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Detection of Ketamine hydrochloride and its effect on the development of immature stages of a forensically important blow fly *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae)

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Abstract
The present study demonstrated the detection of Ketamine hydrochloride from the immature stages of *Chrysomya megacephala* (Fab.) and observation of its effect on the development of this fly. Larvae of *C. megacephala* were reared on rat tissues administered the half lethal, lethal and twice lethal dosages of Ketamine hydrochloride antemortem. Development of immature stages was estimated on the basis of various parameters like weight, length, width, time of adult emergence and survival rate. Results indicate that an estimate of the postmortem interval based on the normal development of *C. megacephala* could have an error of up to 60-92 hours if based on the age of the larva, and 92-172 hours if based on the age of the pupa sampled from decomposing remains. Larvae of *C. megacephala* feeding upon control treatment demonstrated higher survival rate, pupariation and eclosion success as compared to larvae exposed to the different dosages of Ketamine hydrochloride. The presence of Ketamine hydrochloride could be detected through Gas Chromatography (GC) in all rat samples and in almost all dipteral samples in the experiment.

Keywords: Forensic entomology, Entomotoxicology, Calliphoridae, *Chrysomya megacephala*, Ketamine hydrochloride, Gas Chromatography

1. Introduction
A wide variety of arthropods are attracted by decaying organic material, including corpses. Saprophagous insects colonize a fresh corpse, often within minutes, depending on the level of accessibility and environmental conditions [1-6]. Usually the first taxa to arrive on a dead body are flies (Diptera), especially blowflies (Calliphoridae) which may colonize cadavers within few minutes to hours after death. *Chrysomya megacephala* is a forensically important blow fly distributed in many parts of world and is available throughout the year in northwestern part of India [4, 7, 8]. Larvae of this species have been reported in association with human corpses in several cases [9-12]. Blowflies can locate an odor source with great spatial precision and deposit their eggs on a corpse within a few minutes [13-15]. They are, therefore, the primary and the most accurate forensic indicators of the Postmortem Interval (PMI), the time period between death and discovery of the corpse. Forensic entomologists derive an estimate of the PMI by examining the age of the most advanced developmental stage present on the corpse at the time of discovery. Hence understanding the effect of drugs and toxins on fly development rate is paramount before its use for PMI determination because the presence of such xenobiotics can accelerate or retard fly development [16-19].
Fly larvae and pupae are often found on decomposing bodies and in their surroundings long after tissues (blood, urine or solid organs) traditionally sampled for toxicological analyses have disappeared. In such badly decomposed bodies, these immature stages and their remnants are not only useful for estimating the minimum PMI, but they can also be used as a reliable substrate for toxicological analysis, and can sometimes provide a more suitable biosample without any decomposition interference. Ketamine hydrochloride, [2-(o-chlorophenyl)-2-(methylamino)-cyclohexanone hydrochloride] is a potent short-acting anaesthetic. It is used for the purpose of initial and basal narcosis, as well as for multicomponent anaesthesia. It can be used by drug addicts to produce hallucinogenic and narcotic effects [20, 21]. It has gained notoriety as a “Date-rape drug”.
The objective of this study was to determine the effect of Ketamine hydrochloride on the

development and growth of *C. megacephala* that can affect the estimate of postmortem interval (PMI). It was also intended to evaluate the reliability of different development stages of *C. megacephala* including larval stages, pupae, puparial cases and adults as samples for toxicological investigations of Ketamine hydrochloride by using Gas Chromatography (GC).

2. Materials and Methods

2.1 Rearing of flies and administration of drug

After getting due permission from the Ethical Committee (vide letter no. 107/99/CPCSEA-2010-25), female Sprague-Dawley laboratory rats (100-110 g) were procured from the Central Animal House of Punjab University, Chandigarh, India (30.7580° N, 76.7682° E). They were acclimatized to the laboratory conditions for 15 days and were supplied with pellet diet (Aashirwad Industries, Chandigarh, India) and drinking water *ad libitum*. The rats were starved for 24 hours before performing the experiments. A group of four rats were used for one set of the experiment. One of these was kept as control (K_C), while others were administered half lethal (37.5 mg/kg bw), lethal (75 mg/kg bw) and twice lethal (150 mg/kg bw) doses of Ketamine hydrochloride by intramuscular injection and were labelled as K_1, K_2 and K_3 respectively. While K_2 and K_3 died within 20 minutes, K_C and K_1 were killed by cervical dislocation. Samples of liver, heart, kidney, skeletal muscle tissue and blood were taken from each rat for chemical analysis of Ketamine hydrochloride by Gas chromatography (GC). Wild type (F_0) specimens of *C. megacephala* were collected from Punjabi University, Patiala, India (29°49' and 30°47' north latitude, 75°58' and 76°54' east longitude). Laboratory colonies were established and eggs used in the experiment were F_2 generation. Newly hatched larvae (250-300) were obtained from these laboratory bred colonies and allowed to feed upon the rat carcasses (K_1, K_2, K_3, K_C) and their development time was monitored regularly. Minimum and Maximum temperature and humidity were noted daily by using Electronic Thermohygrometer.

2.2 Sampling

After regular time intervals (8 hours) 10 larvae were randomly collected from each rat sample and divided into two lots. Five larvae were used to determine growth based on body weight increase; and five larvae were measured to determine development based on increase in total length. The larvae used to determine weight were killed by freezing, then thawed, washed and dried with filter paper prior to being weighed. The larvae used to determine length were killed in boiling water and then preserved in 70% ETOH. Time of hatching was noted (as 0hr) and subsequently development time was noted for each larval instar (1^{st}, 2^{nd} and 3^{rd}), postfeeding larvae and pupa. Time of emergence of adults was also noted and each stage was sampled for toxicological analysis.

2.3 Data analysis

Data were analyzed using Arithmetic Mean, Standard deviation, Analysis Of Variance (ANOVA) (ToolPak in Microsoft Excel 2007**)**, Mann-Whitney U test (The Wilcoxon Rank-Sum Test) and Pearson Correlation Coefficient. Graphs and tables were prepared using Microsoft Excel 2007.

2.4 Extraction for Gas Chromatography

5 mL of hexane and 200 µL of 6 M NH_4OH were added to each rat tissue sample, vortexed for 30 sec, shaken for 10 min, and centrifuged for 20 min at 3100 rpm. The supernatant was discarded and 2 mL of 1 M H_2SO_4 was added to the hexane layer. Samples were then vortexed, centrifuged, and the hexane layer was transferred and dried down at room temperature. Samples were reconstituted in 1 mL mobile phase (hexane) and 1 µL of the aliquot was analysed using GC [22]. Before entomotoxicological analyses, cleaned tissues (e.g. larvae, pupae) were washed again with distilled water and dried with filter paper. This multiple washing treatment is necessary to prevent contamination by human fluid exudate or transudate. 5 g of cleaned larvae were weighed and finely chopped with scissors [23-28]. For analysis of pupae, a lower amount, 2.5 g, was used. The entomological samples were homogenized in distilled water. The resulting homogenate was centrifuged at 2000 rpm. Puparial shells (1- 1.5 g) were washed twice with water and twice with acetone [29]. One mL of HCl (0.1 M) was added to the segments and incubated overnight. The resulting digests were adjusted to pH > 10 using 0.4% NaOH and extracted with 3.5 mL of diethyl ether. Extracts were evaporated at 60 °C. The residues were reconstituted in 1 mL hexane. A 1 µL aliquot of the solution was injected into the GC–MS system.

2.5 Gas chromatography analysis

Analysis was performed on a YL (Young Lin) 6100 Gas Chromatograph fitted with a Flame-Ionization Detector (GC-FID). Chromatography separation were done on a 30.0 m, Zebron ZB-130 (100% Dimethyl polysiloxane) capillary column with diameter of 0.25 mm and film thickness of 0.25 µm. Constant pressure of 10 psi and initial flow rate of 1.2 mL/min of carrier gas Nitrogen was maintained. An isothermal oven temperature was used (260 °C). Analytes in hexane were analyzed at an Injector temperature of 260 °C and Flame-ionization detector, with temperature of 275 °C. Hydrogen gas flow of 30.0 mL/min and an air flow of 100.0 mL/min was maintained in the FID.

3. Results and Discussion

3.1 Duration of Development and Weight

The presence of Ketamine hydrochloride in the decomposing rat tissue caused a prolongation of time needed for development for all the immature stages of *C. megacephala*. The total development time, defined as the interval between larval eclosion and adult emergence, was significantly different for *C. megacephala* reared on tissues with different dosages of Ketamine hydrochloride for first instar (F=329.489, P=0.000), second instar (F=167.493, P=0.000), third instar (F=1,965.699, P=0.000), postfeeding stage (F=328.441, P=0.000), pupa (F=418.248, P=0.000) and total development (F=418.248, P=0.000). Previous workers [17, 30-34] using cocaine, heroin, methamphetamine, Diazepam, Sodium methohexital, Escopolamine and Phenobarbital in their experiments, showed that all these drugs could alter the rate of development of dipteral flies. The present results indicate that postmortem interval based on the normal development of *C. megacephala* could have an error of up to 60-92 hours if based on the age of the larva, and 92-172 hours if based on the age of the pupa sampled from decomposing remains.

Weight gain occurs in a sigmoid pattern throughout the development of *C. megacephala* up to third instar after which a slight decrease in weight occurs as the larva enters the post feeding stage. There was significant difference in the weight of larvae feeding upon the different doses of Ketamine hydrochloride (F=99.404, P=0.000) as compared to control. The development curve created from the larval weight data shows that mean maximum weight of larvae feeding upon the K_1 rats was 125.3 mg attained in 128 hours. The maximum weight (66.7 mg) was attained in 104 hours by larvae in the K_2

treatment set. The maximum weight for the larvae feeding upon K_3 rats and K_C was recorded at 112 and 136 hours respectively (Fig.1). Thus the larvae feeding upon decomposing tissues with Ketamine hydrochloride reached their mean maximum weight earlier than the control.

The maximum pupal weight (67.2 mg) was observed in the K_3, while, minimum pupal weight (59.37 mg) was observed in the K_C set (Fig. 2). Heavier pupae (as compared to control) were produced by *C. megacephala* colonies that were reared on rat tissues containing Ketamine hydrochloride. Linhares and Oliveira determined the effect of Escopolamine bromide on the development of *C. megacephala* and there was a significant effect of the drug concentration on larval weight ($F=65.92$; $p< 0.001$). Larvae reared on the drug showed smaller body weight [34]. Similarly Goff *et al.* [35] studied the effect of drugs on the development of *Parasarcophaga ruficornis*. In their study also, an increase in pupal weight was observed for the insect colonies that were reared on rabbit tissues containing amitriptyline and nortriptyline as compared to those reared on the drug-free rabbit tissue.

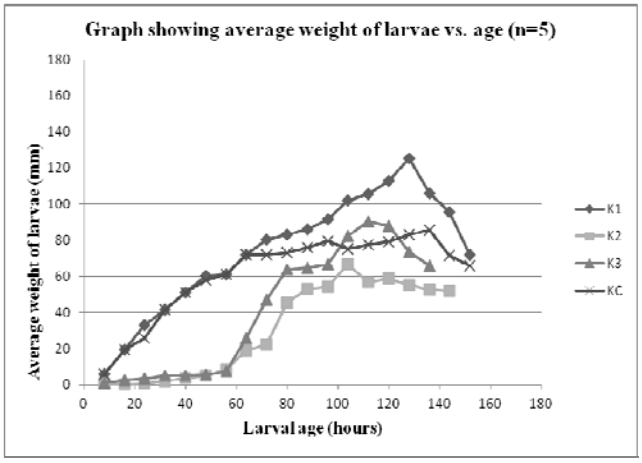

Fig 1: Showing average weight of larvae of *C. megacephala* vs. larval age while feeding upon Ketamine Hydrochloride treated rats antemortem (Maximum daily temp. 18.5 ± 2 °C, Minimum daily temp.15 ± 2 °C, Relative Humidity 72% - 75% and Photoperiod LD 10: 14) (Average of 3 replicates).

Table 1: Duration of each development stages of *C. megacephala* reared on the rat tissues without (K_c) and with (K_1,K_2,K_3) Ketamine hydrochloride (Maximum daily temp. 18.5 ± 2 °C, Minimum daily temp.15 ± 2 °C, Relative Humidity 72% - 75% and Photoperiod LD 10: 14) (Average of 3 replicates).

Dosage of Ketamine hydrochloride (mg/kg bw)	First instar (h)	Second instar (h)	Third instar (h)	Postfeeding stage (h)	Pupal stage (h)	Total development time (h)
37.5 (K_1)	32	40	80	24	136	308
75 (K_2)	24	24	56	48	176	328
150 (K_3)	16	16	120	32	208	392
Control (K_C)	8	24	44	16	128	220

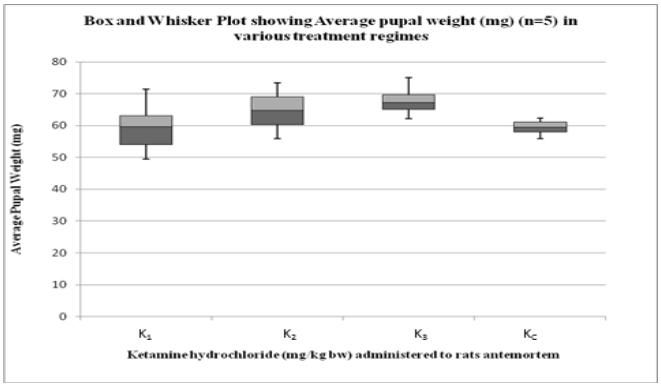

Fig 2: Showing average pupal weight data of *C. megacephala* whose larvae had been feeding upon different dosages of Ketamine hydrochloride treated rats antemortem (Maximum daily temp. 18.5 ± 2 °C, Minimum daily temp. 15 ± 2 °C, Relative Humidity 72% - 75% and Photoperiod LD 10: 14) (Average of 2 replicates). The plot shows the median pupal weight for each type of rearing media at the junction of the two boxes (light and dark). The interquartile range of the pupal weight data for each type of treatment regime is shown as a box. The range of the pupal weight data for each type of treatment regime is illustrated by the thin black bars (whiskers)

3.2 Size: Length and Width

Length of larvae was measured between the head and the tip of the eighth abdominal segment. Larval length increases markedly up to third instar [36]. The development curve created from the length data shows that mean maximum length of larvae, feeding upon the K_1 rats, was 15.69 mm and was attained in 136 hours. The maximum length was also attained within the same time by larvae in the K_C set, though it was only 14.87 mm. The maximum length for the larvae feeding upon K_2 and K_3 rats was recorded at 104 and 112 hours respectively. This corresponded to the end of the larval growth and beginning of the prepupal stage. A sharp decrease in mean larval length was observed afterwards in all of the treatment sets. There were significant differences in the lengths of the larvae feeding upon the different doses of Ketamine hydrochloride (Fig.3) and the time required to reach the maximum length ($F=140.387$;$p=0.000$).

Length has been one of the most frequently measured parameters for successfully estimating larval age [37]. In the study conducted by Goff *et al.* [35] no significant differences were observed in the rate of larval growth among colonies feeding on varying dosages of amitriptyline. However, maximum mean lengths were observed for the colony reared on the liver from rabbit that had received maximum dose of amitriptyline. The same drug amitriptyline was evaluated by Duke and significant differences were observed in both the maximum length achieved and the duration of larval development [37]. In a study involving the effect of escopolamine bromide, larvae showed a significant reduction in the body length [34].

The effect of antemortem ingestion of ethanol by the host on the development of *Phormia regina* maggots in the field showed that there was a significant difference between the distribution of length in maggots feeding on tissues from ethanol treated and untreated pigs. The larvae feeding on tissue that were ethanol treated took 11.9 hours longer to reach the pupal stage than the controls [38]. In a study involving the effect of cadmium on the growth of *Boettcherisca peregrina*, the maggot length was significantly shorter in comparison to control [39].

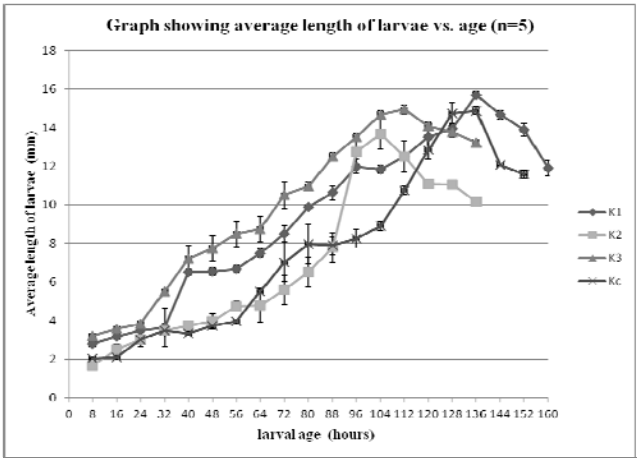

Fig 3: Showing average length of the larvae of *C. megacephala* vs. larval age, feeding upon Ketamine hydrochloride treated rats antemortem (Maximum daily temp. 18.5 ± 2 °C, Minimum daily temp. 15 ± 2 °C, Relative Humidity 72% - 75% and Photoperiod LD 10:14) (Average of 3 replicates)

Larval width has recently been regarded as a valuable parameter for age determination of larvae and consequently PMI estimation [40]. Width of the larvae, viewed laterally, was measured between the ventral and dorsal surfaces at the junction of the fifth and sixth abdominal segments.

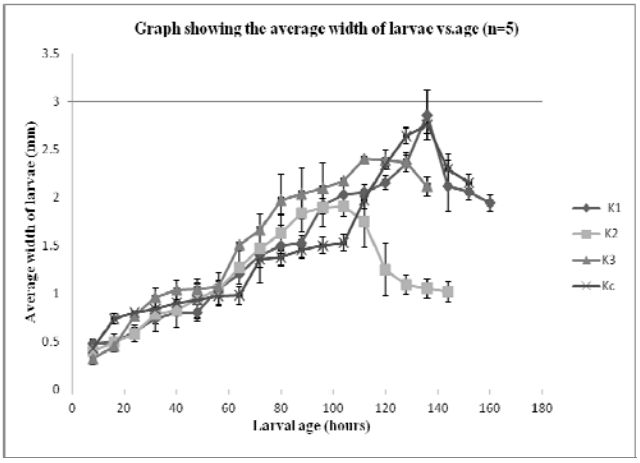

Fig 4: Showing average width of larvae of *C. megacephala* vs. larval age feeding upon Ketamine hydrochloride treated rats antemortem (Maximum daily temp.18.5 ± 2 °C, Minimum daily temp.15 ± 2 °C, Relative Humidity 72% - 75% and Photoperiod LD 10:14) (Average of 3 replicates).

3.3 Pupariation success, larval mortality and eclosion success

Pupariation success denotes the percentage of larvae that successfully undergo pupariation, and is inversely proportional to larval mortality. The larval mortality was highest in the K_2 treatment set (62.5%), whilst lowest mortality was in the K_C group (29%) (Table 2). Pupariation success was higher with increasing the dosage of Ketamine hydrochloride, though it was still lower than the control. The survival of pupae of *B. peregrina* whose larvae had been feeding upon cocaine- or heroin-contaminated tissue was about 90% and not affected by drug presence or dose [30, 31]. Presence of methamphetamine affected survival rate in *P. ruficornis*. Pupal mortality rates for the drug-treated colonies appeared to be inversely proportional to the dosage administered to the animal model [32]. MDMA also affected mortality in *P. ruficornis* at lower concentrations [41], while amitriptyline killed up to half of the larvae [35].

Table 2: Showing Pupariation success, larval mortality and eclosion success of *C. megacephala* after feeding upon different dosages of Ketamine hydrochloride treated rats antemortem (Maximum daily temp. 18.5 ± 2 °C, Minimum daily temp. 15 ± 2 °C, Relative Humidity 72% - 75% and Photoperiod LD 10:14) (Average of 3 replicates)

Percentage	Dosage of Ketamine hydrochloride (mg/kg bw)			
	37.5 (K_1)	75 (K_2)	150 (K_3)	Control (K_C)
Pupariation Success	50.5	37.5	54	71
Larval Mortality	49.5	62.5	46	29
Eclosion success	91.1	80	94.4	95.1

The highest percentage of successful emergence was observed for colonies reared on K_C (95.1%), while the lowest percentage was observed in the K_2 treatment set. The presence of the drug, thus resulted in lower eclosion success percentages as compared to adult but, no clear correlation could be established between the concentrations of the drug and the eclosion success (Table 2). Previously published works [30, 42, 43] indicate that high drug concentrations do not always tend to result in high mortality, as one might expect. In the study by Hecht *et al.* [42] the percentage emergence increased with methadone concentration. In the study conducted by Oliveira *et al.* [44] the mortality rate was greater due to the effect of Buscopan. Gosselin *et al.* [45] have reported a significant difference in the number of adults that emerged between all treatments groups treated with different concentrations of methadone.

3.4 Ketamine hydrochloride analysis in tissue samples and immature stages of *C. megacephala.*

Ketamine hydrochloride was present in the blood, liver, heart, kidney and skeletal muscle tissue samples of the rats that received the injections of the drug, while samples from control rats were negative for the drug. There was a good correlation (Table 3) between the quantity of Ketamine hydrochloride administered and the concentration in tissues. Best correlation between tissue levels and dosage administered (r = 0.99) was observed for kidney as well as skeletal muscle tissue.

Qualitative and Quantitative analyses were made on the samples of larvae (1st, 2nd and 3rd instar) from the colonies of *C. megacephala* feeding upon the drug administered dead rats. Postfeeding larvae, pupae and puparial shells originating from the same colonies were also similarly analyzed. Ketamine hydrochloride was positive in all the samples of the immature stages (Table 4). However, it could not be quantified in puparial shells originating from the K_1 and K_2 rats. Ketamine hydrochloride concentrations in larvae, pupae and puparial cases were considerably lower than those in the rat tissues.

These results differ from those presented by Introna *et al.* [19] where concentrations in larvae were very similar to the liver tissues used. Other studies have found the concentrations in larvae to be significantly lower than those observed in tissues [24, 26]. During the most active period of their growth (2nd and 3rd instar) the larvae accumulate the drug in proportion to the concentrations present in tissues. Following the cessation of feeding in the postfeeding larvae, the concentration decreases until the puparial stage is reached. Thus the postfeeding larvae seem to eliminate the drug until pupariation.

Table 3: Showing Quantification of Ketamine hydrochloride in tissue samples of antemortem dosed rats after GC analysis (Average of 3 replicates)

Tissue Sample	Dosages of Ketamine hydrochloride injected in rats (mg/kg bw)			Control	Correlation Coefficient r
	37.5	75	150		
Blood (ng/ml)	1876	3401	3976	Negative	0.90
Liver (ng/g)	5432	9013	11754	Negative	0.96
Kidney (ng/g)	5721	7564	12748	Negative	0.99
Heart (ng/g)	1799	3373	4234	Negative	0.93
Skeletal muscles (ng/g)	3013	4121	6373	Negative	0.99

r is the correlation coefficient between the injected dosage and the concentration in organs

Table 4: Showing Quantification of Ketamine hydrochloride (ng/g) in life stages of *C. megacephala* sampled from antemortem dosed rats after GC analysis (Average of 3 replicates)

Immature stages of *C. megacephala*	Dosages of Ketamine hydrochloride injected in rats (mg/kg bw)			Correlation coefficients				
	37.5	75	150	r_1	r_2	r_3	r_4	r_5
First instar larvae	30.4	61.3	163.3	0.99	0.93	0.99	0.89	0.99
Second instar larvae	84.4	23.1	83.6	-0.26	-0.08	0.25	-0.17	0.18
Third instar larvae	64.5	72.3	184.3	0.75	0.85	0.99	0.80	0.96
Postfeeding larvae	9.3	15.6	55.6	0.79	0.88	0.98	0.84	0.97
Pupae	1.2	2.5	3.7	0.99	0.99	0.99	0.98	0.97
Puparial shell	NQ	NQ	1.2	-	-	-	-	-

NQ- Ketamine hydrochloride detected but not quantified

r_1 is the correlation coefficient between the blood and the concentration in the fly immature stage

r_2 is the correlation coefficient between the liver and the concentration in the fly immature stage

r_3 is the correlation coefficient between the kidney and the concentration in the fly immature stage

r_4 is the correlation coefficient between the heart and the concentration in the fly immature stage

r_5 is the correlation coefficient between the skeletal muscles and the concentration in the fly immature stage

This elimination has also been observed by several earlier workers [27, 28, 46-49]. Though the drug is excreted by the larvae, some of it is sequestered in the cuticle of the puparium. Such samples present an important toxicological sample of interest, because puparial cases are imputrescible and can be recovered even several years after death. There was significant correlation between the 1st instar larvae and pupae when compared with all the tissues. However, in 3rd instar larvae, postfeeding larvae and pupae correlation existed only with the kidney and the skeletal muscle tissue samples.

4. Conclusion

The work deals with the detection of Ketamine hydrochloride in fly tissues and its impact on larval growth, pupariation, adult emergence and mortality of *C. megacephala*. The drug could be detected not only in maggot samples but also in puparia. The presence of the drug in tissues which the larvae were feeding upon changed the rate of development of the blow fly. This factor must be taken into account while deriving forensic conclusions on the basis of larval development rate. The analysis of immature stages of the fly, including puparia, could point towards suspected poisoning if the drug is detected in suspicious cases.

5. Acknowledgement

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