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Comparison of acute effects of heroin and Kerack on sensory and motor activity of honey bees (*Apis mellifera*)

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ABSTRACT

Objective(s): Previous studies demonstrated a functional similarity between vertebrate and honey bee nervous systems. The aim of the present study was to compare the effects of heroin and Iranian street Kerack, a combination of heroin and caffeine, on sensory threshold and locomotor activity in honey bees.

Materials and Methods: All drugs were given orally to honey bees 30 min before each experiment. The levels of these drugs and their metabolites in brain samples of honey bees were determined by GC/MS. The sucrose sensitivity test was used for evaluation of changes in honey bees' sensory threshold. Following the administration of both drugs, the honey bees' locomotor activity changes were evaluated in open fields.

Results: 6-acetylmorphine had a higher concentration in comparison with other heroin metabolites in honey bees' brains. Concentration of the compound in the brain was directly proportional to the amount ingested. Heroin reduced the sensory threshold of honey bees, but Kerack increased it in the same doses. Locomotor activity of honey bee in open field was enhanced after the administration of both drugs. However, immobility time of honey bees was only affected by high doses of heroin.

Conclusion: Acute effects of heroin andKerack on the sensory and motor functions of honey bees were different. Findings of this research suggest that these differences originated from the activation of different neurotransmitter systems by caffeine together with activation of opioid receptors by heroin.

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Introduction

Although heroin is one of the most addictive and dangerous drugs (1), few experiments have examined its effects on animal sensory and motor activity. Most of the current information has been obtained from the addicts who abused heroin chronically (2). Nowadays, different illicit drugs are being produced from heroin; nevertheless, little information is available about the acute sensory and motor effects of these drugs in people who used it for the first time (3). These findings have practical importance for the estimation of tendency for heroin abuse and its new combinations (4). According to the existing reports, illicit heroin and Kerack are two forms of heroin-containing substances consumed by addicts in Iran (5). For the first time, Kerack sachets were produced in south-east Asia and used by a method called "chasing the dragon" (6). Subsequently, its use spread to other parts of the world (7). These sachets usually contain 75% heroin and 25% caffeine (8). Heroin addicts often shift from one form of heroin to another. Such shift probably results from the difference in their biological effects or the intensity of drug dependence (9, 10). In support of this idea, it is shown that caffeine simultaneously potentiates stimulatory effects of morphine and on the other hand, it attenuates its inhibitory effects on CNS function (11). Therefore, each form of heroin-containing drug with particular additives and formulation may have unique effects on sensory and motor performance of abusers. Thus, a simple animal model for the assessment of such behavioral effects seems essential. Recently, honey bees have been successfully used as a model for assessing the sensory and motor effects of addictive drugs (12). The small brain of honey bee has shown high functional similarity with the vertebrate's brain (13). For example, the interactions of endogenous opioid and dopaminergic systems have been observed in the CNS of honey bee (14, 15). These systems have a fundamental role in mediating the behavioral effects of addictive drugs in both vertebrates and invertebrates (16). Honey bee is also a suitable model for studying lipophilic drug effects which are administered by different routes. It is reported that the distribution rate of such drugs in CNS tissue of honey bee after oral administration or injection is the same (17). In the present study the

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metabolites of heroin and Kerack in honey bees' brain were first determined following oral administration by gas chromatography/mass spectrometry (GC/MS). Then, the acute sensory effects of these two forms on honey bees were assessed by sucrose responsiveness threshold test. Finally, after pretreatment with these two forms of heroin, changes in locomotor activity of honey bees were compared in an open-field. The results of this study can also provide a novel assay method for evaluation of acute effects of other heroin-based drugs.

Materials and Methods

Animal

All experiments were performed on a hybrid of Asian honey bee (*Apis mellifera*), commercially available in Iran (18). The experiments were conducted from April to September in the garden of Shahed University of Iran. Adult honey bees were collected in a plastic box by a suction device at hive entrance. They were transported to the laboratory in the box and maintained for 24 hr at 24 ˚C and fed 40% sucrose solutions. The change in sensory threshold of restrained honey bees was assessed by sucrose sensitivity threshold test 30 min after drug ingestion. The effect of the drugs on locomotor activity in the freely moving honey bees in an open field was measured 30 min after administration of each drug. Different honey bees were used for GC/MS measurements, sucrose sensitivity test and locomotor activity evaluation; each group contained 30 honey bees.

Drugs

Heroin and Kerack were donated by the research laboratory of Anti-narcotics Police of Iran. The so called Kerack used in this study contained 75% heroin and 25% caffeine. The exact chemical composition of both drugs has been reported previously (19, 20). Other chemicals and solvents used in the present study were of analytical reagent grade and were purchased from Merck. Drug samples were crushed and homogenized in a mill. Solutions of the samples were prepared by weighing the homogenized powder and dissolving it in methanol with 1 mg/ml concentration. After evaporation of methanol under nitrogen stream, different doses of drugs were prepared from this stock solution by adding a 40% sucrose solution. Thirty minutes before experiments, honey bees orally received 5 µl of a sucrose solution that contained different doses of heroin or Kerack. The control honey bees only received 5 µl of 40% sucrose solution.

Experimental design

Measurement of heroin metabolites in honey bees' brain

Honey bees were frozen by dipping into liquid nitrogen thirty minutes after feeding, and kept at -20 ˚C freezer until the time of brain dissection. Total brain of the honey bee was removed from head capsule and homogenized in 100 µl chilled normal saline by sonicator. 100 µl chloroform was then added to the final solution and mixed for one minute (21). The presence of heroin and its metabolites were determined by GC/MS in chloroform phase. The component was firstly extracted by a gas chromatograph (Agilent 7890A, Palo Alto, CA) with HP5MS capillary column (30 m×0.25 mm×0.25 µm), and helium gas was used as a carrier. The output of GC was then detected and analyzed by an Agilent Mass Selective Detector (5975C, Palo Alto, CA). The output components of GC were recognized based on their unique retention times, compared to the internal calibration of the instrument with standard diacetylmorphine. The concentration of 6-monoacetylmorphine (6-MAM) in honey bees' brain tissue was determined in treated groups using the proposed methods (22, 23). In practice**,** the calibration curves of peak area versus concentration (ng/ml) of the analytes were plotted. Least-squares regression parameters for the calibration curves were calculated, and the concentrations of the test samples were interpolated from the regression parameters.

Determination of sucrose sensitivity or responsiveness threshold

Sucrose responsiveness threshold, defined as the lowest concentration of sucrose, can induce PER after applying the sucrose solution to the honey bees' antennae (24). In practice, the honey bee's antennae are stimulated with incremental concentrations (30%, 10%, 3%, 1%, 0.3%, and 0.1%) of sucrose solutions. Sucrose stimulation interval is 2 min. To avoid sensitization or habituation of the animals from repeated sucrose presentation, a 0.6 ml drop of distilled water was presented to both antennae between sucrose presentations (1 min before the next sucrose presentation). Furthermore, an interval of 2 min between sucrose presentations did not promote habituation to sucrose (25). Honey bees were restrained in a thick aluminum tube, 24 hr before beginning of the experiments. Then, the harnessed honey bees were divided into the following groups: the control group, only orally treated with 40% sucrose solution; the heroin group, which was treated with sucrose solution containing (100 µg, 10 µg, 1 µg, 100 ng, 10 ng, and 1 ng) of heroin; and the Kerack group treated orally with 40% sucrose solution containing (100 µg, 10 µg, 1 µg, 100 ng, 10 ng, and 1 ng) of Kerack. Extension of proboscis to each concentration of sucrose was considered a positive response.

Locomotor activity test

The honey bees' locomotor activity was measured in an open field box (30 × 30 × 4 cm) made from Plexiglas following the administration of each drug (26). The box was placed vertically and illuminated

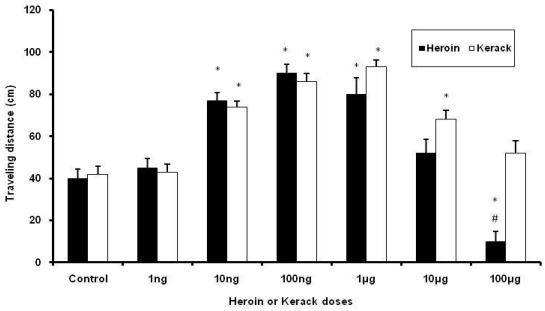

Figure 1. The effect of pretreatment with different doses of heroin and Kerack on traveled distance of honey bees (n=30) in an open field. Control honey bees treated with only 40% sucrose solution. Control vs. Doses (**P*<0.05), Heroin vs. Kerack (#*P*<0.05)

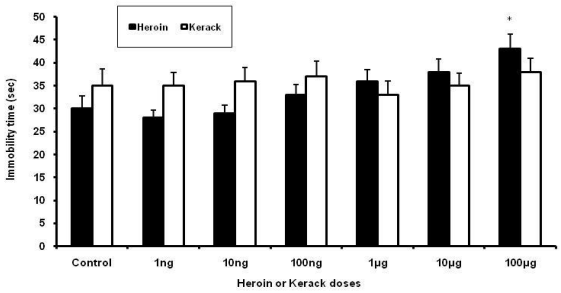

Figure 2. Effects of heroin and Kerack pretreatment on change in immobility time of honey bees in an open field. Control vs. doses (two way ANOVA, **P*<0.05)

from above with a 15 W lamp, which induced the upward motion of the honey bees. The transparent front panel of the box allowed the honey bees' movement to be easily seen in the chamber. The honey bees' locomotor activities in the arena were recorded for three minutes by a camera. The rear panel of the chamber was divided into 36 squares with parallel lines with 5 cm distance from each other. A small hole for entrance of the honey bees was made in lower right side of the front panel. In all groups, locomotor activity of honey bees was measured 30 min after oral administration of different doses of drugs dissolved in sucrose solution. The position of each honey bee in the box was recorded every 3 sec during the three min period of movement. Immobility time denoted the period that the bee stayed in the same square over two consecutive periods of 3 sec each. The total distance traveled by each honey bee and the time spent in each level during 3 min of presence in the box was also measured. Those honey bees that showed abnormal behavior were discarded from the experiments.

Statistical analysis

PER of the honey bees following the administration of different concentrations of sucrose solution were recorded and compared among the groups using the McNemar's test. The locomotor activity of each honey bee was determined by measuring the following parameters: traveling distance, immobility time and the time spent in each level of open field. The recorded variables for locomotor activity of each group were compared to the control matched group using the two-way ANOVA.

Results

Metabolites of heroin and Kerack in honey bee's brain

The GC/Ms results showed that 6-monoacetyl-morphine (6-MAM) constitutes the highest percentage of heroin and Kerack metabolites in the honey bee's brain. In addition to 6-MAM, caffeine was found in brain extract of the honey bees that were treated with Kerack.

Furthermore, the 6-MAM concentrations in the honey bees' brain samples indicated the existence of a direct relation with drug concentrations in feeding solutions in heroin and kerack-treated groups (Table 1).

Drug effects on sucrose sensitivity thresholds

Pretreatment of honey bees (n=30) with 100 µg and 10 µg heroin caused a significant (*P*<0.05) reduction in the PER rate, while PER rates were significantly increased after the administration of 10 ng heroin (Table 2).

The administration of kerack at doses of 10 and 100 µg, significantly (*P*<0.05) increased PER rate of honey bees (n=30). On the contrary, PER rates of honey bees were reduced after the administration of 1 ng Kerack (Table 2).

Locomotor activity changes after the administration of drugs

The administration of heroin at doses of 10 ng to 1 µg significantly (*P*<0.05) increased the traveled distance of honey bees (Figure 1). The traveled distance of honey bees was also enhanced after treatment with the same doses of Kerack. Locomotor stimulatory effect of Kerack continued after increasing the dose to 10 µg. Finally, heroin significantly (*P*<0.05) reduced this parameter at 100 µg.

Compared to all other doses, heroin could only increase (*P*<0.05) immobility time of honey bees at 100 µg. Kerack had no significant effect on immobility time of honey bees at any dose (Figure 2).

Table 1. The calculated GC/MS peak area for 6-MAM in honey bees brain extract samples that treated with heroin and Kerack (mean±SEM, n=30)

Drug concentration	AUC for 6-MAM in heroin groups	AUC for 6-MAM in Kerack group
100 µg	2057098208±35590	1745320815±42667
10 µg	3186607131±3677	335390796±46669
1 µg	52038912±9689	63289229±10848
100 ng	6269748±6150	12480243±12801
10 ng	1333989±1842	1996838±2216
1 ng	261566±324	298035±412

Table 2. Change in numbers of proboscis extension reflex of honey bees (n=30) after pretreatment with different doses of heroin and Kerack. Control group only treated with water. Number of responses before and after drug treatment was compared with the McNemar test (**P*<0.01, **P*<0.05). Abbreviation: D: decreased, I: increased, ns: non significant

Sucrose concentration (%)		0.1	0.3	1	3	10	30
	Control	ns	ns	ns	ns	Ns	ns
	10 mg	D**	D**	D**	D**	D**	D**
	1 mg	D**	D**	D**	D**	ns	ns
Heroin	100 µg	ns	ns	ns	ns	ns	ns
	10 µg	ns	ns	ns	ns	ns	ns
	1 µg	I*	I*	ns	ns	ns	ns
	100 ng	ns	ns	ns	ns	ns	ns
	Control	ns	ns	ns	ns	ns	ns
	10 mg	ns	I*	ns	ns	ns	ns
	1 mg	I*	ns	ns	ns	ns	ns
Kerack	100 µg	ns	ns	ns	ns	ns	ns
	10 µg	ns	ns	ns	ns	ns	ns
	1 µg	ns	ns	ns	ns	ns	ns
	100 ng	I*	ns	ns	ns	ns	D*

It was observed that after taking 1 µg or 10 µg of heroin (Figure 3) or Kerack (Figure 4), the honey bees spent more time in the upper level of the open field. However, the administration of 100 µg of either drug reduced the tendency of honey bees for going to the upper level of the open field.

The results showed that the oral administration of low and high doses of heroin to honey bees, reduced and stimulated PER rates. Inversely, PER rate of honey bees was increased after pretreatment with low doses of Kerack and decreased after high doses.

Discussion

The findings of this investigation indicate that heroin decreased the sucrose taste sensitivity of honey bees. To our knowledge, little information is available about acute effects of heroin on invertebrate sensory function (27). However, change in taste threshold sensation was reported following opioid receptor antagonist treatment in vertebrates (28). Human studies have also shown that activation of central opioid receptors can induce alteration in sweet flavor preference (29). Moreover, a direct relationship was indicated between the duration of heroin abuse and visual and auditory reaction time in heroin addicts (30). Based on pharmacological studies, GABA-ergic system plays a role in acute effect of opioids in vertebrates (31). On the other hand, the regulatory action of GABA on the activity of sensory neurons in antennal lobe of honey bee's brain has been established. GABA-ergic neurons can affect the taste sensory processing in honey bees (32). Considering the functional similarity of the insect antennal lobe and vertebrate olfactory bulb (33), it seems that acute effects of heroin on sucrose sensitivity threshold of honey bee could be mediated through manipulation of GABA-ergic neurons in antennal lobe of honey bees. The current data show that unlike heroin, the same doses of Kerack increase sucrose sensitivity threshold in honey bees. According to the recent molecular findings, activity of dopaminergic neurons in honey bee's brain was significantly affected by caffeine (34). Dopaminergic and GABA-ergic systems exert opposing effects on some behaviors in insects (35). These findings can provide an explanation for opposite effects of heroin and Kerack on sensory threshold of honey bees.

In vertebrates, most of the biological effects of caffeine are related to blockage of all types of adenosine receptors (36). The role of adenosine was also revealed in caffeine effects on the sensory function of honey bees (37, 38). Thus, adenosine can be considered another mediator of caffeine effects in the honey bee's brain. Effect of caffeine was also

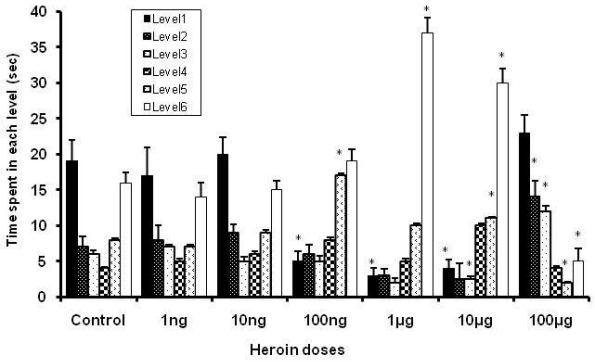

Figure 3. The time (mean+SEM) spent by honey bees in each level of open field after pretreatment with different doses of heroin (Control vs. doses, **P*<0.05, two way ANOVA)

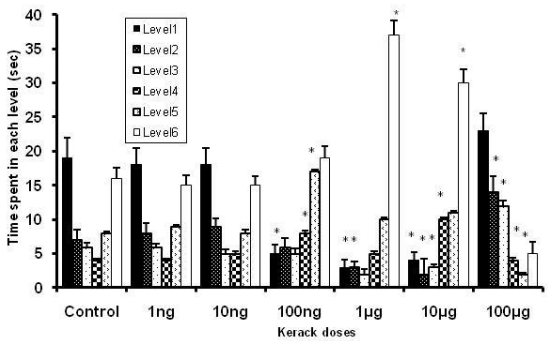

Figure 4. The time (mean+SEM) spent by honey bees in each level of open field after pretreatment with different doses of Kerack (Control vs. doses, **P*<0.05, two way ANOVA)

established on different second messenger systems such as cAMP and calcium within the neurons of honey bee's brain (39). Therefore, it can be concluded that besides the opioid system, dopamine and adenosine were also involved in the effects of Kerack on sensory activity of honey bees.

It was also shown that heroin and Kerack (10 µg–10 ng) resulted in a bell-shaped dose-related increase in traveling distance of honey bees. Vertical displacement of honey bees was increased after receiving high doses of drugs, whereas, the immobility time of honey bees only was decreased after treatment with a high dose (100 µg) of heroin. It was demonstrated that heroin or its combination with other drugs can stimulate locomotor activity in vertebrates (40, 41). In contrast, it was shown that morphine decreased locomotor activity in honey bees (14). It appeared that 6-MAM was responsible for rapid release of striatal dopamine after acute administration of heroin in vertebrates (42). In addition, a dopamine-containing neuron called VUMmx1 was found in the honey bees' brain, which is considered a neural correlate of dopaminergic neurons, originating from the ventral tegmental area in vertebrates (43). Dopamine could stimulate the locomotor activity in honey bees (44). Consequently, the researchers hypothesized that dopamine could be a candidate for mediating the stimulatory effects of heroin and Kerack's active metabolite, possibly 6-MAM, on locomotor activity of honey bees, since other biogenic amines play a fundamental role in regulation of locomotor activity in vertebrates and honey bees (45, 46). Stimulatory effects of these drugs on locomotor activity of honey bees might result from the increased release of these biogenic amines, too.

Conclusion

The results indicate that heroin and Kerack induced different effects on the taste sensitivity threshold of honey bees. The administration of certain doses of heroin and Kerack resulted in an increase in locomotor activity of honey bees. Combination of caffeine and heroin in Kerack caused alteration of its sensory effects on honey bees. Honey bees, therefore, seem to be a practical behavioral model for comparison of heroin-based drug effects.

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