CHAPTER FOUR

Animal Experiments and Weight Studies

4.1. Introduction

The use of animals for modelling human diseases has been considered in the literature review chapter and the various procedures for inducing airway reaction to allergenic stimulation have been reported in literature. The wide variety of available sensitization and challenge protocols as well as the time point of assessment and readout systems has been reviewed by Kumar and Foster (2002). In addition, a comprehensive review of murine models of asthma by Kips et al., (2003) considered a number of critical factors that may affect experimental modelling of asthma in animals by introducing variation in results obtained.

The age of animals or differential response of the airways to various chemical agents used during the sensitization and challenge (nebulization) stages of the experiment could cause such variation. Also important as other sources of variation are the strain of mice used as well as the route and timing of exposure to allergen (Kips et al., 2003).

The procedures used in evaluating the experimental outcomes of modeled asthma also vary slightly depending on researcher discretion and aim of experiment, all targeted at establishing the usefulness of the asthma mouse model. Epithelial shedding, airway smooth muscle hypertrophy and hyperplasia, overproduction of mucus and airway inflammation have all been examined in different previous studies. In some of the review articles studied, there seemed to be suggestions that separate modelling of the various phenotypes or traits of asthma may be better than attempting to model the entire disease in a single experiment. Kips et al., 2003 concluded that the specific characteristics of the various asthma phenotypes could be clearly defined if modeled separately.

Besides the plethora of research on the inflammation aspect of asthma, studies that focus on other phenotypes of asthma such as studies on the ultrastructure of airway epithelium, shedding of epithelium (Shahana et al., 2003; Shebani et al., 2005) as well as airway remodelling (Bischof et al., 2003; Snibson et al., 2005) have been reported.

4.1.1. Aim of study

The aim of this present study was therefore to induce asthma in BALB/c mice using a previously described procedure (albeit with slight variations) and attempt to treat the condition with the aqueous extract of *E. hirta*. The focus in this chapter will be to determine the effects of hydrocortisone as well as the aqueous extract of *E. hirta* on progressive changes in the body weights of the BALB/c mice as this reflects the general wellbeing of the animals.

4.2. Materials and methods

4.2.1. Materials

4.2.1.1. BALB/c Mice

A total of sixty (60) six-week-old male BALB/c mice (mean weight 20g) were used in this study. These mice have previously been used to investigate a number of parameters involved in asthma (Bice et al., 2000).

4.2.1.2. Hydrocortisone (HC)

HC was used in this study as a pharmaceutical control for the extracts of *E. hirta* as used in the cell culture study. One hundred milligrams (100mg) of the sterile powder (Brand name Solu-Cortef®) which contains HC sodium succinate as the active ingredient was dissolved in 2 ml of bacteriostatic injection water, giving a

concentration of 50mg/ml. In this study, a high dose (125mg/kg) and a low dose (100mg/kg) of the 50mg/ml HC preparation were used. Fresh solution was prepared for each injection.

4.2.1.3. *Euphorbia hirta*

Only the aqueous plant extract of *E. hirta* was used for the animal studies. The plant material was collected in the Gezina region of Pretoria South Africa. A herbarium specimen was prepared and compared to an authentic specimen in the HGJW Sweikerdt herbarium at the University of Pretoria. The aboveground parts of the plant were allowed to dry at room temperature for one week in the Cell Biology laboratory of the Department of Anatomy, University of Pretoria, and the material was grounded into a fine powder. Shortly after, 50 grams of the sample was extracted in 500ml of double distilled water after which it was filtered, and dried on a rotary evaporator at 40 °C. A stock aqueous solution of 50mg/ml plant extract was prepared and stored in a fridge until used.

The doses of 62,5mg/kg and 25mg/kg were prepared from the stock solution and administered orally to the mice. This implied that each animal (average weight 20g) received 0.01 ml of plant material. This dose was decided upon after studying literature that mentioned physiological doses suggested by herbalists. Typically, a teaspoon of the herb is added to a teacup volume of water and allowed to simmer for 20 minutes (Lindsey et al., 2002). Alternatively, an extract of the plant could be prepared and the recommended adult dose range of the fluid extract is 0.2-0.3ml, taken three times daily and of the infusion, 120-300mg three times daily (Skidmore-Roth, 2001).

4.2.1.4. Reagents and equipment

The reagents and equipments used for this study include ovalbumin (OVA) purchased from Sigma-Aldrich Co., Phosphate Buffered Saline (PBS), alum, NaCl, KCl, $Na_2HPO_4.H_2O$, KH_2PO_4, DPSS, KLAVA ultrasonic nebulizer, oral-pharyngeal canula, injection syringes and needles.

4.2.2. Methods

This study was divided into short-term (ST) and long-term (LT) phases to assess both initial and advanced effects of the induced asthma on body weight changes, blood cell count, airway activity, airway morphology, among other characteristics.

4.2.2.1. Animal care and grouping

All the mice used in this study were obtained from the Biomedical Research Centre (Faculty of Veterinary Sciences of the University of Pretoria) and maintained in a pathogen-free environment at the Onderstepoort Animal Care facility. A temperature range of 20-24°C, a relative humidity of 40-60% and a 12-hour day light and 12-hour night were maintained. Polycarbonate Type III cages were obtained from Tecniplast and only one mouse was housed per cage containing autoclaved wood shavings as bedding and elite white facial tissue paper as enrichment (to reduce male mouse aggressive behaviour during handling). Animals were provided with OVA-free food (Balanced EpolT mice cubes and pellets obtained from EPOL - a division of Rainbow Farms PTY LTD, South Africa) and pre-boiled tap water was made available *ad libitum,* one bottle per cage. Mice were allowed to acclimatize for seven days before the experiments commenced. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee (UPAUCC).

The mice were randomly assigned to two main groups, each for the short-term (ST) and (LT) long-term phases of the study. Each main group consisted of thirty animals and further assigned randomly into six sub-groups (each containing 5 mice) according to the treatment to be given – control (CT), asthma (AS), high HC-treated (HHC), low HC-treated (LHC), high *E. hirta* – treated (HEH) and low *E. hirta* - treated (LEH) groups respectively. Altogether, there were twelve groups as listed in *table 4.1*

Table 4.1: Groups for the short-term and long-term studies.

Groups for the short-term studies	
Control (CT)	5 mice; pure controls; neither sensitized nor challenged.
Asthma (AS)	5 mice; asthmatic controls; sensitized and challenged but not treated.
High HC (HHC)	5 mice sensitized and challenged, treated with high dose of HC.
Low HC (LHC)	5 mice sensitized and challenged, treated with low dose of HC.
High EH (HEH)	5 mice sensitized and challenged, treated with high dose of *E. hirta* plant extract.
Low EH (LEH)	5 mice sensitized and challenged, treated with low dose of *E. hirta* plant extract.
Groups for the long-term studies	
Control (CT)	5 mice; pure controls; neither sensitized nor challenged.
Asthma (AS)	5 mice; asthmatic controls; sensitized and challenged but not treated.
High HC (HHC)	5 mice; sensitized and challenged mice, treated with high dose of HC.
Low HC (LHC)	5 mice; sensitized and challenged mice, treated low dose of HC.
High EH (HEH)	5 sensitized and challenged mice, treated with high dose of *E. hirta* plant extract.
Low EH (LEH)	5 mice; sensitized and challenged, treated with low dose of *E. hirta* plant extract.

4.2.2.2. Experimental procedure

The process of inducing asthma involved sensitization (immunization) and nebulization (allergen challenge). The study was extended beyond day 18 (the long-term phase) to evaluate the effects of a much longer exposure of the mice to

the test agents (HC and *E. hirta* extract). All animals except those in the CT group were sensitized and nebulized before treatment with the two test agents. Asthma was induced in the AS group and the animals were left untreated whereas animals in all other test groups were treated with HC or *E. hirta* extract after exposure to the same asthma-inducing conditions as the animals in the AS group.

4.2.2.2.1. Sensitization

Sensitization (also described as immunization) is the procedure that prepared the immune system of the animals for the subsequent exposure to allergens in the nebulization chamber. Sensitization was done on days 0 and 5 respectively via intraperitoneal injection of a solution of 25mg OVA emulsified in 2mg aluminium hydroxide [Al $(OH)_3$] and dissolved in 0.5ml of 0.9% saline solution. All mice except those in the control and asthma groups (CT for LT and ST) were sensitized and all sensitized animals were allowed a duration of one week before exposure (challenge).

4.2.2.2.2. Nebulization

The nebulization procedure served to challenge the sensitized immune system of the animals to the presence of allergens. This procedure was carried out one week after immunization and involved placing the mice (except those in the pure control groups) in a Plexiglas chamber and exposing them to fumes generated via a KLAVA ultrasonic nebulizer from a 1% OVA in PBS solution (1mg OVA in 100 ml PBS).

In order to induce acute onset of asthma, eligible mice were nebulized for two consecutive 30-minute periods daily with one-hourly intervals on days 13, 14 and

15 for the short-term groups; and repeated on days 34, 35 and 36 for the long-term groups. The animals were carefully observed and monitored for basic asthmatic symptoms (wheezing and difficulty to breath) shortly after nebulization up to 10 minutes before treatment. Blood cell count was carried out as an additional source of proof of the presence of asthma.

4.2.2.3. Administration of the test agents

HC dosages of 75mg/kg (LHC) and 125mg/kg (HHC) were used in this study and administered to the mice was used in this study and administered to eligible short-term and long-term animal study groups via intraperitoneal injection. On the other hand, the HEH (62.5mg/kg) and LEH (25mg/kg) dosages of the plant extract were administered orally to the mice in designated groups. Each animal received the same dose of the treatment agents twice daily, with an hour' interval allowed between treatments.

For groups involved in the short-term studies, administration of treatment agents was done on days 15 (about 30 minutes after last nebulization), 16 and 17. Animals involved in the short-term studies, were terminated on day 18 to end the short-term studies. For groups involved in the long-term studies, administration of treatment agents was on days 15 (about 30 minutes after last nebulization), 16, 17, 18, 22, 25, 29 and 32. Treatment was stopped after day 32 to repeat nebulization (on days 34, 35, 36) and was continued daily for one week from days 39, 40, 41, 42, 43, 44 and 45.

A number of factors informed the choice of reference days on which to analyze and study the possible effects of the different experimental interventions. The selection took into account the days on which animals were sensitized (days 0 and

5), nebulized (days 13-15) as well as treated (days 15-17 for ST studies; days 22, 25, 29, 32, 39, 40, 41, 42, 43, 44, 45 for the LT studies respectively).

The reference days chosen for analysis included days 6, 13, 15, 18, 26, 32, 37, 42 and 46, as shown in *table 4.2*.

Table 4.2: Day of weighing and expected effects on weight changes

DAY	Expected effects of procedures on weight changes
1	Baseline weight
6	Early effects of immunization
13	Late effects of immunization (just before first nebulization)
15	Effects of first nebulization
18	Effects of first batch of treatment on nebulization (just before sacrifice)
26	Midterm effects of continuous treatment
32	Late effects of continuous treatment
37	Acute effects of repeated nebulization on weight changes
42	Early effects of post-nebulization treatment on weight changes
46	Terminal effects of post-nebulization treatment on weight changes

All treated animals were also observed for basic asthmatic symptoms (wheezing and difficulty to breath) shortly after nebulization, 10 minutes before treatment as well as one hour after each treatment exposure. As one of the criteria for the assessment of general health, animals were weighed at the beginning of the experiment and daily before any other routine procedures were carried out. Weighing continued until day 46 of the long-term study when the last batch of animals was terminated. The mean weights for all the groups on each day were expressed in grams ± standard deviation.

Mean weight values on day 1 were considered baseline weights and a 2-tailed paired-sample t-test was used to determine differences between the baseline

weights and other weight values recorded on all the reference days in each group. Since the starting weights varied between the groups, mean weight values per group per day were converted into percentages of the starting weights and the respective percentage weight differences (PWDs) were determined relative to the starting or baseline weights. This offered a more appropriate means of determining weight differences between groups as opposed to using the original weight values. Values for the percentage weight differences from baseline weights (PWDs) were obtained using the formula:

$$\textbf{PWD} = \frac{[\textbf{Mean weight}_n - \textbf{Mean weight}_b]}{\textbf{Mean baseline weight}_b} \times 100;$$

Where:

Mean weight_n = mean weight of specified group on selected day and

Mean weight_b = mean baseline weight on the specified group.

At the end of the different stages of the experiment, animals were terminated by skilled UPBRC technical personnel on the morning of days 18 (ST) and 46 (LT) either by bleeding them to death or via cervical dislocation depending on whether a lot of blood was required for coagulation studies or not. During the course of the experiment, only one out of the five animals in the AS (LT) group died on day 1 and pathology report did not associate the cause of death with the experimental procedures. A second animal in the low HC (ST) group was perceived to be unhealthy by the technical personnel and was thus excluded from the experiment and terminated on experimental day 4.

4.3. Results and discussion

4.3.1. General effects of HC and *E. hirta* on asthmatic mice

Asthma was induced in BALB/c mice using OVA, the mice were then treated with high and low dosages of HC and *E. hirta.* Basic asthmatic symptoms including reduced physical activity, general discomfort, difficulty of breathing and wheezing, were made throughout the study by the principal investigator.

Although these symptoms were present in most of the animals just after the nebulization, these symptoms gradually eased out. Greater improvement was however observed after about 30 minutes following treatment with either HC or the plant extract.

4.3.2. Effects of HC and *E. hirta* on body weights of asthmatic mice

The co-existence or relationship between airway inflammation (including that seen in asthma) with either excessive body weight, anomalous body mass index, or obesity has been reported (Camargo et al., 1999; Hakala et al., 2000; Aaron et al., 2004; Weiss and Shore 2004; Beuther et al., 2006), but the underlying mechanisms remain obscure. Although it is known that obesity worsens asthmatic conditions, the cause-effect relationship (i.e. whether one of these two conditions can lead to the other) is not understood.

Shore (2006) in a review article, argued that the relationship between asthma and weight changes as reported in most previous studies, does not address the direction of causality, adding that one possible interpretation of these studies could be that asthma leads to obesity, perhaps because asthmatics adopt a sedentary lifestyle to avoid respiratory symptoms during exercise. A different opinion by

Hayman (2006) was that any factor that causes inflammation could lead to weight gain that could in turn lead to more inflammation.

In adult humans, a reduction in excessive body weight by medical treatment and surgical procedures has resulted in a reduction of asthma symptoms, medication usage, and severity, and an improvement of lung function, indicating a possible causal relationship (Macgregor and Greenburg, 1993; Dixon et al., 1999; Stenius-Aarniala et al., 2000).

Animal models of mice, guinea pigs, rats, dogs, cats, monkeys, sheep, and horses have been developed to study disease pathogenesis and for drug discovery (Epstein, 2004a, b). Since the first demonstration of allergic mouse asthma was reported in 1994, mice have become one of the most extensively studied model systems (Epstein, 2006). BALB/c mice were used in this study primarily because so much is already known about their immune responses and genetics from literature. Results from previous studies have shown that despite a few shortcomings, the BALB/c mouse model still manages to paint a better pattern of the human airway disease than any other model (Gleich et al., 1988, Zhao et al., 2000, Blyth et al., 2000; Leigh et al., 2002; McMillan and Lloyd, 2004; Johnson et al., 2004; Jungsuwadee et al., 2004).

Animal models of weight studies have also been reported in literature (Harris et al., 1998; Retana-Ma´rquez et al., 2003; Snibson et al., 2005) and the use of different plant extracts or herbal preparations in managing weight changes have also been reported. For instance, a study using plant extracts showed that the leaves of *Syzygium cordatum* did not cause any weight changes in diabetic rats (Musabayane et al., 2005) unlike the significant weight gains reported in

ovariectomised mice treated with extracts from the plant *Onobrychis ebenoides* (Dontas et al., 2006). Another study by Kyungah et al., (2004) showed that different medicinal plant extracts did not have significant effect on the body weight compared to the control group.

In this study, the possible effects of the plant extract *E. hirta* on asthma and weight change was investigated. *E. hirta* (Euphorbiacea) is found worldwide and in many parts of Africa. Extracts or a decoction of the flowering and fruiting plant have long been used (and are still being used) in East and West Africa and elsewhere for the treatment of many conditions (Oliver 1959; Hazleton and Hellerman 1954; Watt and Breyer Brandwijk 1962; Kokwaro, 1976; Le Strange 1977; Wong 1980; Lanhers 1990; 1991). The medicinal properties of *E. hirta* are possibly due to its content of many active ingredients including alkaloids, flavonoids, glycosides, sterols, tannins and triterpenoids (Gupta and Garg, 1966; Atallah and Nicholas, 1972; Sofowora, 1984; Galvez et al., 1993). Flavanoids are well known to have a high antioxidant activity (Kandaswami and Middleton, 1994).

The (bio) flavonoid in *E. hirta*, Quercitrin (3-rhamnosylquercetin) is usually converted to Quercetin (3-O-alpha-L-rhamnopyranoside - Quercetrin) in the alimentary canal and appears to be the compound that has given this plant its great therapeutic potential. Quercitrin is the glycosylated form of Quercetin and possesses antioxidant as well as anti-inflammatory properties (Comalada et al., 2005). Another flavonoid in *E. hirta,* Myricitrin also seems to be a powerful Nitric Oxide Synthase-inhibiting anti-oxidant. In addition, the sterols 24-methylene-cycloartenol and β-sitosterol have been reported to exert significant and dose-

dependent anti-inflammatory activity while the triterpene β-amyrin also showed anti-inflammatory effects (Martinez-Vazquez et al., 1999).

Free radicals are created in the body as a by-product of energy released by cells. Excessive amounts of free radicals can cause a wide range of diseases but antioxidants help the body fight and neutralize these reactive groups. Asthma has long been associated with an overall increase in reactive groups and oxidative stress (Barnes, 1990; Kharitinov et al., 1994; Nadeem et al., 2003). It is possible that one way by which *E. hirta* functions for the treatment of asthma is through synergistic anti-inflammatory and antioxidant activities of especially the flavonoids, sterols and triterpenoids (Park and Lee, 2006). Since many weight dynamics in especially asthmatics are related to oxidative stress (Fenster et al., 2004; Johnson et al., 2007), the presence of antioxidants in *E. hirta* could have positive effects in eliminating free radicals associated with weight processes in animals treated with extracts of this plant.

The direct or indirect effects of drugs on weight changes have been reported. Sixty percentage of male wistar rats that received cortisone (5 mg/day) were reported to have diminished gain of body weight and total body protein Hausberger and Hausberger (1958). Similarly, a marked decrease in weight was observed in another study in mice receiving HC (Borovitskaya et al., 1971). In addition, following administration of a high-dose of systemic dexamethasone for 3 days, Kumar et al., (1997) reported a marked catabolic effect with weight loss in rats. Another study with Prednisolone showed that the drug caused reduced body weight in mice and guinea pigs (Nagao et al., 2004) while other studies with antipsychotic drugs showed varying results (Ganguli 1999; Goudie et al., 2002). In

line with the above, this current study investigates the possibility of HC and *E. hirta* having effects on weights of asthmatic BALB/c mice.

Day 6 was one day after the last immunization and therefore the acute effects of immunization could be determined on this day. Day 13 was analyzed because any possible effects of the immunization procedure on changes in animal weights could manifest on this day. Similarly, day 15 was considered because it is the last day of the first batch of nebulization and the day when administration of HC and the plant extract commenced. On this day, the acute effects of nebulization on weight changes could be determined. Finally, the weights recorded on day 18 (the last day of the ST studies) could provide information about the effects of all treatments given on days 15, 16 and 17.

Days of analysis for the LT studies included days 18, 26, 32, 37, 42 and 46 since administration of treatment agents continued on days 22, 25, 29, 32 and later days 39, 40, 41, 42, 43, 44, 45. Day 18 was studied to assess the early effects of treatments on animal weights. Day 26 was chosen to assess the midterm effects while day 32 was chosen to assess the late effects of treatments. Since treatment was suspended while the second batch of nebulization was done on days 34-36, treatment effects evaluated after day 36 will be related to the reciprocal effects of nebulization and treatment on the animal weights.

4.3.2.1. Analysis of intra-group weight changes on selected days

Tabulated summaries for mean weights of animals in the ST and LT study groups are given in (*Table 4.3a and b*). The complete daily data are given in the appendix. The mean weights in each experimental group that are significantly lower ($p \leq$

0.05) than the mean baseline or starting weights of same group on Day 1, are indicated by the asterisk symbol in *tables 4.3a* and *b*.

Table 4.3a: Intra-group mean weights on selected reference days (ST study)

DAY	Mean Weights (g) ± SD					
	CT	AS	HHC	LHC	HEH	LEH
1	22.47±0.71	18.29±1.85	21.43±1.53	18.47±2.82	18.89±0.76	17.95±2.56*
6	22.59±0.75	19.57±1.50*	22.23±1.26*	20.69±0.98*	19.59±0.66*	19.42±2.36*
13	23.01±0.64	20.62±1.42*	22.87±0.93*	21.19±0.72*	20.23±0.76*	20.26±2.25*
15	23.40±0.61*	20.98±1.33*	23.43±0.97*	21.72±0.64*	20.83±0.49*	20.84±2.24*
18	23.63±0.57*	21.10±1.81*	22.18±0.97*	19.85±0.82	20.22±0.27*	20.51±2.41*

Table 4.3b: Intra-group mean weights on selected reference days (LT study)

DAY	Mean Weights (g) ± SD					
	CT	AS	HHC	LHC	HEH	LEH
1	19.86±2.36	19.08±2.36	18.40±2.40	18.78±1.71	18.07±1.44	20.09±2.34
6	19.92±2.30	20.17±2.09*	19.47±2.00*	19.69±1.20*	19.19±1.54*	20.82±2.05*
13	20.53±2.46*	21.16±1.80*	20.39±1.77*	20.19±1.27*	19.88±1.55*	21.87±2.07*
15	20.74±2.19*	21.61±1.73*	21.07±1.93*	20.51±1.08*	20.27±1.55*	22.14±2.08*
18	21.11±2.12*	21.90±1.73*	19.79±1.44*	19.64±1.03	20.34±1.48*	22.15±2.13*
26	21.83±2.00*	22.63±1.42*	19.83±1.71*	19.94±1.09*	20.80±1.42*	21.96±1.78*
32	22.56±1.92*	23.52±1.43*	20.73±1.58*	20.72±1.15*	21.78±1.46*	23.50±2.16*
37	22.69±2.08*	23.53±1.43*	21.13±1.57*	21.00±0.87*	22.10±1.56*	23.79±2.15*
42	23.04±2.18*	23.87±1.43*	21.26±1.76*	21.54±1.28*	22.61±1.49*	24.21±2.40*
46	22.86±1.98*	24.46±1.64*	20.90±1.22*	21.31±1.32*	23.09±1.32*	24.37±2.31*

*difference significant compared with weight on day 1 (baseline weight) in same group

The absolute mean weight values were not used when comparing data by group since the starting weights vary between groups. Instead, respective percentage values (PWD) were determined as described in Section 4.2.2.3. Thus, all baseline weights became equivalent to 100% and all original mean weight values that were greater than the baseline weights became percentage values higher than 100% and vice versa for all lower original mean weight values. The respective change in percentage weights between groups on the same reference day were then compared by evaluating their closeness to (or their comparability with) the percentage weight values of the group under reference (*Table 4.4*).

Table 4.4: Percentage weights relative to baseline weights

ST Study						
Days	**CT**	**AS**	**HHC**	**LHC**	**HEH**	**LEH**
1	100.00	100.00	100.00	100.00	100.00	100.00
6	100.54	106.99	103.70	112.03	103.72	108.18
13	102.39	112.72	106.70	114.73	107.11	112.82
15	104.15	114.73	109.31	117.60	110.29	116.07
18	105.15	115.39	103.47	107.45	107.08	114.28
LT study						
Days	**Control**	**Asthmatic**	**HHC**	**LHC**	**HEH**	**LEH**
1	100.00	100.00	100.00	100.00	100.00	100.00
6	100.30	105.69	105.82	104.86	106.22	103.60
13	103.34	110.86	110.79	107.50	110.04	108.87
15	104.42	113.25	114.52	109.23	112.21	110.19
18	106.28	114.74	107.55	104.57	112.56	110.22
26	109.92	118.60	107.76	106.16	115.13	109.31
32	113.56	123.23	112.64	110.33	120.54	116.97
37	114.21	123.31	114.83	111.84	122.32	118.43
42	115.97	125.06	115.55	114.72	125.13	120.51
46	115.08	128.18	113.60	113.46	127.81	121.30

Line and column graphs were used to illustrate weight change trends within groups and to compare patterns between the different groups (*figure 4.1).* Intra-group comparisons of weight changes on selected days showed that most of the mean weights in each experimental group were significantly lower ($p \leq 0.05$) than the mean baseline (or starting weights) of the same group on Day 1 as indicated by the asterisk symbol (*table 4.3a and b*).

4.3.2.1.1. Early and late effects of immunization

Any possible effects of immunization on weight change could be evaluated on day 6. Since all animals received treatment only on a later day, any exaggerated experimental effects on day 6 could only be due to the immunization procedure. Analyses of animal weights in all the groups show that the mean weights on day 6 were significantly higher than baseline weights (*table 4.3a and b*). Unlike all other

weight values on same day, values for the controls in both the short-term and long-term study groups did not differ significantly from the respective baseline weights. Thus, the segment in the histograms for the controls corresponding to the period after immunization is relatively linear (*figure*s *4.3 and 4.4*). Since histograms for all other groups show remarkable percentage weight increase, it could be deduced that the early sensitization (immunization) procedure caused some weight gain in the mice.

Similarly, it is reasonable to examine the possible late effects of immunization on animal weights on day 13 because one week‘s interval was allowed between the first immunization procedure on day 5 and the next nebulization on day 13. Results show that on this day, there was remarkable weight gain in most of the groups but since a similar pattern was observed in the (unexposed) long-term controls, it could imply that the general weight gain observed may not be due to factors other than the immunization procedure. Previous studies involving different forms of animal sensitization showed mixed results, most of which seemed to be species and strain-specific (Curtis et al., 1990; Huneau et al., 1991; Saldanha et al., 2004).

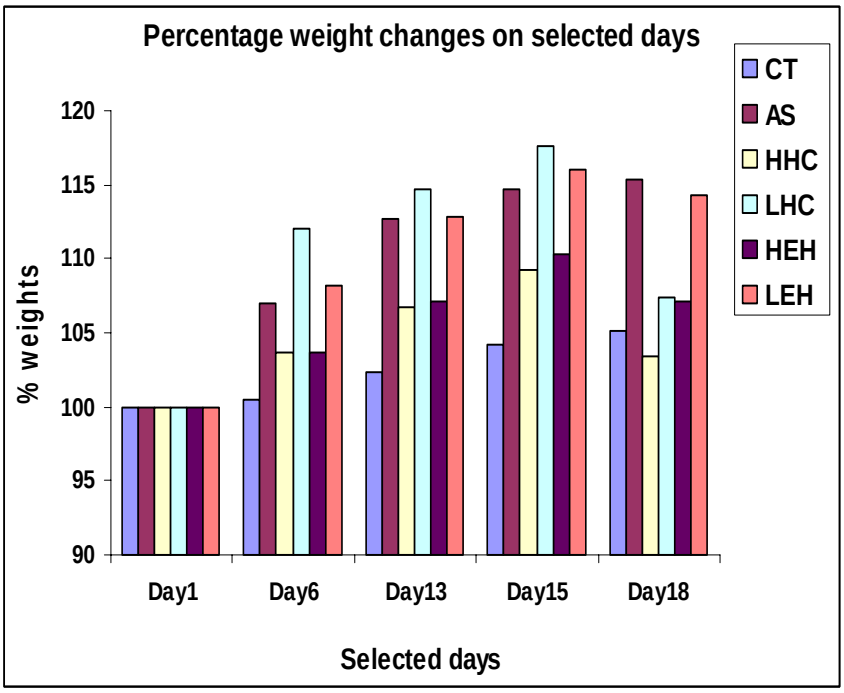

Figure 4.1: Histogram illustrating weight changes during the ST study for the control, asthma, high and low hydrocortisone, high and low E. hirta treatment groups.

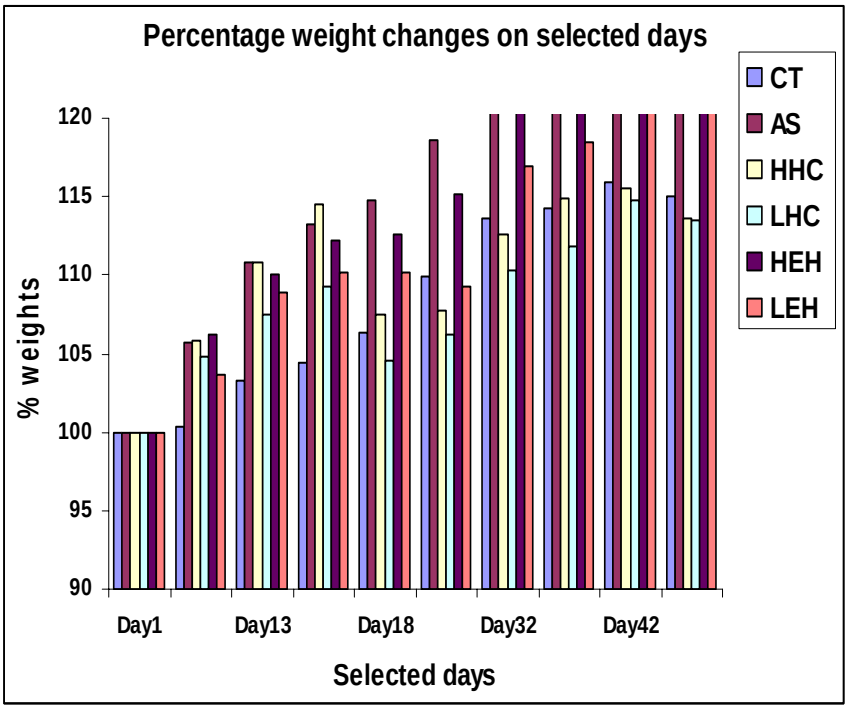

Figure 4.2: Histogram illustrating weight changes during the LT study for the control, asthma, high and low hydrocortisone, high and low E. hirta treatment groups.

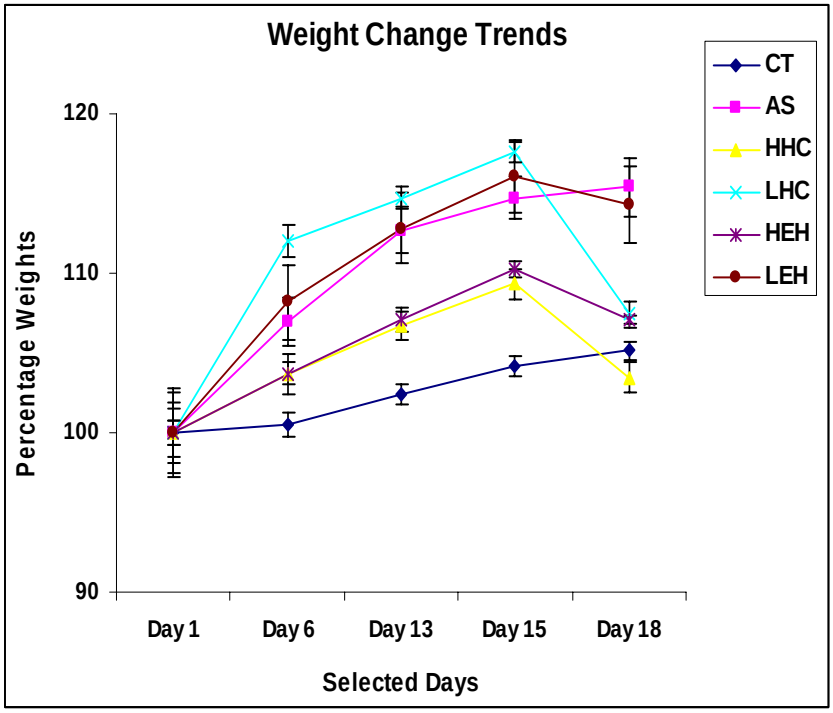

Figure 4.3: Line graph illustrating the trend of changes in weight during the ST study for the control, asthma, high and low hydrocortisone, high and low *E. hirta* treatment groups.

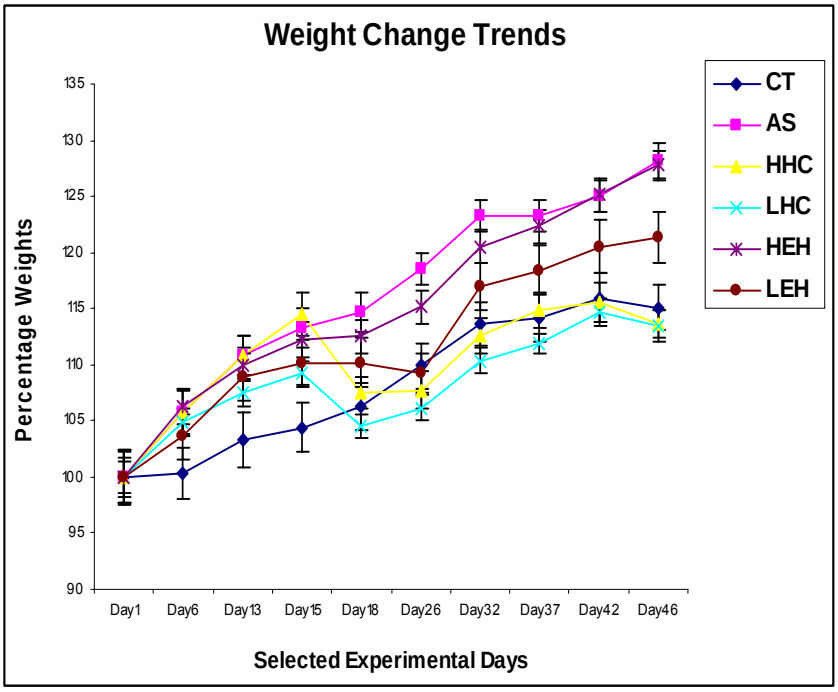

Figure 4.4: Line graph illustrating the trend of changes in weight during the LT study for the control, asthma, high and low hydrocortisone, high and low *E. hirta* treatment groups.

Nebulization was a very important experimental procedure in this study intended to induce the onset of asthma. The first batch of nebulization was on days 13, 14 and 15 before treatment on day 15. The acute effects of nebulization on animal weights could therefore be determined from the weight data taken on day 15 before treatment commenced. Results from *tables 4.3a and b* show that weights in all the groups on this day were significantly ($p \leq 0.05$) higher than baseline weights.

Figures 4.3 and 4.4 show that the histograms for all the groups in both the short and long-term studies were almost linear between day 13 and 15 in most of the groups although slight weight increases were observed. It could therefore be suggested that nebulization had minimal weight gain effects compared to immunization.

4.3.2.1.2. Effects of first time treatment on nebulization

The early effects of HC and *E. hirta* treatment on the nebulization procedure could be evaluated on day 18. Treatment on day 18 was expected to alter the 'asthmatic' effects of nebulization.

Results from *table 4.3a and b* and the histograms (*figures 4.3* and *4.4*) indicate that animals treated with HC and *E. hirta* in both the short and long-term studies lost varying amounts of weight on day 18, most of which was statistically significant. However, the short and long-term control and asthma groups continued to gain relatively small amounts of weight on this day, indicating that treatment with HC remarkably reduced the cumulative weights gained following prior experimental procedures (immunization and nebulization), and in some cases to values approximate to those of Day 6. On the other hand, weights of the two *E.*

hirta groups remained relatively similar to values on Day 15 in the LT study group. During the LT study, weights in the low *E. hirta* group reduced on Day 26 and later increased steadily along with weights in other groups. All these results tend to suggest that the weight gain induced mostly by prior experimental procedures like immunization and nebulization could be reduced remarkably by treatment with HC.

4.3.2.1.3. Midterm and late effects of continuous treatment

Treatment continued during Days 22, 25, 29 and 32 of the long-term study and was suspended during days 34, 35 and 36 in order to repeat the nebulization procedure. The midterm effects of continuous treatment could be assessed from data obtained on Day 26. Except for the LEH group, animals in most of the groups re-gained weight (albeit slightly) on this day after the plunge on day 18. The rate of weight gain was however more marked between days 26 and 32, indicating that a longer period of continuous treatment had remarkable cumulative effects on 'weight recovery'.

Although treatment with HC was observed to cause a sharp weight loss on day 18, prolonged treatment appeared to initiate and sustain an increase in the rate of weight gain as observed between days 26 - 32 (*figures 4.3* and *4.4*). HC is known to enhance improvement in the asthmatic condition (Landstra et al., 2003) while prednisolone was found to reduce body weight in mice and guinea pigs (Nagao et al., 2004).

4.3.2.1.4. Effects of repeated nebulization on weight changes

Effects of initial nebulization observed on Day 15 had minimal weight gain effects compared to immunization. Repeated immunization ended on day 36 of the LT term study and effects of repeating this process were determined on Day 37.

Results from table 2 and the graphs show that weight increase was only very slightly in almost all the test groups, confirming the minimal effects of nebulization on weight gain.

4.3.2.1.5. Early and terminal post-nebulization treatment effects after nebulization on weight changes

After nebulization, treatment resumed during days 39, 40, 41, 42, 43, 44, 45. Acute post-nebulization effects could be determined on day 42. Results from the histograms show that weights increased sharply between day 37 and 42 instead of causing a sharp reduction following treatment after repeated nebulization. This effect was however not sustained after day 42 as animals in especially the two HC groups lost weight slightly while *E. hirta* animals gained weight slightly. The reason for the weight disparity between the treatment groups on Day 46 is not clear but the weight loss effect observed in the HC-treated animals is consistent with one previous finding (Bernick and Zipkin, 1967).

4.3.2.2. Comparison of progressive inter-group weight changes

4.3.2.2.1. Control versus asthma group

Throughout the study, percentage mean weights in the control groups were generally lower than in the asthma group as shown in *Figures 4.3 and 4.4*. The weight gain in the controls was at a relatively slower rate compared to other groups since animals in this group were not exposed to any procedure. The rate of weight gain in the asthma group was faster than in other groups at different periods during the study indicating that the effects of the different experimental procedures on weight change were more pronounced in the asthma group.

4.3.2.2.2. Control versus treatment groups

Percentage mean weights in all the treatment groups were higher than the control weights during most of the ST study period. During the LT study period however, weights in the two HC groups were lower than control weights from Day 26 following prolonged treatment. Weights remained relatively low until the end of the study albeit at about the same rate of weight change. The weight gain in the two *EH* groups were consistently higher than the controls throughout the study. These results suggest that long-term administration of HC causes a low but sustained weight gain pattern while prolonged administration of *E. hirta* extracts causes only a slight but consistent weight gain.

4.3.2.2.3. Asthma versus other groups

The percentage weight values for the asthma group were higher than all other groups by Day 18. During the short-term study, the weights were lower than some of the groups and almost of same value as others. *Figures 3* and *4* shows that there is inconsistent weight change patterns during the short-term period of the experiment but the weights in the asthma group were highest during the long-term study period.

The higher weight values in the asthma group and the consistent rate of weight gain in this group throughout the study indicate that immunization causes a high steady and sustained rate of weight gain in mice, which can only be reduced by treatment. On the other hand, challenge causes only a mild effect on weight change.

During the ST study, administration of the low doses of both HC and *E. hirta* appeared to be less effective in reducing weights to values below the weights in

the asthma group but the rate of weight gain in the two *E. hirta* groups appears to approximate that in the asthma group during the long-term period. This implies that treatment with *E. hirta* causes less reduction in the weight gained due to induced asthmatic conditions, than treatment with HC. This finding is in line with a previous study using extracts of six medicinal plants *Cordyceps militaris* (CM), *Paecilomyces japonia* (PJ), *Phellinus linteus* (PL), *Ganoderma lucidum* (GL), *Grifola frondosa* (GF), and *Panax ginseng* (PG).

In this particular study, body weight, weight gain and food efficiency ratio (FER) were found to have no significant effect on the in treated mice compared to the control group (Jung et al., 2004). Also, extracts of the Brazilian plant *Cissus sicyoides* were found to further reduce the weight loss caused by alloxan in diabetic animals (Viana et al., 2004) implying that *Cissus sicyoides* extracts are less effective in weight reduction.

In *figures 4.1* and *4.2,* the lower the bars in the graph, the more effective is the treatment represented by the bar likely to cause reduction in weight. It was found that the lower doses of the treatment agents used in this study were more effective than the higher doses. The reason for this is unclear but the “low dose of 100mg/kg” used in this study is only described in the context of a relative weight, when considering the higher dose of 125 mg/kg. In a previous study by Hausberger and Hausberger (1958) variable results were reported produced following a 5 mg/day cortisone dose.

4.3.2.2.4. Low dose versus high dose groups

During the short-term study period, there appeared to be no clear weight change pattern as seen in the long-term period. Weight gain was much higher in the "high dose groups" implying that the weights tended towards the values observed in the asthmatic group. The low dose weight data for both the low HC and low *E. hirta* groups were however closer in value to the control group, indicating a low rate of weight gain compared to the corresponding high dose groups. As mentioned previously, these results indicate that 100mg/kg body weight dose of HC and 25mg/kg dose of *E. hirta* had a more effective impact on weight loss than the corresponding higher doses (125 mg/kg HC and 62.5 mg/kg *E. hirta*) respectively.

4.3.2.2.5. Cortisone versus *E. hirta* groups

Weight patterns were generally irregular during the short-term period. However, the plant extract groups appeared to have higher weight values that closely approximated the asthma group for most of the long-term study duration. These results again showed that cortisone administration produces a lower rate of weight gain than does administration of *E. hirta* extracts, indicating that after the animals became asthmatic, the HC-treatment was more effective in restoring conditions to normal (near control) states than *E. hirta*-treatment.

A number of factors including motivation, eating behaviour, amount of activity (especially exercise), overall health, metabolism and stress can cause weight loss or gain. Excessive body weight increases the risk of asthma (Camargo et al., 1999) and obese individuals with asthma may improve their lung-function symptoms and overall health status by engaging in a weight loss program. A controlled study found that weight loss resulted in significant decreases in

episodes of shortness of breath, increases in overall breathing capacity, and decreases in the need for medication to control symptoms (Stenius-Aarniala et al., 2000).

The effects of various forms of activity (experimental procedures, food intake, exercise, physical and chemical stress) on weight changes have been studied in humans and animals alike. Findings from these studies tend to suggest that weight changes that occur especially in relation to stress are usually in response to internal changes in animal physiology as induced by the stress exposure (Retana-Ma´rquez et al., 2003). In one study using restraint stress, rats lost weight and remained hypophagic until a few days after the stressor had ended (Harris et al., 1998).

In yet another study, stress effects on body weight were observed only with repeated exposure to the stressors, and less body weight gain (but not body weight loss) was observed compared to the control group in animals subjected to stress by immobilization or by immobilization plus tail shocks during three days. The loss in body weight observed in other studies by Ottenweller et al., (1992) and Marti et al., (1994) was due to a decrease in food intake.

During most of this study, animals experienced consistent weight increase but there were periods of weight reduction after weight gain. Some of the observed weight gain in all the groups in this study could be attributed to a number of factors including the various experimental interventions. Specifically, any weight changes in the control groups at any time during the study were assumed to be due to changes in normal body metabolic responses, growth as well as changes in food

and fluid consumption patterns. The control animals had no form of experimental intervention and were provided food and water *ad libitum*.

In yet another study, stress effects on body weight were observed only with repeated exposure to the stressors, and less body weight gain (but not body weight loss) was observed compared to the control group in animals subjected to stress by immobilization or by immobilization plus tail shocks during three days. The loss in body weight observed in other studies by Ottenweller et al., 1992 and Marti et al., 1999 was due to a decrease in food intake. One interesting observation was that weights in the control and asthma groups were generally among the lowest throughout the study, suggesting that the progressive higher animal weights observed in all other groups were caused by the different experimental procedures.

4.4. Conclusion

A guided stage-to-stage analysis of all data was undertaken to avoid excluding any possible contributions to weight change by the different experimental interventions, especially because the causes of weight changes are multifactorial. Data obtained show that animals in the control groups initially experienced weight losses that steadily became marginal weight increments (see appendix tables). The reason for the early weight losses is not clear but beyond day 6, mean weights in the control groups became regular and progressively higher throughout the remaining duration of the study. Data also show that animals in both the ST and LT control groups experienced slow but steady progressive weight increments throughout the duration of the study. In addition, mean weights in all other groups also increased progressively in value. After day 6, none of the animals lost weight

below their starting (baseline) weights on the selected days and only at certain stages during the study did animals lose weight relative to the weight values recorded on the previous study days.

It could be concluded that both immunization and nebulization had positive weight gain effects on the animals but the effects were more pronounced following immunization but were only minimal following nebulization. These effects were however modulated variously by treatment with the test agents (HC and *E. hirta* extracts). Prolonged treatment with HC remarkably reduced the cumulative weight gained following prior experimental procedures (immunization and nebulization), followed by a slow and sustained increase in the rate of weight gain. On the other hand, prolonged administration of *E. hirta* causes only a minimal reduction in weight gained due to induced asthmatic conditions. In addition, the lower doses were found to be more effective in lowering weights than the high doses.

The above conclusions are based on the assumption that the control group values represented animals with normal, uninterrupted, physiological states and the asthma group values represented the animals with “the asthmatic symptoms”. Further studies with specific defined weight-related experimental goals would be required to clearly determine the possible effects of varying doses of especially the *E. hirta* plant extracts on animal weights, as well as to confirm the effects of HC observed in this study.