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Effects of morphine on the biomass and development rate of *Chrysomya albiceps* (Diptera: Calliphoridae), a forensically important species

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Abstract. The aim of this study was to determine the effects of morphine on the biomass and development rate of *Chrysomya albiceps* (Diptera: Calliphoridae). *C. albiceps*, a well-known forensically important species which is among the first wave of faunal succession on human cadavers, which makes it a valuable source of information for the estimation of postmortem interval (PMI). Antemortem exposure to substances such as drugs and toxins may have an effect on the biomass and/or on the development rate of insects that feed on carcass, which may directly affect PMI estimation. In this study, three rabbits were administered 12.5, 25 or 50 mg/ml of morphine sulfate via ear perfusion over a period of 3 hours, and a fourth rabbit, which did not receive morphine, was used as a control. The rabbits were sacrificed using chloroform 30 minutes after morphine administration. The tissues were analyzed for the presence of morphine using HPLC-UV. Morphine was detected in all tissues of rabbits that received morphine, except in the bile and spleen of the rabbit which received 12.5 mg/ml dose of morphine. The presence of morphine in rabbit tissues retarded larval development rate, but accelerated the puparial development rate. The rate of development of *C. albiceps* larvae that fed on rabbits which received 25 and 50 mg/ml dosages of morphine was 9 days each. However, the rate of larval development was similar in the 12.5 mg/ml morphine group and the control; 6 days. Results of this study show that an underestimation of the postmortem interval of 72 h based on larval development and an overestimation of 24 to 48 h based on puparial development is possible if the presence of morphine in tissues is not considered. Moreover, the decreased larval development rate caused an increase larval length and weight compared with the control group. In this study, we found a strong correlation between the concentration of morphine administered and concentrations in rabbit tissues. In the estimation of PMI, it is recommended that effects of drugs such as morphine on the development of carcass colonizers be considered.

INTRODUCTION

Necrophagous insects can be used as alternate specimens for toxicological analysis when conventional postmortem samples are not available (Beyer *et al.*, 1980; Goff *et al.*, 1994; Hedouin *et al.*, 1999; Bourel *et al.*, 2001; Gagliano-Candela & Aventaggiato, 2001; George *et al.*, 2009). The effects of drug/toxin on body weight and body length as well as the rate of development of necrophagous insects have been investigated in several studies (Bourel *et al.*, 1999; Gosselin *et al.*, 2010; Sadler *et al.*, 1997; Hedouin *et al.*, 1999; Goff *et al.*, 1989; Rashid *et al.*, 2008). The presence of drugs in

the tissues of carcass can directly influence estimation of PMI, and can lead to an error in calculation based on development data. Many substances such as drugs and their metabolites can affect the development of arthropods of forensic importance when present in the cadavers that they colonize. Currently, two main approaches exist that are used to determine the minimum PMI: one approach explores insect succession patterns in the estimation of PMI, whilst the other approach takes into account the insect growth rate (George *et al.*, 2009). Several studies have been conducted to analyze the impact of antemortem ingestion of drugs and toxins on insect development and behavior. Majority of these studies have shown that antemortem use of various drugs and toxins affects maggot development rate and/or biomass of insects that are exposed to the postmortem cadaver, leading to an inaccurate PMI estimation based on insect development data (Goff *et al.*, 1992; Kintz *et al.*, 1994; Tabor *et al.*, 2004; Ferraz *et al.*, 2016). In some cases, antemortem exposure to drugs or poisons was found to cause deformities and unusual sizes of insects, including organs of the animal models studied (Fathy *et al.*, 2008; Maskell *et al.*, 2016). In general, the effects may differ depending on the type of drug, the species and developmental stage of the insect, and the animal model studied.

C. albiceps is one of the most forensically important flies found in the Afro-tropical and oriental regions. This species is recognized as among the first wave of insects that visit and colonize human cadavers. Currently, this species utilized to indicate the PMI in human deaths. The aim of this study was to evaluate the effects of morphine on the development, weight and length of immature stages of *C. albiceps*. This study also attempts to compare the results of the present study with the results of other similar studies.

MATERIAL AND METHODS

Experiment design

This is an animal model study which included four domestic rabbits with approximate weights as follows: R1=2.500 kg, R2=2.700 kg, R3=2.900 kg (treatment group), and R0=2.600 kg (control). 12.5, 25 and 50 mg/ml of morphine sulfate were administered to rabbits R1, R2 and R3, respectively, via infusion over 3 h. Each rabbit in the treatment group was also infused with 150 ml of isotonic saline solution over 3 h. The control rabbit received only 150 ml of isotonic saline solution without morphine using the same method and duration of inoculation. All rabbits were killed 0.5 h after inoculation with chloroform. An autopsy was immediately carried out in the treatment group, and 10 mg of internal tissue was collected from each rabbit and maintained in distilled water at 4°C in a refrigerator until quantitative analysis. Autopsied rabbits were stitched and weighed together with the control group and immediately transferred to the study field. The samples were transferred to Kermanshah Forensic Medicine Laboratory for analysis. The field of study, situated in the West of Iran, is 13 km from Kermanshah city of Bisestoon protected area (Chalabae), with a geographical location between N 34 24 37 and E 47 14 42. The average annual precipitation in this area is between 400 and 450 mm. Rabbit samples were placed inside cages with dimension 90 by 90 by 150 cm, in direct contact with the ground and closed with fabric lace to prevent interference from other flies. The base of the cages was about 2-3 cm above the surface of the ground to prevent access by beetles. The adults of *C. albiceps* used in this study were kept and the larvae previous study. The study began on June 3, 2017, and data on temperature, humidity and wind speed were measured twice daily in the morning and afternoon. Following egg laying by the adults, several clusters of eggs were collected and transported to transparent plastic containers with dimension of 10 by 10 by 15 cm and covered with a fabric lace lid, containing a layer of 3 cm of sawdust and a piece of internal tissue of rabbits that had received different concentrations of morphine in aluminum foil. Few drops of water were sprayed daily to keep the substrates moist. In the present study, the first day of breeding was considered as T_0. Following hatching, 20-30 larvae at each growth stage were

randomly selected from each plastic container for weighing. The larvae were washed several times with distilled water, and then dried with a paper towel and stored at -20ºC until weighing. In order to measure body length, the same number of larvae at each development stage were randomly selected and fixed in boiling water for 2-3 minutes, and then maintained in 70% alcohol.

Quantitative analysis of tissue samples using HPLC-UV

In the laboratory, morphine was extracted from the specimens by liquid-liquid and acid digestion methods. Specimen from each rabbit was first homogenized, and 100 ml of ammonium sulfate solution (250 g of ammonium sulfate per liter of 20% chloride acid) was added. The pH of the solution was adjusted to 8.9 using ammonia. Next, 100 ml of chloroform-isopropanol (80:20 v/v) was added to the solution and shook for 20 minutes. The sample was then poured into a decanter and the lower organic phase containing morphine was removed. After drying, 1 ml of methanol was added to each sample, and using a 0.2-micron nozzle filter, each sample was filtered and the filtrate was collected in a special vial. 20 µL of each filtrate was injected into the HPLC system. Specifications of the HPLC device include:

KNAUER, Made in Germany, dimension: 250 by 4.6 mm with precolumn, Batch NO: B195975,
Column SN: WH62, Packing Eurospher 100-5 C18, PDA detector

The flow rate was 0.8 ml / min and the mobile phase consisted of phosphate buffer and acetonitrile in a ratio of 3:97. Method validation was performed according to the existing guidelines (Ruzilawati & Miran, 2015). To determine the calibration curve, dilutions of 5, 10, 50, 100, 500 and 1 000 ng/ml morphine were prepared from the original samples at a concentration of 10 mg/ml. 20 µl of each diluted solution was injected into the machine in three replicates (Total run time=15 min).

Statistical analysis

ANOVA and Tukey HSD were used to investigate potential differences in length, weight and development period of immature stages of *C. albiceps* immature stages, as well as weight loss in rabbit carcasses between the different groups. $p \leq 0.05$ was considered significant.

RESULTS

The specificity of the method was determined by comparing the chromatograms of morphine-containing specimens with that of the morphine-free specimen (control). According to Figure 1 and 2, morphine-containing specimens peaked at a retention time of 7,167 min whereas the morphine-free specimen had no interference peaks.

Linearity method was performed using standard diluted solutions 5, 10, 50, 500 and 1 000 ng/ml in three replicates. Calibration curve and the line equation Y = 979.56 X + 3356.2 were obtained using a correlation coefficient R2 > 0.99. The recovery after the injection of dilutions 5, 50, 500, 1 000 ng/ml to a morphine-free matrix was estimated as > 90% using the initial linear concentration equation.

The LOD (Limit of detection) and LOQ (Limit of quantitation) of the method were calculated as 8.36 and 25.3 ng/ml, respectively. Inter and Intra-day precision rates were calculated at less than 8 by computing the coefficient of variation value or CV% for the dilutions 30, 50, 100 ng/ml. Bias was determined using the following formula (Table 1):

Bias = [(found concentration n added concentration)/added concentration] * 100%

Through tissue analysis in the treatment group, we observed that the concentrations of morphine in the carcasses were a function of the initial concentrations of the administered dosage. The concentration of morphine was also different in tissues of the rabbits in the treatment group. All

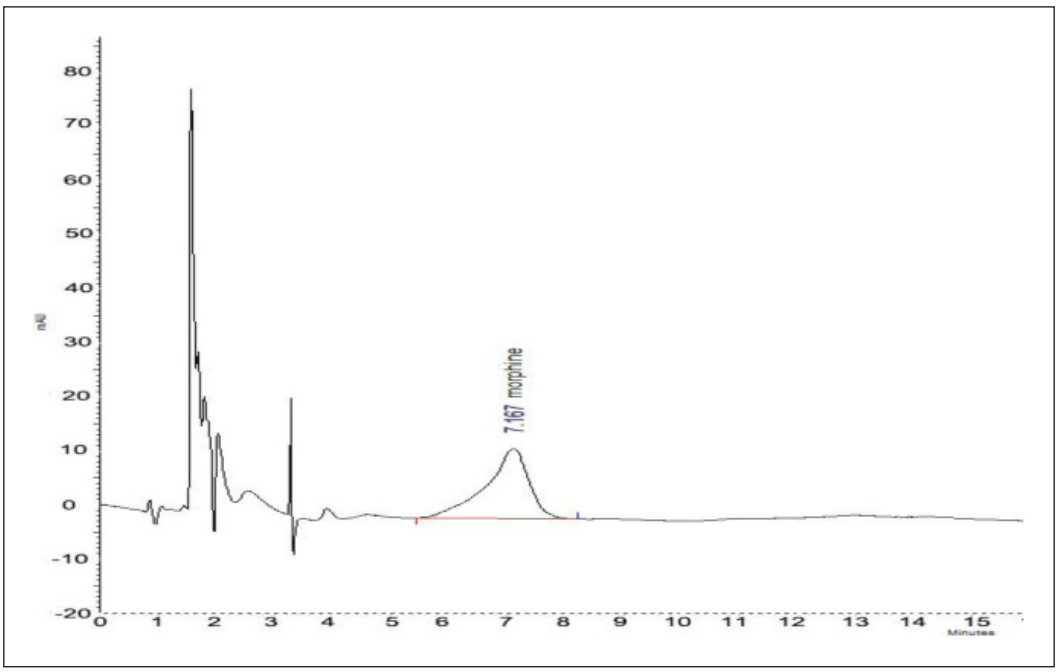

Figure 1. Chromatograms of morphine for standard sample (500ng/ml) with Retention time 7.167 min and Total run time 15 min.

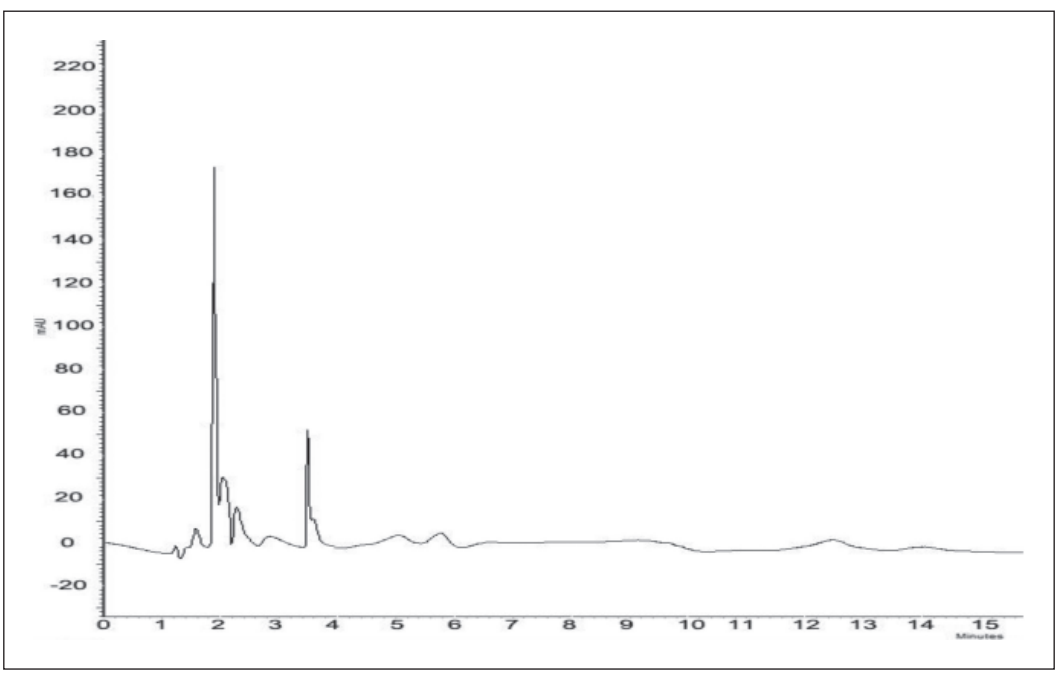

Figure 2. Blank sample without interferential peak in retention time = 7.167.

Table 1. Results of intra and inter-day validation of morphine sulfate

Concentration added (ng/ml)	Concentration founded (ng/ml)	SD	CV %	Bias %	Recovery	n
Intra-day						
30	28.3	2.00	7.14	4.3	93.3	3
50	51.1	1.13	2.23	2.2	102	3
100	101	2.35	2.37	1	101	3
Inter day						
30	27.6	2.08	7.52	4.3	92	3
50	50.51	1.13	2.23	1.02	101	3
100	99.4	2.35	2.37	-0.6	99.4	3

SD = standard deviation; CV = coefficient of variation

Table 2. Concentrations of morphine in rabbit samples administered different dosages of morphine

Rabbit carcasses samples	R0 Concentration (ng/gr)	R1 Concentration (ng/gr)	R2 Concentration (ng/gr)	R3 Concentrating (ng/gr)
Liver	0	47	215	2695
Kidney	0	28	505	2390
Spleen	0	0	722	1191
Bile	0	0	629.5	1411
Mesenteric muscle	0	31	500.7	1666
Urine	0	655.4	891	1969

Sulfate: R0=control, R1=12.5 mg/ml; R2=25 mg/ml; R3=50mg/ml.

samples from the control were negative. At a concentration of R1=12.5 mg/ml, the highest morphine levels were recorded in the urine sample > liver > mesenteric muscle > kidney, in order of decreasing concentration. In this group, morphine was not detected in the bile and spleen. In R2=25 mg/ml, the concentration of morphine was highest in urine samples followed by spleen, bile, kidney, mesenteric muscle, and liver. In the R3=50 mg/ml group, the highest morphine levels were detected in the liver > kidney > urine > mesenteric muscle > bile > spleen, in order of decreasing concentration (Table 2).

In the present study, percentage weight loss was similar in all carcasses which received different doses of morphine, and One-way ANOVA showed no statistically significant difference between the weight of the different carcasses (*P-value* > 0.05). The average minimum and maximum daily temperatures in the study area were 16°C and 33°C, respectively. The R3 group had the maximum larval length which was 15 mm. The larval length in R2, R1 and R0 concentrations were 14.9, 13.2, 13 mm, respectively. The maximum larval weight (61.4 mg) was recorded in the R3 concentration. The larval weight recorded in the other morphine concentrations was as follows: R2=63.3 mg, R1=50.1 mg and R0=49.3 mg (Tables 3-6). One-way ANOVA showed no statistically significant difference in larval growth rates between the treatment group and the control. Maggots were normal morphology in all groups. In Figure 3, peak-feeding larval, post-feeding larval and pupal stages of *C. albiceps* in different concentrations of morphine are shown. The duration of Prepuparial period increased with increasing morphine concentration; prepuparial period was 9 days in R2 and R3 concentrations whereas it was 6 days in R0 and R1 concentrations. The duration of the Puparial period in the concentrations R2 and R3 was 4 and 5 days, respectively, and

Table 3. Average body length and weights of *Chrysomya albiceps* larvae on rabbit carcasses administered R_3=50 mg/ml dosage of Morphine Sulfate (Mean Tmin=16°C. Mean Tmax=33°C)

Day	Length $_{mean}$+/-SD (mm)	Weight $_{mean}$+/-SD (mg)	Carcass weight loss %
1	2.5+/-1.7	3.3+/-2.4	4
2	3.2+/-3.0	6.4+/-3.5	5
3	4.6+/-2.3	12.2+/-5.2	8.6
4	6.7+/-3.1	21.1+/-3.3	22.56
5	9.2+/-2.1	33.5+/-1.9	38
6	12.1+/-4	49.3+/-4.1	50.5
7	15+/- 3.3	61.4+/-2.3	64.3
8	13.6+/-1.7	56+/-3.5	72
9	12+/-1.5	49.3+/-2.9	77.3

Table 4. Average body length and weights of *Chrysomya albiceps* larvae on rabbit carcasses administered R_2=25 mg/ml dosage of Morphine Sulfate. (Mean Tmin=16°C. Mean Tmax=33°C)

Day	Length $_{mean}$+/-SD (mm)	Weight $_{mean}$+/-SD (mg)	Carcass weight loss %
1	1.8+/-2.1	4.2+/-3.5	10
2	4.2+/-1.8	7.5+/-1.3	15
3	5.5+/-3.5	13.6+/-4.0	20.5
4	7.1+/-2.6	20.1+/-1.3	33
5	9.3+/-4.1	31.6+/-3.3	45
6	10.8+/-1.7	41.4+/-2.8	62
7	14.9+/-3.3	63.3+/-2.1	68
8	12.2+/-4.0	57.3+/-5.0	76
9	11.1+/-2.2	51.9+/-3.6	80

Table 5. Average body length and weights of *Chrysomya albiceps* larvae on rabbit carcasses administered R_1=12.5 mg/ml dosage of Morphine Sulfate. (Mean Tmin=16°C. Mean Tmax=33°C)

Day	Length $_{mean}$+/-SD (mm)	Weight $_{mean}$+/-SD (mg)	Carcass weight loss %
1	1.9+/-4.0	1.9+/-6.0	2
2	3.3+/-2.8	6.1+/-2.3	4.3
3	5.1+/-1.7	11.8+/-3.2	17.44
4	6.9+/-1.6	18.3+/-1.7	27
5	8.1+/-5.0	21.1+/-2.1	37
6	11.2+/-3.0	31.3+/-5.5	56
7	13.2+/-2.4	50.1+/-1.9	67
8	12.3+/-1.3	42.3+/-3.0	74.5
9	11.2+/-0.7	38.6+/-2.5	77.5

6 days in R0 and R1 concentrations. The developmental period from the larval stage to adult emergence in R2 and R3 was 13 and 14 days, respectively, and 12 days in R1 and R0 (Table 7, Figure 4).

Using ANOVA test, we found a significant difference between the developmental period of prepuparial and puparial stages of *Chrysomya albiceps* on rabbit carcasses. Also, Tukey HSD analysis showed homogeneity of variance between all groups.

According to Table 7 which is based on the Tukey HSD test, we can distinguish similar concentrations. Based on the *P-value*, in the subset (R0, R1) there is no significant difference together (*P-value* 0.958), while R2

Table 6. Average body length and weights of *Chrysomya albiceps* larvae on Control rabbit carcasses (R_0). (Mean Tmin=16°C. Mean Tmax=33°C)

Day	Length $_{mean}$+/-SD (mm)	Weight $_{mean}$+/-SD (mg)	Carcass weight loss %
1	1.6+/-6.0	2.1+/-4.3	2.1
2	3.5+/-3.3	6.4+/-4.1	5
3	4.9+/-4.2	12.1+/-3.0	20.3
4	5.6+/-3.0	18.3+/-6.0	40
5	7+/-1.8	22.8+/-1.7	50
6	8.2+/-1.6	29+/-3.0	51.1
7	13+/-1.3	49.3+/-2.6	74
8	11.9+/-3.0	44.3+/-5.2	76.5
9	10.9+/-2.6	33.2+/-0.7	78.5

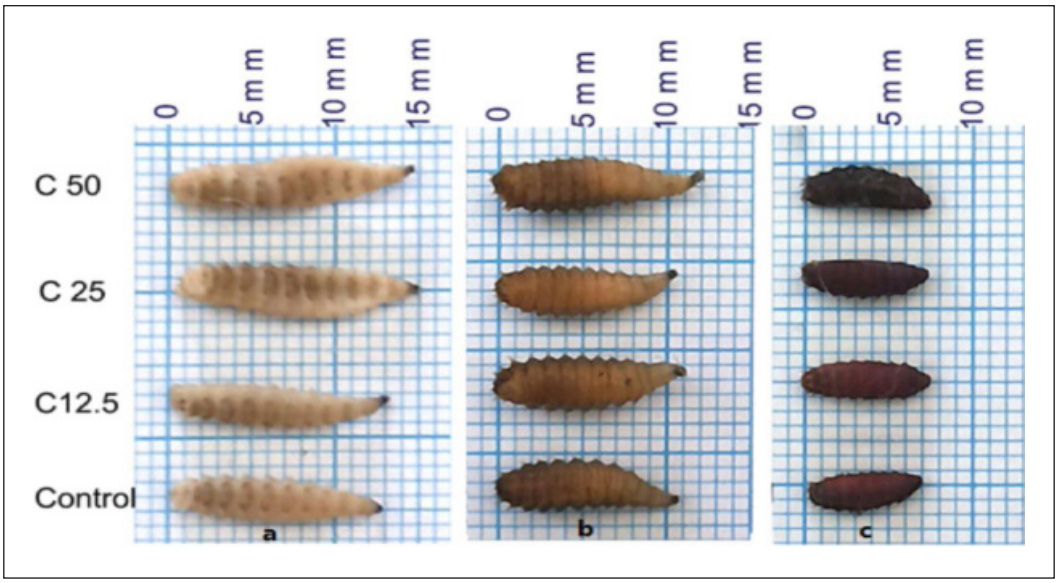

Figure 3. Comparison of body length and morphology in Peak feeding larvae (a), Post feeding larvae (b) and Pupal stage (c) of *C. albiceps* at different concentrations of morphine (12.5, 25, 50 mg/ml) showed that no significant difference between treatment and control groups.

Table 7. Homogeneity of variance in different groups (R0, R1, R2 and R3)

Tukey HSD[a] Concentration	N	Subset of alpha = 0.05		
		1	2	3
R0	5	5		
R1	5	5	5.8	
R2	5			
R3	5			6.1
P-value		0.958	1.000	1.000

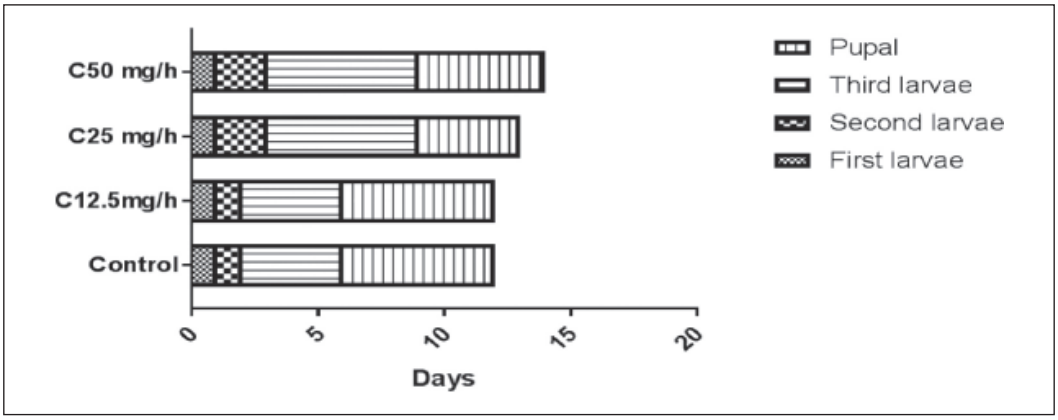

Figure 4. Comparing the development period of *C. albiceps* immature on rabbit carcasses administered Morphine sulfate with different dosages.

is different from another subset (*P-value*= 1.000). The same issue, as R2, holds true for R3, i.e. also R3 is different from another subset (*P-value*=1.000)

DISCUSSION

Morphine is one of the deacetylate metabolites of heroin. Postmortem morphine detection can be attributed to a number of sources including the legitimate prescriptive use or recreational use of opioids (Gagliano-Candela & Aventaggiato, 2001; Crandall *et al*., 2006; Gottas *et al*., 2016). The main reason why we have chosen morphine as our initial drug of study in this research is that heroin addiction is a major public health problem worldwide, moreover, deaths through heroin overdose are frequent. Pharmacokinetically, the elimination of morphine from the body is similar to that of heroin (Hedouin *et al*., 1999). The presence of drugs in carcass tissues can alter the rate of development and biomass of insects that colonize them, resulting in an incorrect estimate of PMI. In the present study, we found a positive correlation between morphine concentration in the tissues of rabbit and antemortem doses of morphine. This result is consistent with that of other studies (Bourel *et al*., 1999; Gosselin *et al*., 2010; Maskell *et al*., 2016; Bourel *et al*., 2001 a, b; Introna *et al*., 1990). Insect growth is affected by temperature, humidity, competition and most importantly, food. The presence of drugs and other toxic substances in food substrates can directly alter the growth and development rate of these insects. Depending on the type of drug, growth and development can be accelerated or retarded. Morphine is among the drugs that are highly distributed throughout the body, and it is mainly metabolized in the liver and excreted through urine and bile. Numerous studies have been conducted on the effects of opioid drugs such as morphine, heroin, codeine, methadone, and tramadol on the growth rate of insect species (Carvalho *et al*., 2004; Goff *et al*., 1989; Bourel *et al*., 2001 a, b; Fathy *et al*., 2008; Gosselin *et al*., 2010; El-Samad *et al*., 2011; Gunn *et al*., 2006; George *et al*., 2009). Most of these studies pointed to the effects of these drugs on the growth and development of insects.

In this study, we selected *Chrysomya albiceps* which is a common initial colonizer of carcasses in Afrotropical regions, Oriental regions, Central and South America, and Southern Europe. *C. albiceps* is well-known as among the first wave of faunal succession on human cadavers, which makes it a valuable tool for the estimation of minimum postmortem interval (PMImin). In this study, we observed a difference in the rate of larval growth in *C. albiceps* that fed on rabbit tissues containing morphine. The duration of the larval stages was longer compared with the puparial stages. Similar to our results, Bourel *et al*. (1999) found that the time taken for morphine-fed larvae to operate

was 28 h longer than the time taken by the control group. Also Kharbouche *et al*. (2008) indicated that the pupal period of *L. sericata* was between 21 and 29 h shorter in colonies that had been codeine-morphine fed compared with the control group. In our study, we found that morphine has an effect on the development and growth rate of *C. albiceps*, but contrary to our results, George *et al*. (2009) pointed out in their study that the presence of morphine in the feeding substrate of *C. stygia* did not alter the development and growth rate, and there was no significant difference between treatment group and control in terms of length and width of larvae and pupae. This difference may be due to the different species studied between the two studies; species response to drugs may differ from each other. According our results, concentration of morphine in various tissues and third-feeding larval stage significantly increased with increasing the initial dose. El-Samad *et al*. (2011) in their study on the effect of Tramadol (from the category of opioids) on the development of *L. sericata* indicated that there was a positive correlation between initial dose and Tramadol concentration in tissues and feeding larval stages. In the present study, we found that morphine concentration in the larvae was lower than in the tissues. These results are consistent with other studies such as Hedouin *et al*., 1999, 2001; Knitz *et al*., 1994; El-Samad *et al*., 2011. Our data also show that larvae that fed on rabbits receiving morphine were larger and attained their maximum length compared with those that fed on the control. Based on the results of Bourel *et al*. (1999) if the presence of morphine in the tissues is not considered then an underestimation of the postmortem interval of 24 h is possible for larvae of *L. sericata* measuring from 8 to 14 mm total length. El-Samad *et al*. (2011) indicated that larvae which fed on rabbits that had received Tramadol were larger than control colony. In the present study, we observed that the larvae exposed to the control rabbit (R0) and the rabbit which received 12.5 mg/ml of morphine were similar in terms of their rate of development, whilst larvae exposed to rabbits which received 25 mg/ml (R2) and 50 mg/ml (R3) of morphine developed at a slower rate, but were larger. This result is consistent with the result of Bourel's study (1999) about the effects of Morphine in decomposing bodies on the development of *Lucilia sericata*. Contrary to this finding, George *et al*. (2011) indicated that the development of *C. stygia* was unaffected by morphine. The difference between these results may be due to differences species and analytical methods used between the two studies. In our study, despite the long period of development in treatment (R3, R2, R1) compared to with the control (R0), the percentage of carcass weight loss in R3, R2, and R1 was approximately similar to that of the control carcass. The abundance of *C. albiceps* larvae on carcasses of the treatment group was found to be lower than that of the control. These results are in agreement with that of a study conducted by Charabidze *et al*. (2015) who indicated that the presence of a more evolved olfactory system in blowflies may be the reason for a lower tendency to reproduce on carcasses containing high drug concentrations, whereas the abundance of beetles was similar in all carcasses.

CONCLUSION

This study and previous studies indicate that different species appear to have different responses to drugs/toxins and rate of growth can be either retarded, increased or unchanged. This study demonstrated that an opiate (e.g., morphine) can alter the rate of development in the *Chrysomya albiceps*. This study also, indicated that the time taken for morphine-fed larvae to pupariate was longer and for puparial stage was shorter, than the time taken by control colonies. Overall, results of this study show that an underestimation of the postmortem interval of 72 h based on larval development and an overestimation of 24 to 48 h based on puparial development is possible if the presence of morphine in tissues is not considered.

Ethical approval. Necessary ethical approval was obtained from School of Public Health, Tehran University of Medical

Sciences Ethics Committee with code No. IR.TUMS.SPH.REC.1395.1634, 2017-02-04.

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