Plasma Cocaine Metabolite and Liver CYP450 3A4 Isoenzyme Levels as Indicators of Cocaine Dependence in Rats Treated with Nutritional Supplements

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ABSTRACT

The effects that chronic cocaine administration (CCA) have on craving, cocaine metabolite concentrations and cytochrome P450 3A4 isoenzyme (CYP450 3A4) activities in Sprague-Dawley rats following the administration of Salako Nutritional Supplements (SNS) were examined. Five groups of fifty rats were used to assess the effect of the SNS following CCA. Craving was analyzed for each rat using a Conditioned Place Preference protocol. Blood samples were obtained at regular intervals and used to measure cocaine plasma metabolite levels. CYP450 3A4 activity was determined in the liver. Administration of the SNS reduced craving of cocaine significantly, upon discontinuing cocaine in the rats. Blood plasma analysis showing higher benzoylecgonine equilibrium and the CYP450 3A4 levels demonstrated that the SNS possibly aided in the removal of the stored metabolites indicative of increased metabolism of cocaine, enhanced by the Supplements. Results indicate that the SNS formulation reduces craving caused by CCA by increasing the liver CYP450 3A4 activity, resulting in better plasma clearance.

Keywords Cocaine Metabolite, Conditioned Place Preference, Drug Abuse Liver CYP450 3A4 Isoenzyme, Nutritional Supplement Formulation, Plasma, Rats

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INTRODUCTION

Cocaine ($C_{17}H_{21}NO_4$), classified as a stimulant, is described as being the most potent, powerfully addictive stimulant of natural origin. It is an alkaloid that is extracted from the leaves of the Coca plant (*Erythroxylum coca*), which originates in South America, and to a lesser extent, in Africa, Indonesia and India (UNODC, 2010). Cocaine is one of the oldest known psychoactive substances. Coca use has been traced as far back as around 5000 B.C. wherein the leaves of the plant were continually chewed in the mouth. Pure cocaine was isolated in the 1880's (National Institute on Drug Abuse, 2008). In the early 1900s, pure cocaine was the main active ingredient in numerous pharmaceutical and recreational formulations due to their properties that enhanced general activity and decreased fatigue.

Cocaine use varies widely with drug users. The duration of the euphoric effects produced by cocaine is dependent on the route of administration. The faster the absorption of cocaine, the more rapid and intense the high produced, but the shorter the duration of action. Toxic amounts of cocaine can be absorbed by any route of administration, which can have a wide range of effects which can include sudden death.

Metabolism of Cocaine

Cocaine is rapidly absorbed in the blood following the snorting, smoking and intravenous administration. Cocaine is metabolized by several enzymatic and non-enzymatic pathways (Heard et al., 2008) rapidly and extensively in animals to yield benzoylecgonine and ecgonine as the principal products, along with other metabolites, particularly ecgonine methyl ester, which are all generally inactive. Norcocaine is a minor metabolite that is formed, but is active and found to be neurotoxic. Cocaine is converted to norcocaine via the cytochrome P450 3A4 isoenzyme by means of N-demethylation. Norcocaine has shown to be equal in potency to cocaine (Hawks et al., 1974; Misra et al., 1975). The pharmacology of cocaine is complex, which exhibits simultaneous effects in several organ systems in the body including the brain, cardiovascular, immune and hematological systems (Espinoza, 2012; Heard et al., 2008 and Levine et al., 1990). Cocaine has been shown to be extremely hepatotoxic (Boyer & Peterson, 2005; Kanel et al., 1989; Pasanen et al., 1995) and also results in fatty infiltration responsible for increases in lipid content of the liver (Affi et al., 1998; MachLachlan & Hodge, 1938). Developing an effective, safe and cost-effective means of treating substance abuse is of paramount importance in society.

Chronic use of cocaine results in the metabolite being present in the urine for a prolonged period following introduction of a large amount into the body. There is still a paucity of information which exists regarding the effects that cocaine has on the body as a complete system, including the toxicity that exists during chronic usage and the general basic mode of action following varying modes of administration.

Conditioned Place Preference

Conditioned place preference is one popular method of an animal model of drug dependence. The apparatus used to carry out the experiment is called a conditioned place preference box, which consists of two chambers separated by a neutral chamber. One chamber differs from the other by tactile clues such as colour and light intensity. Using a biased procedure, the non-preferred compartment is paired with the drug and the CPP is a measurement of overcoming the initial aversion for that compartment (Sanchis-Segura and Spanagel, 2006). In adult rats, place preference for cocaine is effectively established for cocaine at a dosage of 20 mg/kg (Bardo et al. 1986; and Brenhouse and Andersen, 2008).

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Treating Cocaine Addiction and Nutritional Supplements Administration

Presently there are no pharmacological methods of treating cocaine misuse (Garcia-Rodriguez et al., 2009; Kennedy et al., 2012). The medications that are used currently treat symptomatically the effects produced as a consequence of the discontinuation of cocaine use. There are numerous research areas that are investigating possible treatment methods for cocaine misuse however the key areas that show promise operate on the premise of the cocaine-induced adaptations that occur which disturb the balances between excitatory and inhibitory neurotransmission which primarily involve the glutamate and gamma-amino-butyric acid (GABA) (Hu, 2007; Volkow, 2009).

The main modality of the treatment of cocaine misuse involves behavioural interventions, the most effective being Cognitive-behavioral Therapy (CBT) (Garcia-Rodriguez et al., 2009; Maude-Griffin et al., 1998; Weiss R. D. et al., 2005). CBT is focused on the abstinence of the user from cocaine and is based on the assumption that the development and continuation of cocaine misuse are influenced by learning processes (Center for Substance Abuse Treatment, 1999). The integration of both behavioural and pharmacological treatments will seemingly make the most effective approach. However, at present there is no medication that treats cocaine misuse (Kennedy et al., 2012). There is therefore the need for effective pharmacological method of treatment of cocaine misuse.

The Salako Nutritional Supplements formulation used in this study (Provisional Patent# 60/528,755) was developed in the clinics of the Getwele Treatment and Rehabilitation Services Incorporated in Maryland, USA by Dr. Ajibike Salako-Akande for use in reducing craving for alcohol and illicit drugs. Cocaine treatment, with this proprietary formulation, involves the use of multi-nutritional formulation fortified with vitamins and minerals, which has been shown to be effective in relapse management and prevention. It is being screened in our laboratories to check if it may represent a safe and cost effective secondary prevention approach to cocaine addiction.

MATERIALS AND METHODS

Cocaine hydrochloride was obtained from Health Cooperation Limited, Kingston, Jamaica with permission from the Ministry of Health of Jamaica.

Kits for Cocaine hydrochloride determination were purchased from Neogen Cooperation Ltd., USA.

Kits for Cytochrome P450-3A4 isoenzyme determination were purchased from Promega Corporation, USA.

Salako Nutritional Supplements formulation was provided by Dr. Ajibike Salako-Akande of Getwele Foundation International, USA.

Animal Study

Design of Feeding Experiment

Cocaine hydrochloride acquisition and usage were with the approval of the Ministry of Health Dangerous Drugs Inspector. The animal study was performed with the approval of the University of the West Indies, Mona Ethics Committee. Fifty (50) adult Sprague-Dawley rats (25 males and 25 females) between the ages of 6-8 weeks were obtained from the University of the West Indies Animal House. The rats were separated, weighed, labeled for differentiation; and placed into five groups (shown in Table 1), with an average body weight of 238.5 g. The stainless-steel

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Table 1. Rat groups and their assigned diets

Group	Group Description
ND	Normal rats fed normal rat diet.
NS	Normal rats fed supplemented rat diet.
CC	Rats dependent on cocaine and continually given cocaine throughout the entire study period, fed normal rat diet.
CDND	Rats dependent on cocaine, cocaine use discontinued, fed normal rat diet.
CDNS	Rats dependent on cocaine, cocaine use discontinued, fed supplemented rat diet

cages that they were housed in were maintained daily and were placed in a room that was kept on a 12/12-hour light/dark cycle. Throughout the entire study, the rats had access to food and water *ad libitum.* The rats were acclimatized for a week prior to the start of the study.

Inducing Chronic Cocaine Dependence in Animal Models Using Conditioned Place Preference (CPP)

Chronic drug dependence was induced in the animal models according to a modified method of Martin et al. (2000) by means of a conditioned place preference paradigm. The conditioned place preference paradigm utilized a conditioned place preference (CPP) box. The CPP box consists of two compartments (15” x 15” x 15”) that differed in both colour (white versus black) and light intensity (brightly lit versus dark and covered), separated by a neutral area. The rats were habituated to the conditioned place preference (CPP) box for 3 days by allowing them to run freely throughout the two compartments via the neutral area.

Rats in groups CC, CDND and CDNS were brought to a stage of drug dependence by use of the CPP box. The rats in groups ND and NS were used as controls and were brought through the CPP method, but instead using only vehicle (normal saline). This CPP paradigm occurred in three phases and outlined as described below:

A. **Preconditioning Phase:** Each rat was placed in the neutral compartment and allowed to explore both compartments freely. During this stage, the time spent in each compartment was recorded out of a total 18 minutes and used in order to pair the compartments for association with either the drug or the vehicle (normal saline). The preferred compartment was paired to vehicle and the non-preferred compartment was paired with the cocaine hydrochloride solution.
B. **Conditioning Phase:** Rats were treated with alternate injections of cocaine hydrochloride (20 mg/kg in 1cc. normal saline) or vehicle (normal saline) intra-peritoneally (i.p.) for 6 consecutive days. They were confined to the corresponding compartment immediately following injection using guillotine doors which matched the walls for 25 minutes. Drug was administered on days 1, 3 and 5 whereas the vehicle was administered on days 2, 4 and 6. Control subjects received vehicle every day. Following the drug pairing phase, rats in groups CC, CDND and CDNS were chronically administered cocaine at a dosage of 25 mg/kg in 1 cc. normal saline i.p. for 20 consecutive days. This occurred as stated above in the drug pairing phase. Control animals received vehicle every day and treated in the same manner.
C. **Testing Phase:** This phase was carried out as described previously in the preconditioning phase. Free access was given to the rats of both compartments via the neutral chamber and

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the time spent in each compartment was recorded out of a total 18 minutes. This step occurred after every phase of the feeding trial, and weekly throughout the supplement administration phase.

Supplement Administration

The SA supplement formulation had three constituents and was introduced to the rats at dosages specified in its proprietary form based on body weights. Rat chow (PMI Nutrition Inc., Lab diet 5008) was milled to a powder to facilitate the homogenous mixing of the supplement constituents obtained from Getwele Foundation International (USA).

Rats in group CC were continually subjected to the drug throughout the entire course of the study at the same dosage and method as described previously. After the 20 day chronic drug administration period, rats in groups CDND and CDNS had their cocaine use discontinued and fed normal rat diet and supplemented rat diet respectively for a period of three months according to the SA supplement guidelines. Rats in group NS were fed supplemented rat diet henceforth. Other groups were continued with the normal rat diet.

Sample Collection

Blood was obtained from the rats after acclimatization (baseline), after chronic drug dependence, at the mid-point and the end of the supplement administration period. Serum was obtained for analyses following the centrifugation (x 5000 rpm for 5 minutes, Centrifuge 5402 (Eppendorf, Germany)) of the blood samples.

Analysis of Cocaine Metabolite Plasma Concentrations

Cocaine hydrochloride and its metabolites were determined by a direct competitive ELISA according to manufacturer's guidelines (Neogen Cooperation Cocaine/ Benzoylecgonine ELISA kit, 2006). The principle of the test was based on competition between a horseradish peroxidase enzyme conjugate and the cocaine metabolite that is present in the sample, with a limited number of specific binding sites on the microplate precoated with antibody. A colorimetric method was then employed to compare the absorbance readings of the samples with the standards using a microplate reader at 650 nm (ElX800 Universal Microplate Reader, Bio-Tek Instrument Inc., USA).

Liver Cytochrome P450 3A4 Isoenzyme Determination

Cytochrome P450 3A4 isoenzyme was determined in the liver utilizing a luminescent method following manufacturer's instructions in a kit from Promega, P450-Glo V9002 Assays (2011). The available cytochrome P450 isoenzymes present in the samples were reacted with a luminogenic cytochrome P450 substrate. The cytochrome P450 isoenzymes converted the substrate to luciferin, which in turn reacted with luciferase to produce light. The amount of light produced was directly proportional to the cytochrome P450 activity on the samples.

Statistical Analysis

The results obtained were expressed as mean value ± the standard error of the mean (SEM). The differences among all the groups at $p \leq 0.05$ were tested using the Analysis of the variance (ANOVA) test. To determine whether the significant difference lies among the groups, the Duncan's multiple range test was used to compare the means. All statistical analyses were done using the statistical program SPSS version 16 (2007).

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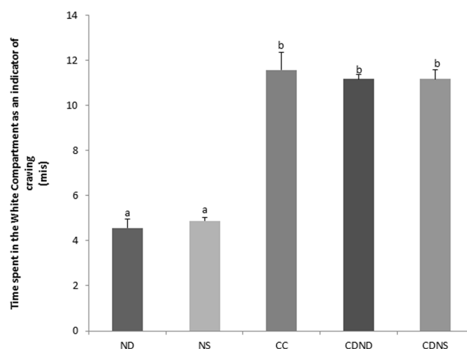
RESULTS

Effect of Supplement Administration on Craving Indicated by Time Spent in the White Compartment of the CPP Box

Figures 1-4 shows the time spent in the white compartment of the conditioned place preference (CPP) box as an indicator of craving for cocaine at varying phases of the supplement administration period. There were no significant differences ($p \geq 0.05$) in the time spent in the white compartment of the CPP box between any of the groups during the acclimatization period. Groups ND and NS maintained a time of between 4 to 6 minutes for the remainder of the study. These groups were the normal groups not exposed to cocaine. Groups CC, CDND and CDNS showed remarkable increase ($p \leq 0.05$) in time spent in the white compartment during the drug pairing exercise and after chronic cocaine treatment (Figure 1). Group CC was maintained on cocaine throughout the remainder of the study and fed normal diet which resulted in a time constant at just below 13 minutes throughout the remainder of the study, indicating sustained craving for cocaine.

Groups CDND and CDNS both had their cocaine use discontinued following the chronic cocaine administration period. Both groups showed signs of reduced craving for the drug given the moderate time spent in the white compartment ($p \leq 0.05$) of the CPP box at around week 5 of the supplement administration period. By week 6 (Figure 3), group CDNS spent significantly less time ($p \leq 0.05$) in the white compartment of the CPP box than that of group CDND. This trend continued until the end of the supplement administration phase, where the time spent in the white compartment of the CPP box for groups CDND and CDNS was recorded at approximately 10 minutes and 6 minutes respectively.

Figure 1. Time spent in the white compartment of the CPP box (minutes) as an indicator of craving following chronic cocaine administration in rat model



ND - *Normal rats fed normal rat diet*; NS - *Normal rats fed supplemented rat diet*; CC - *Rats dependent on cocaine and continually given cocaine throughout the entire study period, fed normal rat diet*; CDND - *Rats dependent on cocaine, cocaine use discontinued, fed normal rat diet*; CDNS - *Rats dependent on cocaine, cocaine use discontinued, fed supplemented rat diet.*
The values shown represent mean ± standard error of the mean.
Figures with different superscripts are significantly different ($p \leq 0.05$).

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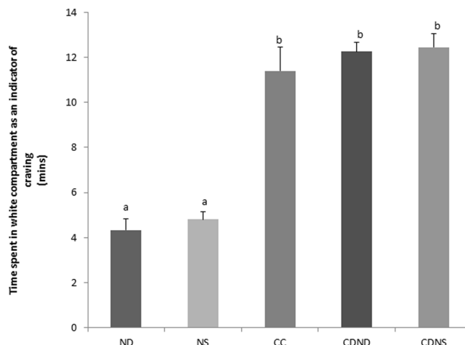
Figure 2. Time spent in the white compartment of the CPP box (minutes) as an indicator of craving one week following the start of nutritional supplement administration in rats



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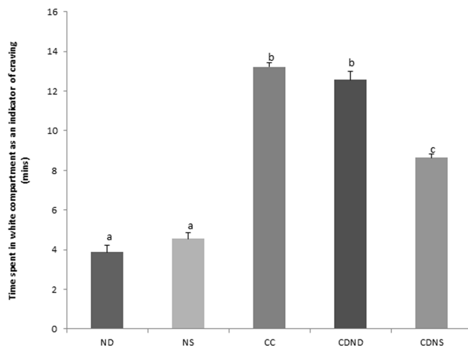
The values shown represent mean ± standard error of the mean.
Figures with different superscripts are significantly different ($p \leq 0.05$).

Figure 3. Time spent in the white compartment of the CPP box (minutes) as an indicator of craving mid-way following the start of nutritional supplement administration period in experimental rat groups NS and CDNS



ND - *Normal rats fed normal rat diet*; NS - *Normal rats fed supplemented rat diet*; CC - *Rats dependent on cocaine and continually given cocaine throughout the entire study period, fed normal rat diet*; CDND - *Rats dependent on cocaine, cocaine use discontinued, fed normal rat diet*; CDNS - *Rats dependent on cocaine, cocaine use discontinued, fed supplemented rat diet.*
The values shown represent mean ± standard error of the mean.
Figures with different superscripts are significantly different ($p \leq 0.05$).

Effect of Supplement Administration on Serum Cocaine Metabolite Levels

Table 2 illustrates the cocaine metabolite concentration in plasma of rats at varying intervals of the animal study. There was no cocaine metabolite observed in the groups not exposed to cocaine. Following chronic cocaine dependence, there was significant increase in the metabolite

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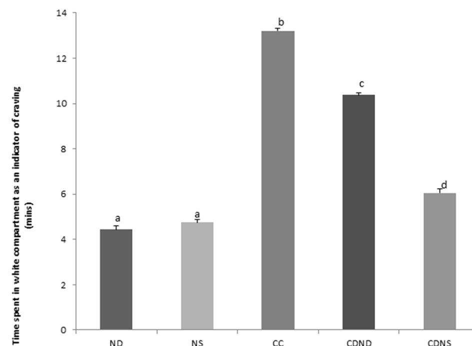
Figure 4. Time spent in the white compartment of the CPP box (minutes) as an indicator of craving at the end of the nutritional supplement administration period in experimental rat groups NS and CDNS



ND - *Normal rats fed normal rat diet*; NS - *Normal rats fed supplemented rat diet*; CC - *Rats dependent on cocaine and continually given cocaine throughout the entire study period, fed normal rat diet*; CDND - *Rats dependent on cocaine, cocaine use discontinued, fed normal rat diet*; CDNS - *Rats dependent on cocaine, cocaine use discontinued, fed supplemented rat diet.*
The values shown represent mean ± standard error of the mean.
Figures with different superscripts are significantly different ($p \leq 0.05$).

Table 2. The cocaine hydrochloride metabolite (benzoylecgonine) concentration in plasma of rats at varying intervals of the animal study

	Cocaine hydrochloride metabolite (benzoylecgonine) (ng/mL)			
	Phase of Animal Study			
Group	Baseline	After Chronic Cocaine Dependence	Mid-way Supplement Administration	End of Supplement Administration
ND	None detected	None detected	None detected	None detected
NS	None detected	None detected	None detected	None detected
CC	None detected	1090.32 ± 13.56^{a}	1141.20 ± 30.11^{a}	251.78 ± 31.95^{b}
CDND	None detected	1099.32 ± 25.45^{a}	203.89 ± 37.18^{b}	None detected
CDNS	None detected	1147.88 ± 31.41^{a}	361.83 ± 91.88^{c}	None detected

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Results are expressed as mean ± standard error of the mean.
Figures with different letters are significantly different ($p \leq 0.05$).

concentration in the groups of rats exposed to cocaine (CC, CDND and CDNS). Mid-way the supplement administration phase, there was a high level of cocaine metabolite in the circulating plasma in the group continually given cocaine throughout the entire study period. There was significant increase ($p \leq 0.05$) in the circulating cocaine metabolite concentrations in the groups that had their cocaine use discontinued and fed nutritionally supplemented diet in comparison to the group given normal diet.

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Cytochrome P450 3A4 Isoenzyme Liver Activity

Figure 5 illustrates cytochrome P450 3A4 isoenzyme activity in the liver at the end of the study period. Increased activity ($p \leq 0.05$) was observed in the group NS (fed the SA supplement formulation) when compared with the normal control group, ND. Groups CC, CDND and CDNS (the groups exposed to cocaine) exhibited increased enzyme activity ($p \leq 0.05$) where group CDNS in turn showed a significant increase ($p \leq 0.05$) in enzyme activity.

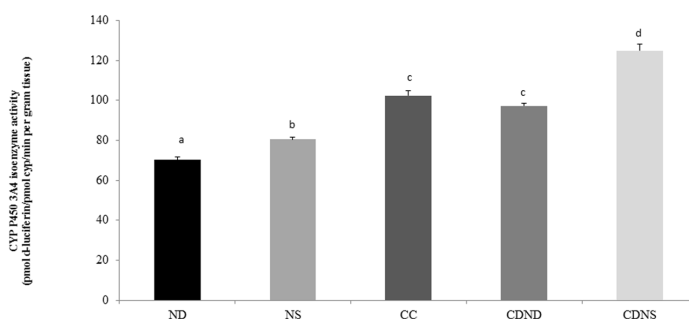
DISCUSSION

Conditioned Place Preference (CPP)

In this study rats with chronic cocaine dependence were developed using the conditioned place preference (CPP) paradigm. These rats were used as indicators of craving, in order to determine the efficacy of the SA supplement with discontinued cocaine use, in order to elucidate the proposed mechanism of action of the supplements.

CPP has been developed and is used as one of the most effective experimental protocols to measure drug reward when using laboratory animals (Bardo & Bevins, 2000; Cunningham et al., 2006) and has been used in numerous studies as a standard measure of craving in animal models (Carboni & Vacca, 2003; Tzschentke, 1998). The principle underlying the use of a CPP box is based on the choice for an otherwise 'unpreferred' compartment as an indication of craving. In this experiment, the pre-conditioning phase indicated that the 'preferred' compartment was the black, dark chamber in which the animal models spent the majority of the time naturally, as opposed to the white, brightly lit chamber which was the 'unpreferred' compartment, and where significantly less time was spent. The white, brightly lit chamber was paired with cocaine subsequently and a craving was noted by the significant increase in time spent in this once 'unpreferred' compartment. A marked craving for the drug was demonstrated by a significant increase in the amount

Figure 5. CYP450 3A4 isoenzyme analysis following chronic cocaine exposure and subsequent treatment with SA supplements in rats



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Figures represent mean ± standard error of the mean.
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of time spent in the white compartment after chronic drug administration in comparison to the periods before any cocaine was administered.

Other studies have consistently used this method where time spent in the chambers was indicative of craving for the drug (Maldonado & Rodriguez de Fonseca, 2002; Martin et al., 2000; Sora et al., 1998). Results indicate that there was significant craving produced for the drug prior to supplement administration in the groups that were exposed to cocaine chronically, namely groups CC, CDND and CDNS.

The effectiveness of the supplements on craving was tested in one group, and compared with normal rat diet given to another group following chronic cocaine administration. The rats that were continually exposed to cocaine throughout the study consistently exhibited a craving for the drug throughout the study. At week 5 of the supplement administration period, group CDNS showed evidence of reduced craving for the drug as seen by a reduction in the time spent in the white compartment from 12.46 ± 0.18 minutes at the start of the administration to 11.38 ± 0.18 minutes. Over time, this value decreased until the end of the administration period. At week 13, the time spent in the white compartment was 6.05 ± 0.18 minutes, which was comparable to the group's time at acclimatization 5.98 ± 0.48 minutes. Group CDND exhibited a slight decrease in time spent in this compartment, but not as marked as group CDNS whereas group CC exhibited a continued indication of craving for the drug.

These results demonstrate that the administration of the supplements reduced craving of the drug significantly following discontinued cocaine use during the supplement administration period.

Cocaine Metabolism/Plasma Metabolites

Cocaine is metabolised in the body in numerous ways. The two-primary means of metabolism incorporate the esterases which are found in various tissues and organs and the oxidative pathway, performed by the liver. Cocaine can release epinephrine and norepinephrine into the circulating system which may give way to glutathione depletion in the liver (James et al., 1983; Register & Bartlett, 1954). Catecholamines seem to be elevated by some cocaine metabolites, which may lead to cocaine toxicity (Misra & Pontani, 1977; Williams et al., 1977)

The primary cocaine metabolite, benzoylecgonine, was measured in the circulating plasma of rats throughout the entire study period. There was a high circulating plasma concentration of benzoylecgonine following chronic cocaine administration which was maintained over a period of time by the group continually administered cocaine throughout the study period. Cocaine as previously mentioned has been found to be stored in organs in the body when administered chronically (Bystrowska et al., 2012; Colucci et al., 2010). Circulating plasma concentrations are often an indicator of equilibrium between these organs operating as storage and the blood, which gives an indication of the concentrations remaining in the body (Chaing & Hawks, 1986; Rook et al., 2006). The concentrations were significantly higher in the treatment group (CDNS) when compared to the untreated group (CDND). The higher equilibrium that appears to exist in the treatment group could possibly be an indicator of a gradual, slow release of the stored metabolites which may have contributed to better management of craving in the animal models.

Cytochrome P450 3A4 Isoenzyme Concentration

The cytochrome P450 system is one major system that carries out the metabolism of cocaine (Shuster et al., 1983), particularly the cytochrome P450 3A4 isoenzyme activities which were determined at the end of the animal study using liver cells. Increased activity of the cytochrome P450 3A4 isoenzyme which is partly responsible for cocaine metabolism is suggestively indicative of increased metabolism of cocaine into its varying metabolites. The activities were significantly

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increased in the rats that were exposed to cocaine. This increased activity was further enhanced in the treated group (CDNS) which leads to the possible premise that the SA supplement formulation increases the breakdown of the cocaine into its metabolites and somehow providing a protective effect on the liver.

SUMMARY

The study was to investigate the effects of chronic cocaine administration and subsequent administration of nutritional supplements in reducing craving in this rat model. It was seen that the supplements reduced craving of cocaine significantly following discontinued cocaine in the animal model. The cytochrome P450 3A4 isoenzyme is partly responsible for cocaine metabolism and is suggestively indicative of increased metabolism of cocaine, which was further enhanced by the supplements. Blood plasma analysis showing higher benzoylecgonine equilibrium demonstrated that the supplements possibly have aided in removing the stored metabolites and this may have contributed to better management of craving in the rats.

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