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The effect of cocaine on the development rate of immatures and adults of *Chrysomya albiceps* and *Chrysomya putoria* (Diptera: Calliphoridae) and its importance to postmortem interval estimate

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ABSTRACT

This study aimed to determine the effect of cocaine on the development and growth of immature and adult blowflies, in an attempt to better understand the impacts of such effects on postmortem interval (PMI) estimation. Twice the lethal dose of cocaine was injected into rabbits. The control animals were injected only with saline solution. Experimental and control rabbits were autopsied, and portions of their livers were exposed to newly eclosed larvae of *Chrysomya putoria* and *Chrysomya albiceps*. Larvae were weighed individually every 6 h, up 54 h of exposure. The larvae were then placed on an artificial diet to continue their development. Pupariation time, adult emergence and adult longevity were also analysed. The larvae of both species that fed on the cocaine-containing livers developed faster than those that fed on the livers of the control animals, leading to the conclusion that cocaine influences and stimulates larval growth. The difference in growth between the control and treated flies was best observed from 12 h of exposure onward. This finding has important implications for forensic investigations.

1. Introduction

Drugs used in society for recreational and other purposes might differ between countries depending on availability, social and cultural aspects, life style and police and government control [1].

Cocaine is one of the most widely consumed drugs of abuse in the world, with high rates of its use in North America [2] and South America [3]. Spain is considered to be the most important entryway for South American cocaine into Europe, where the use of this drug is also widespread. Between 1990 and 1992 alone, 533 people died because of the use of the drug; of these, 84% were found to have both cocaine and heroin in their bodies and 16% were positive for only cocaine [4]. In another survey conducted in the United States, more than 50,000 cocaine-related deaths occurred in a period of 10 years according to government sources [5], and there were 2.4 million cocaine users aged ≥12 years between 2002 and 2006 [6].

Drivers testing positive for cocaine ranged from 0.3% to 9.8% in the USA, 0.1% in Australia and 6% to 7% in Canada and Spain [7]. In Brazil, cocaine is one of the most abused drugs and is frequently associated with unexpected deaths. Seventy-five percent to 90% of a dose is converted to the inactive metabolites ecgonine and benzoylecgonine, which can be excreted for several days after use of the parent drug [8]. Cocaine was the second most frequently found substance in postmortem materials screened for the drug, present in 22% of the samples [9]. The annual mortality rate amongst crack users admitted to a hospital (Taipas General Hospital) in the São Paulo city (Brazil) was 2.5%, which was 7 times higher than the overall mortality rate in the city during the same period [10].

Cocaine can be considered one of the most potent stimulants of the central nervous system and has been frequently associated to traffic accidents and violent crime [11]. Its absorption and action in the body depend on the form of intake. The drug is quickly metabolised when taken orally. Because of this, it is usually inhaled through the nose or is injected intravenously. Other studies have also noted that some users, particularly homosexuals, administer cocaine through the anus for faster absorption and effects. Death may occur by applying just 20–30 mg of this drug to the nasal mucosa, but 1 g ingested orally may not be fatal. Tolerance varies between individuals but the typical usual intravenous dose is approximately 100 mg. However, habitual users may tolerate

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higher doses. Absorption via the nasal mucosa is less effective than intravenous injection, thus requiring higher doses to achieve similar effects. Death caused by cocaine overdose or hypersensitivity to the drug can occur very rapidly, as dramatic increases in blood pressure can lead to a brain haemorrhage. Blood cocaine levels in fatal cases vary greatly, but typically range from 1 to 21 mg l^{-1} (average 5.2 mg l^{-1}) [12]. In Italy, Bertol *et al.* [13] found that in overdose cases, the mean ratios of cocaine/benzoylecgonine were 10.28 μg ml^{-1} in the brain and 0.69 μg ml^{-1} in the blood, whereas in incidental cases, the means in the brain and blood were 0.71 and 0.21 μg ml^{-1}, respectively. Usually, the concentrations of benzoylecgonine in blood are higher than that of the parent drug cocaine and the frequency distribution of cocaine concentrations can vary according to the tissue [1].

A shift has been observed in the route of cocaine administration by drug abusers in several countries. Smoking of the drug, in the form of crack, has increased in prevalence, which may be the result of the low cost of this form of the drug, the heightened behavioural responses compared to other routes of administration and the avoidance of needle-transmitted diseases [14]. Because of the many forms and routes of administration of the drug, there is a need for the development of versatile methods for determining cocaine concentrations [2].

There are many ongoing studies seeking to determine the effect of drugs on the development of insects, particularly blowflies and fleshflies. These studies aim to minimise potential errors that can rise during postmortem interval (PMI) estimation, by using techniques that measure drug-related alterations of insect larval stage durations, pupariation and emergence times [15–18]. The purpose of this research was to determine the influence of cocaine on the life cycles of *Chrysomya albiceps* and *Chrysomya putoria*, two flies that are of forensic interest in Brazil.

2. Materials and methods

Ten male white domestic rabbits (five control and five treatment animals), with weights ranging from 3 to 4 kg were used in the experiments. The method was modified as per Goff *et al.* [15] The animals were kept in an animal care facility for 1 week before the start of the experiment, and then weighed, placed into separate cages and given food and water.

2.1. Drug purification

In forensic science laboratories, cocaine is rarely found in its pure form. Adulterants are commonly added to prepared cocaine, resulting in low concentrations of the actual drug [19], It was therefore necessary to purify the drug before starting the experiment. One gram of cocaine was diluted in 100 ml of 2 N ammonium hydroxide. A chloroform extraction (5× 100 ml) was performed in order to separate the aqueous from the organic phase. The chloroform phase was recovered, and sodium sulphate was added to it for water adsorption. The solution was filtered, recovered and dried in a rotary evaporator at a low temperature. After this procedure, 1 ml of methanol was added, and the solution was once again placed in the rotary evaporator to obtain the cocaine crystals. A total of 485 mg of pure cocaine was produced.

2.2. Fly experiment

The drug used in this study was diluted and homogenised with five drops of Tween-60 emulsifier. The drug was administered intravenously in 2 ml of saline solution. In the experiment, each replicate received a dose of 138 mg (2× the LD50), given that the lethal dose 50 (LD50) for rabbits is 17 mg kg^{-1} [15]. Each control rabbit received only 2 ml of the saline solution. Thirty minutes after the drug had been administered, the controls and the treatment animals that had not died from the drug were sacrificed mechanically by cervical disjoint with no superficial wounding so as to avoid blood loss. Following this, the rabbits were autopsied for the removal of their livers. Pieces of liver tissue were collected, weighed and submitted to qualitative analysis using gas chromatography–mass spectrometry (GC–MS) to confirm the presence of the drug in the sample. Two samples from the livers of each rabbit were collected and exposed to larvae of *C. albiceps* and *C. putoria* obtained from laboratory-stock colonies.

The two species were placed separately on the liver samples. One hundred larvae from a single oviposition of each species were used. These materials were placed in plastic jars with organza caps, and then taken to a rearing room kept at room temperature (±27 °C). At 6-h intervals, up to 54 h of exposure, 10 larvae of each species from all replicas and controls were weighed individually. The larvae were then removed from the livers, counted to determine their mortality rate and given an artificial diet of beer yeast, powdered milk, casein, agar, methylparaben and water [20] to continue and complete their development. Pupariation time (start and end) was observed, as well as adult emergence time. The longevity and mortality of each species were also verified.

The data were analysed with the SAS statistics application [21]. The Proc GLM (general linear models) procedure was used for the analysis of variance (ANOVA); the Proc TTest procedure was used for Student's *t*-test, and the Proc Corr was used for correlation. For the ANOVA results, Duncan's multiple comparisons *F*-test was used to verify possible differences between the means, for each stipulated factor or variable. The following factors were analysed: the growth (weight) of the larvae after their exposure to drug-containing and control liver tissues; the species of the fly; the drug used; the time of exposure on the liver, in hours; and the species × drug, species × time and drug × time interactions. The output variables were larva weight (mg), pupariation time (h), emergence time (h) and mortality.

2.3. GC–MS analysis

One gram of the liver samples was homogenised using homogenator equipment in 5 ml of methanol. The ethanolic extract was centrifuged at 3000 rpm for 20 min. The supernatant was recovered, and 1 N acetic acid (4 ml) was added, followed by vortexing. Ether:hexane (3:1, 3 ml) was then added, and the sample was vortexed. Samples were centrifuged at 1500 rpm for 20 min, the organic layers were taken to waste and the aqueous phases were pipetted onto cation-exchange resin (Amberlite–Sigma) columns. These columns were previously packed with 0.8 g of resin, and conditioned by rinsing, in sequence, with water (3 ml), 1 N acetic acid (12 ml), water (12 ml), ethanol (3 ml) and water (6 ml). After applying the samples, the columns were sequentially washed with water (3 ml), hexane (2 ml), methylene chloride (2 ml) and ethanol (3 ml) [22]. The analytes were then eluted using 7 ml of aqueous ammonium hydroxide:ethanol (1:3) and collected in cap vials. The eluates were evaporated to dryness under nitrogen. The resulting residues were recovered in 10 ml of methanol to GC–MS analysis. An aliquot of cocaine standard solution was diluted in methanol and 1 μl was injected, and served as control for GC–MS analysis.

The analysis was performed on a Hewlett Packard model-5890 series II, coupled to a selective mass detector HP-5970. Chromatographic separations were achieved on an S-5 30 m × 0.25 mm × 0.25 μm capillary column (Sigma–Aldrich). A programmed column temperature was used initially set at 150 °C and increased to 300 °C at 6 °C min^{-1}. The GC injection port was operated at 250 °C, without reason of division during 0.25 min, and the detector was set to 280 °C. The helium carrier gas linear velocity was 1 ml min^{-1}. Detection was carried out by multiple ion monitoring (*m/z* 82, 182 and 303).

3. Results

All of the liver samples submitted to analysis were positive for cocaine.

No weight differences between the control and treated flies were found in either species after the first hour of exposure. At both the 6th- and 18th-hour observations and weigh-ins, the control larvae of *C. albiceps* had developed faster than those exposed to the drug, but the opposite was observed in the larvae of *C. putoria*.

At 24 h of contact with the livers, *C. albiceps* larvae exposed to cocaine were less developed than *C. albiceps* exposed to the control livers. No significant difference was observed between the control and experimental treatments for *C. putoria* larvae.

At the 30- and 42-h weigh-ins, no significant developmental differences were observed for *C. albiceps*, but the drug-exposed *C. putoria* larvae had developed significantly faster than larvae in the control treatment.

At 54 h post-exposure, the cocaine-exposed larvae of both species had grown more than their controls. The weight of the *C. putoria* larvae that fed on cocaine treated livers was twice that of the control larvae (Fig. 1).

Larvae exposed to cocaine started and ended the pupariation process before the larvae in the control treatment, with a difference of approximately 13 h in the duration of this stage (Fig. 2). In both species, pupariation time was significantly different between cocaine-exposed and control larvae. In this way, both the *C. albiceps* and the *C. putoria* larvae exposed to the drug presented a much earlier pupariation time (start and end)