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DEVELOPING CHRONIC UNPREDICTABLE/ALTERNATING STRESS MODEL IN WISTAR ALBINO RATS

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Abstract:
In order to study stress a lot of animal models have been developed so as to copy as closely as possible the stress experienced by humans in everyday life. Some of these models are very harsh and cause permanent physical damage or psychological diseases in animals. Some of the models are time consuming and are not strong enough leading to adaptation. Some models are too expensive and not visibly advantageous. To contribute to this field, we designed a chronic alternating stress model aimed at examining the impact of stress on both parent rats and their initial offspring generation. This model involved subjecting the rodents to a three-week regimen of chronic alternating stressors, encompassing disruptions to circadian rhythms, cold water immersion and restraint-induced stress. This manuscript presents an in-depth account of our methodology and draws comparisons with existing stress models. In conclusion chronic alternating stress model, administered over a 3-week period, effectively induced stress related behavioral changes and activated the Hypothalamic Pituitary Adrenal (HPA) axis by raising Corticosterone and Adrenocorticotropic Hormone (ACTH).

Keywords: Chronic Alternating Stress, Wistar Albino Rats, Cold Water Immersion Stress, Restraint Stress, Circadian Rhythm, Corticosterone, Adrenocorticotropic Hormone, Hypothalamic Pituitary Adrenal axis, Hole Board Behavioural Test.

1.Introduction

Long-term stress creates dysfunctional reactions that may lead to heart disease, stomach ulcers, sleep disturbances and mental health issues. There is an increased risk of hypertension, coronary artery disease and stroke in those with psychiatric illnesses connected to stress (Budzyński and Kłopocka, 2014). Persistent stress may have a deleterious effect on menstruation, sperm maturation, pregnancy, and sexual desire in the reproductive system (Chu et al., 2023).

The establishment of stress protocols in rats is of paramount importance in comprehending the physiological and neurological impacts of stress. The significance of stress in relation to human health is noteworthy due to its influence on a variety of ailments, including both mental and immunologic problems (Glaser and Kiecolt-Glaser, 2005). Rats, due to their physiological similarities with humans, are excellent models for research purposes (Bryda, 2013). When used in conjunction with neuroscience methodologies, these procedures provide a comprehensive comprehension of the brain's responses to stress. The observation of behavioral modifications in rats also offers valuable information into possible stress-related reactions in human beings (Demeter et al., 2008). Moreover, it is essential to comprehend the impacts of stress to help in advancement and evaluation of therapeutic interventions. Furthermore, these methods provide a comprehensive insight into the complex interplay between genes and environment in the context of stress responses (Atrooz et al., 2021).

There has been extensive use of animal models especially rodent models of chronic stress. Numerous protocols have been established to develop chronic stress in rat models. Some of the models are not robust enough and lead to early adaptation, with some of the animals' not developing stress. While others may be too severe causing alterations and damage to organs (Xu et al., 2019). Moreover, some of the models are time consuming and not cost effective (Finke et al., 2018). One of the approaches used frequently is chronic alternating stress. However, there are a lot of variations in chronic alternating stress models each with its own advantages and disadvantages. Keeping this in mind we devised a model of chronic alternating stress using wistar albino rats and checked its efficacy in two generations of rats, both old and young rats.

2. Materials and methods

2.1. Experimental design

Our study was an Experimental Case-Control Study of two-year duration. 130 healthy rats were taken in the parent group. Randomization was done by lottery method. Ethical approval was given by the Ethical Committee of Peshawar Medical College (Prime/IRB/2023-207) and Khyber Medical University (DIR-KMU-EB/HS/000675). The free availability of food (standard laboratory diet) and water was ensured (Radahmadi et al., 2017). Ethical guidelines for animal care UK were followed.

2.2. Animals

A total of 130 adult specific pathogen-free Wistar rats weighing 280-300 g, age 11 weeks, male to female ratio 1:1 were raised at the Peshawar Medical College Animal House. The rats were kept in cages with 4-6 rats per cage at 25±2˚C temperature and a humidity of 40-60% under a 12hour light/dark cycle. Preliminary behavioral testing on hole board apparatus was done of all the rats. 10 rats showed stress and were removed. At this point 10 rats were grouped as parental control group (P1B) and were sacrificed for blood and histological samples. The remaining 110 rats were split into a control group (P1B) with 40 rats and the case group (P1A) with 70 rats. Only the case group were given 3 weeks of chronic alternating stress after which behavioral testing was done. We only included those rats which were stressed. There were 10 rats which were removed as they did not show the induction of stress. Out of 60 rats in case group we sacrificed 10 for blood and tissue sampling. The remaining 50 stressed rats were distributed in cages containing 4 to 6 rats per cage with 2:2 or 3:3 male to female ratio. The 40 rats in the control group were not given any stress however the males were kept separate from the females to avoid pregnancy. Behavioral tests were done for control group of rats to check if keeping males and females separate had caused any stress which was not found to be different from the previous tests. After 3 weeks the male and female rats in both groups were kept together and permitted to mate. The offspring of both of these parent groups were exposed to stress

and then subjected to behavioral tests and blood sampling at 5 weeks, 8 weeks and 14 weeks of age the details of which have been reported in another paper by Usman et al "Development of protocol for transgenerational stress in wistar rats" submitted for publication.

2.3. Chronic Stressors

The stress protocol comprised of three stressors alternating with each other. On day 1 was alteration of day-night cycle, on day 2 was exposure to cold water immersion stress and on day 3 was restraint stress. This cycle was repeated for a total of 21 days (in total 7 cycles of three stressors) and due to the alternating nature of the stressors, adaptation to stress did not occur (Willner, 1997). The details of the stressors are as follows: -

2.3.1. Alterations in circadian rhythm

The first stressor was alteration in circadian rhythm. Lights in the cage of rats were turned on from 7 o'clock in the evening till 7 o'clock in the morning and were put off from 7 o'clock in the morning till 7oclock in the evening. Thus the night and day phase of the circadian cycle were reversed (Fonken et al., 2009),(Munn et al., 2011). Dark room environment was created by covering the windows and door edges with black covers.

2.3.2. Cold water immersion stress

On day 2 the rats were dipped in a water tub at 15-18 degree Centigrade with the height of water column of 15 to 18 cm for the duration of 5 minutes (Agrawal et al., 2011). The rats either remained in an upright position keeping their heads above the water level or they swam. The height of the tub was such that the rat could not climb out of the container. An alcohol thermometer was used to check the temperature of water in the tub.

2.3.3. Restraint stress and immobilization

Restraint stress was applied on day 3. The rats were kept in a cylindrical tube having ventilation holes for a duration of 120 min. The size of the cylindrical tubes was such that the limbs could not be moved. The rats were kept restrained in their natural posture and they could not turn or escape (Padovan and Guimaraes, 2000).

Table 1 Sequence of applying stressors.

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
A	**B**	**C**	**A**	**B**	**C**	**A**
Day 8	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**
B	**C**	**A**	**B**	**C**	**A**	**B**
Day 15	**Day 16**	**Day 17**	**Day 18**	**Day 19**	**Day 20**	**Day 21**
C	**A**	**B**	**C**	**A**	**B**	**C**

A=Alteration of Circadian Rhythm, **B**= Cold water immersion stress, **C**= Restraint stress

2.4. Behavioral tests and Blood Sampling:

The 70 rats in the case parent generation were given the above protocol of chronic alternating stress for 3 weeks / 21 days. Behavioral testing was done on the 22nd day. We checked the induction of stress via open field and hole board behavioral test. 10/70 (14%) of the rats belonging to the case generation of the parents were not stressed so these were removed. All behavioral tests were conducted by the same person within the time duration of 9 a.m in the morning till 4 p.m in the evening. Out of the 60 stressed rats 10 were sacrificed through exsanguination via intra cardiac puncture (Supplementary-A) after been given Isoflurane anesthesia (Supplementary-B). The sera were separated from the blood via centrifugation at 4,000 revolutions per minute for 20 minutes and stored in a minus 80-degree freezer. These were later taken out for Elisa Testing. This research work is a part of my PhD thesis titled, "Hormonal and structural changes in the reproductive system of a chronic stress rat model and their offspring."

2.5. Hole Board Behavioral Test:

The hole board behavioral test is utilized to check for anxiety or exploratory behavior (Brown and Nemes, 2008).We used a modified apparatus for the hole board test consisting of a grey wooden box measuring 72 cm × 72 cm. The wall was of height 40 cm while the box was elevated 28 cm above the ground with the help of a metal stand. The number of holes cut into the floor were 9. The central area was the area in the center which was bounded by the holes. The periphery was the area near to the walls and outer to the holes. Anxiety was assessed by the number of head dippings which occurred when a rat dipped its head into the hole with the ears at the floor level. Increased head dipping was considered an exploratory behavior and the rat was considered less anxious. Secondly, anxiety was assessed by rearing; when a rat uses hind limbs to stand. Thirdly by locomotion which means when a rat moved with all its 4 paws into a different area. The entire distance travelled by the rat was added up and recorded as locomotion (Bailey and Crawley, 2009). If a rat went into the center, it was considered as relaxed while keeping to the walls in the periphery (thigmotaxis) revealed anxiety (Kallai et al., 2007), (Ohl, 2003). Increased percentage of time in the center was considered as exploratory behavior and higher occurrence was considered as less anxious behavior in rats (Brown and Nemes, 2008).

A 5-min test session was recorded to capture general locomotion (Bailey and Crawley, 2009). Testing session lasted for 5 min done by direct observation and recorded by a digital camera (Schmatz et al., 2009). At the start the rat was held by its tail and was placed in the hole board's central area at which time the video was also started. Videos were recorded by Sony super steady shot D5C-H50 camera, and the video was analyzed manually by two independent researchers. Floor was swept with 30% ethanol and dried with a dry paper towel to clean the area for the next rat (Ramos et al., 2002),(Ramos et al., 2008).

2.6. Blood tests:

After collection, blood samples were cold (-4) centrifuged at 4000 revolutions/minute for 20 minutes. Serum was separated and transferred to eppendorf tubes, which were then stored at -80°C for further testing. Cortisol was estimated through ELISA from Elabscience USA (Catalog number: E-EL-R0269). Adrenocorticotropic hormone (ACTH) was estimated through ELISA from Elabscience USA (Catalog number: E-EL-R0048).

2.7. Statistical Analysis:

Data was analyzed by SPSS statistical software version 25. The distribution of the data was checked using test of normality; Shapiro-Wilk test. The data which showed non-normal distribution was log transformed. The log transformed data was presented as Geometric mean [(lower bound to upper bound confidence interval (CI)]. For comparison between different groups showing normal distribution, we used independent sample t test and MANN-Whitney U test was used for data not normally distributed. Graphs were made through Graph Pad Prism version 9.5.1 for the blood markers and variables of behavioral tests. P-value ≤ 0.05 was taken as a cutoff point for significance.

3. Results: -

3.1. Serum Corticosterone levels:

Corticosterone levels were higher in stressed rats; P1A. The geometric mean (Confidence Interval-lower bound to upper bound) of P1A [155.09 (101.15-237.79) ng/ml] was substantially increased ($p = < 0.001$) as opposed to the unstressed rats; P1B [49.22 (35.88- 67.54) ng/ml].

3.2. Serum Adrenocorticotropic hormone levels:

Adrenocorticotropic hormone (ACTH) levels were also higher in P1A [Geometric mean (CI) 176.49(108.10-288.17) pg/ml] as compared to P1B= [39.36(27.62-56.09) pg/ml] and was statistically significant ($p = < 0.001$).

3.3. Comparison of variables of Hole Board Behavioral test:

Locomotion (cm) was much higher in unstressed; P1B (2441.4 ± 351.924) compared to stressed; P1A rats (380.00 ± 261.876 cm, p = <0.001). Head dipping (n) was higher in P1B (5.91±4.23) than P1A (1.58 ±1.38, p = <0.001). Number of rearing and rearing time (sec) was higher in P1B as compared to P1A (Figure 2). Central entries (n) were increased in P1B (1.45 ±1.508) as compared to P1A (0.08±0.289, p<0.001). Moreover, time in seconds for central entries (sec) were higher in P1B as compared to P1A (Figure 2). Time spent in center (percentage %) was greater for P1B compared to P1A. However, time spent in periphery (percentage %) was lesser for P1B as compared to P1A (Figure 2).

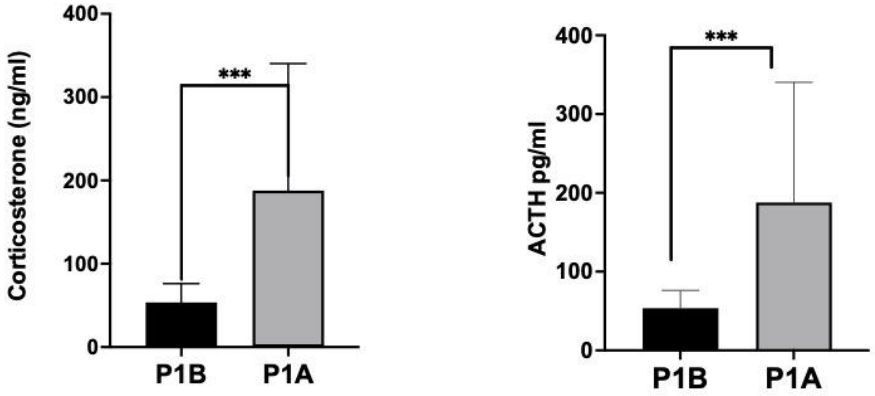

Figure 1 Corticosterone (ng/ml) and ACTH (pg/ml) levels in unstressed parents P1B and stressed parents P1A. (***) denotes p-value < 0.001.

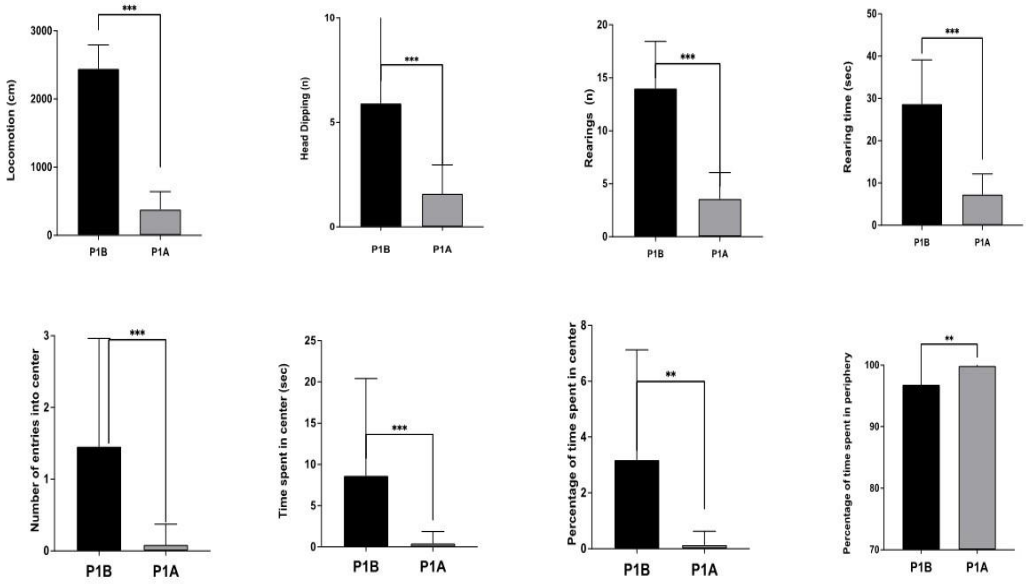

Figure 2: Comparison of parameters of hole board behavioral test between unstressed parents P1B and stressed parents P1A.

(***) denotes p-value < 0.001
(**) denotes p-value < 0.01
(*) denotes p-value < 0.05

4. Discussion:

Animal models, particularly those involving rodents, have been widely used to study chronic stress. Numerous protocols have been established to develop chronic stress in rat models. Some of the models are not robust enough and lead to early adaptation, with some of the animals' not developing stress.

While others may be too severe causing alterations and damage to organs (Xu et al., 2019). Moreover, some of the models are time consuming and not cost effective (Finke et al., 2018). One of the approaches used frequently is chronic alternating stress. However, there are a lot of variations in chronic alternating stress models each with its own advantages and disadvantages. Keeping this in mind we devised a model of chronic alternating stress using wistar albino rats and checked its efficacy in two generations of rats, both old and young rats.

The modern times and competitive lifestyle have created a lot of stress for people. There has been a constant increase in diseases both in adults and in children with the passage of time. Most of these are linked directly to stress. Consequently, we developed a rat model of chronic alternating stress in order to get a more comprehensive insight into the impact of stress on people.

We planned this study to model an animal chronic stress model, which is robust, cost effective, not damaging other organs and not much time consuming. Our chronic stress model constituted a chronic alternating stress protocol for three weeks. We evaluated our model after three weeks and found that 60 out of 70 rats developed stress (85.7%). However, 10 out of 70 rats (14.3%) did not develop stress. Dariusz Zurawek et al used 110 rats in the stress group out of which 78 were stressed or anhedonic while 32 were resilient and did not develop stress (Zurawek et al., 2017). This study was similar to ours in which 10 rats (14 percent) were resilient in the parent generation whom we dropped out as we wanted the parent generation of cases to be stressed so as to check the impact of the stress in the succeeding generation. Theilmann et al subjected stress to 26 rats and anhedonic behavior was seen in 53.8% (14/26) of the animals which were classified as stressed , while hedonic behavior was seen in the rest 46% of the animals (12/26) which were classified as resilient (Theilmann et al., 2020). Theilmann et al., used the chronic mild stress comprising of exposure to continuous light , water and food deprivation, swimming sessions in water once at a temperature of 40°C for 10 minutes and also at a temperature of 15°C for 5 minutes, wet bedding and 30 min restraint stress followed by social crowding (Theilmann et al., 2020). This study was similar to ours in that they used changes in light dark cycle, restraint stress and water immersion stress which was the same as ours. The attrition rate of our study was 14.6% which was remarkably less than theirs which was 46%. However contrary to our study, Christiansen et al gave stress to 48 rats and all developed stress and anhedonic behavior as checked by sucrose preference test and their attrition rate was 0% (Christiansen et al., 2016). This study used a weekly stress regimen which was different from ours and comprised of exposure to light flashes, grouping, restriction of food and water, two occasions of cage soiling and three periods of cage tilting (Christiansen et al., 2016). This study used the water and food deprivation stressor and soiled cage along with box tilt which might be more powerful stressors as compared to ours.

Our stress protocol had a duration of three weeks. There are many stress protocols which are less than 3 weeks duration like Zurawek et al who gave chronic mild stress for two weeks (Zurawek et al., 2017).Theilmann et al reportedly used stress protocol for a duration of three weeks (Theilmann et al., 2020). SL Christiansen et al used a 3.5-week stress protocol (Christiansen et al., 2016). Meina Quan et al also designed a 3-week research study on wistar albino rats (Quan et al., 2011). There were other studies which gave stress for a more extended duration like 5 weeks (Matisz et al., 2021) and 12 weeks (Jiang et al., 2022). We chose a duration of three weeks so as to give chronic stress and at the same time prevent habituation. According to a meta-analysis by Antoniuk-S et al., Wistar Albino Rats have been found to have increased Corticosterone levels at 3 weeks after stress, while the levels decreased at 4 to 5 weeks exhibiting habituation. Wistar albino rats also exhibited very low inter group heterogeneity (Antoniuk et al., 2019). Human chronic stress is usually thought of to be a few years exposure to physical and social stresses. In animal models when translated to human life span a chronic stress has to be at least 10 days of the animals life. If it is applied for weeks it can causing permanent behavioral and physiological effects(Tran and Gellner, 2023).

The number of stressors given per day by various studies varied a lot but typically most studies used one to two stressors per day (Zurawek et al., 2017),(Theilmann et al., 2020),(Christiansen et al., 2016),(Quan et al., 2011). Very few used a maximum of three stressors per day (Harkin et al., 2002). Katz et al., in 1982 created a three-week chronic intermittent stress protocol in which they used one

to two stressors per day (Katz, 1982). Jiang X et al also used one stressor per day but never used the same stressor successively for twelve weeks (Jiang et al., 2022). Wei et al., also devised a stress model in which they used one stressor per day (Wei et al., 2017). So, most of these studies used one stressor per day which were the same as ours. The studies that wanted a more intense stress protocol used more than two stressors per day. We used one stressor per day because we wanted to give moderate stress that more accurately reflected the day-to-day stressful events of human life.

The types of stressors used in various studies comprised of reversal of light/dark cycle, restraint stress, cold water immersion stress, electric foot shock, cage tilt, cage soiling, predator odor, food and water restriction, tail pinch, exposure to cold temperature and heat waves and stroboscopic light and noise. Some studies used a large variety of stressors amounting to seven in all (Quan et al., 2011). Matisz C et al devised a stress model using a total of 4 stressors including elevated platform stress, restraint stress, food and water deprivation stress and isolation stress so as to produce chronic unpredictable stress for a duration of 5 weeks. (Matisz et al., 2021). Moustafa Algamal et al. introduced a 21-day repeated unpredictable stress paradigm using 5 stressors (Algamal et al., 2018). We used a total of three stressors and our model was effective in the implementation of stress in the rats using one stressor per day. We alternated the stressors so as to avoid habituation. Most of the studies that we evaluated used one to two stressors per day and repeated the cycle weekly. Three stressors cycle was repeated twice weekly in our study (Table 1).

We used the hole board and open field behavioral tests along with serum levels of Corticosterone and ACTH to check for induction of stress. A lot of studies used the sucrose consumption test to check for induction of stress. A rat was stressed or anhedonic if there was a decrease in sucrose consumption of over 20 percent before and after the stress period (Zurawek et al., 2017), (Theilmann et al., 2020), (Christiansen et al., 2016; Quan et al., 2011), (Harkin et al., 2002; Katz, 1982; Matisz et al., 2021). Schweizer et al included an array of behavioral tests like the forced swim test, modified hole board test, open field test, box test having light and dark areas and saccharin consumption test in order to assess potential alterations in behavior (Schweizer et al., 2009). Jiang X et al in 2022 used the sucrose consumption test and open field behavioral test to assess the induction of stress (Jiang et al., 2022). In our study the rats in the case group were stressed as shown by increased Corticosterone and ACTH hormones along with decreased locomotion, head dipping, central time and rearing in the hole board behavioral test. These effects were also mirrored by open field test however we have described only the hole board test in this paper. Both hormonal and behavioral tests reinforce the confirmation of induction of stress. Using both hormones and behavioral tests for evaluation of stress have also been used by Matisz et al who also used fecal corticosterone to check for stress levels (Matisz et al., 2021). Algamal et al used the behavioral tests of elevated plus maze and open field along with ACTH and Corticosterone levels to assess stress in mice (Algamal et al., 2018). Wei et al in 2017 used the open field behavioral test and the sugar preference test to establish the induction of stress along with blood samples for corticosterone and epinephrine (Wei et al., 2017)**.**

The stress protocol in our study used 3 different stressors for a minimum duration of three weeks in wistar albino rats and was successful in mimicking chronic stress. We tried to keep this stress protocol of moderate intensity because we wanted to mimic the human stress that an average person experiences.

5. Conclusion

This study's chronic alternating stress model, administered over a 3-week period, effectively induced stress related behavioral changes and activated the HPA axis by raising levels of corticosterone and ACTH.

6.Future Implications

This model offers a versatile platform for investigating the impact of stress on performance, hormonal regulation and potential interventions, contributing to a deeper understanding of stress-related disorders and therapeutic strategies. Further investigations utilizing this model could elucidate

underlying mechanisms and provide insights into developing targeted interventions for stress-related diseases.

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Study Design, lab work, data collection and analysis, manuscript writeup.

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Manuscript writeup, idea of study, statistics, critical reading.

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Idea of study, critical reading.

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Lab work, critical reading.

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Supplementary A:
Blood sampling through cardiac puncture
A 10 ml syringe equipped with a 23 Gauge needle was used. The rat was positioned in a supine position with its ventral side facing us. The position of the heart was determined by placing the index finger of the left hand at the level of the lowest ribs, without exerting any pressure. The heart is situated slightly to the right and about one cm above. The syringe was held at a 45-degree angle. A needle was inserted until a droplet of blood was seen in the syringe. Subsequently, the plunger of the syringe was retracted to draw as much blood as possible into barrel and completely fill the syringe, which was then cautiously detached. The blood was transferred into a tube. If more blood was needed, the syringe was once again attached to the needle. A volume of 5-10 ml of blood was extracted from each rat (Beeton, Garcia and Chandy, 2007)

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Supplementary B:
Open drop method
1. After donning the gloves, gauze was soaked with 1cc of a 30% v/v isoflurane in propylene glycol solution for every 500cc volume of the anesthesia container.
2. The gauze piece was placed in a wire mesh placed at the bottom of the glass container to avoid direct contact with isoflurane. This precaution is necessary since isoflurane may cause irritation and be absorbed through the skin.
3. Rats were placed individually in the container and the lid was carefully secured. The rat was anaesthetized within around 2 minutes which was confirmed by the absence of the righting reflex.
4. The subject was kept under deep anesthesia for a duration of 10 seconds before being removed from the container, which was immediately closed with a lid.
5. The procedure began if there was no response to toe pinch; otherwise, it was placed back into the container.

Using the open drop method, rats were briefly anaesthetized, during which blood was obtained by heart puncture which eventually euthanized the rat by exsanguination (Risling, Caulkett and Florence, 2012)

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