CYP2D6 and as such may be subject to genetic variations. Benetton et al. report that any P450 phenotyping variations would be insignificant for the metabolism of Selegiline to MA, and this was further extended to include the metabolism of MA and MDMA.[66,79] Regardless, there are variations in CYP2D6 activity and not just in hepatic P450 systems. An understudied area is a role that P450 enzymes have in the brain in the metabolism of centrally-acting drugs.[80,81] The pharmacogenetic impact on the metabolism of MDMA is more pronounced with allele-dependency dictating the rate and extent of metabolism.[82] Although, the mechanisms are not clear, there still exists the change for drug-drug interactions leading to toxicity. Since MA and MDMA are popular and easily obtainable, drug- drug interactions are probable. This study determined that the inhibitor potency of all test compounds and quinidine were relatively the same. It was determined that some maximum test compounds inhibition decreased significantly compared to maximum quinidine and CMT/ MDMA inhibition. This suggests that all the test compounds inhibited CYP2D6 activity; one or all of the drugs may not be metabolized as quickly resulting in toxicity of those drugs. The quinidine IC_{50} value was consistent with reported values. This indicates that the CYP2D6 was performed in accordance with the manufacturer's specifications. The V_{max} value in the CMT treated group increased significantly compared to naïve. The K_m values in the CMT and saline treated group increased significantly compared to naïve. Both kinetic parameters showed there was an increase after the MA challenge but no effects due to the OTC drugs. This suggests that the low affinity/high capacity CYP2D2 isoform was upregulated meaning that more CYP2D2 was present, suggesting that MA is an inducer *via* CYP2D2. Understanding that MA can induce CYP2D2 and CYP2D6 is important considering the co-administration of other drugs such as DEX, CMT, or MDMA. This information is vital since many other drugs can be used recreationally to achieve a 'high' or to combine drugs to potentiate or prolong the high associated with an illicit drug. Increasing our understanding of these drug-drug relationships will aid in our interpretation of forensic findings as well as provide a better foundation for understanding toxicological relationships between various drugs.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest involved with this manuscript.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

REFERENCES

1. Badyal DK, Dadhich AP. Educational forum cytochrome P450 and drug interactions. *Indian J Pharmacol*. 2001; 33: 248-259.

2. Brown C. Overview of drug interactions modulated by cytochrome P450. *US Pharm*. 2001: HS26-HS45.

3. Nishimura M, Yaguti H, Yoshitsugu H, Naito S, Satoh T. Tissue distribution of mRNA expression of human cytochrome P450 isoforms assessed by high sensitivity real time reverse transcription PCR. *Yakugaku Zasshi*. 2003; 123(5): 369-375. doi: 10.1248/yakushi.123.369

4. Drummer O, Odell M. Pharmacokinetics and duration of action. In: Bentliff G, McAllister L, eds. *The Forensic Pharmacology of Drugs of Abuse*. 1st ed. Abingdon, UK: Taylor & Francis; 2001: 27-29.

5. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*. 2013; 138(1): 103-141. doi: 10.1016/j.pharmthera.2012.12.007

6. Raunio H, Kuusisto M, Juvonen RO, Pentikäinen OT. Modeling of interactions between xenobiotics and cytochrome P450 (CYP) enzymes. *Front Pharmacol*. 2015; 6(123): 1-14. doi: 10.3389/fphar.2015.00123

7. Shen H, He MM, Liu H, et al. Comparative metabolic capabilities and inhibitory profiles of CYP2D6.1, CYP2D6.10, and CYP2D6.17. *Drug Metab Dispos*. 2007; 35(8): 1292-1300. doi: 10.1124/dmd.107.015354

8. Qu Q, Qu J, Han L, et al. Inhibitory effects of phytochemicals on metabolic capabilities of CYP2D6*1 and CYP2D6*10 using cell-based models in vitro. *Nat Publ Gr*. 2014; 35(10): 685-696. doi: 10.1038/aps.2013.202

9. Cupp M, Tracy T. Cytochrome P450: New nomenclature and clinical implications. *Am Fam Physician*. 1998; 57(1): 107-116.

10. Van LM, Heydari A, Yang J, et al. The impact of experimental design on assessing mechanism-based inactivation of CYP2D6 by MDMA (Ecstasy). *J Psychopharmacol*. 2006; 20(6): 834-841. doi: 10.1177/0269881106062902

11. Van LM, Hargreaves JA, Lennard MS, Tucker GT, Rostami-Hodjegan A. Inactivation of CYP2D6 by methylenedioxymethamphetamine in different recombinant expression systems. *Eur J Pharm Sci*. 2007; 32(1): 8-16. doi: 10.1016/j.ejps.2007.05.002

12. Van LM, Swales J, Hammond C, Wilson C, Hargreaves JA, Rostami-Hodjegan A. Kinetics of the time-dependent inactivation of CYP2D6 in cryopreserved human hepatocytes by methylenedioxymethamphetamine (MDMA). *Eur J Pharm Sci*. 2007; 31(1): 53-61. doi: 10.1016/j.ejps.2007.02.005

13. Brown C. Overview of Drug–Drug Interactions with SSRIs. *US Pharm*. 2008: HS3-HS19.

14. Cupp MJ, Tracy TS. Cytochrome P450: New nomenclature and clinical implications. *Am Fam Physician*. 1998; 57(1): 107-116.

15. Martinez C, Albet C, Agundez JA, et al. Comparative in vitro and in vivo inhibition of cytochrome P450 CYP1A2, CYP2D6, and CYP3A by H2-receptor antagonists. *Clin Pharmacol Ther*. 1999; 65(4): 369-376. doi: 10.1016/S0009-9236(99)70129-3

16. Clemens KJ, Cornish JL, Li KM, Hunt GE, McGregor IS. MDMA ('Ecstasy') and methamphetamine combined: Order of administration influences hyperthermic and long-term adverse effects in female rats. *Neuropharmacology*. 2005; 49(2005): 195-207. doi: 10.1016/j.neuropharm.2005.03.002

17. Maréchal J-D, Kemp C, Roberts G, Paine M, Wolf C, Sutcliffe M. Insights into drug metabolism by cytochromes P450 from modelling studies of CYP2D6-drug interactions. *Br J Pharmacol*. 2008; 153: 82-89. doi: 10.1038/sj.bjp.0707570

18. Drummer O, Odell M. Stimulants. In: Bentliff G, McAllister L, eds. *The Forensic Pharmacology of Drugs of Abuse*. 1st ed. Abingdon, UK: Taylor & Francis; 2001: 49-102.

19. Levine B. *Principles of Forensic Toxicology*. 4th ed. Washington, DC, USA: AACC Press; 2013.

20. Logan B. Methamphetamine-effects on human performance and behavior. *Forensic Sci Rev*. 2002; 14(1-2): 133-151.

21. Shoblock JR, Sullivan EB, Maisonneuve IM, Glick SD. Neurochemical and behavioral differences between d-methamphetamine and d-amphetamine in rats. *Psychopharmacology (Berl)*. 2003; 165: 359-369. doi: 10.1007/s00213-002-1288-7

22. Logan B, Couper F. 3,4-Methylenedioxymethamphetamine — effects on human performance and behavior. *Forensic Sci Rev*. 2003; 15(1): 11-28.

23. Oesterheld JR, Armstrong SC, Cozza KL. Med-Psych Drug-Drug Interactions Update Ecstasy: Pharmacodynamic and Pharmacokinetic Interactions. *Psychosomatics*. 2004; 45(1): 84-87. doi: 10.1176/appi.psy.45.1.84

24. Casco C, Forcella MC, Beretta G, Grieco A, Campana G. Long-term effects of MDMA (Ecstasy) on the human central nervous system revealed by visual evoked potentials. *Addict Biol*. 2005; 10(2): 187-195. doi: 10.1080/13556210500123340

25. Escobedo I, O'Shea E, Orio L, et al. A comparative study on the acute and long-term effects of MDMA and 3,4-dihydroxy-

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

methamphetamine (HHMA) on brain monoamine levels after i.p. or striatal administration in mice. *Br J Pharmacol*. 2009; 144(2): 231-241. doi: 10.1038/sj.bjp.0706071

26. De Letter EA, Belpaire FM, Clauwaert KM, Lambert WE, Van Bocxlaer JF, Piette MH. Post-mortem redistribution of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") in the rabbit. Part II: Post-mortem infusion in trachea or stomach. *Int J Legal Med*. 2002; 116(4): 225-232. doi: 10.1007/s00414-002-0293-z

27. Jantratid E, Prakongpan S, Dressman J, et al. Biowaiver monographs for immediate release solid oral dosage forms: Cimetidine. *J Pharm Sci*. 2006; 95(5): 974-984. doi: 10.1002/jps.20614

28. Liska DJ. The detoxification enzyme systems. *Altern Med Rev*. 1998; 3(3): 187-198.

29. Madeira M, Levine M, Chang TKH, Mirfazaelian A, Bellward GD. The effect of cimetidine on dextromethorphan O -demethylase activity of human liver microsomes and recombinant Cyp2D6. *Drug Metab Dispos*. 2004; 32(4): 460-467. doi: 10.1124/dmd.32.4.460

30. Park E, Cho H, Lee Y. Effect of Cimetidine and Phenobarbital on metabolite kinetics of Omeprazole in rats. *Arch Pharm Res*. 2005; 28(10): 1196-11202.

31. Draper AJ, Madan A, Parkinson A. Inhibition of coumarin 7-hydroxylase activity in human liver microsomes. *Arch Biochem Biophys*. 1997; 341(1): 47-61. doi: 10.1006/abbi.1997.9964

32. Suzuki T, Chiang H-JF, Misawa M. Effects of quinidine and cimetidine on the methamphetamine level in the rat brain. *Jpn J Pharmacol*. 1987; 43: 103-106. doi: 10.1254/jjp.43.103

33. Levine M, Bellward GD. Effect of cimetidine on hepatic cytochrome P450: Evidence for formation of a metabolite-intermediate complex. *Drug Metab Dispos*. 1995; 23(12): 1407-1411.

34. Larsson R, Erlanson P, Bodemar G, Norlander B, Fransson L, Strouth L. Pharmacokinetics of cimetidine and its sulphoxide metabolite during haemodialysis. *Eur J Clin Pharmacol*. 1982; 21(4): 325-330. doi: 10.1007/BF00637621

35. Ogbru O. Cimetidine, Tagamet HB. http://www.medicinenet.com/cimetidine/article.html. Accessed June 30, 2017.

36. Manap RA, Wright CE, Gregory A, et al. The antitussive effect of dextromethorphan in relation to CYP2D6 activity. *Br J Clin Pharmacol*. 1999; 48: 382-387. doi: 10.1046/j.1365-2125.1999.00029.x

37. CESAR. Dextromethorphan (DXM). Dextromethorphan (DXM). http://www.cesar.umd.edu/cesar/drugs/dxm.asp. Accessed June 30, 2017.

38. Nicholson K, Hayes B, Balster R. Evaluation of the reinforcing properties and phencyclidine-like discriminative stimulus effects of dextromethorphan and dextrorphan in rats and rhesus monkeys. *Psychopharmacology (Berl)*. 1999; 146(1): 49-59.

39. Ehret GB, Daali Y, Chabert J, et al. Influence of CYP2D6 activity on pre-emptive analgesia by the N-Methyl-D-Aspartate antagonist dextromethorphan in a randomized controlled trial of acute pain. *Pain Physician*. 2013; 16: 45-56.

40. Lotrich F, Rosen J, Pollock B. Dextromethorphan-induced delirium and possible methadone interaction. *Am J Geriatr Pharmacother*. 2005; 3(1): 17-20.

41. Frank D, Jaehde U, Fuhr U. Evaluation of probe drugs and pharmacokinetic metrics for CYP2D6 phenotyping. *Eur J Clin Pharmacol*. 2007; 63(4): 321-333. doi: 10.1007/s00228-006-0250-8

42. Glick SD, Maisonneuve IM, Dickinson HA, Kitchen BA. Comparative effects of dextromethorphan and dextrorphan on morphine, methamphetamine, and nicotine self-administration in rats. *Eur J Pharmacol*. 2001; 422(1-3): 87-90. doi: 10.1016/S0014-2999(01)01066-4

43. Witherow LE, Houston JB. Sigmoidal kinetics of CYP3A substrates: An approach for scaling dextromethorphan metabolism in hepatic microsomes and isolated hepatocytes to predict in vivo clearance in rat. *J Pharmacol Exp Ther*. 1999; 290(1): 58-65.

44. Min DI, Ku Y-M, Vichiendilokkul A, Fleckenstein LL. A urine metabolic ratio of dextromethorphan and 3-methoxymorphinan as a probe for CYP3A activity and prediction of cyclosporine clearance in healthy volunteers. *Pharmacotherapy1*. 1999; 19(6): 753-759. doi: 10.1592/phco.19.9.753.31536

45. Kerryi NL, Somogyit AA, Bochner F, Mikus G, Somogyi AAA. The role of CYP2D6 in primary and secondary oxidative metabolism of dextromethorphan: In vitro studies using human liver microsomes. *Br J clin Pharmac*. 1994; 38(3): 243-248. doi: 10.1111/j.1365-2125.1994.tb04348.x

46. Magarey J. Dextromethorphan. INCHEM. http://www.inchem.org/documents/pims/pharm/pim179.htm. Published 1996. Accessed June 30, 2017.

47. Couper FJ, Logan BK. *Drugs and Human Performance Fact Sheets – Methamphetamine and Amphetamines*. 2014. https://www.nhtsa.gov/sites/nhtsa.dot.gov/files/809725-drugshuman-performfs.pdf. Accessed August 1, 2017.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

48. Chauret N, Dobbs B, Lackman RL, et al. The use of 3- [2- (N , N-Diethyl-N-Methylammonium) Ethyl] -7-Methoxy-4- methylcoumarin (AMMC) as a specific Cyp2D6 probe in Human liver microsomes. *Drug Metab Dispos*. 2001; 29(9): 1196-1200.

49. Riley RJ, Grime K. Metabolic screening in vitro: Metabolic stability, CYP inhibition and induction. *Drug Discov Today Technol*. 2004; 1(4): 365-372. doi: 10.1016/j.ddtec.2004.10.008

50. Crespi CL, Miller VP, Penman BW. Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Anal Biochem*. 1997; 248(1957): 188-190. doi: 10.1006/abio.1997.2145

51. Palamanda JR, Favreau L, Lin C, Nomeir AA. Validation of a rapid microtiter plate assay to conduct cytochrome P450 2D6 enzyme inhibition studies. *Drug Discov Today*. 1998; 3(10): 466-470. doi: 10.1016/S1359-6446(98)01248-3

52. Turpeinen M, Korhonen LE, Tolonen A, et al. Cytochrome P450 (CYP) inhibition screening: Comparison of three tests. *Eur J Pharm Sci*. 2006; 29(2): 130-138. doi: 10.1016/j.ejps.2006.06.005

53. Nelson AC, Huang W, Moody DE. Variables in human liver microsome preparation: Impact on the kinetics of L-acetylmethadol (LAAM) N-demethylation and dextromethorphan O-demethylation. *Drug Metab Dispos*. 2001; 29(3): 319-325.

54. Florence VM, Di Stefano EW, Sum CY, Cho AK. The metabolism of (R)-(-)-amphetamine by rabbit liver microsomes. *Drug Metab Dispos*. 1982; 10(4): 312-315.

55. Bradford MM. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72: 248-254.

56. Stresser DM, Turner SD, Blanchard AP, Miller VP, Crespi CL. Cytochrome P450 fluorometric substrates: Identification of isoform-selective probes for rat CYP2D2 and human CYP3A4. *Drug Metab Dispos*. 2002; 30(7): 845-852. doi: 10.1124/dmd.30.7.845

57. Pelkonen O, Puurunen J. Effect of cimetidine on microsomal drug metabolism in man. *Biochem Pharmacol*. 1980; 29(22): 3075-3080. doi: 10.1016/0006-2952(80)90448-7

58. Levine M, Law EY, Bandiera SM, Chang TK, Bellward GD. In vivo cimetidine inhibits hepatic CYP2C6 and CYP2C11 but not CYP1A1 in adult male rats. *J Pharmacol Exp Ther*. 1998; 284(2): 493-499.

59. Klotz U, Reimann IW. Drug Interactions through binding to cytochrome P450: The experience with H2-receptor blocking agents. *Pharm Res*. 1984; 1(2): 59-62.doi: 10.1023/A:1016347229923

60. Schmider J, Greenblatt DJ, Fogelman SM, Von Moltke LL, Shader RI. Metabolism of dextromethorphan in vitro: Involvement of cytochromes P450 2D6 and 3A3/4, with a possible role of 2E1. *Biopharm Drug Dispos*. 1997; 18(3): 227-240. doi: 10.1002/(SICI)1099-081X(199704)

61. Lin LY, Di Stefano EW, Schmitz DA, et al. Oxidation of methamphetamine and methylenedioxymethamphetamine by CYP2D6. *Drug Metab Dispos*. 1997; 25(9): 1059-1064.

62. Wu D, Victoria Otton S, Inaba T, Kalow W, Sellers EM. Interactions of amphetamine analogs with human liver CYP2D6. *Biochem Pharmacol*. 1997; 53(11): 1605-1612. doi: 10.1016/S0006-2952(97)00014-2

63. Sorkin E, Darvey D. Review of cimetidine drug interactions. *Drug Intell Clin Pharmacol*. 1983; 17(2): 110-120.

64. Taavitsainen P, Kiukaanniemi K, Pelkonen O. In vitro inhibition screening of human hepatic P450 enzymes by five angiotensin-II receptor antagonists. *Eur J Clin Pharmacol*. 2000; 56(2): 135-140. doi: 10.1007/s002280050731

65. Yamamoto T, Suzuki A, Kohno Y. Application of microtiter plate assay to evaluate inhibitory EŠects of various compounds on nine cytochrome P450 isoforms and to estimate their inhibition patterns. *Drug Metab Pharmacokinet*. 2002; 17(5): 437-448. doi: 10.2133/dmpk.17.437

66. de la Torre R, Yubero-Lahoz S, Pardo-Lozano R, Farré M. MDMA, methamphetamine, and CYP2D6 pharmacogenetics: What is clinically relevant? *Front Genet*. 2012; 3(NOV): 1-8. doi: 10.3389/fgene.2012.00235

67. Orishiki M, Matsuo Y, Nishioka M, Ichikawa Y. In vivo administration of H2 blockers, cimetidine and ranitidine, reduced the contents of the cytochrome P450IID (CYP2D) subfamily and their activities in rat liver microsomes. *Int J Biochem*. 1994; 26(6): 751-758. doi: 10.1016/0020-711X(94)90104-X

68. Martinez C, Albet C, Agundez JA, et al. The genetic polymorphisms of drug-metabolizing enzymes are a common determinant of interindividual differences in the therapeutic effect and toxicity of many drugs. *Clin Pharmacol Ther*. 1999; 65(4): 369-376. doi: 10.1016/S0009-9236(99)70129-3

69. Rendić S. Drug interactions of H2-receptor antagonists involving cytochrome P450 (CYPs) enzymes: From the laboratory to the clinic. *Croat Med J*. 1999; 40(3): 357-367.

70. Ruffalo R, Thompson J, Segal J. Diazepam-cimetidine drug interaction: A clinically significant effect. *South Med J*. 1981; 74(9): 1075-1078.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-120

71. Dostalek M, Jurica J, Pistovcakova J, et al. Effect of methamphetamine on cytochrome P450 activity. *Xenobiotica*. 2007; 37(12): 1355-1366. doi: 10.1080/00498250701652877

72. Dostalek M, Hadasova E, Hanesova M, et al. Effect of methamphetamine on the pharmacokinetics of dextromethorphan and midazolam in rats. *Eur J Drug Metab Pharmacokinet*. 2005; 30(3): 195-201. doi: 10.1007/BF03190620

73. Yang PP, Huang EYK, Yeh GC, Tao PL. Co-administration of dextromethorphan with methamphetamine attenuates methamphetamine-induced rewarding and behavioral sensitization. *J Biomed Sci*. 2006; 13(5): 695-702. doi: 10.1007/s11373-006-9096-4

74. Kreth KP, Kovar KA, Schwab M, Zanger UM. Identification of the human cytochromes P450 involved in the oxidative Metabolism of "Ecstasy" - related designer drugs. *Biochem Pharmacol*. 2000; 59: 1563-1571. doi: 10.1016/S0006-2952(00)00284-7

75. Meyer MR, Peters FT, Maurer HH. The role of human Hepatic cytochrome P450 isozymes in the metabolism of racemic 3, 4-methylenedioxy- methamphetamine and its enantiomers. *Pharmacology*. 2008; 36(11): 2345-2354. doi: 10.1124/dmd.108.021543

76. Downey C, Daly F, O'Boyle KM. An in vitro approach to assessing a potential drug interaction between MDMA (ecstasy) and caffeine. *Toxicol In Vitro*. 2014; 28(2): 231-239. doi: 10.1016/j.tiv.2013.10.021

77. Shin EJ, Nah SY, Kim WK, et al. The dextromethorphan analog dimemorfan attenuates kainate-induced seizures via sigma1 receptor activation: comparison with the effects of dextromethorphan. *Br J Pharmacol*. 2005; 144(7): 908-918. doi: 10.1038/sj.bjp.0705998

78. Shin EJ, Nah SY, Chae JS, et al. Dextromethorphan attenuates trimethyltin-induced neurotoxicity via σ1 receptor activation in rats. *Neurochem Int*. 2007; 50(6): 791-799. doi: 10.1016/j.neuint.2007.01.008

79. Benetton SA, Fang C, Yang Y-O, et al. P450 Phenotyping of the metabolism of selegiline to desmethylselegiline and methamphetamine. *Drug Metab Pharmacokinet*. 2007; 22(2): 78-87. doi: 10.2133/dmpk.22.78

80. Cheng J, Zhen Y, Miksys S, et al. Potential role of CYP2D6 in the central nervous system. *Xenobiotica*. 2013; 43(11): 973-984. doi: 10.3109/00498254.2013.791410

81. Haduch A, Bromek E, Daniel WA. Role of brain cytochrome P450 (CYP2D) in the metabolism of monoaminergic neurotransmitters. *Pharmacol Rep*. 2013; 65(6): 1519-1528.

82. Ramamoorthy Y, Yu A, Suh N, Haining RL, Tyndale RF, Sellers EM. Reduced (±)-3,4-methylenedioxymethamphetamine ("Ecstasy") metabolism with cytochrome P450 2D6 inhibitors and pharmacogenetic variants in vitro. *Biochem Pharmacol*. 2002; 63(12): 2111-2119. doi: 10.1016/S0006-2952(02)01028-6

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-121

Research

*Corresponding author
Balik Dzhambazov, PhD
Professor
Department of Developmental Biology
Plovdiv University
24 "Tsar Assen" Str., Plovdiv 4000
Bulgaria
Tel. +359-32-261535
Fax: +359-32-261-566
E-mail: balik@uni-plovdiv.bg

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2121

Article History
Received: September 15th, 2017
Accepted: September 26th, 2017
Published: September 27th, 2017

Citation
Batsalova T, Teneva I, Belkinova D, Stoyanov P, Rusinova-Videva S, Dzhambazov B. Assessment of cadmium, nickel and lead toxicity by using green algae *Scenedesmus incrassatulus* and human cell lines: Potential in vitro test-systems for monitoring of heavy metal pollution. *Toxicol Forensic Med Open J*. 2017; 2(2): 63-73. doi: 10.17140/TFMOJ-2-121

Assessment of Cadmium, Nickel and Lead Toxicity by Using Green Algae *Scenedesmus Incrassatulus* and Human Cell Lines: Potential *In Vitro* Test-Systems for Monitoring of Heavy Metal Pollution

Tsvetelina Batsalova, PhD[1#]; Ivanka Teneva, PhD[2#]; Detelina Belkinova, PhD[2,3]; Plamen Stoyanov, PhD[2,4]; Snezhana Rusinova-Videva, PhD[2]; Balik Dzhambazov, PhD[1*]

[#]These authors have contributed equally

[1]Department of Developmental Biology, Plovdiv University, Plovdiv 4000, Bulgaria

[2]Department of Botany, Plovdiv University, Plovdiv 4000, Bulgaria

[3]Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

[4]Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University of Plovdiv, Plovdiv 4000, Bulgaria

ABSTRACT

Background: Pollution of aquatic ecosystems with heavy metals leads to decrease of the biodiversity and accumulation of toxicants in the food chain. Species of the genus *Scenedesmus* are sensitive indicators of environmental changes and have been used for the evaluation of risk factors for contamination of aquatic ecosystems. The microalga *Scenedesmus incrassatulus* can remove chromium and cadmium from the growth medium. Also, mammalian cell lines are another type of test system that has been used to study the mechanisms of heavy metal toxicity. However, little is known about the sensitivity and potential application of different human cell lines for bio-monitoring of heavy metal contamination.

Aim: To investigate the toxicity of increasing concentrations of cadmium, nickel and lead on the green microalga *Scenedesmus incrassatulus* and the human cell lines HeLa, A549, FL, and Caco-2.

Materials and Methods: To evaluate the toxic effects of Cd, Ni, and Pb, two test systems were used: an algal culture of *S. incrassatulus* and four human cell lines. For the algal system, the growth of the algae and the features such as "cell number in the coenobium/single cells", "position of the inner cells in the coenobium", and "shape of the peripheral cell", were assessed. For the human cell cultures, the methyl-thiazol-tetrazolium (MTT) and neutral red assays were performed. In both the systems, the effects were measured at different time points (24, 48, and 72 hours) of treatment.

Results: The experimental observations showed that lead exposure in maximal permissible levels (MPL) inhibited the growth of the green algae *S. incrassatulus*, while cadmium had a stimulating effect even at lower test concentrations. Cadmium and nickel treatment affected the morphological features "cell number in the coenobium/single cells" and "position of the inner cells in the coenobium". Regarding "shape of the peripheral cell", lead had the most significant effect after 24 h of treatment, which was expressed in reduction of the type "incrassatulus" compared to the type "obliquus". Cytotoxic effects of the heavy metals were also observed for all tested human cell lines. HeLa, FL and Caco-2 were most sensitive to cadmium compared to lead, while A549 cells showed equal sensitivity to all three heavy metals.

Conclusion: The reported data presented specific impacts on the studied parameters for both the test-systems. The present study concluded that the green alga *S. incrassatulus* could be used as an effective test system for the biomonitoring of lead pollution. The cell line A549 can serve as a sensitive test system for the presence of cadmium, nickel and lead.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

KEY WORDS: Cadmium; Lead; Nickel; *In vitro* cytotoxicity; Biomonitoring; *Scenedesmus incrassatulus*; Human cell lines.

ABBREVIATIONS: MPL: Maximal Permissible Levels; MTT: methyl-thiazol-tetrazolium; IARC: International Agency for Research on Cancer; NR: Neutral Red; DNA: Deoxyribonucleic acid.

INTRODUCTION

Heavy metals are the main contaminants of soil and water basins. Their high concentration in nature affects the biodiversity and poses a serious threat to human health.[1,2] Anthropogenic activity is the general cause underlying the constantly increasing levels of different toxicants including heavy metals. The rapid development of the industry is an important factor attributed to the elevated concentrations of heavy metals in the environment. Direct disposal of industrial wastewater from factories associated with the production of fertilizers, textile, batteries, paper, installations for metal covering and mining, is responsible for causing a significant percent of the pollution on account of heavy metal contamination. In contrast to the organic pollutants, heavy metals are not biodegradable and accumulate in living organisms. Because of their high degree of toxicity, these elements rank among the priority metals that are of great significance to public health. These are all systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. Ions of many known heavy metals (zinc, copper, nickel, mercury, cadmium, lead, chromium) are carcinogenic. According to the International Agency for Research on Cancer (IARC), these metals are also classified as either "known" or "probable" human carcinogens based on existing research. Epidemiological and experimental studies demonstrate an association between heavy metal exposure and incidence of cancer in humans.[3] Undoubtedly, these pollutants pose a serious threat that needs to be resolved and scientists are actively working to find solutions.[4] It is necessary to develop sensitive methods for biomonitoring of heavy metals in the environment in addition to the conventional chemical analysis because essential parameters like bioavailability, cytotoxicity and genotoxicity can be examined only in live cells.

There is a growing interest in the application of algae for the elimination of organic and inorganic contaminants. Biotechnological tools and techniques based on the activity of growing algae cultures could be applied effectively in the process of heavy metal extraction from industrial and wastewater. The ability of microalgae, including species from the genus *Scenedesmus*, to remove heavy metals from the environment by accumulating these chemicals is currently being investigated.[5-7]

On the other hand, exposition to heavy metals induces synthesis of specific products (pigments, lipids, phytohormones, exopolymers and others) in some algal species. Stimulation of such production depends on many factors: the type of heavy metal and its concentration, strain specificity, culture conditions and growth medium composition.[8] A promising new approach is the combination of processes of heavy metal removal and biosynthesis of specific products of interest induced by heavy metals exposition. Many studies are directed to explore the microalgae phenotypic plasticity in response to the exposition to heavy metals. Their general objective is to clarify the potential application of microalgae as bioindicators of this type of pollution.[9-13] Species from the genus *Scenedesmus* (Chlorophyta) are widely spread in freshwater basins and are convenient test-objects for scientific research including the evaluation of the impact of heavy metals.[14-16] The algal growths, morphology, pigment content, photosynthetic and biochemical activities are affected by heavy metals.

The potential application of algal cultures for the detection of heavy metal pollution is unambiguous. Investigation of specific responses of different algal strains against grading concentrations of heavy metals is needed to enhance our knowledge on algal test-systems and apply it in some biotechnological manufactured products.

In addition to algal cultures, mammalian cell lines present a useful research model for the examination of the mode of action of toxic agents and monitoring their levels. The use of this model organism allows for the visualization of the uptake and distribution of heavy metals, identification of cellular targets and metabolic processes that are affected by the toxicants. Also, it facilitates the examination of the level of response to the toxicant concentration and thus, could serve as a tool for biomonitoring environmental pollution. In cell culture systems, the humoral, neural and other control mechanisms are absent, thus, an examination of the concentration gradient and secondary effects of bioaccumulation are not applicable. Cell lines constitute a homogenous system comprised of specific cell types allowing for the direct investigation of different cellular processes under well-defined and reproducible experimental conditions excluding secondary effects induced in specific locations of the whole organism.[17] Mammalian cell lines have been used to analyze the target organelles and cellular molecules for heavy metal toxicity.[18] Heavy metals like nickel (Ni), lead (Pb) and cadmium (Cd) have been shown to induce reactive oxygen species production and oxidative stress in cultured mammalian cells.[19-21] Different types of heavy metal ions have been found to interact with deoxyribonucleic acid (DNA) and nuclear proteins eliciting conformational changes and damage to the key cellular molecules. Overall, these processes affect all the phases of cell cycle but mostly the synthetic phase[17] leading to carcinogenesis or apoptosis.[18,22] Depending on the studied cell type, specific effects of heavy metals at the cellular level can be elucidated using different cell lines.[3]

In the present study, the cytotoxic and growth inhibitory effect of cadmium, nickel and lead were analyzed using the two assays—methyl-thiazol-tetrazolium (MTT) test which

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

is based on the ability of metabolically active mitochondria to reduce tetrazolium salts, and Neutral red (NR) assay used to assess cell membrane integrity and cellular viability. These assays were standardized in numerous studies and were used to assess cytotoxicity.[23-25]

The discussed experiments aimed to investigate the impact of increasing concentrations of cadmium, nickel and lead on the green alga *Scenedesmus incrassatulus* and the human cell lines HeLa, A549, FL, and Caco-2. The choice of these two test-systems allowed for the comparison of the effects of heavy metals on the density and growth of the plant and mammalian cells. The observed results raised interesting conclusions about the influence of Cd, Ni and Pb on the morphology of *S. incrassatulus*, the affected physiological processes and their targeted structures in both plant and human cells and elucidated their potential application like tools for biomonitoring of heavy metal pollutants as lead, cadmium or nickel .

MATERIALS AND METHODS

Algal Culture and Exposition to Different Concentrations of Heavy Metals

Scenedesmus incrassatulus BOHLIN used in the present study was obtained from the algological collection of the Plovdiv University (Plovdiv Algal Culture Collection, PACC). The algal strain was stored under the reference number PACC 7069. The algae were grown in BBM1-Bold`s Basal Medium[26] under optimal growth conditions for the species: 28 °C temperature and light regimen 15:9 hours (light:dark period). A 72-hours intensive cultivation was conducted for the experiment involving exposition to heavy metals. *Scenedesmus incrassatulus* cultures were grown in BBM1 medium containing different concentrations of Cd^{2+}, Ni^{2+} and Pb^{2+}. Stock solutions of nitrate salts of each heavy metal ($Cd(NO_3)_2$, $Ni(NO_3)_2$, $Pb(NO_3)_2$) were used. The heavy metal concentrations were selected according to the maximal permissible levels (MPL) established by the Bulgarian law (in accordance with the National regulation for standards of environmental quality for priority substances and some other contaminants), which was based on the European legislation for water quality.[27] 100%, 75%, 50% and 25% of the MPL for cadmium, lead and nickel were tested in the experiments. Overall, 13 main samples were assayed: control with standard growth medium; growth medium with 0.45 µg/L Cd^{2+} (corresponding to maximum permissible level /MPL/); growth medium containing 0.3375 µg/L Cd^{2+} (corresponding to 75% MPL); growth medium with 0.225 µg/L Cd^{2+}(50% MPL); growth medium containing 0.1 µg/L Cd^{2+} (25% MPL); growth medium with 20 µg/L Ni^{2+} (corresponding to MPL); growth medium with 15 µg/L Ni^{2+} (75% MPL); growth medium containing 10 µg/L Ni^{2+} (50% MPL); growth medium with 5 µg/L Ni^{2+} (25% MPL); growth medium with 7.2 µg/L Pb^{2+} (corresponding to MPL); growth medium containing 5.4 µg/L Pb^{2+} (75% MPL); growth medium with 3.6 µg/L Pb^{2+} (50% MPL); growth medium containing 1.4 µg/L Pb^{2+} (25% MPL). All tested concentrations represent the free heavy metal content corrected from the nitrate formula weight.

All experimental variants were assayed in Ackerman's vials with 25 ml algal suspension, consisting of 20 ml of growth medium or growth medium plus heavy metal followed by a 5 ml algal inoculum (Density of 200×10^4 cells/mL).

Measurement of Algal Growth

The growth of algal cultures was determined spectrophotometrically at three time points: 24 h, 48 h and 72 h. Extinction at 663 nm wavelength was measured using the Visible Spectrophotometer M107 (Spectronic Camspec Ltd., Leeds, UK).

Morphological Analysis of *Scenedesmus incrassatulus*

The morphological analysis was performed on a standard light microscope Magnum-T equipped with high definition digital camera Si-3000 and software (Medline Scientific, Chalgrove, Oxon, UK). The following taxonomical parameters were analyzed at a maximal magnification of 400x: number of cells in the coenobium/single cells, position of the inner cells in the coenobium, morphology of peripheral cells. One hundred coenobia/single cells were examined in order to determine the first taxonomical sign (number of cells). To evaluate the parameters "position of the inner cells" and "morphology of the peripheral cell", 50 coenobia were analyzed. The morphological variability of *S. incrassatulus* was documented using the digital camera.

Assessment of Cytotoxicity on Human Cell Lines *In Vitro*

MTT and NR assays were performed in order to evaluate the toxic effects of Cd^{2+}, Ni^{2+} and Pb^{2+}. Four different human cell lines were used for performing the experiments, namely: FL (ATCC CCL 62) derived from human amniotic cells; A549 (ATCC CCL 185) isolated from lung carcinoma; HeLa (ATCC CCL-2) established from cervical adenocarcinoma, and Caco-2 (ECACC 86010202) derived from colorectal adenocarcinoma. The cells were incubated in the presence of different concentrations of heavy metals corresponding to 100% MPL, 75% MPL, 50% MPL and 25% MPL for 24, 48 and 72 h at 37 °C, 5% CO_2 and high humidity. Stock solutions of nitrate salts of each heavy metal ($Cd(NO_3)_2$, $Ni(NO_3)_2$, $Pb(NO_3)_2$) were added to the cell culture medium (Dulbecco's modified Eagle's medium, DMEM) supplemented with 10% fetal calf serum and antibiotics (all from Sigma-Aldrich, Germany) to achieve the appropriate concentration of Cd^{2+}, Ni^{2+} and Pb^{2+} in the assayed samples. The control cells were incubated for the same time periods in supplemented DMEM devoid of heavy metals. All samples were assayed in triplicates.

All cell lines were expanded in 75 cm^2 culture flasks (TPP, Trasadingen, Switzerland) following which the cells were detached from the culture vessel. The resulting suspensions

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

were adjusted to 1×10^5 cells/ml concentration and the cells were seeded on 96-well plates (TPP, Trasadingen, Switzerland) using a 200 mL suspension/well. The cells were cultured in standard supplemented DMEM for 24 h at 37 °C, 5% CO_2 and high humidity. Then, the culture medium was replaced with complete DMEM containing 100%, 75%, 50%, 25% and 0% MPL of Cd, Ni or Pb. At the end of each test-period (24 h, 48 h, 72 h), MTT (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide) (Sigma-Aldrich, Germany) solution in a final concentration of 0.5 mg/ml was added to all samples. The cells were incubated for 3 h in a humidified incubator at 37 °C with 5% CO_2 content. During this period, live cells with functional mitochondria were able to reduce MTT to an insoluble formazan product. The quantity of accumulated formazan corresponded to the number of viable cells in the sample. Subsequently, the MTT containing medium was removed and 100 mL DMSO were pipetted into each test-well. The cells were incubated for 15 minutes at room temperature on a shaker in order to dissolve the accumulated formazan crystals in the cells. Absorbance was measured at 570 nm wavelength using Synergy-2 reader (BioTek, Winooski, VT, USA). The percent rate of inhibition of cell growth was calculated using the absorbance units from each test sample and the data from the control cells incubated in the absence of heavy metals.

For the Neutral red (NR) assay, FL, HeLa, A549 and Caco-2 cells were expanded, subcultured, seeded on 96-well plates and treated with heavy metals in the same way as described for the MTT assay. The NR stain bound to the lysosomal matrix only in live cells. Therefore, the NR assay was used to evaluate the cell culture viability and growth after treatment with the test-agent. In the described experiments, after 24, 48 and 72 hours treatment with heavy metals, the cells were stained with 0.5 mg/mL NR solution for 3 h at 37 ºC and high humidity. Then, the medium containing unbound stain was aspirated and 100 mL extracting solution (50% ethanol-1% acetic acid) was added to all samples. The culture plates were incubated for 15 min on a shaker and then absorption at 540 nm was measured using Synergy-2 reader (BioTek, Winooski, VT, USA). Similar to the MTT assay, percent inhibition was calculated based on the detected absorbance units.

Statistics

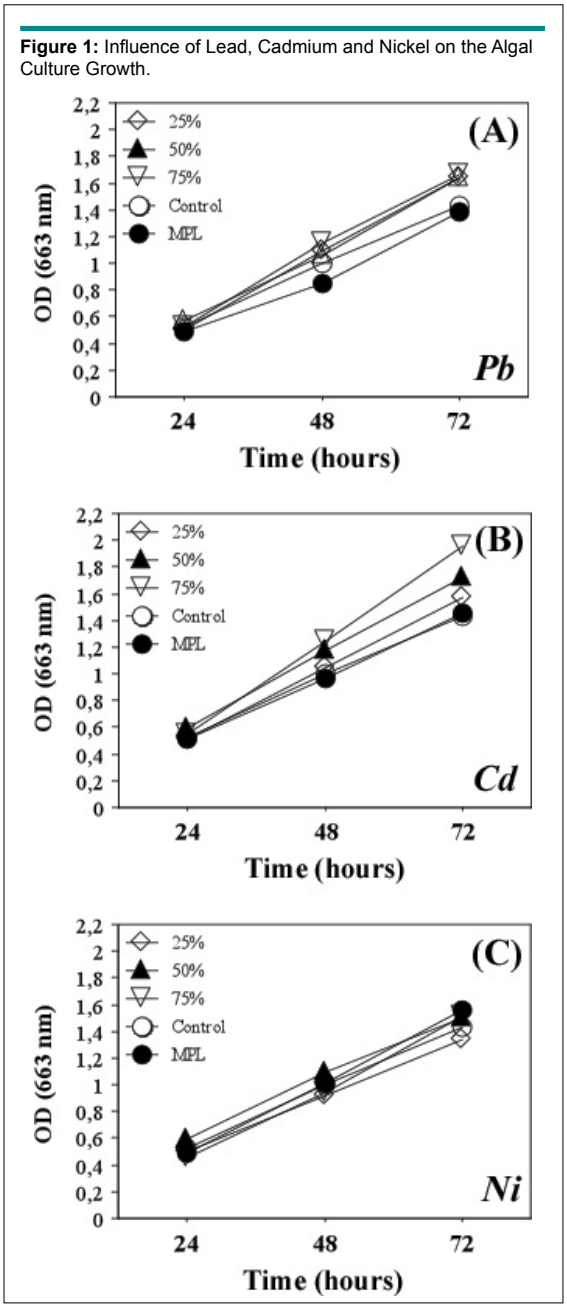
The non-parametric Mann-Whitney *U*-test was applied to determine statistically significant differences between the assayed samples using StatView software (SAS Institute, USA). The Mann-Whitney *U*-test compares the median of two groups of data, i.e., control group and treated group. It is used to test the null hypothesis whether two sample groups come from the same population/have the same median and it converts the scores on the continuous variable to ranks, across the two groups. Calculated *p* values lower than 0.05 were considered statistically significant.

RESULTS

Cd^{2+}, Ni^{2+} and Pb^{2+} Effects on the Growth and Development of *Scenedesmus incrassatulus*

The studied heavy metals exerted different effects on the growth of *S. incrassatulus*. The presence of lead in the culture medium in concentrations corresponding to MPL inhibited the growth and development of algae (Figure 1A). Low concentrations of cadmium (corresponding to 25%, 50% and 75% of MPL) had a stimulatory effect on *S. incrassatulus* growth (Figure 1B); however, all tested concentrations of nickel did not affect the density of the algal culture (Figure 1C).

Figure 1: Influence of Lead, Cadmium and Nickel on the Algal Culture Growth.



ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

Cd^{2+}, $Ni^{2+}$and Pb^{2+} Influenced the Morphology of *S. incrassatulus*

The changes in *S. incrassatulus* morphology were evaluated by analyzing the taxonomical characteristics "cell number in the coenobium/single cells", "position of the inner cells in the coenobium" and "shape of the peripheral cell".

S. incrassatulus reacted to the presence of cadmium and nickel by formation of single cells and reduction of the percent coenobia. These alterations were detected already 24 h after treatment with the heavy metals (Figure 2). The effect was stronger when the algal cultures were treated with 100% MPL concentrations of Cd (Figure 2B) and Ni (Figure 2C). Dissociation to single cells has been shown on Figure 3.

Figure 2: Reaction of *S. incrassatulus* Concerning the Taxonomical Sign "Cell Number in the Coenobium/Single Cells".

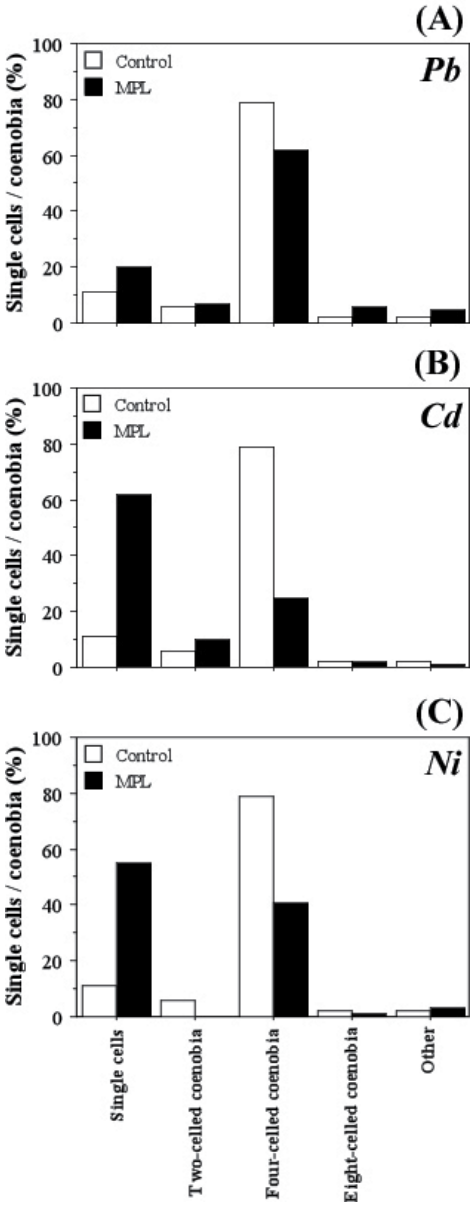
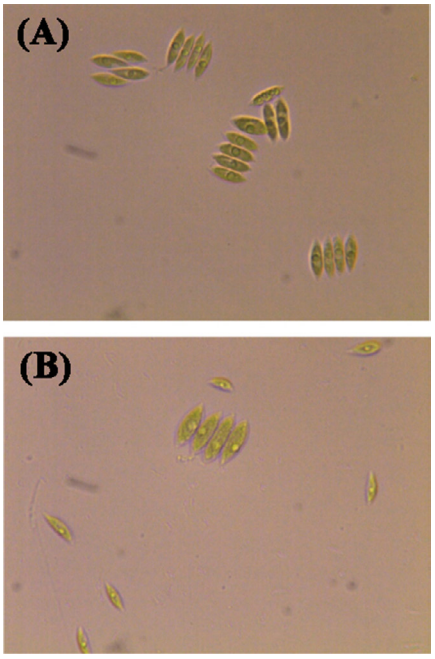


Figure 3: Dissociation of *S. incrassatulus* to Single Cells after Exposure to Cd. Control Culture (24 h) with Predominance of Coenobia (A); in Growth Medium with Cd^{2+} (MPL, 24 h) with Predominance of Single Cells (B).



Cadmium and nickel affected the arrangement of inner cells in coenobia, increasing the percentage of irregularly positioned inner cells compared to the linearly arranged cells. In the presence of nickel, this effect is observed even at 24 hours (Table 1).

The reaction of *S. incrassatulus* to MPL concentrations of cadmium and nickel 24 and 48 h after treatment concerning the sign "shape of the peripheral cell" led to the predominance of the typical species shape "*incrassatulus*" (Table 2). On the other hand, 24 h treatment with Pb reduced the type "*incrassatulus*" and led to an increase in the occurrence of the morphology type "*obliquus*". At longer exposition to Pb, percent cells with "other location" were detected while the type "*incrassatulus*" decreased (Table 2).

Treatment with Cd^{2+}, Ni^{2+} and Pb^{2+} Reduced the Growth and Viability of Human Cell Lines

MTT and NR assays were performed using different human cell lines. Accumulation of formazan and NR in the cells were measured after three time points (24, 48 and 72 h) of exposition to Cd^{2+}, Ni^{2+} and Pb^{2+}. The recorded results demonstrated significant cytotoxic effects of heavy metals, which were most prominent at the highest test-concentrations (100% MPL) of Cd^{2+}, Ni^{2+} and Pb^{2+} for all cell lines.

A549 cells showed well established dose-dependent response to treatment with different concentrations of heavy met-

TOXICOLOGY AND FORENSIC MEDICINE
Open Journal
Openventio PUBLISHERS
ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

Table 1: Reaction of *S. incrassatulus* Concerning the Taxonomical Sign "Arrangement of the Inner Cells".

Exposure time	Arrangement of internal cells	Control (%)	Lead	Cadmium	Nickel
			MPL (%)	MPL (%)	MPL (%)
24 h	Linearly	58	46	64	26
	Alternately	24	10	22	12
	Irregularly	18	44	14	62
48 h	Linearly	42	30	8	22
	Alternately	28	32	26	28
	Irregularly	30	38	66	50
72 h	Linearly	56	26	16	26
	Alternately	26	34	28	36
	Irregularly	18	40	56	38

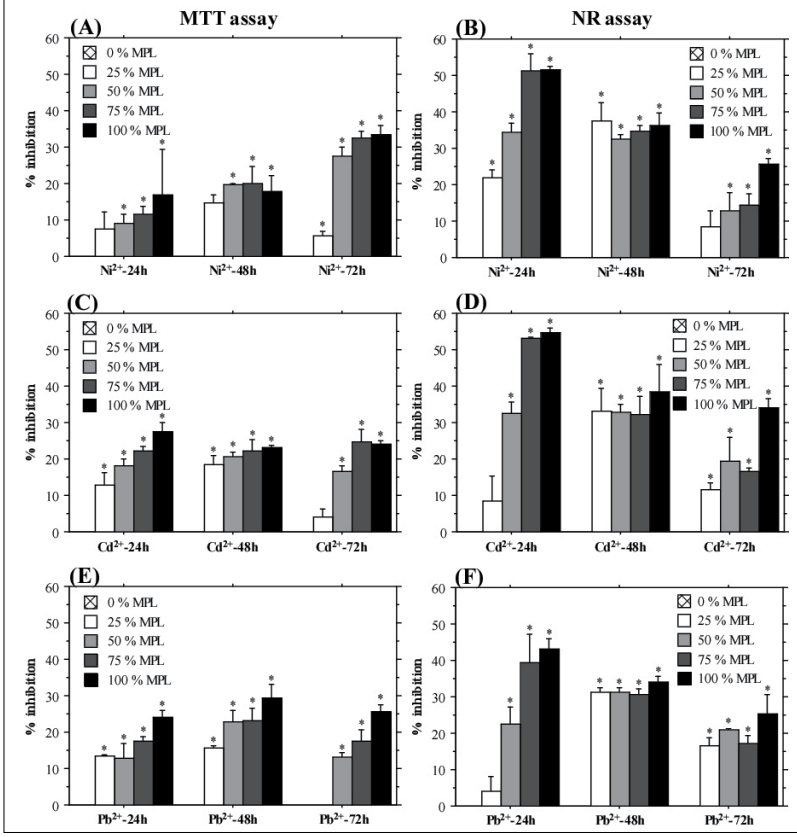
MPL: Maximal Permissible Levels; h: Hours.

Table 2: Reaction of *S. incrassatulus* Concerning the Taxonomical Sign "Shape of the Peripheral Cell".

Exposure time	Shape of peripheral cell	Control (%)	Lead	Cadmium	Nickel
			MPL (%)	MPL (%)	MPL (%)
24 h	Type"*incrassatulus*"	38	30	56	56
	Type"*obliquus*"	40	68	30	36
	Other type	22	2	14	8
48 h	Type"*incrassatulus*"	52	44	60	94
	Type"*obliquus*"	36	40	26	4
	Other type	12	16	14	2
72 h	Type"*incrassatulus*"	10	0	6	6
	Type"*obliquus*"	50	30	74	60
	Other type	40	70	20	34

MPL: Maximal Permissible Levels; h: Hours.

Figure 4: Inhibition of A549 Cells Growth after Treatment with Cadmium, Nickel and Lead. MTT Assay (A), (C), (E); NR Assay (B), (D), (F). The Graphs Display Mean±SE. *p<0.05.



ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

als (Figure 4). The highest percent inhibition of cell growth was determined after treatment with 100% and 75% MPL concentrations already at the shortest exposition period (24 h). The results from the NR assay demonstrated an interesting trend—higher inhibition of cell growth at 24 h test-period compared to the longer exposition times (48 and 72 h). On the other hand, the results from the MTT assay did not show time-dependent level of response. These data indicated a stronger effect of heavy metal exposition on cellular lysosomes, which was partly reduced by developed compensatory mechanisms when the cells were treated for a longer period. Conversely, such tendency was not observed in HeLa cells, which showed the highest percent inhibition and clear dose-dependent response after 72 h treatment with all three heavy metals when measured by NR assay (Figure 5). Caco-2 cells also displayed stronger inhibition after 72 h of exposition measured by MTT assay (Table 3). The NR assay did not show a time-dependent response. Overall, cadmium and lead treatment induced a stronger inhibition of Caco-2 cells compared to

Figure 5: Influence of Nickel, Cadmium and Lead Exposition on HeLa Cells. MTT Assay (A), (C), (E); NR Assay (B), (D), (F). The Graphs Show Mean±SE. *p<0.05.

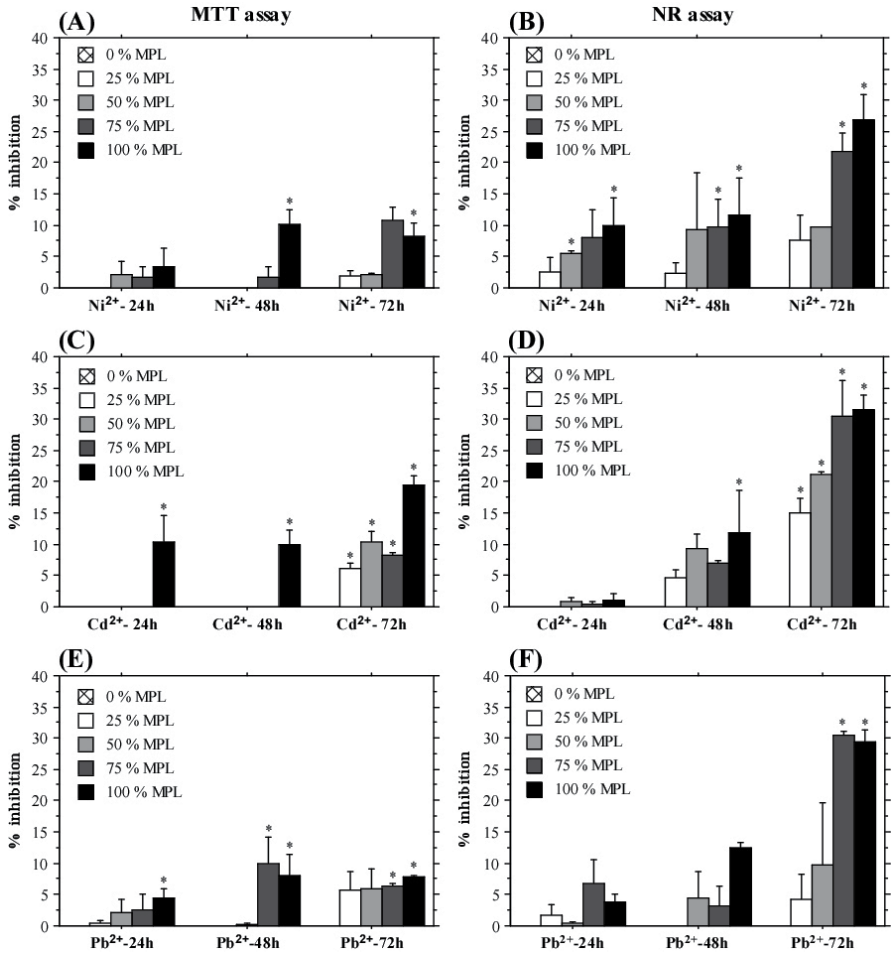


Table 3: Inhibition of Caco-2 Cells Growth Induced by Cadmium, Nickel and Lead Treatment. Data has been Displayed as Mean Percent Inhibition.

Caco-2		Nickel			Cadmium			Lead		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
MTT assay	100% MPL	12.94	8.74	22.16	12.59	11.19	38.92	12.59	12.24	28.41
	75% MPL	2.80	3.15	18.19	7.35	7.00	29.83	7.00	3.50	25.57
	50% MPL	2.45	7.70	15.63	6.65	10.49	21.31	8.50	2.45	25.86
	25% MPL	2.45	4.55	11.94	5.60	6.99	19.60	5.59	1.40	23.87
NR assay	100% MPL	24.60	30.67	21.09	34.20	40.10	31.96	34.60	22.48	31.96
	75% MPL	23.20	18.56	14.13	33.20	36.41	21.74	34.00	32.59	24.57
	50% MPL	22.60	11.92	10.87	29.20	18.63	18.27	27.60	0	21.52
	25% MPL	11.60	10.94	5.92	21.60	2.85	14.78	0	5.74	13.74

MPL: Maximal Permissible Levels; h: Hours; MTT: methyl-thiazol-tetrazolium; NR: Neutral Red.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

Table 4: Inhibitory Effects on FL Cell Line Determined after Exposition for Different Time-Periods to Nickel, Cadmium and Lead. Data has been Ddisplayed as mean Percent Inhibition.

FL		Nickel			Cadmium			Lead		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
MTT assay	100% MPL	5.29	7.28	4.97	18.81	5.55	9.15	16.18	9	4.05
	75% MPL	7.52	1.92	4.97	6.43	1.34	0.78	5.32	4.98	1.83
	50% MPL	6.10	1.34	4.18	6.43	0	2.16	4.59	1.34	3.01
	25% MPL	4.58	0	1.96	5.95	0	0.59	2.41	2.3	2.87
NR assay	100% MPL	13.82	15.3	10.78	14.91	19.09	35.18	14.07	10.75	23.25
	75% MPL	7.79	9.24	3.79	13.57	8.79	22.36	9.80	15.23	0
	50% MPL	6.53	0.76	6.59	14.91	5.00	19.96	11.56	9.09	1.30
	25% MPL	6.37	0.46	4.19	5.19	5.23	10.38	3.18	6.97	0

MPL: Maximal Permissible Levels; h: Hours; MTT: methyl-thiazol-tetrazolium; NR: Neutral Red.

treatment with nickel. Interestingly, only the MTT assay results showed time-dependent response with a prominent increase in percentage inhibition after 72 h exposition to heavy metals.

Table 4 presents the data from MTT and NR assays performed with FL cells. The recorded observations demonstrated a higher sensitivity of the NR assay. The highest percent inhibition was induced by exposition to cadmium. MPL concentrations of lead and cadmium induced significant inhibition after 24 h exposition measured by MTT assay, but this effect was not evident at longer test-periods. Therefore, heavy metal toxicity was predominantly directed to cellular lysosomes in the FL cell line similar to A549. Lead and nickel treatment resulted in lower inhibition of cell growth. Like HeLa cells, the strongest percent inhibition of FL and Caco-2 growth was measured after 48 and 72h exposition to Cd^{2+}. These results demonstrate higher sensitivity of HeLa, FL and Caco-2 cell to cadmium treatment, while A549 cells exhibited similar level of response to all three heavy metals.

DISCUSSION

The present paper demonstrated the specific effects of selected concentrations of cadmium, nickel and lead on the green algae *Scenedesmus incrassatulus* and four human cell lines. Exposition to Cd^{2+}, $Ni^{2+}$and Pb^{2+} led to a different influence on the growth and development of *S. incrassatulus*. Lead treatment inhibited the algal growth, exposition to cadmium-induced stimulatory effect, and neither of the tested nickel concentrations influenced the growth and development of algae. Data from existing reports discuss about the influence of heavy metals on species from the genus *Scenedesmus* which supported the observations of the study and suggested species specificity with respect to algal reaction to heavy metals. Mercury (Hg) in concentrations 2.5-5 mg/L inhibited completely the growth of *Scenedesmus acutus*.[15] Cadmium caused 50% inhibition of *Scenedesmus armatus* growth after 24 h exposition to concentrations ranging from 0.46 to 0.54 mg/L.[14] On the other hand, low concentrations of Cd and Pb induced stimulatory effects on the growth of *Scenedesmus quadricauda*, *Scenedesmus pectinatus* and *Scenedesmus acuminatus*.[9,12,16]

In some algal species, heavy metals influenced specific morphological characteristics. For example, *Scenedesmus bernardii* underwent morphological changes in response to Zn, Cd and Pb exposition: up to 82% dissociation to single cells after exposure to zinc, formation of unusual coenobia, extensive secretion of mucilage and clustering of cells and coenobia as a result of Cd and Pb treatment.[11]

The present study demonstrated the influence of three types of heavy metals on the morphology of *Scenedesmus incrassatulus* BOHLIN. The chosen test-object generated coenobia composed of 2, 4 or 8 cells. The inner cells in four- and eight-cellular coenobia acquired a serial, alternative or irregular orientation. The shape of the cells ranged from narrow to wide spindle-shaped, gradually thinning with sharp edges of the cells, bent to the center of the coenobium (more prominent in the outer cells compared to the inner ones). Intercellular connections spanned from 1/2 to 2/3 through the length of adjacent cells. The variation in the taxonomical characteristics of *S. incrassatulus* have been described previously.[28] A stable taxonomical sign for the discrimination of *S. incrassatulus* from other closely related species was the "shape of peripheral cells". Typical *S. incrassatulus* peripheral cells were spindle-shaped gradually thinning with pointed ends bent to the center of the coenobium. Except the shape of peripheral cells, the experiments conducted in the present study aimed to analyze two other taxonomical characteristics—number of cells in the coenobium/single cells and position of the inner cells in the coenobium.

Concerning the sign "cell number", *S. incrassatulus* reacted to the presence of cadmium and nickel which supported the formation of single cells and reduced the coenobia composition. This effect was evident even after 24 h of treatment, at MPL concentrations and was more pronounced with cadmium treatment (Figure 2). Similar reaction of *Scenedesmus incrassatulus* after exposition to divalent copper and cadmium was detected.[13] On the basis of related experiments, the divalent Cu and Cd-induced single cells commonly from four-cellular *Scenedesmus incrassatulus*, while being exposed to hexavalent chromium (Cr) led to the appearance of coenobia without sheath that corresponded to the formation of autospores.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

MPL concentrations of cadmium and nickel affected also the position of inner cells in the coenobium by increasing the percent of coenobia with irregularly positioned cells and decreasing the composition of coenobia with linearly positioned cells. In the presence of nickel, these changes appeared already after 24 h. These observations demonstrated that the heavy metals Cd and Ni influence the development of *S. incrassatulus* even after a short exposition (24 h) by varying the characteristics "cell number" and "position of inner cells in the coenobium". But these taxonomical traits can be influenced also by environmental factors and vary considerably. Therefore, these morphological reactions cannot serve as reliable markers specific to the presence of cadmium and nickel.

The morphology of peripheral cells of *S. incrassatulus* was influenced by the exposition to lead. The effect of lead was the strongest compared to the other two heavy metals. The peripheral cell type "*incrassatulus*" decreased already following 24 h of exposition and after 72 h at MPL concentration of Pb^{2+}, typical for the species in which peripheral cells were not present. This finding supported the conclusion that *Scenedesmus incrassatulus* manifested highest sensitivity to lead exposition, which negatively influenced the algal growth and significantly changed the morphology of the species in concentrations corresponding to MPL. Based on these data, *S. incrassatulus* can be recommended as a test-object for the presence of lead in fresh water basins.

A general objective of the present study was to suggest new sensitive test-systems for biomonitoring of Cd, Ni and Pb pollution that react to MPL and even lower than MPL concentrations of the heavy metals. Therefore, in addition to *Scenedesmus incrassatulus* other test-objects were also examined. Three cancer cell lines (HeLa, A549, Caco-2) and a cell line (FL) derived from normal amniotic cells were used to evaluate the effect of Cd, Ni and Pb on human cell growth *in vitro*. These experiments aimed to investigate whether the heavy metals exert cytotoxic and growth inhibitory effects on different cell lines and determine the most sensitive cell type.

Different assays were performed for the evaluation of cytotoxic effects *in vitro* – MTT assay, XTT assay, LDH assay, NR test, assays determining cell number and total protein content, etc. Several of these assays were compared and it was suggested that MTT and NR tests showed the highest sensitivity.[29] This accounted for the reason to choose NR and MTT assays for the evaluation of *in vitro* growth inhibitory and cytotoxic effects of heavy metals.

Mammalian cell lines have been extensively used to study the mechanisms of heavy metal toxicity[17,20,21,29] and data from *in vitro* cytotoxicity assays have been reported for the cell lines A549, HeLa and Caco-2 treated with different heavy metals and heavy metal compounds.[17,25,30] However, to date, evaluation of the inhibitory effect of MPL and lower than MPL heavy metals concentrations have not been reported and little is known on the potential use of human cell lines for detection of heavy metals. The results led to the conclusion that all tested cell lines (A549, HeLa, FL and Caco-2) could be used for detection of cadmium, nickel and lead MPL concentrations after 72 h of exposition. Among the four cell lines, A549 showed almost equal sensitivity to all three heavy metals, while the other cell lines were more sensitive to cadmium relative to lead. Moreover, A549 demonstrated the highest percent inhibition and this strong response was evident even at 24 h exposition to either Cd^{2+}, Ni^{2+} or Pb^{2+}. Therefore, it was concluded that A549 cell line showed the highest sensitivity to MPL concentrations of the three heavy metals compared to HeLa, FL and Caco-2 cell lines.

CONCLUSION

The present study provided an insight for the potential application of new test-systems (algal culture of *Scenedesmus incrassatulus* and human cell lines) for biomonitoring of pollution with cadmium, nickel and lead. The reported data demonstrated that these heavy metals have different effects on the tested cells, and these effects were dose and time-dependent. The cadmium, nickel and lead influenced different morphological features of the algal system during the exposure. Based on the reaction of the algal culture by changing the evaluated morphological features some of the heavy metal pollutants could be detected very early, especially when the reaction was metal-specific. All four tested human cell lines may be used as test-systems for assessment of heavy metal toxicity or for biomonitoring of pollution with cadmium, nickel and lead. The observations of the present study demonstrated that the cell line A549 was the most sensitive for the tested heavy metals.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. He M, Wang Z, Tang H. The chemical, toxicological and ecological studies in assessing the heavy metal pollution in Le An River, China. *Water Research.* 1998; 32(2): 510-518. doi: 10.1016/S0043-1354(97)00229-7

2. Narula P, Mahajan A, Gurnani C, Kumar V, Mukhija S. Microalgae as an indispensable tool against heavy metals toxicity to plants: A review. *Int J Pharm Sci Rev Res.* 2015; 31(1): 86-93.

3. Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ. Heavy metal toxicity and the environment. *EXS.* 2012; 101: 133-164. doi: 10.1007/978-3-7643-8340-4_6

4. Fu F, Wang Q. Removal of heavy metal ions from wastewaters: A review. *J Environ Manage.* 2011; 92(3): 407-418. doi: 10.1016/j.jenvman.2010.11.011

5. Franklin NM, Stauber JL, Lim RP, Petocz P. Toxicity of metal

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

mixtures to a tropical freshwater alga (*Chlorella* sp): The effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. *Environ Toxicol Chem.* 2002; 21(11): 2412-2422. doi: 10.1002/etc.5620211121

6. Pena-Castro JM, Martinez-Jeronimo F, Esparza-Garcia F, Canizares-Villanueva RO. Heavy metals removal by the microalga Scenedesmus incrassatulus in continuous cultures. *Bioresour Technol.* 2004; 94(2): 219-222. doi: 10.1016/j.biortech.2003.12.005

7. Shanab S, Essa A, Shalaby E. Bioremoval capacity of three heavy metals by some microalgae species (Egyptian Isolates). *Plant Signal Behav.* 2012; 7(3): 392-399. doi: 10.4161/psb.19173

8. Miazek K, Iwanek W, Remacle C, Richel A, Goffin D. Effect of metals, metalloids and metallic nanoparticles on microalgae growth and industrial product biosynthesis: A review. *Int J Mol Sci.* 2015; 16(10): 23929-23969. doi: 10.3390/ijms161023929

9. Belkinova D, Mladenov R. Influence of lead on the taxonomical characteristics of *Scenedesmus pectinatus* Mayen (Chlorophyta, Chlorococcales). *Scientific researches of the Union of Scientists in Bulgaria - Plovdiv, series B. Natural sciences and humanities.* 2003; IV: 158-163.

10. Dimitrova-Dyulgerova I, Belkinova D, Mladenov R. Influence of Cu^{2+} on the development and morphology of *Scenedesmus bernardii*. *Trav. Sci. Univ. Plovdiv, Plantarum.* 2007;40:79-88.

11. Dimitrova-Dyulgerova I, Ivanova D, Stoyanov P, Teneva I, Belkinova D. *Scenedesmus bernardii* G.M. Smith – a sensitive bioindicator for heavy metals in water. *Trav Sci Univ Plovdiv Plantarum.* 2011; 41(6): 125-140.

12. Mladenov R, Kiryakov I, Dimitrova I. Influence of lead on the morphosystematic parameters of *Scenedesmus acuminatus* (Chlorophyta, Chlorococcales). *Scientific researches of the Union of Scientists in Bulgaria - Plovdiv, series B. Natural sciences and humanities.* 2003; III: 96-101.

13. Pena-Castro JM, Martinez-Jeronimo F, Esparza-Garcia F, Canizares-Villanueva RO. Phenotypic plasticity in *Scenedesmus incrassatulus* (Chlorophyceae) in response to heavy metals stress. *Chemosphere.* 2004; 57(11): 1629-1636. doi: 10.1016/j.chemosphere.2004.06.041

14. Bascik-Remisiewicz A, Tukaj Z. Toxicity of inorganic cadmium salts to the microalga *Scenedesmus armatus* (Chiorophyta) with respect to medium composition, pH and CO_2 concentration. *Acta Physiologiae Plantarum.* 2002; 24(1): 59-65. doi: 10.1007/s11738-002-0022-8

15. Capolino E, Tredici M, Pepi M, Baldi F. Tolerance to mercury chloride in *Scenedesmus* strains. *Bio Metals.* 1997; 10(2): 85-94. doi: 10.1023/A%3A1018375005791

16. Starodub ME, Wong PTS, Mayfield CI. Short term and long term studies on individual and combined toxicities of copper, zinc and lead to *Scenedesmus quadricauda*. *Science of The Total Environment.* 1987; 63: 101-110. doi: 10.1016/0048-9697(87)90039-8

17. Fischer AB, Skreb Y. *In vitro* toxicology of heavy metals using mammalian cells: An overview of collaborative research data. *Arh Hig Rada Toksikol.* 2001; 52(3): 333-354. doi: 10.1007/s11738-002-0022-8

18. Wang S, Shi X. Molecular mechanisms of metal toxicity and carcinogenesis. *Molecular and Cellular Biochemistry.* 2001; 222(1/2): 3-9. doi: 10.1023/A:1017918013293

19. Das KK, Das SN, Dhundasi SA. Nickel, its adverse health effects & oxidative stress. *Indian J Med Res.* 2008; 128(4): 412-425.

20. Tchounwou PB, Ishaque AB, Schneider J. Cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells (HepG2) exposed to cadmium chloride. *Mol Cell Biochem.* 2001; 222(1-2): 21-28. doi: 10.1007/978-1-4615-0793-2_3

21. Yedjou CG, Tchounwou PB. N-acetyl-l-cysteine affords protection against lead-induced cytotoxicity and oxidative stress in human liver carcinoma (HepG2) cells. *Int J Environ Res Public Health.* 2007; 4(2): 132-137.

22. Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Arch Toxicol.* 2008; 82(8): 493-512. doi: 10.1007/s00204-008-0313-y

23. Combes RD, Balls M. Integrated testing strategies for toxicity employing new and existing technologies. *Altern Lab Anim.* 2011; 39(3): 213-225.

24. Walum E, Balls M, Bianchi V, et al. ECITTS: an integrated approach to the application of in vitro test systems to the hazard assessment of chemicals. *Altern Lab Anim.* 1992;20:406–428.

25. Zapor L. Evaluation of the toxic potency of selected cadmium compounds on A549 and CHO-9 cells. *Int J Occup Saf Ergon.* 2014; 20(4): 573-581. doi: 10.1080/10803548.2014.11077080

26. Archibald PA, Bold HC. Phycological Studies XI. The genus *Chlorococcum* MENEGHINI. Austin, Texas, USA: University of Texas; 1970:115.

27. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives. Directives 2000/60/EU and 2008/105/EU as regards priority substances in the field of water policy. *Official Journal of the European Union.* 2013; L 226: 1-17. Web site. http://www.fao.org/faolex/results/details/en/c/LEX-FAOC127344/. Accessed September 14, 2017.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-121

28. Belkinova D, Ilieva G. Variability of the sign "cell number" in *Scenedesmus incrassatulus* Bohl. (Chlorophyta). *Scientific researches of the Union of Scientists in Bulgaria - Plovdiv, series B. Natural sciences and humanities*. 2000; I: 333-336.

29. Fotakis G, Timbrell JA. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett.* 2006; 160(2): 171-177. doi: 10.1016/j.toxlet.2005.07.001

30. Calabro AR, Gazarian DI, Barile FA. Effect of metals on β-actin and total protein synthesis in cultured human intestinal epithelial cells. *J Pharmacol Toxicol Methods*. 2011; 63(1): 47-58. doi: 10.1016/j.vascn.2010.04.012

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-122

Research

*Corresponding author
Sujata Maiti Choudhury, PhD
Department of Human Physiology with Community Health
Vidyasagar University
Midnapore 721102, West Bengal, India
Tel. 9474444646
E-mail: sujata.vu2009@gmail.com

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2122

Article History
Received: October 17th, 2017
Accepted: December 14th, 2017
Published: December 15th, 2017

Citation
Das T, Ghosh R, Paramanik A, et al. Dose-dependent hematological, hepatic and gonadal toxicity of cypermethrin in Wistar rats. *Toxicol Forensic Med Open J*. 2017; 2(2): 74-83. doi: 10.17140/TFMOJ-2-122

Dose-Dependent Hematological, Hepatic and Gonadal Toxicity of Cypermethrin in Wistar Rats

Tuhina Das, MSc; Rini Ghosh, MSc; Anurag Paramanik, MSc; Ananya Pradhan, MSc; Surya Kanta Dey, MSc; Tamanna Roy, MSc; Debjani Chatterjee, MSc; Sujata Maiti Choudhury, PhD*

Department of Human Physiology with Community Health, Vidyasagar University, Midnapore 721102, West Bengal, India

ABSTRACT

Background: Pesticides are used frequently and may have various adverse effects on human health in different ways. Cypermethrin (CYP) is a synthetic type II pyrethroid pesticide that has been used extensively to control a wide variety of pests in agriculture, forestry, horticulture, and public health.
Objectives: This study aimed to investigate the dose-dependent hematological, hepatic and gonadal toxicity of CYP in mature male and female Wistar rats.
Methods: Rats were randomly divided into nine groups, different doses of CYP were administered for 14 consecutive days and different hematological, hepatic and gonadal parameters were assayed.
Results: Erythrocyte count, hemoglobin percentage, hepatic reduced glutathione (GSH) content and sperm count were significantly diminished. Serum aspartic and alanine transaminase, hepatic malondialdehyde (MDA), testicular cholesterol content were increased following CYP treatment in male rats at 40 and 80 mg/kg body weight (1/9 and 1/4.5 LD_{50}). Elevated ovarian cholesterol content and decreased 17β hydroxy steroid dehydrogenase (HSD) levels were also observed in CYP-exposed female rats at a dose level of 34.33 and 51.5 mg/kg body wt. (1/9 and 1/6 LD_{50}).
Conclusion: Taken together, CYP initiated hematological, hepatic and gonadal toxicity in mature male rats with a body weight of 40 mg/kg (1/9 LD_{50}) and gonadal toxicity in mature female rats with a body weight of 34.33 mg/kg (1/9 LD_{50}) and above.

KEY WORDS: Cypermethrin; Hematological; Serum aspartic and alanine transaminase; Testicular and ovarian cholesterol.

ABBREVIATIONS: CYP: Cypermethrin; SGOT: Serum glutamate-oxalacetate transaminase; SGPT: serum glutamate-pyruvate transaminase; GSH: Glutathione; MDA: Malondialdehyde; HSD: Hydroxy Steroid Dehydrogenase; EC: Emulsifiable Concentrate; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals.

INTRODUCTION

Synthetic pyrethroids have been widely used for more than three decades to manage pests in a variety of crops,[1] but they have become increasingly popular following a complete ban on the use of cholinesterase-inhibiting insecticides.[2,3] The pyrethroids constitute a high share of the insecticide market because their activity profiles indicate high efficiency, low mammalian and avian toxicity, and biodegradability.[4] Today, pyrethroids are used in agriculture, forestry, horticulture, public health, and are active ingredients of many insect-controlling products intended for indoor use.[3] Based on the symptoms generated in animals, pyrethroids fall under two distinct classes: Types I and II. Type I pyrethroids affect the sodium channels in nerve membranes and type II pyrethroids cause an even longer delay in the sodium channel inactiva-

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

tion leading to persistent depolarization of the nerve membrane without monotonous discharge. The pyrethroids are more hydrophobic in nature,[5] and their target site of action is the biological membrane. In addition, type II pyrethroids affect the central nervous system (CNS), while type I affects the peripheral nerves.[6] To analyze the effects of suspected toxic substances, hemato-biochemical studies are essential. Recent studies have reported that pyrethroids lead to significant alterations as was observed on the basis of hematological findings.[7] Moreover, the liver, being the primary site for pyrethroid metabolism ultimately led to liver damage, and was found to accumulate large amounts of pyrethroid residues.[8] Several investigations have reported that pesticides adversely affect testicular functions in experimental animals[6,9-11] and function as potent endocrine disrupters.[12,13] Few reports have demonstrated the induction of oxidative stress by pyrethroids.[12-15] CYP is an insecticide that belongs to the family of synthetic pyrethroids. Its chemical formula is alpha-cyano-3-phenoxybenzyl ester of 2,2 dimethyl-3- (2,2 dichlorovinyl)-cyclopropane carboxilic acid,, the most widely used type II pyrethroid pesticides. It is commonly used to control moths and pests of cotton, soyabean and other crops. It was believed that CYP showed low mammalian toxicity so it was also used for controlling household pests. However, a number of studies have proven that CYP and other pyrethroids have hepatotoxic, carcinogenic, neurotoxic, and immunosuppressive potential in mammals.[16-18] Synthetic pyrethroids also have the ability to disrupt biochemical, hematological, and reproductive parameters.[19] The toxicity of pyrethroid insecticides in mammals have received great attention in the recent years because animals exposed to these insecticides have exhibited changes in their physiological activities besides other pathological features.[20] Oxidative stress and reactive oxygen species so produced in response to stress, as well as endocrine disturbance are among the most significant effects of pesticide toxicology.[21-25] CYP may also induce oxidative stress and endocrine disturbance in organisms. Giray et al[14] also reported that oral administration of CYP to rats induce significant oxidative stress in cerebral and hepatic tissues. Recently, it was proven that exposure to CYP during lactation significantly decreased the layer of spermatogenic cells, increased the inside diameter of seminiferous tubules, and disturbed the array of spermatogenic cells in the testes of pups on postnatal day 21.[26]

For any type of pesticide, the exposure concentration is important in analyzing the variation of its toxicity. So, in this piece of work, Wistar rats were exposed to different concentrations of CYP to study the impact of CYP on hematological, hepatic and gonadal parameters in rats.

MATERIALS AND METHODS

Chemicals and Reagents

A commercial formulation of cypermethrin [(RS) -α -cyano-3-phenoxybenzyl (1RS)-cis-trans-3-(2,2-dichlorovinyl) 2,2-dimethylcyclopropanecarboxylate] 10% emulsifiable concentrate (EC), named ''Ustad'' (United Phosphorus Limited) was used in the experiments. H_2O_2, red blood cell dilution fluid, white blood cell dilution fluid, Drabkin's diluents, aspartic acid, α-ketoglutaric acid, NaOH, DL-alanine,2,4-dinitrophenylhydrazine hydrochloride (DNPH), sodium dodecyl sulfate (SDS), thiobarbituric acid, n-Butanol-pyridine, acetate buffer, sulfosalicylic acid, dithionitrobenzoic acid (DTNB), HCl, Tris-HCl, para-nitrophenol phosphate, $ZnSO_4$, ferric chloride, glacial acetic acid, cholesterol, NaCl, phosphate buffer, Na_2HPO_4, KH_2PO_4, NaH_2PO_4, NaOH, DL-alanine, α-Ketoglutaric acid, glycerol, ethylenediaminetetraacetic acid, pentobarbital sodium, TNaPP, nicotinamide adenine dinucleotide, testosterone, dehydroepiandrosterone, and other chemicals were procured from Sigma-Aldrich, St. Louis, MO, USA; Merck India, Ltd., Mumbai, India and Himedia India, Ltd., Mumbai, India.

Animal Care and Maintenance

For the present study, mature Wistar male and female albino rats (weighing 130-150 g) were taken and the animals were accommodated in labeled cages with solid plastic sides and stainless-steel grid tops and floors, in a temperature controlled room (approximately 25±2 °C), and light cycle (12 h light, 12 h dark). The rats were acclimatized for one week prior to the different modes of treatment. Animals were fed a standard laboratory pellet diet and water ad libitum. This study was permitted by the Institutional Animal Ethical Committee (IAEC), registered under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Experimental Design

This study was undertaken in accordance to the OECD-423 guidelines. Mature male and female rats were divided into nine groups where each group contained six animals. Group I was designated as the control and Groups II to IX were CYP-treated, were administered with 1/11, 1/10, 1/9, 1/7, 1/5, 1/4.5, 1/4 and 1/3 LD_{50} of CYP-EC respectively. According to the Food and Agriculture Organization (FAO) report,[27] an oral LD_{50} dose of CYP for male and female rats were of the body weight 360 and 309 mg/kg respectively.[28] Following the oral treatment of CYP for 14 consecutive days, all animals were anesthetized with pentobarbital sodium on the 15th day and sacrificed by cervical dislocation. Tissue samples (liver, testis and ovary) were collected and stored at -80 ^{0}C until analysis. For sperm collection, epididymis was collected and washed immediately.

Blood Withdrawal Technique

Blood samples were drawn from hepatic vein of all animals using a syringe and collected in graduated centrifuge tubes containing anticoagulant ethylenediaminetetraacetic (EDTA) for the estimation of hematological parameters. For the estimation of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT), serum was prepared

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

from blood which was not treated with EDTA.

Study on Body Weight

During the treatment schedule, the body weight of each animal that was put under fasting overnight was measured regularly before each treatment. Following 24 h of the last dose, the weight of all the animals were recorded, following which they were sacrificed.

Study of Haematological Parameters in Male Rat

Blood cell count and Hb%: The total erythrocyte count was recorded after diluting the blood sample at 1:200 with the RBC dilution fluid. The total RBC counts[29] were recorded using the Neubaur hemocytometer and expressed as $x10^6$ mm^{-3}. Blood was diluted in a ratio of 1:20 with the WBC dilution fluid. Using Neubaur hemocytometer, the total count for leukocytes was recorded,[29] and the counts were expressed as $x10^3$ mm^{-3}.

Using the cyanomethemoglobin method,[30] the hemoglobin percentage was measured. 20 µl of blood and 5 ml of Drabkin's solution were added in a test tube and the optical density was measured at 540 nm.

Estimation of serum glutamate-oxalacetate transaminase (SGOT) and serum glutamate-pyruvate transaminase (SGPT): Briefly, to 1.0 ml of the buffered substrate (200 mM/L of DL-aspartate and 2 mM/L of α-ketoglutarate, pH=7.4), 0.1 ml of serum was added and incubated at 37 °C for 1 h. Then, 1 ml of dinitrophenyl hydrazine (DNPH) was added and kept for 20 minutes at room temperature. After 20 minutes, 10 ml of 0.4 N sodium hydroxide (NaOH) was added, and the color intensity was measured at 520 nm in a spectrophotometer (UV-Shimadzu-245, Japan) after 10 minutes against the blank. For the estimation of SGPT, to 1.0 ml of the buffered substrate (200 mM/L of DL-alanine and 2 mM/L of α- ketoglutarate pH=7.4), 0.1 ml of serum was added and incubated at 37 °C for 1 h. The reaction was arrested by the addition of 1.0 ml of DNPH and left aside for 20 min at room temperature. The color developed by the addition of 10 ml of 0.4N NaOH was read at 520 nm in spectrophotometer (UV-Shimadzu-245, Japan)[31] against the blank. The enzyme activity in serum was expressed as IU/lit.

Estimation of Malondialdehyde (MDA)

Malondialdehyde estimation was also performed according to the modified method of Ohkawa et al.[32] Briefly, 1 ml of the sample was mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetate buffer (pH-3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid and the mixtures were boiled for 60 min at 95 ˚C. The red pigment produced after heating was extracted with 5 ml of n-butanol-pyridine (15:1) mixture and centrifuged at 5000 rpm for 10 min at room temperature. The absorbance of the supernatants was recorded at 535 nm.

Assay of Reduced Glutathione

Reduced glutathione estimation was performed using the method of Griffith.[33] First, 100 µl of sulfosalicylic acid was mixed with 200 µl of liver homogenate, and the mixture was centrifuged for 10 min at 3000 rpm. Then, 1.8 ml of DTNB was added to the supernatant and was shaken well. The reading was taken at 412 nm. The glutathione level in tissue was expressed as µg/mg protein.

Sperm Count

Sperm suspension from the caudal epididymis was diluted using a sperm dilution fluid and added to the counting chamber. The count of spermatozoa was recorded[34] using a hemocytometer under the light microscope. The sperm concentration was expressed as $x10^6$ ml^{-1}.

Determination of Testicular and Ovarian Cholesterol

Tissue homogenate (20 mg/ml) was prepared with 0.5% $FeCl_3$ solution and was centrifuged at 2000 rpm to collect the supernatant. Next, to 0.1 ml of supernatant, 6 ml of glacial acetic acid was added to prepare the sample. Simultaneously, 5.9 ml of glacial acetic acid was also taken in a separate test tube with 0.1 ml of the working standard (0.2 mg/ml) and 0.1 ml of distilled water was used to prepare the standard. The blank was prepared by mixing 6 ml of glacial acetic acid and 0.1 ml of distilled water in another test tube. Then, 4 ml of colored reagents were added to each test tube and mixed vigorously, and was allowed to stand for 20 minutes for the spectrophotometric reading at 570 nm against the blank.[35]

Estimation of testicular Δ^5 3β-hydroxy steroid dehydrogenase (Δ^5, 3β-HSD): The estimation of testicular Δ5, 3β-hydroxy steroid dehydrogenase (Δ5, 3β-HSD) was performed according to the protocol of Talalay.[36] Testicular Δ^5, 3β-HSD homogenizing media was prepared by adding 20 ml of glycerol, and 0.01 M EDTA in 0.05 M phosphate buffer to 100 ml of redistilled water. Following the centrifugation of testicular homogenate at 10,000 rpm for 30 min at 4 °C in a cold centrifuge, 1 ml of the supernatant was mixed with 0.9 ml of distilled water, 1 ml of sodium pyrophosphate buffer, and 40 µl of dehydroepiandrosterone (DHEA). The activity of Δ^5,3β-HSD was measured following the addition of 0.1ml of NAD, at 340 nm against a blank (without NAD).

Estimation of ovarian 17β HSD: Tissue homogenate (20 mg/ml) was centrifuged at 10,000 rpm for 30 min at 4 °C in a cold centrifuge and the supernatant was collected. The supernatant (1ml) was mixed with 1 ml of sodium pyrophosphate buffer and 40 µl of dehydroepiandrosterone (DHEA). For 17ß HSD activity measurements,[37] the same supernatant fluid was added into 440 µM of sodium pyrophosphate buffer, 960 µl of bovine serum albumin, and 40 µl of ethanol containing testosterone. The en-

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

zyme activity was measured following the addition of NAD to the tissue supernatant mixture in a spectrophotometer at 340 nm against a blank (without NAD). Result is expressed as one unit of enzyme activity which is equivalent to a change in the absorbance of 0.001/min at 340 nm.

Statistical Analysis

The result was expressed as mean±SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA) using Origin 6.1 software. The difference was considered as statistically significant when $p<0.05$.

RESULTS

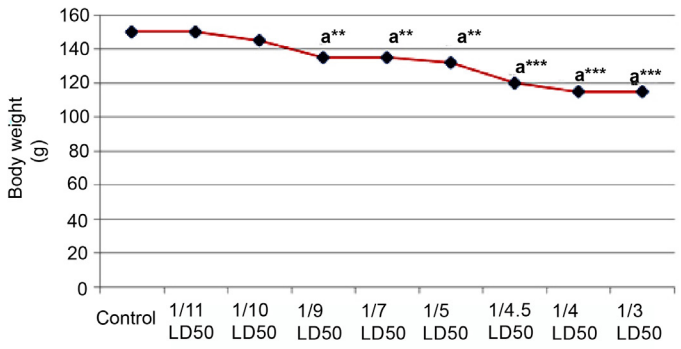
Alterations in Body Weight, Food and Water Intake

Significant alterations in the final body weight were observed at the dose level of 80 mg/kg body weight (1/4.5 LD_{50}) in male rats (Figure 1) and at the dose level of 51.5 mg/kg body weight (1/6 LD_{50}) in female rats (Figure 2).

No change in food and water intake was observed in the last consecutive four days of treatment period but slight alterations were seen in food consumption at 1/4 and 1/5 LD_{50} concentration. The animals were under observation at least once in 30 minute intervals following each treatment. Rats exposed to CYP over a period of 14 days experience ataxia, distress, rolling, and also tremors which were considered as signs of toxicity ranging from a dose of 40 mg/kg body weight (1/9LD_{50}) to 80 mg/kg body weight (1/4.5 LD_{50}) up to the 10th day of treatment following which the symptoms were minimized. There was no evidence of mortality in response to the above mentioned doses.

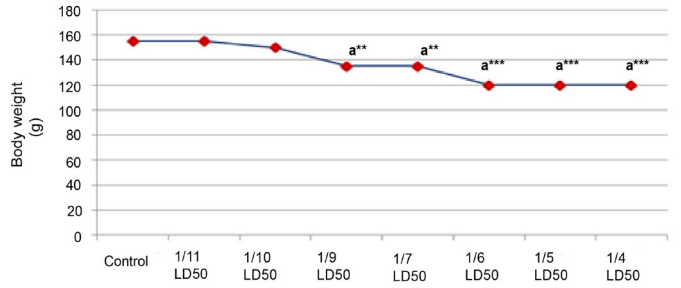
Before beginning with different systemic studies, some clinically significant biochemical parameters from each system were studied to identify the toxic levels of CYP, From the study, it was so observed that the total RBC count was decreased significantly ($p<0.05$) from the dose level of 1/9 LD_{50} to1/4 LD_{50} in male rats. It was seen that the total leukocyte count was enhanced significantly ($p<0.01$) at the dose level of 1/9 LD_{50} but decreased significantly ($p<0.01$) from the dose level of 1/4.5 LD_{50} to1/3 LD_{50} in male rats. No significant changes were shown below 1/9 LD_{50} dose. The maximum toxic effect of CYP was observed at the 1/4.5LD_{50} (Table 1) without showing any signs of mortality. From the 1/4 LD_{50} onwards, the rate of mortality was significantly increased. Similar types of results were observed in the case of hemoglobin percentage (Table 1).

Figure 1: Effect of Cypermethrin on Body Weight of Male Rat.



Results are Expressed as Mean±SEM. The Data has been Analyzed using one way ANOVA. Superscript a, Control Group *Versus* All Other Groups (**indicates $p<0.01$ and ***indicates $p<0.001$).

Figure 2: Effect of Cypermethrin on Body Weight of Female Rat.



Results are Expressed as Mean±SEM. Data Analysis has been Performed Using One Way ANOVA. Superscript a, Control Group V*ersus* Aall Other Groups (**Indicates $p<0.01$ and ***Indicates $p<0.001$).

TOXICOLOGY AND FORENSIC MEDICINE
Open Journal
Openventio PUBLISHERS
ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

Table 1: Effect Cypermethrin on Haematological and Hepatic Biomarkers at Different Dose Levels.

	RBC Count	WBC Count	Hb Percentage	SGOT	SGPT	Hepatic MDA
Control	6±0.577	5367±76	12.5±0.76	54.66±0.881	44.83±1.79	0.255±0.007
1/11 LD_{50}	6±0.577	5367±76	12.5±0.76	54.66±0.881	45.83±1.93	0.255±0.007
1/10 LD_{50}	6±0.577	5367±76	12.5±0.76	54.66±0.881	45.83±1.93	0.255±0.007
1/9LD_{50}	3.833±0.3 a^{***}	7233±54 a^{**}	10.16±0.6 a^{**}	85.6±0.881 a^{*}	54.3±1.28 a^{**}	0.49±0.007 a^{***}
1/7LD_{50}	3.833±0.47a^{***}	7117±60 a^{**}	10.16±0.6 a^{**}	87±0.96 a^{*}	54.3±1.28 a^{**}	0.49±0.007 a^{***}
1/5LD_{50}	3.333±0.44a^{***}	7117±60 a^{**}	10.16±0.6 a^{**}	88±0.96 a^{*}	60±0.96 a^{**}	0.721±0.008 a^{**}
1/4.5LD_{50}	3±0.36 a^{***}	6300±58 a^{**}	9.16±0.41 a^{***}	98.6±0.8 a^{***}	73.6±1.15 a^{***}	1.24±0.012 a^{***}
1/4 LD_{50}	3±0.36 a^{***}	6300±58 a^{**}	9.16±0.4 a^{***}	98.6±0.8 a^{***}	73.6±1.11 a^{***}	1.24±0.01 a^{***}
1/3LD_{50}	3±0.36 a^{***}	6300±58 a^{**}	9.16±0.4 a^{***}	98.6±0.8 a^{***}	73.6±1.11 a^{***}	1.24±0.008 a^{***}

Results are expressed as Mean±SEM. Data has been analyzed using one way ANOVA. Superscript a Control group versus all other groups (*indicates $p<0.05$, **indicates $p<0.01$, ***indicates $p<0.001$).

Hepatoxicity

Serum SGOT and SGPT: In Table-1, the effects of CYP on SGOT and SGPT have been exhibited respectively. With an increase in the concentration of CYP, the activity of these two important hepatic enzymes were enhanced significantly ($p<0.001$) as compared to the control rats. No significant variations were noted below the 1/9 LD_{50}.

Effect on hepatic MDA and glutathione content: In Table 1, hepatic MDA and glutathione content in the control and the CYP-induced experimental groups of the rats have been shown. MDA was seen to be significantly augmented ($p<0.01$) whereas reduced glutathione content was decreased significantly ($p<0.001$) with an increase in the dose levels of CYP.

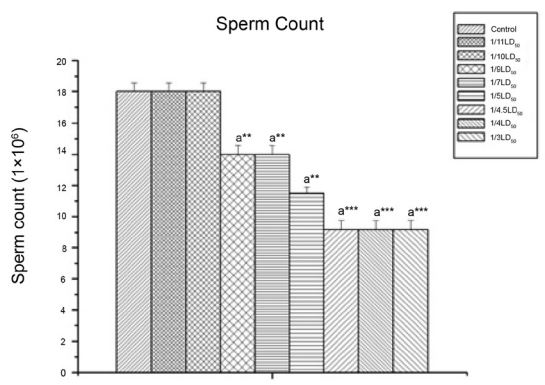
Reproductive Toxicity

Male reproductive toxicity

Effect on sperm count and testicular cholesterol: As shown in figure 3 and 4, CYP resulted in reproductive toxicity as was reflected by the reduced epididymal sperm count ($p<0.001$), and by an increase in the testicular cholesterol level ($p<0.001$).

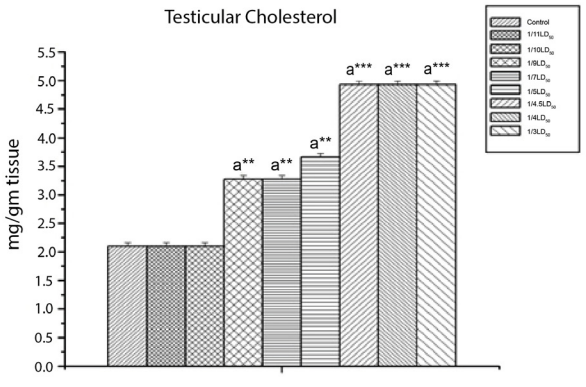
Effect on testicular Δ5, 3β HSD activity and MDA content: Figure 5 shows that CYP caused a significant decrease ($p<0.001$) in testicular Δ^5,3β HSD activity. Testicular MDA (Figure 6) increased significantly ($p<0.01$) in response to CYP exposure. From the dose level of 40 mg/kg body wt. ($1/9^{th}$ LD_{50} dose) onwards, significant changes were detected.

Figure 3: The Effect of Cypermethrin on Sperm Count.



Results are expressed as Mean±SEM. Data analysis has been performed using one way ANOVA. Superscript a Control group *versus* all other groups (**indicates $p<0.01$, ***indicates $p<0.001$).

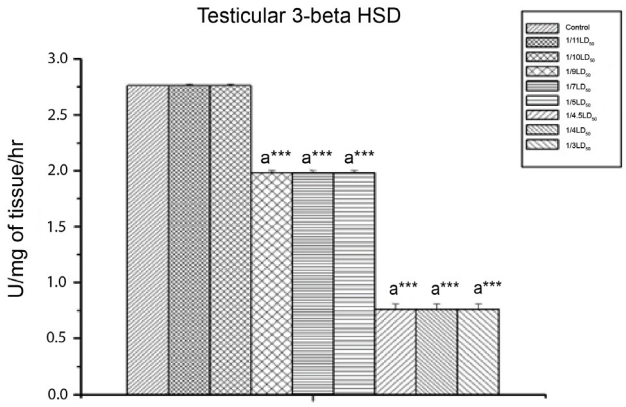
Figure 4: The Effect of Cypermethrin on Testicular Cholesterol.



Results are expressed as Mean±SEM. Data analysis has been performed using one way ANOVA. Superscript a Control group *versus* all other groups (***indicates $p<0.001$).

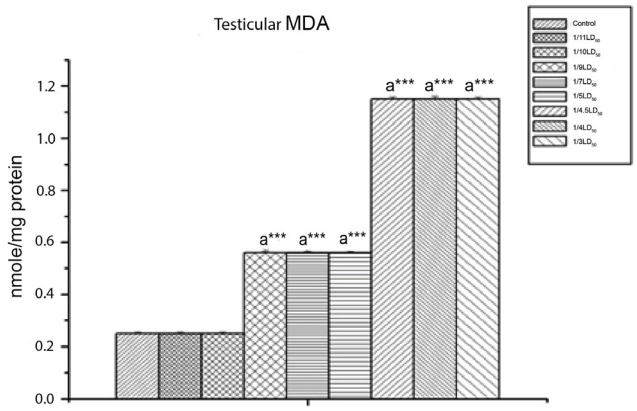
ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

Figure 5: The Effect of Cypermethrin on Testicular 3β HSD Activity.



Results are expressed as Mean±SEM. Data analysis has been done by one way ANOVA. Superscript a Control group *versus* all other groups (***indicates $p<0.001$).

Figure 6: The Effect of Cypermethrin on Testicular MDA.



Results are expressed as Mean±SEM. Data analysis has been performed using one way ANOVA. Superscript a Control group *versus* all other groups (***indicates $p<0.001$).

Female Reproductive Toxicity

Effect on ovarian cholesterol and 17β HSD: Increased ovarian cholesterol levels and a significant decrease in the ovarian 17β HSD enzyme activity were observed at 1/9LD_{50} to1/6 LD_{50} dose level which indicated that CYP induced ovarian toxicity (Table 2).

Effect on ovarian MDA and GSH content: A noticeable dose-dependent increase ($p<0.001$) in the MDA level and decline in the GSH content were seen in the CYP-induced rat from the 1/9th LD_{50} dose level onwards.

Experimental dose selection: From these above results for male rats, 1/9 and 1/4.5 LD_{50} dose and for female rats, 1/9 and 1/6 LD_{50} dose were considered as effective doses for further studies. As the 1/9 LD_{50} dose caused minimum significant changes in different measures of systemic biochemical parameters in male rats whereas the effect of 1/11, 1/10 doses did not significantly differ from control and in female rats, the first significant response was observed at the 1/9 LD_{50} dose. So, in case of male rats, 1/4.5 LD_{50} dose and for female rats 1/6 LD_{50} dose were selected as the high doses. Minimum response was recorded at the 1/9 LD_{50} dose for male and female rats. Therefore, this dose was considered as an effectively low dose for systemic studies.

DISCUSSION

The present study was conducted to investigate the toxic effect of CYP on the different systems of male and female Wistar rats. In toxicity-related studies, body weight is an important parameter for the assessment of organ toxicity. In the current study,

Table 2: Effect of Cypermethrin on Ovarian Cholesterol, 17β HSD, MDA and GSH at Different Dose Levels.

	Ovarian cholesterol	Ovarian 17 β HSD	Ovarian MDA	Ovarian GSH
Control	1.32±0.001	2.7±0.006	2.65±0.009	1.9±0.01
1/11 LD_{50}	1.32±0.001	2.708±0.006	2.65±0.009	1.9±0.01
1/ 10 LD_{50}	1.32±0.001	2.708±0.006	2.65±0.009	1.9±0.01
1/ 9 LD_{50}	1.511±0.0013 a*	1.2±0.005 a***	3.74±0.01 a***	1.4±0.009 a***
1/ 7 LD_{50}	1.511±0.0013 a*	1.2±0.005 a***	3.74±0.01 a***	1.4±0.009 a***
1/ 6 LD_{50}	1.72±0.0013 a**	0.531±0.006 a***	4.13±0.16 a***	1.03±0.007 a***
1/5 LD_{50}	1.72±0.0013 a**	0.531±0.006 a***	4.13±0.16 a***	1.03±0.007 a***
1/4 LD_{50}	1.72±0.0013 a**	0.531±0.006 a***	4.13±0.16 a***	1.03±0.007 a***

Results are expressed as Mean±SEM. Analysis has been performed using one way ANOVA. Superscript a Control *versus* all other groups (*indicates $p<0.05$,**indicates $p<0.01$, ***indicates $p<0.001$).

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

body weight was reduced significantly following the oral administration of CYP in both the male and female rats.

The decrease in RBC count after CYP treatment could be due to hemolysis as type II pyrethroid exposure causes hemorrhage and reduction in erythropoiesis.[38,39] Some erythrocytes are absorbed by lymphatic vessels due to internal hemorrhages, particularly hemorrhages in the body cavities and the remaining RBCs are lyzed or phagocytosed.[40] Various authors have reported similar results in CYP-treated rats,[41] sheep,[42] rabbit[19,43] and goats.[44] In the present study, hemoglobin concentration significantly decreased in rats treated with CYP concentrations from 1/9LD_{50} onwards. Reduction of Hb content could be due to the impaired biosynthesis of heme in the bone marrow.[45] Due to the disruptive action of pesticides on the erythropoietic tissue, a decrease in the red blood cell count and hemoglobin content may be observed which could considerably affect the viability of the RBC cells. In addition, the reduction in blood parameters (Hb and RBC) may have been attributed to the hyperactivity of the bone marrow leading to the production of RBCs with impaired integrity which could be easily destroyed in circulation by the reticuloendothelial system. Manna et al[41] suggested that a decrease in the RBC count and Hb concentration was indicative of depressed erythropoietin in rats.

The increased WBC counts were noted in CYP-treated groups and it may have been due to the activation of defense and immune system of the body.[19] These findings were also supported by the findings of Yousef et al in sheep,[9] and Yousef et al[46] in rabbits. This increase in leukocyte count may indicate an activation of the animal's defense mechanism and immune system. This may also result in an increase in the release of WBC from the bone marrow storage pool into the blood. The primary function of WBCs is to defend the body from foreign bodies, which are generated by leukocytosis and antibody production. Pathological leukocytosis may occur due to exposure to chemicals or acute hemorrhages and hemolysis. Leukocytosis may result due to a resistance developed by the animal towards the localization of the inflammatory response. Another possible cause of leukocytosis may be severe hemorrhages in the liver and lungs.[40] This increase may be related to an increase in the lymphocyte percentage.

A significant increase in the SGOT and SGPT levels following CYP treatment at different concentrations in the present study may indicate active utilization of amino acids in energy-yielding metabolic processes like gluconeogenesis. On account of an elevated level of lipid peroxidation products, pyrethroids may induce oxidative stress.[47] The observation concerning an increase in the serum glutamate-oxalacetate transaminase (SGOT) and serum glutamate-pyruvate transaminase (SGPT) activities (Table 1) is in agreement with the findings of Yousef et al[9,10] and El-Demerdash et al.[12] The increase in these serum enzyme activities is suggestive of liver dysfunction. Hence, cellular damage caused by toxic substances is frequently accompanied by increasing cell membrane permeability. The current results showed that CYP led to an increase in MDA and a decrease in the level of GSH which might be attributed to CYP-induced oxidative stress in rat hepatocytes.[48]

The results have shown reproductive dysfunction following CYP exposure at different doses. The reduction of epididymal sperm count in CYP intoxicated rats drew our attention towards the impairment of spermatogenesis.

The increase in the cholesterol, the main hormone involved in the control of fertility of male animals including rats, level in the testes may be due to its non-utilization. A decrease in the androgenic key enzyme (Δ5, 3β-HSD) activity was highlighted in the CYP-treated group which indicated an inhibition of testicular androgenesis.[49] The low steroidogenic enzyme ($^{\Delta}$5,3βHSD) activity also reflected from the above findings indicated the low gonadotrophin levels as the activity of the associated enzymes was regulated by gonadotrophins.[49] Oxidative stress defines an imbalance between the production of reactive oxygen species (ROS) and antioxidative defense mechanisms. During pyrethroid metabolism, ROS were generated and caused oxidative stress in intoxicated animals. In oxidative stress, lipid peroxidation occurred due to excessive free radical production and was considered a primary mechanism of cell membrane destruction and cell damage. MDA is the end product of lipid peroxidation. Increased MDA levels in testis suggested that CYP caused testicular damage.[50] In the present study, a significant rise in the ovarian cholesterol level of CYP-treated rats indicated less consumption of cholesterol towards the biosynthesis of ovarian steroid hormones. Thus, it resulted in the malfunctioning of ovarian steroidogenic activity of CYP-treated rats. The low steroidogenic activity was also confirmed by the decreased activity of steroidogenic enzymes (17βHSD) which indicated an inhibition of ovarian steroidogenesis.[50] Reduction in GSH levels in ovary following CYP treatment may be indicative of oxidative stress, whereas GSH was utilized for the detoxification of reactive toxic substances. As one of the most essential biological molecules, GSH played a key role in the detoxification of the reactive toxic metabolites. Normal cellular function was executed based on a balance between ROS production and antioxidant defense mechanisms existing in the cell.[51]

CONCLUSION

The present study suggested that CYP intoxication was responsible for hematological, hepatic and gonadal toxicity at 40 mg/kg body weight (1/9 LD_{50}), and 80 mg/kg body weight (1/4.5 LD_{50}) in male rats, and at 34.33 mg/kg body weight (1/9 LD_{50}) and 51.5 mg/kg body weight (1/6 LD_{50}) in female rats.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

REFERENCES

1. Maund SJ, Travis KZ, Hendley P, Giddings JM, Solomon KR. Probabilistic risk assessment of cotton pyrethroids: V. Combining landscape-level exposures and ecotoxicological effects data to characterize risks. *Environ Toxicol Chem.* 2001; 20: 687-692.

2. Luo Y, Zhang, M. Environmental modeling and exposure assessment of sediment-associated pyrethroids in an agricultural watershed. *PLoS ONE*. 2011; 6: e15794. doi: 10.1371/journal.pone.0015794

3. Feo ML, Eljarrat E, Barcelo' D. Determination of pyrethroid insecticides in environmental samples. *Trends Anal Chem.* 2010; 29: 692-705. doi: 10.1016/j.trac.2010.03.011

4. Pap L, Bajomi D, Szekely I. The pyrethroids, an overview. *International Pest Council.* 1996; 38: 15-19.

5. Michelangeli F, Robson MJ, East JM, Lee AG. The conformation of pyrethroids bound to lipid bilayers. *Biochim Biophys Acta*. 1990; 1028: 49-57. doi: 10.1016/0005-2736(90)90264-O

6. Lawrence LJ, Casida JE. Pyrethroid toxicology: Mouseintracerebral structure-toxicity relationships. *Pestic Biochem Physiol*. 1982; 18: 9-14. doi: 10.1016/0048-3575(82)90082-7

7. Khan A, Faridi HAM, Ali M, et al. Effects of cyper-methrinon some clinico-hemato biochemical and pathological parametersin male dwarf goats. *Exp Toxicol Pathol.* 2009; 61: 151-160. doi: 10.1016/j.etp.2008.07.001

8. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci*. 2002; 65(2): 166-176.

9. Yousef MI, Ibrahim HZ, Yacout HM, Hassan AA. Effects of cypermethrin and dimethoate on some physiological and biochemical parameters in Barki sheep. *Egypt J Nutr Feeds.* 1998; 1: 41-52.

10. Yousef MI, El-Demerdash FM, Al-Salhen KS. Protective role of isoflavones against the toxic effect of cypermethrin on semen quality and testosterone levels of rabbits. *J Environ Sci Health B*. 2003; 38: 463-478. doi: 10.1081/PFC-120021666

11. Yousef MI, El-Demerdash FM, Kamel KI, Al-Salhen KS. Changes in some hematological and biochemical indices of rabbits induced by isoflavones and cypermethrin. *Toxicol*. 2003; 189: 223-234. doi: 10.1016/S0300-483X(03)00145-8

12. El-Demerdash FM, Yousef MI, Kedwany FS, Baghdadi HH. Role of α-tochopherol and β-carotene in ameliorating the fenvalerate-induced changes in oxidative stress, hematobiochemical parameters and semen quality of male rats. *J Environ Sci Health B*. 2004; 39: 443-459. doi: 10.1081/PFC-120035929

13. Kale M, Rathore N, Jone S, Bhatnagar D. Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: A possible involvement of reactive oxygen specie. *Toxicol Lett.* 1999; 105: 197-205. doi: 10.1016/S0378-4274(98)00399-3

14. Giray B, Gurbay A, Hincal F. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by Vitamin E and allopurinol. *Toxicol Lett*. 2001; 118: 139-146. doi: 10.1016/S0378-4274(00)00277-0

15. Prasanthi K, Muralidhara Rajini PS. Fenvalerate-induced oxidative damage in rat tissues and its attenuation by dietary sesameoil. *Food Chem Toxicol*. 2005; 43: 299-306.

16. Carriquiriborde P, Diaz J, Mugni H, Bonetto C, Ronco AE. Impact of cypermethrinon stream fish population under field use in biotech-soybean production. *Chemosphere*. 2007; 68: 613-621. doi: 10.1016/j.chemosphere.2007.02.051

17. Santoni G, Cantalamessa F, Sperghini E, Sagretti O, Staffolani M, Pivvoli M. Alternation of T cell distribution and function in prenatally cypermethrinexposed rats: Possible involvement of cateholamines. *Toxicol*. 1999; 138: 175-187. doi: 10.1016/S0300-483X(99)00103-1

18. Shukla Y, Yadova A, Arora A. Carcinogenic and carcinogenic potential ofcypermethrin on mouse skin. *Cancer Lett*. 2002; 182: 33-41. doi: 10.1016/S0304-3835(02)00077-0

19. Nasuti C, Rosita G, Maria LF, Antonio DS, Franco C. Dopaminergic system modulation, behavioral changes and oxidative stress after neonatal administration of pyrethroids. *Toxicol*. 2007; 229: 194-205. doi: 10.1016/j.tox.2006.10.015

20. Yousef M, Abdallah G, Kamel K. Effect of ascorbic acid and vitamin esupplementation on semen quality and biochemical parameters of male rabbits. *Anim Reprod Sci*. 2003; 76: 99-111. doi: 10.1016/S0378-4320(02)00226-9

21. Sakr S. Pyrethroid inhalation-induced hepatotoxicity in Albino Rat. [Thesis] submitted to Biology Department, Faculty of Applied Science, Umm Al-QuraUniversity, Makkah, Saudi Arabia. 2003.

22. Nakamura Y, Sugihara K, Sone T, Isobe M, Ohta S, Kitamura S. The *in vitro* metabolism of a pyrethroid insecticide, permethrin, and its hydrolysis products in rats. *Toxicology*. 2007; 235: 176-184. doi: 10.1016/j.tox.2007.03.016

23. Mckinlay R, Plant JA, Bell JNB, Voulvoulis N. Endocrine disruptingpesticides: Implication for risk assessment. *Environ Int*. 2008; 34: 168-183. doi: 10.1016/j.envint.2007.07.013

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

24. Jin YX, Wang WY, Xu C, Fu ZW, Liu WP. Induction of hepatic estrogen responsive gene transduction by permethrin enantiomers in male adult zebra fish. *Aquat Toxicol*. 2008; 88: 146-152. doi: 10.1016/j.aquatox.2008.04.004

25. Badrawi R, Abdelmaoula NB, Feki N, Naser HB, Rebai T. Endocrine disruption and ovarian morphometric responses in rats following exposure to tetradifon. *Gen Comp Endocrinal*. 2010; 166: 268-272. doi: 10.1016/j.ygcen.2009.09.013

26. Wang H, Wang Q, Zhao XF, et al. Cypermethrin exposure during puberty disrupts testosterone synthesis via down regulation. [This article can be downloaded from www.ijpbs.net B - 951 StAR in mouse testes. *Arch Toxicol*. 2010; 84: 53-61]. *Int J Pharm Bio Sci*. 2013; 4(2): 942-953. doi: 10.1007/s00204-009-0479-y

27. FAO specifications and evaluations for agricultural pesticides. Food and Agriculture Organization of the United Nations, 2006: 37.

28. DeeAn J. Environmental fate of cypermethrin, Environmental Monitoring & Pest Management, Department of Pesticide Regulation, Sacramento, Data from EPA's Pesticide Fact Sheet Database. Sacramento, CA 95814-3510, USA: 1992:

29. Wintrobe MM. Clinical hematology. Lea and Febiger, Philadelphia, Library of Congress, Print USA; 1967.

30. Dacie JV, Lewis SM. *Practical Haematology*. 5th ed. London, UK: Churchill Livingstone; 1975: 628.

31. Goel BK. Routine biochemical tests. In: Mukherjee KL, ed. *Medical Laboratory Technology.* Vol.III. New Delhi, India: Tata McGraw-Hill publishing company Ltd; 1988: 985-1079.

32. Ohkawa H, Onishi N, Yagi K. Assay for lipid per-oxidation in animal tissue by thiobarbituric acid reaction. *Anal Biochem*. 1979; 95: 351-358. doi: 10.1016/0003-2697(79)90738-3

33. Griffith OW. Glutathione turnover in human erythrocytes. *J Biochem*. 1981; 256: 4900-4904.

34. *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction*. 4th ed. NewYork, USA: Cambridge University Press; 1999.

35. Zlatkis A, Zak B, Boyle AJ. A new method for direct determination of serum cholesterol. *J Lab Clin Med*. 1953; 41: 486-492.

36. Talalay P. Hydroxysteriod dehydrogenase. In: Colowick SP, Kaplan NO, eds. *Methods in Enzymology*. New York, USA: Academic Press; 1962: 512-516.

37. Jarabak J, Adams JA, Williams-Ashman HG, Talalay P. Purification of 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transdehydrogenase function. *J Biol Chem*. 1962; 237: 345-357.

38. Mandal A, Chakraborty S, Lahiri P. Hematological changes produced by lindane (-HCH) in six species of birds. *Toxicol*. 1986; 40: 103-111. doi: 10.1016/0300-483X(86)90050-8

39. Morgan DP, Stockdale EM, Roberts RJ, Walter HW. Anaemia associated with exposure to' lindane. *Arc Environ Hlth*. 1980; 35: 307-310. doi: 10.1080/00039896.1980.10667510

40. Latimer KS, Mahaffey EA, Prasse KW. *Clinical Pathology: Veterinary Laboratory Medicine*. 4th ed. Iowa State University Press, Ames, Iowa, USA. 2004.

41. Manna S, Bhattacharyya D, Basak DK, Mandal TK. Single oral dose toxicity study of acypermethrinin rats. *Indian J Pharmocol*. 2004; 36: 25-28.

42. Yousef MI, Ibrahim HZ, Yacout HM, Hassan AA. Effects of cypermethrin and dimethoate on some physiological and biochemical parameters in Barkisheep. *J Nutr Feeds*. 1998; 1(1): 41-52.

43. Basir A. Evaluation of haematological, histopathological and genotoxic effects of lambdacyhalothrinin rabbits. [MSc (Hons) Thesis]. Department of Pathology - University of Agriculture Faisalabad (UAF), Pakistan. 2005.

44. Faridi HAM. Haematological and histopathological changes with cypermethrin treatment in male dwarf goats (*Capra hircus*). [MSc (Hons) Thesis]. Department of Pathology - University of Agriculture Faisalabad (UAF), Pakistan. 2005.

45. Shakoori AR, Aslam F, Sabir M. Effect of prolonged administration of insecticide (cyhalothrin/karate) on the blood and liver of rabbits. *Folia Biol*. 1992; 40: 91-99.

46. Yousef MI, Ibrahim HZ, Salem HM, Hassan GA, Helmi S, Bertheussen K. Hematological and biochmical change induced by carbofuran and glyphosate in rabbits. *Environ Nutr Interac*. 1999; 3: 179-194.

47. Banerjee BD, Seth V, Bhattacharya A, Pasha ST, Chakraborty AK. Biochemical effects of some pesticides on lipid peroxidation and free radical scavengers. *Toxicol Lett*. 1999; 107: 33-47. doi: 10.1016/S0378-4274(99)00029-6

48. Al-Amoudi W. Ameliorative role and antioxidant effect of propolis against hepatotoxicity of fenvalerate in Albino rats. *J Cytol Histol*. 2015; 6: 303. doi: 10.4172/2157-7099.1000303

49. Murono EP, Payne AH. Testicular maturation in the rat. In-vivo effect of gonadotrophins on steroidogenic enzymes in the hypophysectomized immature rat. *Biol Report*. 1979; 20: 911-918. doi: 10.1095/biolreprod20.4.911

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-122

50. Gupta A, Nigam D, Gupta A, Shukla GS, Agarwal AK. Effect of pyrethroid based liquid mosquits repellent inhalation on the blood-brain barrier function and oxidative damage in selected organs of developing rats. *J Appl Toxicol*. 1999; 19: 67-72.

51. Ghosh R, Maiti Choudhury S. Alteration in estrous cycle, lipid peroxidation and antioxidant status in female rat after exposure to lambda cyhalothrin and its attenuation by taurine. *Int J Bioassays*. 2015; 4526-4532. doi: 10.21746/ijbio.2015.11.0018

TOXICOLOGY AND FORENSIC MEDICINE

Openventio PUBLISHERS

ISSN 2474-8978 Open Journal http://dx.doi.org/10.17140/TFMOJ-2-123

Commentary

*Corresponding author
Bashir M. Rezk, PhD
Associate Professor
Department of Natural Sciences
Southern University at New Orleans
6400 Press Drive
New Orleans, LA 70126, USA
Tel. 504-286-5405
E-mail: batteia@suno.edu

Volume 2 : Issue 2
Article Ref. #: 1000TFMOJ2123

Article History
Received: December 10th, 2017
Accepted: December 21st, 2017
Published: December 22nd, 2017

Citation
Rezk BM, Lucchesi PA. Hyperhomocysteinemia and alcoholism: A double hit? *Toxicol Forensic Med Open J*. 2017; 2(2): 84-87. doi: 10.17140/TFMOJ-2-123

Hyperhomocysteinemia and Alcoholism: A Double Hit?

Bashir M. Rezk, PhD[1*]; Pamela A. Lucchesi, PhD, FAHA[2]

[1]Department of Natural Sciences, Southern University at New Orleans, New Orleans, LA 70126, USA

[2]Department of Basic Sciences, Geisinger Commonwealth School of Medicine, Medical Sciences Building, Scranton, PA 18509, USA

Homocysteine (HCY) is a non-protein sulfur-containing amino acid. It has received a great deal of attention over the last two decades within the scientific community for its unique role in the induction of several diseases ranging from atherosclerotic cardiovascular disease to neural tube defects (NTD). The hypothesis that hyperhomocysteinemia (HHCY) causes atherosclerosis was proposed by McCully[1] in 1969, when he observed that children with homocysteinuria had atherosclerotic plaques of the peripheral, coronary, and cerebral vasculature. In 1976, the initial epidemiological study of Wilcken and Wilcken [2] supported this hypothesis and provided the first evidence of the pathogenic role of HCY in atherosclerotic cardiovascular disease and alcoholism. HCY strictly associated with alcohol consumption and enhanced the alcohol consumption that leads to alcohol-related disorders such as brain atrophy, epileptic seizures during withdrawal, and mood disorders.[3] Although, HHCY involves both genetic and nutritional causes,[4] the mechanism of HCY toxicity is not completely understood.

In vascular biology and under physiological conditions, the proliferation and apoptosis of cells are balanced as a symphony in which the cell death triggers cell migration and proliferation. However, in the pathogenic conditions, a selective increase in cell proliferation induces hyperplasia and a selective elevation of apoptosis leads to atrophy. As early as 1973, Ross and Glomset[5] proposed that proliferation of smooth muscle cells within the wall of the artery was the key event in the genesis of the lesions of atherosclerosis. Atherosclerosis is a multifactorial process including endothelial dysfunction, and vascular smooth muscle cells (VSMCs) apoptosis, proliferation and migration from the media to the intima, and subsequently, formation of the atherosclerotic plaques.[5-10] Indeed, apoptosis and proliferation of VSMCs are the most prominent hallmarks of atherosclerotic plaque instability. A stable atherosclerotic plaque possesses a thick fibrous cap with excessive numbers of VSMCs, whereas unstable atherosclerotic plaques have a thin fibrous cap with limited numbers of VSMCs. Apoptosis of VSMCs is substantially elevated in unstable plaques which promotes plaque rapture and subsequently led to heart attacks.[9] On the other hand, proliferation of VSMCs is associated with stable plaques.[8]

A clinical study by Guang et al[11] reported that HCY might convert a stable plaque into an unstable plaque due to the elevation of oxidative stress and chemokines in patients with acute coronary artery disease. To explain these phenomena, one possibility considered is that HCY initiates monocyte, T-lymphocyte, endothelial cell and VSMCs activities that interact with other inflammatory stimuli (such as C-reactive protein and ox-LDL), sensitize the inflammatory responsiveness leading to an enhanced production of reactive oxygen species (ROS) and a chronic inflammatory condition at the site of atherosclerotic lesions.[11] In this condition, the production of proinflammatory chemokines and ROS are enhanced further by monocyte, T-lymphocyte, endothelial cell and VSMCs.[11] These chemokines increased leukocyte adhesion and transendothelial migration.[11] At the same time, ROS induce further modified LDL into a highly oxidized form, which is taken up by macrophage and results in foam cell formation.[11] Study of Rasmussen et al[12] emphasized that HCY increased VSMC proliferation, whereas there was no change in apoptosis. Similarly, Taha et al[13] reported that HCY enhanced DNA synthesis and proliferation of VSMCs. Although the accumulation of VSMCs is the main cause of intimal thickening of vascular disease, Weissberg and co-workers[14] speculated that VSMC

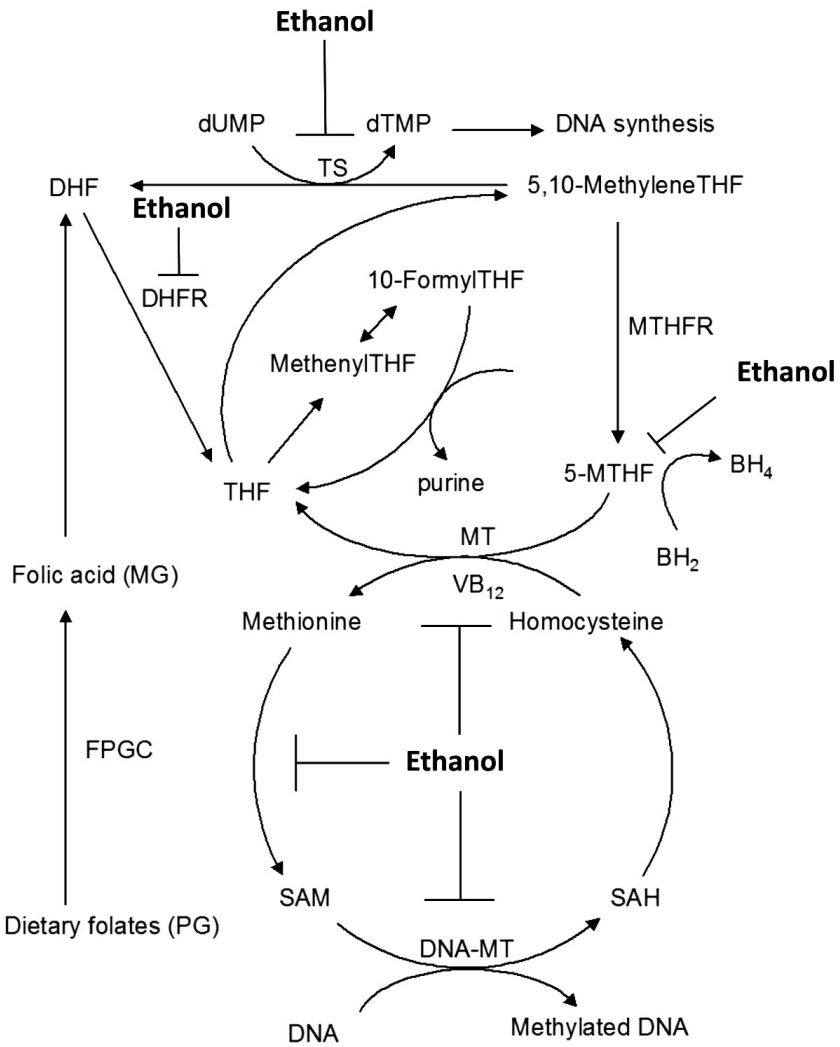
ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-123

proliferation might be beneficial. As such long-term treatment to prevent VSMCs proliferation would rupture the atherosclerotic lesions.[15] In our opinion proliferation of VSMCs is not beneficial but it might delay the rupture of the stable plaques. Importantly, a study by Buemi et al showed that HCY elevated both apoptosis and proliferation of VSMCs *in vitro*.[16] Clinically, atherosclerosis appeared to be associated with HCY levels of approximately 250 μM in hyperhomocysteinemic patients.[17-19] In addition, Yan et al[20] denoted that HCY (0.05-1 mM) elevated apoptosis of VSMCs in a dose-dependent manner. Vascular toxicity of HCY seems to be due to the elevation of oxidative stress after auto-oxidation of homocysteine into homocystine.[4] The mechanisms of induction of oxidative stress by HCY have been exclusively studied by Tyagi et al[21,22] and Moshal et al.[23]

More recent studies suggest the deleterious effects of HCY go beyond oxidative stress. For example, Kobus-Bianchini[24] reported that HCY interfered with the proliferation of neural cells and induced a decrease in neuronal differentiation in the spinal cord. Thus, the molecular networks of homocysteine are multifactorial and also include epigenetic regulation (Figure 1). Alterations in human homocysteine levels can influence DNA-methylation of specific gene sequences that may change the expression and synthesis of proteins important for the genesis and maintenance of alcohol dependence.[25,26]

The metabolism of folates and homocysteine are interrelated (Figure 1). Folic acid catalyzes methylation of homocysteine, leading to a reduction of total plasma homocysteine.[27] Conversion of homocysteine into methionine can occur in a reaction catalyzed by methionine synthetase, which uses 5-methyltetrahydrofolate (5-MTHF) as a methyl donor and cobalamin (vitamin B-12) as an essential co-factor.[28] Consumption of alcohols, negatively, interferes with the physiological and biochemical metabolic pathways of folates and homocysteine (Figure 1) that leads to a double hit. Ethanol

Figure 1: Proposed Mechanism of Interference of Ethanol with Metabolism of Folate and Homocysteine.



PG: Polyglutamate; MG: Monoglutamate; FPGC: Folylpolyglutamate carboxypeptidase; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; THF: Tetrahydrofolate; TS: Thymidylate synthetase; dUMP: Deoxyuridylate; dTMP: Thymidylate; MTHFR: Methylenetetrahydrofolate reductase; 5-MTHF: 5-methyltetrahydrofolate; MT: Methionine synthetase; VB_{12}: Vitamin B_{12}; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; DNA-MT: DNA methyltransferase; BH_2: dihydrobiopterin; BH_4: Tetrahydrobiopterin.[32]

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-123

modulated liver methionine/homocysteine metabolism has been reflected by decreased SAM and increased SAH content.[29] Ethanol impaired the renal conservation of 5-methyltetrahydrofolate in the isolated perfused rat kidney.[30] Chronic alcoholism is known to interfere with one-carbon metabolism, for which folate, vitamin B-12, and vitamin B-6 serve as coenzymes.[31] Mean serum HCY was twice as high in chronic alcoholics compared to that of non-drinkers.[31]

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. McCully KS. Vascular pathology of homocysteinemia: Implications for the pathogenesis of arteriosclerosis. *Am J Pathol*. 1969; 56: 111-128.

2. Wilcken DE, Wilcken B. The pathogenesis of coronary artery disease. A possible role for methionine metabolism. *J Clin Invest*. 1976; 57: 1079-1082. doi: 10.1172/JCI108350

3. Coppola M, Mondola R. Correlation between plasma homocysteine levels and craving in alcohol dependent stabilized patients. *Clin Nutr*. 2017. pii: S0261-5614(17)30165-6. doi: 10.1016/j.clnu.2017.05.004

4. Welch GN, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med*. 1998; 338: 1042-1050. doi: 10.1056/NEJM199804093381507

5. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science*. 1973; 180: 1332-1339. doi: 10.1126/science.180.4093.1332

6. Davies MJ, Richardson PD, Woolf N, Katz DR, Mann J. Risk of thrombosis in human atherosclerotic plaques: Role of extracellular lipid, macrophage, and smooth muscle cell content. *Br Heart J*. 1993; 69: 377-381. doi: 10.1136/hrt.69.5.377

7. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 1999; 340: 115-126. doi: 10.1056/NEJM199901143400207

8. Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: New perspectives and therapeutic strategies. *Nat Med*. 2002; 8: 1249-1256. doi: 10.1038/nm1102-1249

9. Clarke MC, Figg N, Maguire JJ, et al. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med*. 2006; 12: 1075-1080. doi: 10.1038/nm1459

10. Clarke M, Bennett M. Defining the role of vascular smooth muscle cell apoptosis in atherosclerosis. *Cell Cycle*. 2006; 5: 2329-2331. doi: 10.4161/cc.5.20.3383

11. Guang W, Jie-ming M, Xian W, Fu-chun Z. Effect of homocysteine on plaque formation and oxidative stress in patients with acute coronary syndromes. *Chinese Medical J*. 2004; 117: 1650-1654.

12. Rasmussen LM, Hansen PR, Ledet T. Homocysteine and the production of collagens, proliferation and apoptosis in human arterial smooth muscle cells. *APMIS*. 2004; 112: 598-604. doi: 10.1016/S0009-9120(03)00062-6

13. Taha S, Azzi A, Ozer NK. Homocysteine induces DNA synthesis and proliferation of vascular smooth muscle cells by a hydrogen peroxide-independent mechanism. *Antioxid Redox Signal*. 1999; 1: 365-369. doi: 10.1089/ars.1999.1.3-365

14. Weissberg PL, Clesham GJ, Bennett MR. Is vascular smooth muscle cell proliferation beneficial? *Lancet*. 1996; 347: 305-307. doi: 10.1016/S0140-6736(96)90472-9

15. Schwartz SM, Virmani R, Rosenfeld ME. The good smooth muscle cells in atherosclerosis. *Curr Atheroscler Rep*. 2000; 2: 422-429. doi: 10.1007/s11883-000-0081-5

16. Buemi M, Marino D, Di Pasquale G, et al. Effects of homocysteine on proliferation, necrosis, and apoptosis of vascular smooth muscle cells in culture and influence of folic acid. *Thromb Res*. 2001; 104: 207-213. doi: 10.1016/S0049-3848(01)00363-2

17. Clarke R, Daly L, Robinson K, et al. Hyperhomocysteinemia: An independent risk factor for vascular disease. *N Engl J Med*. 1991; 324: 1149-1155. doi: 10.1056/NEJM199104253241701

18. Malinow MR. Hyperhomocyst(e)inemia. A common and easily reversible risk factor for occlusive atherosclerosis. *Circulation*. 1990; 81: 2004-2006. doi: 10.1161/01.CIR.81.6.2004

19. Ueland PM, Refsum H. Plasma homocysteine, a risk factor for vascular disease: Plasma levels in health, disease, and drug therapy. *J Lab Clin Med*. 1989; 114: 473-501.

20. Yan SK, Chang T, Wang H, Wu L, Wang R, Meng QH. Effects of hydrogen sulfide on homocysteine-induced oxidative stress in vascular smooth muscle cells. *Biochem Biophys Res Commun*. 2006; 351: 485-491. doi: 10.1016/j.bbrc.2006.10.058

21. Tyagi N, Moshal KS, Ovechkin AV, et al. Mitochondrial mechanism of oxidative stress and systemic hypertension in hyperhomocysteinemia. *J Cell Biochem*. 2005; 96: 665-671. doi: 10.1002/jcb.20578

22. Tyagi N, Sedoris KC, Steed M, et al. Mechanisms of

ISSN 2474-8978 http://dx.doi.org/10.17140/TFMOJ-2-123

homocysteine-induced oxidative stress. *Am J Physiol Heart Circ Physiol*. 2005; 289: H2649-H2656. doi: 10.1152/ajpheart.00548.2005

23. Moshal KS, Sen U, Tyagi N, et al. Regulation of homocysteine-induced MMP-9 by ERK1/2 pathway. *Am J Physiol Cell Physiol*. 2006; 290: C883-C891. doi: 10.1152/ajpcell.00359.2005

24. Kobus-Bianchini K, Bourckhardt GF, Ammar D, Nazari EM, Müller YMR. Homocysteine-induced changes in cell proliferation and differentiation in the chick embryo spinal cord: implications for mechanisms of neural tube defects (NTD). *Reprod Toxicol.* 2017; 69: 167-173. doi: 10.1016/j.reprotox.2017.02.011

25. Bleich S, Hillemacher T. Homocysteine, alcoholism and its molecular networks. *Pharmacopsychiatry.* 2009; 42(Suppl 1): S102-S109. doi: 10.1055/s-0029-1214396

26. Kamat PK, Mallonee CJ, George AK, Tyagi SC, Tyagi N. Homocysteine, alcoholism, and its potential epigenetic mechanism. *Alcohol Clin Exp Res*. 2016; 40(12): 2474-2481. doi: 10.1111/acer.13234

27. Refsum H, Ueland PM, Nygard O, Vollset SE. Homocysteine and cardiovascular disease. *Annu Rev Med.* 1998; 49: 31-62.

28. Finkelstein JD. The metabolism of homocysteine: Pathways and regulation. *Eur J Pediatr.* 1998; 157: S40-S44. doi: 10.1007/PL00014300

29. Watson WH, Song Z, Kirpich IA, Deaciuc IV, Chen T, McClain CJ. Ethanol exposure modulates hepatic S-adenosylmethionine and S-adenosylhomocysteine levels in the isolated perfused rat liver through changes in the redox state of the NADH/NAD(+) system. *Biochim Biophys Acta.* 2011; 1812(5): 613-618. doi: 10.1016/j.bbadis.2011.01.016

30. Muldoon RT, McMartin KE. Ethanol acutely impairs the renal conservation of 5-methyltetrahydrofolate in the isolated perfused rat kidney. *Alcohol Clin Exp Re*s. 1994; 18(2): 333-339. doi: 10.1016/j.bbadis.2011.01.016

31. Cravo ML, Glória LM, Selhub J, et al. Hyperhomocysteinemia in chronic alcoholism: Correlation with folate, vitamin B-12, and vitamin B-6 status. *Am J ClinNutr.* 1996; 63(2): 220-224.

32. Rezk BM. *Antioxidants and their Metabolites: Some Toxicological Aspects*. Maastricht, Netherlands: *Universitaire Pres Maastricht;* 2004.