Assessment of Formaldehyde-Induced Arthritis (FIA) using Modified Rheumatoid Arthritis Disease Activity Index (RADAI) on Adult Male Wistar Rats

ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune inflammatory and degenerative disease affecting the joints and joint cartilage, with extra-articular manifestations. The chronicity of RA causes irreversible damage to the skeletal system and with a poorly understood pathogenesis, current management modalities are grossly limited. Pre-clinical studies on RA in Africa are plagued with scarcity and inaccessibility of research grants and funding, and this has negatively impacted interest in research. Also, inducting agents that mimic the true pathogenesis of diseases such as RA are limited. Therefore, there is a need to find readily available alternatives to promote research studies on RA in our environment. This study aimed to evaluate the rheumatoid arthritis disease activity index (RADAI) of formaldehyde induction (FIA) in comparison to collagen induction (CIA) of arthritis using twenty adult male Wistar rats which were grouped into 5 (A-E) (n=4). Group A, the control group had feed and water only. Groups B, C, and D had two doses of 0.1% formaldehyde (50µl, 100µl, and 200µl, respectively). Group E had 200µl of bovine CII-CFA emulsion (0.2mg/ml). Booster dosage was performed 9 days later by injecting 100µl of bovine CII-CFA emulsion (0.1mg/ml). Body weights, ankle measurements, and blood samples were taken on days 1, 16, and 48, before sacrificing the animals by cervical dislocation. Both ankles were rapidly dissected and processed for histological assessment using Modified Mankin System. We recorded a 100% mortality rate on high-dose formaldehyde administration (Group D), but no mortalities were seen in other groups. In low and moderate dosages of formaldehyde, RA induction was achieved and when compared to Collagen Induced Arthritis (CIA), there was no significant difference noted. We postulate that Formaldehyde Induced Arthritis (FIA) models can serve as a substitute for CIA mimicking the true pathogenesis seen in rheumatoid arthritis.

Keywords: *Rheumatoid arthritis, Bovine-Collagen II and Complete Freund's adjuvant emulsion (Bovine CII-CFA), Formaldehyde, Arthritis induction.*

1. INTRODUCTION

Rheumatoid arthritis (RA) is one of the commonest diseases whose pathology has been erroneously attributed to aging. It is an autoimmune disease that leads to chronic inflammation of joints and their surrounding tissues. RA is a degenerative and crippling

disease affecting the skeletal system. It is associated with progressive disabilities, socio-economic challenges, and in severe cases; early death [1-3].

RA usually causes polyarthritic joint pains and damage throughout the human body in a symmetrical pattern [4]. It is amongst the frequent joint diseases which is not rigidly ascribed to exhibit only joint inflammation but is characterized by extensive extra-articular manifestations which complicate its therapeutic management [5]. The aetiopathogenesis of RA remains fully unknown, and as such, the prognosis is watched over [1]. Many hospital-based studies in Nigeria have demonstrated that RA is quite a common disease with increased morbidity. [4, 6].

The primary degeneration of cartilages in rheumatoid arthritis is associated with secondary changes which include; the infiltration of synovium and its compartment by leukocytes which migrate from distant sites in response to chemokines and adhesion molecules rather than local proliferation that involves the synovia and the subchondral bone metabolism gives rise to the inflammation of the synovium (synovitis) in the involved joints [7]. Synovitis may also be defined as the result of responses to the hypoxic interior of an inflamed synovium owing to the presumable proliferation of synovial cells and the drastic reduction in capillary flow rate in the core cells of the synovium [8]. It enables cell migration through stimulation of angiogenesis leading to factors that may stimulate vessel formation and endothelial activation thus snowballing the face of adhesion molecules such as selectins, integrins, immunoglobulins, and chemokines. As this progresses, synovial inflammatory tissues accumulate thus resulting in the advancement of progression in rheumatoid arthritis [8, 9]. In the normal synovial milieu, mesenchymal-derived fibroblast-like synoviocytes (FLSs) and resident macrophages are in abundance. However, in rheumatoid arthritis, there exists expansion of the “synovial membrane” and the FLSs undertake a semi-autonomous phenotype pigeon-holed by loss of contact inhibition, anchorage independence, high degrees of disease-relevant chemokines and cytokines expression, matrix metalloproteinases (MMPs), tissue-inhibitors of metalloproteinases (TIMPs), and adhesion molecules [10]. Fibroblast-like synoviocytes thus encourage unswerving chronicity of local synovitis and cartilage damage, while endorsing an accommodating micro-environment that enables T cell and B cell survival, and “adaptive immune pathways” [11]. Synovial hyperplasia is the chief contributor to the destruction of cartilage in rheumatoid arthritis. It promotes FLS invasion and adhesion through the loss of the normal shielding effects of synovium which brings about a “reduction in the expression of lubricin” to alter the protein binding physiognomies of the surface of cartilages [12, 13]. This process alters the metabolism, affecting water retention, and glycosaminoglycan content, thus leading to biomechanical dysfunction and destruction of the surface cartilages and joint space tapering radiographic appearance [1, 14].

The clinical presentation associated with RA includes one or more symmetrical (on both sides of the body) joint swelling and tenderness, and morning stiffness for > 30mins, which progressively worsens. It is also characterized by fever, fatigue, formation of rheumatoid nodules under the skin, generalized body weakness, and weight loss. Physicians have established a complementary tool to assess rheumatoid arthritis disease activity by examining clinical measures (physical characteristics) and laboratory measures (blood parameters) of affected individuals [15]. The contents of this tool; the Rheumatoid Arthritis Disease Activity Index (RADAI) includes a five-item questionnaire that includes; global disease activity, current swollen and tender joints, arthritis pain, morning stiffness duration, and rating joint tenderness from 0-10 [15]. However, pre-clinical assessment of disease activity requires a modification of RADAI to consist of only examinable parameters which include; current swollen and tender joints, morning stiffness duration, and blood parameters (Rheumatoid factor – RF, Erythrocyte Sedimentation Rate - ESR, C-reactive protein - CRP, anti-cyclic citrullinated peptide antibody - ACCPA). Therefore, to study RA pre-clinically, establishing a humanized model of arthritis and its evaluation using modified RADAI is very invaluable [15].

Current RA laboratory-inducing agents include; Bovine-Collagen II, Freund's adjuvant (Complete and Incomplete), Zymosan, *Streptococcus pyogenes* cell wall, Pristane, Cartilage Oligomeric matrix protein (COMP), Antigens, Proteoglycans, and G-6-P isomerase. The Collagen-Induced Arthritis (CIA) model is quite established as the most widely used RA inducer pre-clinically [16, 17]. Its mechanism of action is the destruction of collagen tolerance and auto-antibody production in collagen-induced models [16]. Currently, it is emulsified as bovine collagen II (CII) with Complete Freund's adjuvant (CFA) and injected into the base of the tail/intra-peritoneum of the animal models. It is also termed the best model of RA [16, 18]. However, some researchers have indicated that there is a possibility of the use of 10% formaldehyde to induce rheumatoid arthritis [19, 20]. The bovine Collagen-II arthritis index is remarkable [16 -20], thus comparing its result to formaldehyde induction would give a clear demonstration of both bias and precision.

This study sought an alternative in 10% formaldehyde to the current RA inducers using bovine Collagen-II and Complete Freund's Adjuvant (bovine CII-CFA) emulsion as the gold standard/example. The various characteristics and properties of 10% formaldehyde such as make it a standout candidate to be evaluated for use as a substitute to bovine CII-CFA in the in vivo pre-clinical induction of rheumatoid arthritis

The modified RADAI employed compared the anterior-posterior and the lateral diameters of model limbs, the erythrocyte sedimentation rates (ESR), the anti-cyclic citrullinated peptide antibody assay (ACCPA), the weight of the animals, and mean

histology scores of limbs using the modified Mankin scoring system of FIA to those of CIA.

2. MATERIALS AND METHODS

2.1 Experimental Animals and Design

A total of 20 adult male Wistar rats weighing 180-250g were procured from and housed in cross-ventilated cages, in the animal house of the Department of Anatomy, University of Nigeria, Enugu Campus, Enugu-Nigeria. The animals were afforded 12 hours of dark/light cycle and had access to commercial growers' mash (Guinea Feed Nigeria Limited) and tap water ad libitum. All animals were fed and acclimatized for three weeks. The 20 rats were randomly grouped into five (A-E) of 4 rats each as displayed in Table 1.

Table 1. Experimental Animals and induction of models

	Description	Rats	Induction
A	Normal Control	4	Feed and Water only
B	Low dose FOM	4	two doses of 50µl of 0.1% formaldehyde (0.05mg/ml)
C	Medium dose FOM	4	two doses of 100µl of 0.1% formaldehyde (0.1mg/ml)
D	High dose FOM	4	two doses of 200µl of 0.1% formaldehyde (0.2mg/ml)
E	Bovine CII-CFA emulsion	4	200µl of bovine CII-CFA emulsion (0.2mg/ml). Booster dosage was performed 9 days later by injecting 100µl of bovine CII-CFA emulsion (0.1mg/ml)

2.2 Induction and evaluation of Rheumatoid arthritis

The rats were anesthetized using 5% isoflurane in an induction-bell jar before induction. The route of administration was parenteral by injecting an inducer into the base of the tail using a 27F-gauge needle. Groups B, C, and D received the two doses at an interval of 9 days. Anthropometric data of the animals were recorded on days 1, 16 (1 week after booster/second dose was given), and 48, using a vernier caliper to measure the anterior-posterior diameters (between the posterior strip of elbows and wrists, and ankles and heels) and lateral diameters of both ankles of all animal limbs, and a weighing balance for weight measurement. On day 48, peripheral blood samples from 2 rats chosen at random from each group were investigated for Erythrocyte Sedimentation rate (ESR), and the remaining 2 rats from each group were investigated for Anti-Cyclic Citrullinated Peptide Antibody (ACCPA). At the end of day 48, all rats were sacrificed by cervical dislocation and both ankles were rapidly dissected and processed for histological study.

2.3 Histological Study

On cervical dislocation of the animals, the ankles were carefully dissected by amputating limbs about 10mm away from the joint capsules with the surrounding ligaments intact. This tissue was fixed in 10% buffered formalin and post-fixed in Zenker's fluid for 18 hours. Further tissue processing for paraffin sectioning at 5µ thickness and routine H & E staining were done. The slides were analyzed and scored using the Modified Mankin scoring system.

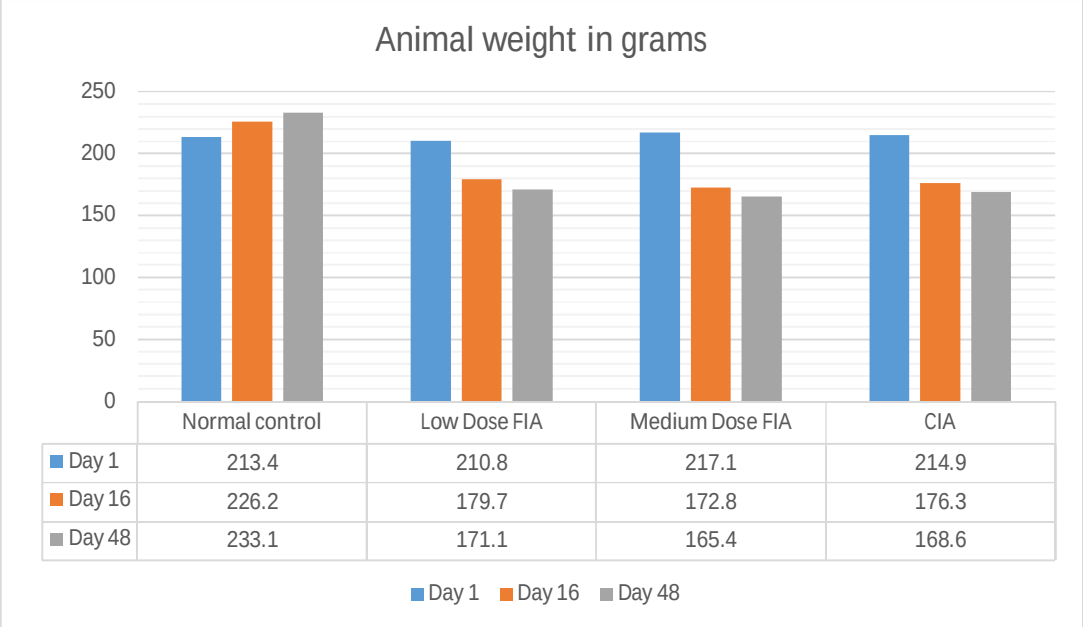
2.4 Statistical Analysis

Results of anthropometric measures, hematological and microscopic analysis were analyzed using SPSS (version 26), expressed as Mean ± Standard Deviation (SD). Statistical differences in mean between groups were analyzed using one way ANOVA (Analysis of variance). P-value less than 0.05 were considered as statistically significant.

3. RESULTS

3.1 Physical attributes and Hematological analysis

In this study, 12 rats were induced using formaldehyde, while 4 were induced using bovine-collagen. 4 rats were not induced, and 20% of the rats ended up dead. Changes observed in physical appearance were spared in the normal control group, but significant in all other groups. This included weight loss and ankle joint swellings. Seventy-five percent (n=12) of induced rats were ACCPA positive and had an ESR exceeding 25mm/hour, as shown in Figure 2. Thus, they had seropositive rheumatoid arthritis.



	Normal control	Low Dose FIA	Medium Dose FIA	CIA
Day 1	213.4	210.8	217.1	214.9
Day 16	226.2	179.7	172.8	176.3
Day 48	233.1	171.1	165.4	168.6

Figure 1: Graphical representation of all groups showing changes observed in the body weights from day 1 to 48. P-value ≤ 0.05 (statistically significant in the weight of experimental animals on days 16 and 48 within the low dose FIA, medium dose FIA and CIA groups compared to that of the normal control group). Values were expressed as Mean ± SD.

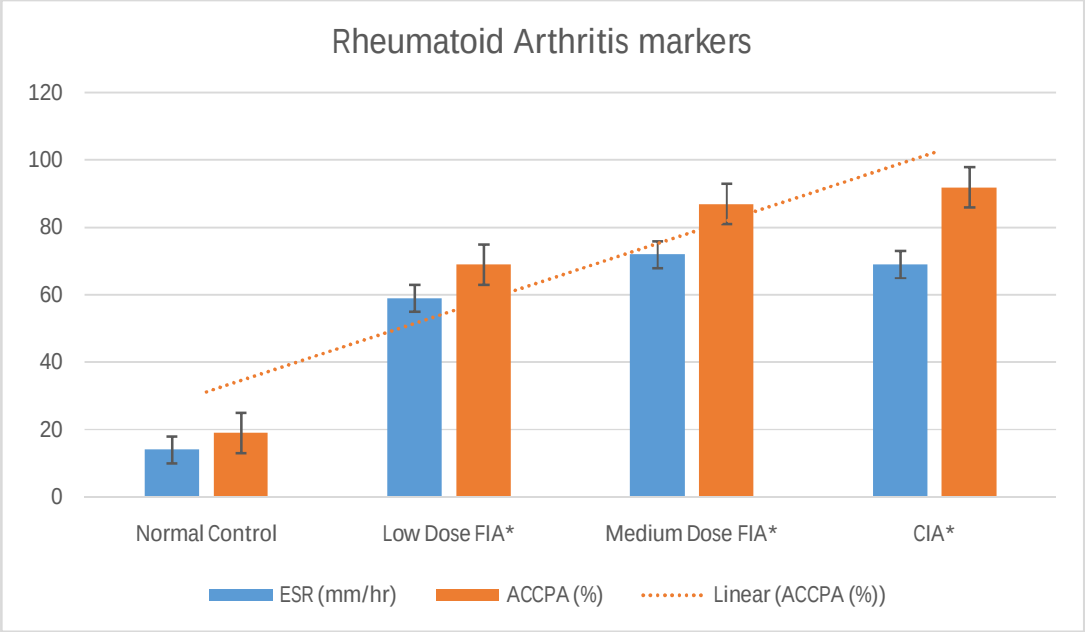


Figure 2: Graphical representation of all groups showing the Erythrocyte Sedimentation Rate (ESR) and anti-cyclic citrullinated peptide antibody (ACCPA). *P-value ≤ 0.05 (statistically significant compared with normal control). Values were expressed as Mean ± SD.

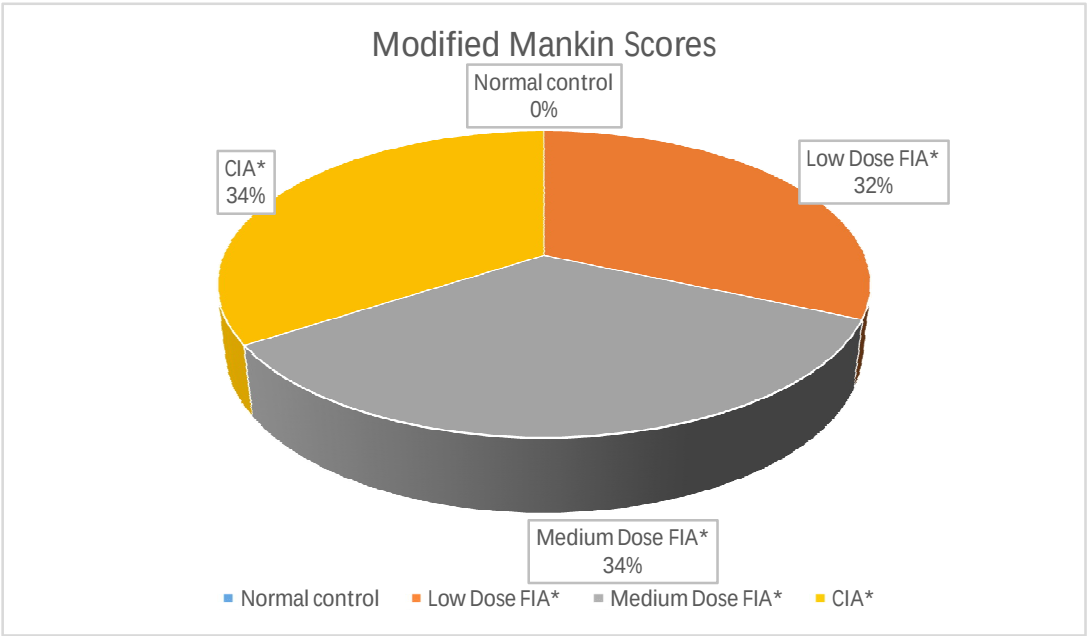


Figure 3: Graphical representation of all groups showing light microscopic analysis using the Modified Mankin Scoring System. *P-value ≤ 0.05 (statistically significant compared with normal control).

3.2 Light Microscopic Analysis with Modified Mankin Scoring System

The normal control group had a Mankin score of zero, which demonstrated nil remarkable features under the microscope. However, the distribution of the Mankin scores in groups B, C, and E demonstrated features suggestive of massive cartilage erosion that extended into calcified cartilages with intensely enhanced periphery staining of sparsely distributed chondrocytes and a poor background staining intensity.

Table 2. Anthropometric records of the limbs of the rats on day 1, 16 and 48.

Ankles	Group	n	Anterior-posterior diameter (mm)			Lateral diameter (mm)		
			Day 1	Day 16	Day 48	Day 1	Day 16	Day 48
Right Ankles	A (Normal control)	4	7.71 ± 0.28	8.21 ± 0.43	8.14 ± 0.27	7.52 ± 0.24	7.82 ± 0.11	7.72 ± 0.38
	B (Low dose FIA)*	4	7.42 ± 0.32	11.53 ± 0.38	14.41 ± 0.35	7.69 ± 0.36	12.11 ± 0.39	14.78 ± 0.48
	C (Medium dose FIA)*	4	6.94 ± 0.21	12.60 ± 0.29	15.10 ± 0.39	7.14 ± 0.38	11.98 ± 0.56	15.10 ± 0.52
	D (High dose FIA)	4	7.32 ± 0.25			7.40 ± 0.73		
	E (CIA)*	4	7.80 ± 0.36	12.01 ± 0.42	14.21 ± 0.28	7.20 ± 0.49	12.27 ± 0.43	14.94 ± 0.19
Left Ankles	A (Normal control)	4	7.31 ± 0.32	7.65 ± 0.36	7.92 ± 0.26	7.39 ± 0.51	7.67 ± 0.62	7.77 ± 0.37
	B (Low dose FIA)*	4	7.89 ± 0.22	12.22 ± 0.41	14.33 ± 0.31	7.47 ± 0.46	11.82 ± 0.48	14.21 ± 0.45
	C (Medium dose FIA)*	4	7.01 ± 0.29	12.31 ± 0.34	15.21 ± 0.20	7.69 ± 0.23	12.72 ± 0.33	15.12 ± 0.51
	D (High dose FIA)	4	7.54 ± 0.25			7.38 ± 0.38		
	E (CIA)*	4	7.22 ± 0.34	11.92 ± 0.27	14.78 ± 0.22	7.64 ± 0.50	12.34 ± 0.29	15.20 ± 0.38

The values are expressed as mean ± SD.

*P ≤ 0.05 statistically significant compared to normal control on days 16 and 48.

Red shows the group with 100% mortality after induction.

4. DISCUSSION

The observed weight loss noted in groups B, C, and E is an important indication of an ongoing chronic pathology suggestive of rheumatoid arthritis. This has been reported in some studies as an attribute of the presence of pro-inflammatory cytokines which may include TNF-α and IL-1β, which are detrimental to the joint tissues in RA, to also exert significant influence on body protein and energy metabolism [21]. In rheumatoid arthritis, there is an increase in the rate of whole-body protein breakdown due to the production and circulation of TNF-α by peripheral blood mononuclear cells [22]. Therefore the observed weight loss is suggestive of successful rheumatoid arthritis induction in the formaldehyde groups. The four biomarkers included by the American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) in the criteria for diagnosing RA are; rheumatoid factor, C-reactive protein (CRP), ESR, and ACCPA [23]. Any of the four is sufficient in making new or confirming a diagnosis of rheumatoid arthritis. However, in this study, we made use of both ESR and ACCPA. Elevated ESR may be associated with a systemic or local inflammatory process or tissue injury [23]. Therefore, the inclusion of ACCPA which is more specific for the confirmation of RA was used in this study. In ACCPA, citrullination is a post-translational

modification of proteins that can generate new epitopes that are foreign to the immune system, thus leading to the creation of new autoantibodies against them [24]. This is highly specific to rheumatoid arthritis [23, 24], and again it was well demonstrated in groups B and C, with no significant difference portrayed when compared to the CIA-induced group. In this study, the changes observed in FIA and CIA groups were similar except for those in group D (High dose). The death of all animals in group D could be attributed to the lethal dose of formaldehyde. However, in low and moderate doses, induction of rheumatoid arthritis was achieved and all measured parameters showed no significant differences.

The analyzing system proposed by H. J. Mankin [25] demonstrated the microscopic features of an arthritic joint [26]. There was no difference noted yet again between the average Mankin scores of the low and medium-dose FIA groups when compared to the CIA group. A score of 13/15 and 12/15 seen in both CIA and medium dose FIA, and low dose FIA respectively signifies severe damage to joint tissues, clearly indicating rheumatoid arthritis induction. This is a positive and strong indication of the potential noted throughout this study for the use of formaldehyde in the induction of rheumatoid arthritis to serve as a clear substitute for bovine CII-CFA emulsion.

The anthropometric records of the anterior-posterior diameters and lateral diameters of the ankles of the rats resound the parameters reported earlier. Group A which served as the control showed no changes, as the joint diameters were fairly constant throughout the study. However, groups B, C, and E showed remarkable changes consistent with joint swellings notable in rheumatoid arthritis. Joint swelling and possible inflammation commenced after induction of rheumatoid arthritis as reported on day 16 measurements and further worsened as evidenced by the increase in the anterior-posterior and lateral diameters of the ankles on day 48.

Analyzing the RADAI for Formaldehyde induced rheumatoid arthritis using CIA as a baseline, it is clear that FIA is reproducible in provoking joint inflammation and tissue destruction evidenced by increased levels of ESR and ACCPA, increasing the anterior-posterior and lateral diameters of joints, inducing weight loss, and positive count in the Modified Mankin Scoring System.

5. CONCLUSION

The study provides a detailed description of the phenotype of a new RA-inducing agent in formaldehyde. This is a readily and easily accessible agent that can serve as a substitute in place of Bovine-Collagen II.

We believe that this substitute would be invaluable in advancing our approach to understanding and studying rheumatoid arthritis via humanized arthritis model as it has been demonstrated to mimic the pathogenesis of rheumatoid arthritis. It also serves as a readily available agent in low-income research economies. Finally, future studies

would be encouraged into understanding the mechanism of action of formaldehyde on joint cartilage as regards their effect on auto-reactive T-cell induction which secrete inflammatory cytokines.

Ethical Approval

Ethical approval was obtained from the Faculty of Basic Medical Science research ethics committee, Enugu State University of Science and Technology College of Medicine (ESUCOM), with the Ethical Right Permission Number: (ESUCOM/FBMS/ETR/2021/007) and the research was conducted according to the guidelines for the care and use of laboratory animals of ESUCOM.

CONFLICT OF INTERESTS

The authors declared no conflict of interest.

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