Journal of Advances in Medicine and Medical Research

32(17): 8-16, 2020; Article no.JAMMR.60733
ISSN: 2456-8899
(Past name: British Journal of Medicine and Medical Research, Past ISSN: 2231-0614, NLM ID: 101570965)

Comparing the Effect of Methods of Rat Euthanasia on the Brain of Wistar Rats: Cervical Dislocation, Chloroform Inhalation, Diethyl Ether Inhalation and Formalin Inhalation

U. S. Aguwa[1*], C. E. Eze[1], B. N. Obinwa[1], S. N. Okeke[1], S. F. Onwuelingo[2], D. I. Okonkwo[2], D. K. Ogbuokiri[1], A. E. Agulanna[1], I. J. Obiesie[1] and A. J. Umezulike[3]

[1]Anatomy Department, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi, Nigeria.
[2]Anatomy Department, Faculty of Basic Medical Sciences, Madonna University, Nigeria.
[3]Physiology Department, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author USA designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors CEE, BNO and AJU managed the literature search. Author SFO, DIO and AEA performed the statistical analysis. Author SNO, DKO and IJO managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMMR/2020/v32i1730636
Editor(s):
(1) Dr. Chan-Min Liu, Xuzhou Normal University, China.
Reviewers:
(1) Robson Chacon Castoldi, Universidade Estadual Paulista - UNESP, Brazil.
(2) Gurram Srinivas, P. V. Narsimha Rao Telangana Veterinary University (PVNRTVU), India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/60733

Original Research Article

Received 28 June 2020
Accepted 01 September 2020
Published 22 September 2020

ABSTRACT

Contentions still exist as to the most humane method of sacrificing experimental animals. Beyond humaneness, there is also the issue of appropriateness of a method of sacrifice with regards to organ safety. This study compared four common methods of rodent sacrifice used in Nigeria and their effects on the brain. Twenty male Wistar rats weighing 150 to 180g were randomly separated into four groups of five rats each. After a two week period of acclimatization, the animals were sacrificed; Group A by Cervical dislocation (CD) which also served as control, Group B by

**Corresponding author: E-mail: usaguwa@gmail.com, us.aguwa@unizik.edu.ng;*

chloroform inhalation (CI), Group C by diethyl ether inhalation (DEI) and group D by Formalin inhalation (FI). The time taken for the animals to pass out was documented and the brains were harvested. Four of the brains from rats in each group were homogenized in phosphate buffer solution and centrifuged at 10000rpm. The supernatant were used for antioxidant studies. The remaining one brain from each group were fixed in 10% formal saline and after 48 hours, the cerebellum (CB) and hippocampus (Hp) were used for histological studies using the H & E method. Our results show although CD was the quickest, it gave the least quantity of blood. Meanwhile CI was the most humane, gave the most blood and saved time compared to DEI and FI. None of the methods of sacrifice showed adverse effect on the antioxidant parameters of the rats. However, histological studies showed that while CD and CI showed no adverse effects on the hippocampus, only CD was completely safe for the CB. The other methods showed various levels of cell death. It is therefore expedient to consider these factors in making the choice of an appropriate method of sacrifice and to use the most humane and safest method with reference to the organ studied.

Keywords: Brain; euthanasia; cervical dislocation; diethyl ether; chloroform.

1. INTRODUCTION

Animal sacrifice is any procedure used in inducing humane death in an animal resulting in rapid loss of consciousness and death of the animal for research purposes [1]. Sacrificing research animals is one of the most challenging tasks in animal studies because of the technicality required [2]. Errors at this time can impact negatively on the outcome of the research. Methods of animal sacrifice include inhalation of anesthesia, cervical dislocation, decapitation, injectable barbiturate and exsanguinations [3]. Regulations exist in different countries on the acceptable methods for animal euthanasia. This study was carried out in Nigeria and highlights common acceptable practices in Nigeria. Researchers are of the opinion that particular methods of animal sacrifice are more suitable for certain research protocol and not for others.

Cervical dislocation has been acclaimed a humane method for the sacrifice of small rodents, but has shown a surprisingly high failure rate [4]. Also, observation of the procedure puts a question mark to its humaneness considering the level of physical exertion on the animals. Some scientists consider the procedure harsh and barbaric. Inhaled anesthetics were first introduced into clinical practice in the 1840s. Chloroform sedation has been used to sacrifice animals reportedly with no undesirable effects although other reports have shown that chloroform has significant toxicity, including hepato- and nephrotoxicity [5]. In the mid-17th century, diethyl ether was first introduced as an anesthetic agent [6]. Formalin is a common chemical used in fixing tissues for histological studies and for embalmment of human and animal remains.

2. MATERIALS AND METHODS

A preliminary research was carried out to ascertain the commonest animals used for research among clinical anatomists and the prevalent method of sacrifice used by them. A questionnaire was distributed at the 18th annual conference and AGM of the Society of Experimental and Clinical Anatomists of Nigeria (SECAN) held at Enugu State University of Science and Technology (ESUT) between 28th and 30th of November, 2019. Participants for this conference were drawn from across Nigerian Universities. A total of 141 questionnaires were distributed randomly among the participants out of which 126 were retrieved, representing 89.4%.

Twenty adult male Wistar rats weighing between 150 to 180g were used for the animal study. The rats were housed in standard wooden cages with wire guaze all around and chipping saw dust as bedding. The rats were fed pelletized rat feed produced by Pfizer. Animals were separated into 4 groups of five (5) rats each based on closeness to weight. The rats were acclimatized for 2 weeks under standard conditions for handling research animals.

After acclimatization, the rats were sacrificed as follows: Group A by cervical dislocation, Group B by chloroform inhalation, Group C by diethyl ether inhalation and Group D by formalin Inhalation.

For cervical dislocation, the animals were suspended by their neck and pulled on the tail

until the crack sound indicating dislocation of the atlantoaxial joint was confirmed.

For the inhalation groups B, C and D exposed to chloroform, diethyl ether and formalin respectively, the animals were introduced into a desiccator containing their corresponding chemical. Prior to exposure, the chemical was soaked in cotton wool and placed in the desiccator for 5minutes to ensure circulation. The time taken for the rats to pass out was recorded using a stop watch. After 30 minutes, the formalin exposed rats were not knocked off and so were sacrificed by cervical dislocation. All chemicals used were manufactured by SIGMA-ADRICH, INC., P.O. Box 14508, St. Louis, MO 63178, USA.

2.1 Anti-oxidant Assay

Brain samples from each group were taken for anti-oxidant tests to examine the activities of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and malondialdehyde (MDA) which is a measure of lipid peroxidation. The brain samples were collected and weighed. Phosphate buffer solution was prepared and poured into the specimen bottle containing each brain sample for homogenization using a homogenizing machine. The sample was centrifuged with cold centrifuge at 10,000 rpm (rounds per minute) and the supernatant was collected. The rest of the assay was done using the supernatants.

The protein concentrations of the samples were determined by the Biuret method as described by Gornal et al., [7]. Assessment of lipid peroxidation was carried out by the method of Varshney and Kale [8]. The level of SOD activity was determined by the method of Misra and Fridovich [9]. Catalase activity was determined according to the method of Sinha A., [10].The level of reduced glutathione (GSH) was estimated using the method of Beutler et al., [11].

2.2 Histopathological Studies

After 48 hours of fixation, the brains were removed and the cerebellum and hippocampus excised from each one for histological assessment using H&E. Tissues were placed in ascending grades of alcohol (70%, 80%, 90%, and absolute). The time for each grade of alcohol is one hours and in absolute (100%) it is put twice, one hour each time.

Xylene was used to remove alcohol which itself removed water from the tissue because paraffin wax used in embedding is not miscible with alcohol. The tissue was passed twice for one hour each time. The tissues were embedded in molten paraffin wax at constant temperatures of 36-60ºC in an oven of paraffin bath, changing it four times, 1 hour for each change. Metal blocks were filed with paraffin wax and tissues were placed in it immediately with forceps, the face to be cut facing downward. When the paraffin cools, a thin scum of solid paraffin is formed on the bottom of the block which is now immersed in water to solidify. Afterwards it was removed, ready for sectioning. The section was cut thin to 5µm using a rotary microtome after excess paraffin wax was trimmed off. A slice of the section was taken and one side of the glass slide was made sticky by rubbing albumin of egg. The section was put in the centre of the slide so that the section starts floating. The section was immersed in water bath, keeping temperature between 50-55ºC so that sections become straightened and wrinkles disappear. Water was drained off and the slide is put in an incubator so that the section is completely fixed on the slide and becomes dry.

Haematoxylin and eosin staining technique: Procedure for H&E stain as described by Drury and Wallington [12] was adopted. The sections were dewaxed in two changes of xylene for 2minutes each, rehydrated in descending grades of alcohol, 100%, 95%, 90%, 70%, 50% ethanol for 2 minutes each, rinsed in distilled water, stained in haematoxylin for 10-15 minutes, washed well in running tap water for 2-3 minutes and examined microscopically to confirm sufficient degree of staining. Tissue was differentiated in 1% HCL acid for a few seconds to remove excess stain. Sections were then washed in running tap water for 15 minutes to regain the blue color and then stained in 1% aqueous eosin for about 5 minutes. Surplus stain was washed off in running tap water and mounted in distrene plasticizer xylene (DPX) using clean glass cover slide. Tissues were then focused under Leica research light microscope and photomicrographs taken from each group and labeled using Microsoft power point.

2.3 Statistical Analysis

Data obtained were analyzed using Microsoft excel and the means were compared using one-way ANOVA and expressed as Mean ± SD of 5

rats in each group. Differences between means were regarded significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Preliminary Results

The result of our preliminary studies shows that 90% of our researchers use rat models for their studies. The other 7.5% and 2.5% use mice and rabbit respectively. This informed our choice of using rats for this study. We also found that 16.25% of our respondents sacrifice their animals by cervical dislocation, 50% by chloroform inhalation, 6.25% by diethyl ether inhalation while 15% reported to use formalin inhalation. Over 55% of the respondents have been in active research for upwards of 6years and have used these methods over time. Majority of the respondents affirmed that their choice of method of sacrifice was based on availability of chemical in the laboratory where they conducted their animal sacrifice.

3.1.1 Physical observation

Physical observation was made following rat sacrifice with reference to humaneness of procedure, time of knockout, blood yield and cost of chemicals.

Our assessment of humaneness was based on the impact of the process of sedation on rat behaviour, and especially the physical manifestation of the rat just before passing out. Based on these criteria, chloroform was the mildest agent as the rats quietly passed out. Sometimes it required a careful look to notice the rats were already knocked out. Cervical dislocation was harsh in our judgment. The rats screeched in pain at the point of dislocation. The inhumaneness is more pronounced if the procedure fails at the first attempt (which is reportedly a frequent occurrence with this method). Formalin quieted the rats and left them gasping for breathe without knocking them off even after 30 minutes. We considered the pain the rats were subjected to within this long exposure period without any accompanying anesthetization inhumane. Diethyl ether was considered the most inhumane in this study as the rats had to writhe and spin and stretch before they pass out. Upon handling after passing out we also observed that rat body was rigid and the furs stood. We considered these hard on the rats.

Blood was collected from each rat after sedation and these constituted our second criteria for assessment of suitability. Cervical dislocation gave the least quantity of blood by cardiac puncture. For some of the rats the blood for congealed at the point of collection. Rats sacrificed by formalin and diethyl ether inhalation gave moderate quantities of blood, higher than what was obtained from cervical dislocation. Chloroform sedation gave the highest quantity of blood as the animals bleed freely. Therefore, if the study in view requires blood collection after sacrifice and much blood is needed, the researcher may have to look towards chloroform inhalation. Note that in this research, the blood had to be collected post-sedation and not before. Assad et al., [13] reported that chloroform and diethyl ether inhalation gave more blood compared to ketamine injection and the process was a lot easier.

Table 1. Result of time lapse for rat knockout

Group	Method	Time taken (in Mins:secs
A	Cervical dislocation	00mins:04secs
B	Chloroform inhalation	00mins:35secs
C	Diethylether inhalation	02mins:98secs
D	Formalin inhalation	30mins:00secs

Table 2. Results of physical observation

Parameter	Cervical dislocation	Chloroform inhalation	Di-ethyl ether inhalation	Formalin inhalation
Humaneness	++	+	+++	++
Time of knockout	+	++	+++	++++
Blood yield	+	+++	++	++
Cost	-	+	++	+

One of the important factors in research is time economy. Due to the numerous procedures being carried out at the same time, researchers seek for methods of sacrifice that saves time while still giving good results. We find that cervical dislocation was the quickest as it required an average of 5 secs to sedate each rat. This was followed by chloroform inhalation which took an average of 35 seconds to sedate each rat. Diethyl ether required an average of 2 mins and 98 secs for each rat. Formalin did not sedate any rat even after 30 minutes of exposure and so is not a recommended method under normal circumstances.

It is therefore important for the scientist to put these several factors into consideration and make the best decisions in the choice of method of sacrifice. Cervical dislocation was more rapid but requires the application of some level of force and exertion on the rat. This threatens its stands as a humane method although it is for a short period. Also, we see that the animal may not die after the first attempt at cervical dislocation, thereby requiring a second induction. This is however the cheapest of all the methods used as no chemical was required. Africa as we know (Nigeria inclusive) is facing serious shortage of research funding such that the average researcher makes use of personal funds in carrying out research. Therefore, it is important to minimize cost and so there may be a natural inclination towards cheaper procedures with less concern on its appropriateness.

Diethyl ether sedation lasted longer than cervical dislocation and chloroform sedation and characteristically produces a spinning and constricting effect on the rats in the last minute of exposure. The spinning is immediately followed by death of the rat. When brought out from the inhalation chamber, the body of the rat is stiffened and the hairs and furs stand. Apart from these, diethyl ether is more expensive than all the other chemicals used in this study. It was about 20% more expensive than its equivalent quantity of chloroform and about 25% costlier than formalin.

3.1.2 Result of antioxidant studies

The result in Table 3 shows that there was no significant impact of the methods of sacrifice compared to the control group. The result obtained from the antioxidant studies shows that there was no statistically significant difference between MDA levels in the experimental groups compared to the control group. Increase in MDA levels indicates increase in lipid peroxidation and presence of oxidative stress. This was found absent for the results of this study [7][14].

The result also shows no statistically significant difference in SOD levels between the experimental groups and the control group. Under oxidative stress, SOD levels are depleted as they are involved in the body antioxidant defense mechanism. This result shows that there was no oxidative stress in the rats [14]. However, this result show statistically significant increase in CAT levels in Group D compared to group A. CAT levels was also increased in group C although the increase was not statistically significant.

Also, we observed that the GSH levels of the experimental animals were not significantly different from those of the control group except for group C where it was statistically increased. GSH is formed in the liver from three amino acids which are: glycine, glutathione and cysterie. Cysterie is the rate limiting step in GSH synthesis. The active form of GSH is a strong antioxidant that protects the brain cells against damage caused by free radicals and it also recycles vitamins (C and E) [15]. GSH depletion under continuous oxidative stress causes oxidation and damage of lipids, proteins and DNA by reactive oxygen [16]. Also, significant decrease in GSH levels in the prefrontal cortex of rats causes mild stress [17]. Stress is characterized by decreased antioxidant status, as evidence by lowered tyrosine, albumin,

Table 3. Result of antioxidant analysis

Parameter	A	B	C	D
MDA	0.70±0.01	0.68±0.02	0.63±0.11	0.65±0.02
SOD	0.22±0.07	0.23±0.05	0.26±0.08	0.22±0.02
CAT	0.85±0.42	0.32±0.08	1.39±0.39	2.22±0.86
GSH	0.48±0.13	0.42±0.08	0.78±0.11	0.61±0.20

zinc and vitamin E [18]. Exposure to stress induces marked disturbances in oxidative parameters and decreases in the activity of antioxidant enzyme [19]. Also GSH dependent enzyme is involved in detoxifying process and responsible for removing hydrogen peroxide and formation of oxidized glutathione [20].

Our results show that chloroform inhalation had no significant effect on the antioxidant parameters of rats in this research. MDA levels were not significantly altered between the experimental groups compared to the control group. This implies that the animals were not under oxidative stress. The same goes for the other parameters studied: SOD, CAT and GSH.

Assad et al., [13] however reported that Hemoglobin (Hb) and red blood cell (RBC) values were significant reduced in animals sacrificed by chloroform inhalation compared with control.

3.1.3 Result of histological studies

The results of our histological studies show that cervical dislocation and chloroform sedation had no adverse effect on the hippocampus of Wistar rats. However, the histological slides of the diethyl ether sedated group showed few necrotic pyramidal cells. The formalin group showed numerous necrotic pyramidal as well as stellate cells. The photomicrographs of the cerebellum shows that apart from cervical dislocation group

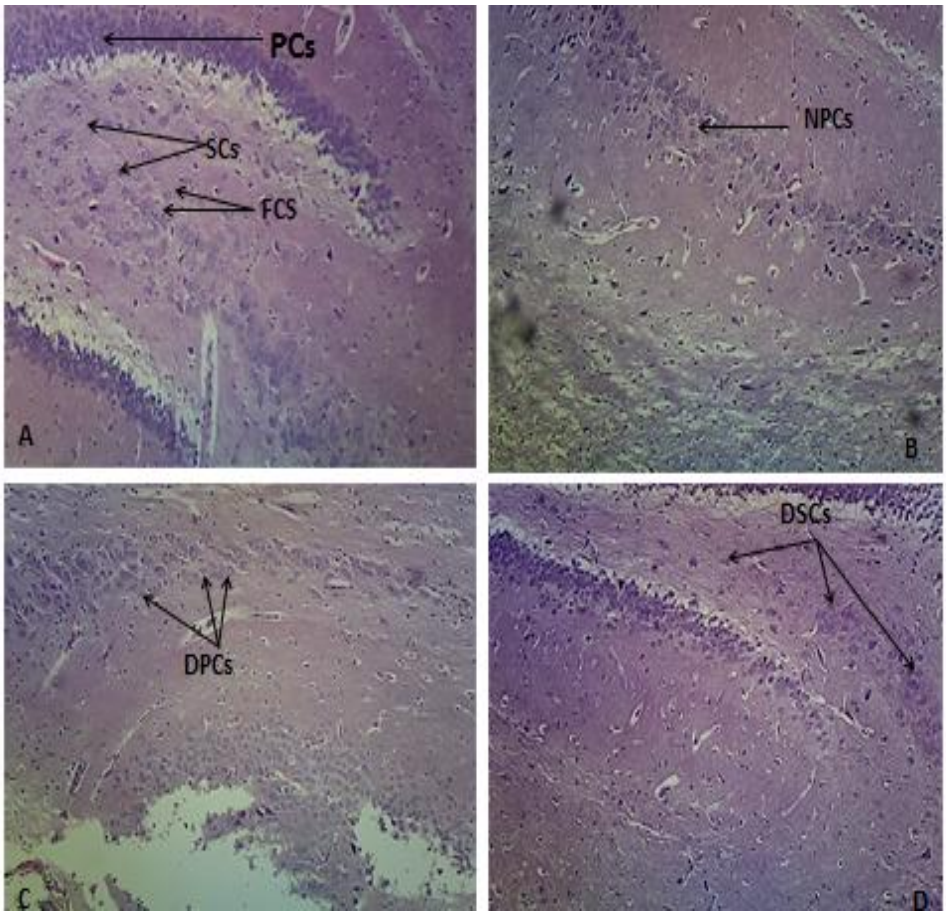

Plate 1. Representative photomicrograph of rat hippocampus from group A showing normal pyramidal (PCs) stellate (SCs) and fusiform (FCs) cells. B represents rat hippocampus from group B sacrificed by chloroform inhalation. It shows normal cellular outline and normal pyramidal cells. C represents micrograph of rat hippocampus in group C showing numerous dying pyramidal cells (DPCs). D shows several dying stellate (DSCs) and pyramidal cells (DPCs) X500

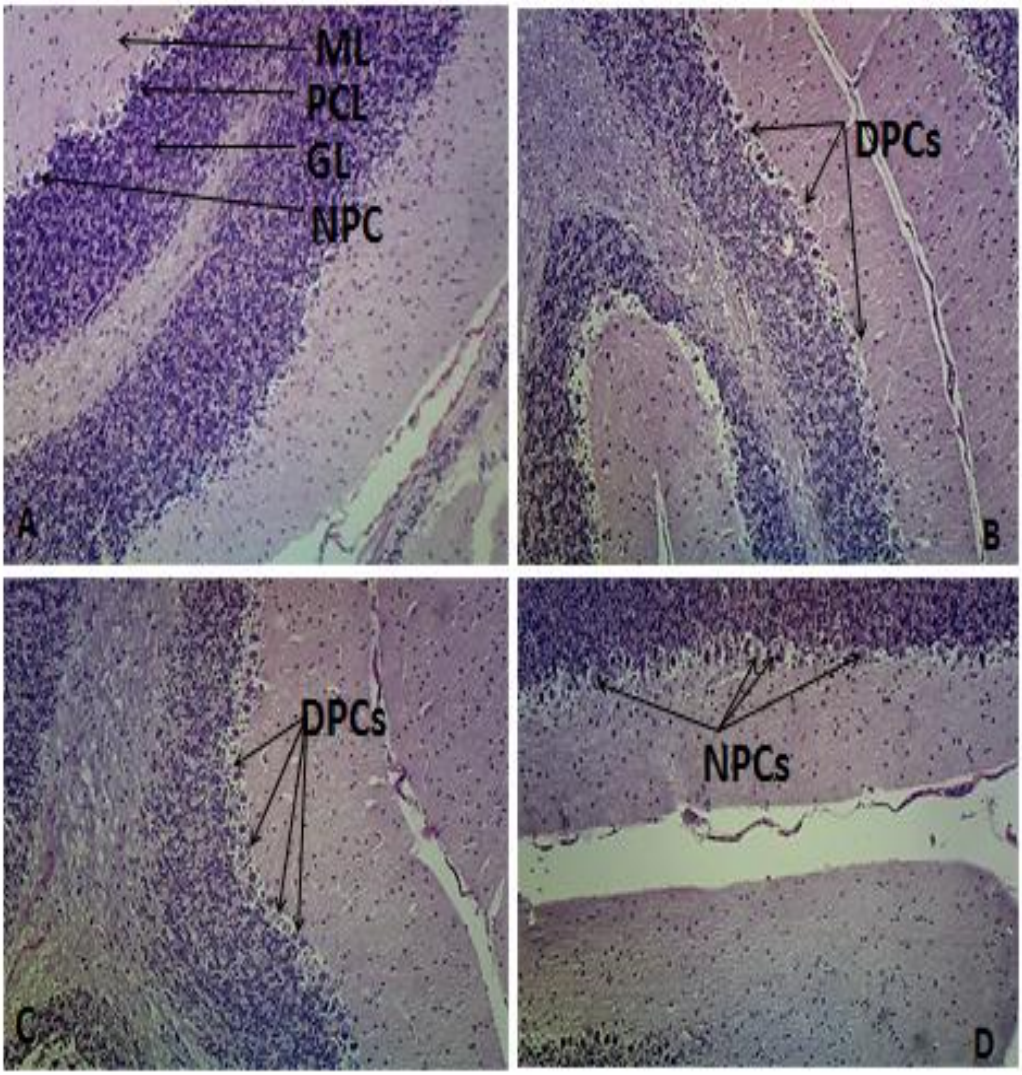

Plate 2. Representative photomicrographs of rat cerebellum. A (control) shows normal cerebellar cortex layers: molecular layer (ML), Purkinje cell layer (PCL) and granular layer (GL) as well as normal purkinje cells (NPCs). B Represents photomicrograph of rat cerebellum in group B showing some dead Purkinje cells (DPCs). C is representative photomicrograph of rat cerebellum in group C showing more dying Purkinje cells (DPCs). D is photomicrograph of rat cerebellum in group D showing numerous necrotic Purkinje cells (NPCs), several of which are pyknotic X500

(control), the rest of the groups B,C and D showed increasing degrees of purkinje cell necrosis. Our result is contrary to the report of Marceau [21] which portrays di ethyl ether as safe as an inhalant anesthetic for rodents. However, they agree that diethyl ether takes time to sedate animals. They considered the slow speed of sedation with diethyl ether an advantage in that there are lesser chances of organ damage. This advantage however was not evident in our study. Assad et al., [13] reported that ether had no adverse effect on the liver and kidney of rats. This also contradicts the report of Aguwa et al., [22]. His work claims di ethyl ether is safer due to its poor volatility and the relatively longer time it takes to anaesthetize rats. Kral [23] reported that the method of sacrifice employed affects lipid and carbohydrate metabolism in experimental rats. Mike Clark [24] also reported that method of sacrifice affects adenosine levels

in rat brain. Therefore, the method of sacrifice adopted in a research protocol must be considered based on the peculiarity of the research protocol and other prevailing circumstances peculiar to the research.

4. CONCLUSION

The result of our studies show that the four methods of sacrifice used for this study showed comparative advantage in different regards except formalin inhalation which showed no particular advantage over the other methods. Caution must however be applied when choosing methods with reported adverse effects on the organ studied.

CONSENT AND ETHICAL APPROVAL

As per international standard or university standard guideline participant consent and ethical approval has been collected and preserved by the authors.

Animal Ethic committee approval has been taken to carry out this study.

ACKNOWLEDGEMENT

The authors wish to appreciate the staff of the Anatomy, Physiology and Biochemistry labs of Nnamdi Azikiwe University, Nnewi.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rai J, Kuldeep K. Reduction of animal sacrifice in biomedical science & research through alternative design of animal experiments. Saudi Pharmaceutical Journal2. 2018;6(6):896-902.
2. Cressey D. Best way to kill lab animals sought. Nature News. 2013;500(7461):130.
Pubmed| Crossref| Others
3. American Veterinary Medical Association Guidelines for euthanasia of animals (AVMA) guidelines for the euthanasia of animal sedition; 2013.
Pubmed| Crossref| Others
4. Carbone L, Carbone ET, Yi EM, et al. Assessing cervical dislocation as a humane euthanasia method in mice. J American Association Laboratory Animal. Sci. 2012;51(3):352-356. Pubmed| Crossref| Others
5. Keijzer C, de Lange JJ, Roberto SG. General introduction: Interactions of inhalational anesthetics and carbon dioxide absorbents. Interactions of inhalational anesthetics and carbon dioxide absorbents. 2007;9.
ISBN/EAN: 978-90-9022049-9
6. Ryder OA, McLaren A, Sydney B, Ya-Ping Z, Kurt B. DNA banks for endangered animal species. Science. 2000;288(5464):275-277.
DOI:10.1126/science.288.5464.275
7. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. Journal of biological chemistry. 1949;177(2):751-766.
Available:http://www.academia.edu/download/7522360/gornall_etal.pdf
8. Varshney R, Kale RK. Effects of calmodulin antagonists on radiation-induced lipid peroxidation in microsomes. International journal of radiation biology. 1990;58(5):733-743.
Available:https://doi.org/10.1080/09553009014552121
9. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biological chemistry. 1972;247(10):3170-3175. The Journal of Biological Chemistry. 1972;247:3170-3175.
10. Sinha KA. Colorimetric assay of catalase. Anal. Biochem. 1971;47:389-394.
11. Beutler E. Improved method for the determination of blood glutathione. J. lab. clin. Med. 1963;61:882-888. J. Lab. Clin. Med. 1963;61(1963):882-888.
NII Article ID (NAID)10005420816.
12. Drury RAB, Wallington EA. Preparation and fixation of tissues. Carleton's histological technique. 1980;5:41-54.
13. Assad HE, Khudhair NA, Haider RA. Effect of different methods of anesthesia on physiobiochemical parameters in laboratory male rats. Bas.J.Vet.Res. 2020;19(1).
14. Aguwa US, Anibeze CP, Ndukwe GU, Olu SI, Ovie FO, Ukoba O. Comparing The Neuroprotective And Antioxidant Effect Of The Aqueous And Ethanolic Leaf Extracts of Vernonia Amygdalina On The Cerebellum Of Mercury Chloride

Intoxicated Adult Male Wistar Rats. 2018;7(9):2277 – 8160. IF : 5.156 | IC Value : 85.78

15. Alkadi H. A review on free radicals and antioxidants. Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders) 2020;20(1):16-26. DOI: https://doi.org/10.2174/1871526518666180628124323
16. Parveen S, Muhammad H, Euphemia L, Kelvin KH, Annie Y, Jonathan A, Gayan HD et al. Anticancer organorhodium and-iridium complexes with low toxicity in vivo but high potency *In Vitro*: DNA damage, reactive oxygen species formation, and haemolytic activity. Chemical Communications. 2019;55(80):12016-12019. Available:https://doi.org/10.1039/C9CC03822A
17. Izumi H, Keita S, Kazuhiro K, Takashi S, Takaomi CS, Kohji F. Oral glutathione administration inhibits the oxidative stress and the inflammatory responses in+ AppNL− GF/NL− GF knock-in mice. Neuropharmacology. (2020); 108026. Available:https://doi.org/10.1016/j.neuropharm.2020.108026
18. Khairnar SI, Umesh BM, Kalpesh RP, Harun MP, Sachin DS, Sameer NG, Sateesh B, Shreesh Ojha, Chandragouda R. Patil. Disulfiram and its copper chelate attenuate cisplatin-induced acute nephrotoxicity in rats via reduction of oxidative stress and inflammation. Biological trace element research. 2020;193(1):174-184. DOI: 10.1113/jphysiol.2004.080564
19. Pastore A, Giorgio FE, Fiorella P. Analysis of glutathione: implication in redox and detoxification. Clinica chimica acta. 2003;333(1):19-39. Available:https://doi.org/10.1016/S0009-8981(03)00200-6
20. Reitz RH, Fox TR, Quast JF. Mechanistic considerations for carcinogenic risk estimation: chloroform. Environmental Health Perspectives. 1982;46:163-168.
21. Marceau JF. How the Animal Welfare Act Harms Animals (May 9, 2018). 69 Hastings Law Journal 925 (2018), U Denver Legal Studies Research Paper No. 18-15. Available:https://ssrn.com/abstract=3176101
22. Aguwa US, Okeke SN, Ezejindu DN, Eze CE, Azurunwa O, Obinwa BN, Ovie FO et al. Evaluating the Effect of Chloroform Inhalation as a Method of Euthanasia on the Cerebellum and Hippocampus of Adult Wistar Rats. Journal of Advances in Medical and Pharmaceutical Sciences. 2020;14-25. Available:https://doi.org/10.9734/jamps/2020/v22i630176
23. Kral JG. The effects of different methods of sacrifice on lipid and carbohydrate metabolism in the rat. Hormone and Metabolic Research. 1974;6(06):483-487. Clark M, Saeed Dar M. The effects of various methods of sacrifice and of ethanol on adenosine levels in selected areas of rat brain. Journal of Neuroscience Methods. 1988;25(3):243-249. Available:https://doi.org/10.1016/0165-0270(88)90139-2
24. Clark M, Saeed Dar M. The effects of various methods of sacrifice and of ethanol on adenosine levels in selected areas of rat brain. Journal of Neuroscience Methods. 1988;25(3): 243-249. Available:https://doi.org/10.1016/0165-0270(88)90139-2

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/60733