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Research Article

Cefixime; a Cephalosporin Antibiotic, Triggers Mitochondrial-mediated Cell Death via Mitochondrial Permeability Transition Pore Opening in Male Wistar Rats

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ABSTRACT

Inducers or inhibitors of opening of the Mitochondrial Permeability Transition (mPT) Pore are targets of drug development for conditions arising from dysregulated apoptosis. Some antibiotics have been shown to effect anticancer property by induction of mitochondrial-mediated cell death via mPT pore opening. This study therefore investigated the effect of cefixime; a cephalosporin antibiotic on mitochondrial-mediated cell death via mPT pore using rat model. Thirty male Wistar strain rats were randomly assigned into five equal groups; group I is the control while groups II, III, IV and V were orally treated with cefixime (10, 20, 30 and 40mg/kg) daily for two weeks. Mitochondria were isolated by differential centrifugation. The opening of the pore, cytochrome c release, mitochondrial ATPase (mATPase) activity, mitochondrial lipid peroxidation (mLPO), caspases 3 and 9 activities and hepatic DNA fragmentation were determined. Varying concentrations of cefixime caused induction of mPT pore opening, cytochrome c release and mATPase activity in a concentration-dependent manner. Similarly, oral administration of cefixime caused dose-dependent induction of mPT pore opening, mATPase activity, mitochondrial lipid peroxidation (mLPO) and caspases 3 and 9 activation and hepatic DNA fragmentation. These results suggest that cefixime; a cephalosporin antibiotic, triggers mitochondrial-mediated cell death via mitochondrial permeability transition pore opening in male Wistar rats. Its anticancer potential should further be explored.

Keywords: *Cefixime, mitochondria, apoptosis, cancer*

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INTRODUCTION

Mitochondrion is a highly dynamic cellular organelle involved in the generation of ATP. Apart from its involvement in bioenergetics, mitochondria also mediate cellular homeostasis, cell proliferation and cell death. Several studies have explored the role of mitochondria in cell death (Newmeyer *et al*., 1994).

Various stimuli such as hypoxia, excess ROS, DNA damage, disruption of the mitochondrial membrane potential homeostasis, increased permeability and release of mitochondrial proapoptotic factors into the cytoplasm could activate the mitochondrial apoptosis pathway and ultimately cell death (Köhler *et al*., 1999; Kim *et al*., 2008). The release of proapoptotic factors; such as Cytochrome c, AIF, and APAF-1 occurs when the mitochondria permeability transition (mPT) pore opens (Dejean *et al*., 2006). The release of Cytochrome c into the cytosol triggers caspase activation and ultimately, apoptosis (Garrido *et al*., 2006; Martins, 2006; Bonora *et al*., 2015). When the mPT pore opens, mitochondrial membrane permeability increases, mitochondria swell, and the outer mitochondrial membrane ruptures. This eventually leads to impaired cell bioenergetics and cell death. (Salvador-Gallego *et al*., 2016). The mPT pore now serves as a promising pharmacological target for the development of drugs relevant in diseased conditions involving dysregulated mitochondrial-mediated cell death (Giampaolo *et al*., 2021). Several compounds have been reported to evoke their chemotherapeutic effect against tumor and cancer by induction of mPT pore opening (Lisa *et al*., 2014; Massimo *et al*., 2020; Olowofolahan *et al*., 2020;

Olowofolahan *et al*., 2021). Some antibiotics have been shown to elicit anticancer property via disruption of mitochondrial membrane potential and Bax/Bcl-2-dependent pathway (Yuan *et al*., 2020). Cefixime is a third-generation cephalosporin antibiotic useful for the treatment of a number of bacterial infections (Panda *et al* 2014). This study intended to investigate the effect of cefixime on mitochondrial-mediated cell death via mPT pore.

MATERIALS AND METHODS

Chemicals and reagents: Mannitol, sucrose, ATP, N-2-hydroxyethyl-pipe-arizine-N-2-ethanesulfonic acid (HEPES), rotenone, spermine, Folin-Ciocalteu reagent, Bovine Serum Albumin (BSA), and all other reagents were of highest purity grade and were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Cefixime was purchased from a reputable Pharmacy in Ibadan, Oyo State, Nigeria and its certainty was verified using the NAFDAC short code system. It was packaged in a 10-tablet sachet containing 400mg each.

Animals: The present experimental study used forty Wistar strain rats that weighed 100 ± 5 g. Animals were obtained from the Preclinical Animal House, University of Ibadan, maintained in special cages under standard conditions of temperature and humidity (a 12-hour dark/light cycle), had free access to standard chow and water and allowed to acclimatize for two weeks. In order to conduct a comparative evaluation, we divided the rats into five groups of 6 animals each. The remaining ten rats were used for the in vitro study. The experiment was conducted following the ethical standards (1964 Declaration of Helsinki).

Experimental design:
Thirty of the male Wistar rats were randomly assigned into 5 equal groups and orally administered for 14 days as follows;
Group 1 (control) received distilled water (1 ml/kg) while groups 2, 3,4 and 5 received CEF at doses of 10 mg/kg, 20 mg/kg, 30 mg/kg and 40 mg/kg respectively. The chosen doses were based on pilot study and literature search (Paget and Barnes, 1969). At the end of the experimental period, the animals were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg) and euthanized by cervical dislocation. It was carried out according to the guidelines of American Veterinary Medical Association (AVMA) for the euthanasia of animals (2020-Edition). Assays were carried out and histological assessment of the liver sections was performed following standard procedure. The remaining animals were used for the in vitro study.

Isolation of rat liver mitochondria: The isolation was carried out as described by Johnson and Lardy (1967), with little modification by Olorunsogo *et al*. (1979). The animals were sacrificed; liver was rapidly excised, trimmed, blotted with blotting paper and weighed. It was rinsed with isolation buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, pH 7.4 and 1 mM EGTA), and homogenized in a 10% w/v of ice-cold isolation buffer. The homogenate was centrifuged in an MSE refrigerated centrifuge (Progen Scientific, UK) at 2,300 rpm for five minutes to sediment nuclear fraction and cell debris. The supernatant obtained was centrifuged at 13,000 rpm for 10 minutes to obtain the mitochondrial pellet, which was washed twice, with washing buffer (210 mM Mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4, 0.5% BSA) at 12,000rpm for 10 minutes. The mitochondrial fraction was resuspended in suspension buffer (210 mM Mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4), dispensed into Eppendorf tubes as aliquots and stored at 4^{o}C. All experiments with isolated mitochondria were performed within 4 hours of the preparation.

Determination of mitochondrial protein: This was done as described by Lowry *et al*. (1951), using bovine serum albumin as standard.

Mitochondrial swelling assay: Mitochondrial Permeability Transition (mPT) was monitored by observing the changes in diffraction of light in the spectrophotometer through absorbance of mitochondria suspension at 540 nm in a T70 UV/Visible spectrophotometer (China) essentially according to the method of Lapidus and Sokolove (1993). Briefly, mitochondria (1 mg protein/ml) were pre-incubated in the presence of 0.8 µM rotenone (10 µl) in a medium containing 210 mM mannitol, 70mM sucrose and 5 mM HEPES-KOH (MSH) buffer (pH 7.4) (2,200 µl) for 31/2 minutes at 27°C prior to the addition of 5 mM succinate (50 µl). The mPT was spectrophotometrically monitored for 12 minutes at 30 seconds interval. The above experiment was repeated with mitochondria (1 mg protein/ml) pre-incubated in the presence of 0.8 µM rotenone (10 µl) in the same medium for 3 minutes at 27°C prior to the addition of 24 µM CaCl2 (25 µl). Thirty seconds later, 5 mM succinate (50 µl) was added and mPT was quantified at 540 nm for 12 minutes at 30 seconds interval. This experiment was again repeated with mitochondria (1 mg protein/ml) pre-incubated in the presence of 0.8 µM rotenone (10 µl), 4 mM spermine (63 µl) in the same medium for 3 minutes at 27°C prior to the addition of different concentrations of cefixime. Thirty seconds later, 5 mM succinate (50 µl) was added and mPT was quantified at 540 nm for 12 minutes at 30 seconds interval.

Assessment of mitochondrial FoF1 ATPase activity: This was determined by a modification of the method of Olorunsogo and Malomo (1985). Each reaction mixture contained 65 mM Tris-HCl buffer (1300 µl) pH 7.4, 0.5 mM KCl (50 µl), 1 mM ATP (40 µl), 25 mM sucrose (50µl) and varying concentrations (8-72 µg/ml) of the drug. The reaction mixture was made up to a total volume of 2 ml with distilled water. Mitochondrial suspension was added to the reaction medium in a shaker water bath and allowed to proceed for 30 minutes at 27°C. Aliquot amount (1mL) of 10 percent sodium dodecyl sulphate (SDS) solution was added to stop the reaction at 30 seconds intervals. 2, 4 Dinitrophenol (2, 4 DNP) (50 µl) was used as a standard uncoupling agent. Aliquot of each solution (300µl) was dispensed into fresh test tubes, followed by the addition of 300 µl of distilled water. To each of the test tube, 1 ml of 5% ammonium molybdate and 1 ml of 9% freshly prepared solution of ascorbic acid were added. The tube was well mixed and allowed to stand for 20 minutes. The

absorbance was read at 680 nm. Water blank was used to set the spectrophotometer at zero.

Determination of Cytochrome c release: The quantitative determination of cytochrome c released from isolated mitochondria was performed by measuring the Soret (γ) peak for cytochrome c at 414 nm (ε=100 mM−1 cm−1), according to method of Appaix *et al*. (2000). Mitochondria (1 mg protein/ml) were preincubated in the presence of 0.8µM rotenone (10 µl) in a medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH (pH 7.4) (2,200 µl) for 30 minutes at 27 °C in the presence of different concentrations (10-90µg/ml) of cefixime, using 24 mM calcium (25 µl) as the standard (Triggering Agent). After the incubation, the mixture was centrifuged at 15,000 rpm for 10 minutes. The optical density of the supernatant was measured at 414 nm which is the soret (γ) peak for cytochrome c.

Measurement of malondialdehyde (MDA) level: The malondialdehyde (MDA) content in mitochondria was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the mitochondria according to the method of Varshney and Kale, (1990). The rat liver mitochondria (0.5 mg protein/ml) were incubated with 1.6ml of Tris- KCL buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75% TBA was added and placed in a water bath for 45minutes at 80ºC. This was then cooled in ice to room temperature and centrifuged at 3000rpm for 10min. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm.
Lipid peroxidation (nmole MDA/ mg protein) = Absorbance × volume of mixture
E532nm × volume of sample × mg protein/ml

Determination of Caspases-9 and 3 activity

Sample Preparation and Analysis of Caspases 9 & 3 using Elisa Technique: The rat liver was excised, weighed and rinsed with phosphate buffered saline thoroughly until a clear wash was obtained. The washed livers were homogenized on ice and the homogenates were centrifuged at 8,000 rpm for 5 minutes. The supernatant thus obtained were then put in sample bottles and freezed. After freezing for two days, the samples were brought out to thaw. This was done twice after which the samples were used for caspases 9 and 3 analysis, respectively.

Analysis of caspases 9 and 3: Analysis of caspases 9 and 3 were carried out using an ELISA kit, a product of Elabscience biotechnology Ltd., Technology Industry Park, WuHan, Peoples Republic of China. This kit uses Sandwich-ELISA as the method. A microplate reader (DNM-9602A from China) was used to read the optical density at 450nm wavelength.

DNA Fragmentation: The percentage hepatic DNA fragmentation was determined according to the method of Wu *et al*. (2006). Liver was sliced with scissors and homogenized in 10 volumes of Tri-EDTA Triton buffer (TET) pH 8.0. Homogenates were centrifuged at 27,000 × g for 20 min to separate intact chromatin (pellet A) from fragmented (pellet B). Pellet A was suspended in Tris EDTA buffer (TE) pH 8.0. An aliquot (1 ml) of each sample (pellet and supernatant) was placed in separate test tubes and then 1 ml of freshly prepared diphenylamine solution was added to each. Reaction mixture was incubated at 37 °C for 20 h. Absorbance of the mixture was then measured at 620 nm.

Calculation: Quantity of fragmented DNA was estimated by using the formula:
% fragmented DNA= {B÷(A+B)}X100 Where A is the intact chromatin and B is the fragmented chromatin.

Statistical analysis of data: The data reported on mPT are representative of multiple (≥3) experiments using microsoft excel 2010. All other data were expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 9 software. Comparison of the variables was made using analysis of variance (ANOVA at α 0.05) followed by Tukey's post-test.

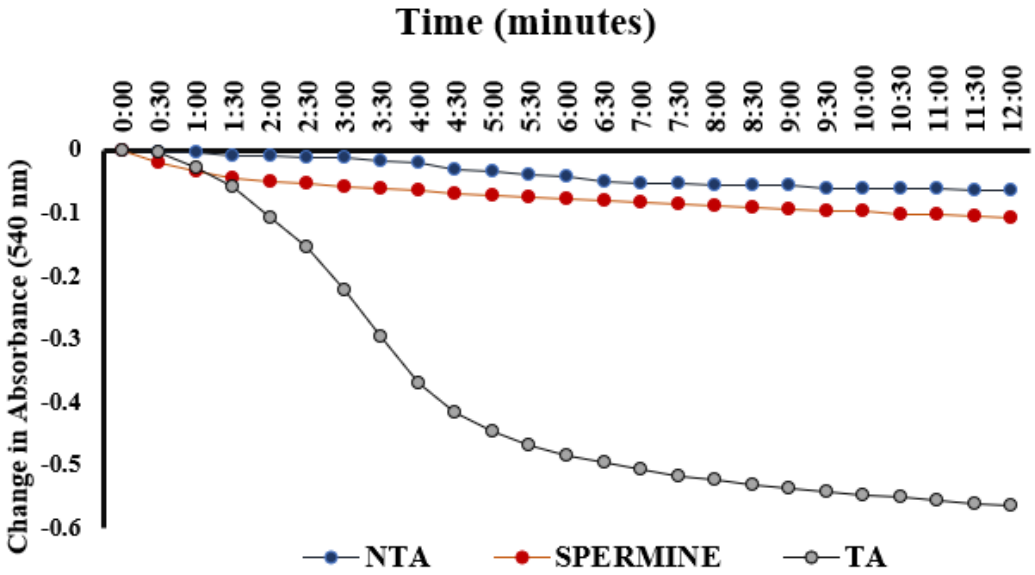

Figure 1:
Determination of mitochondrial intactness
NTA: Non Triggering Agent
TA: Triggering Agent

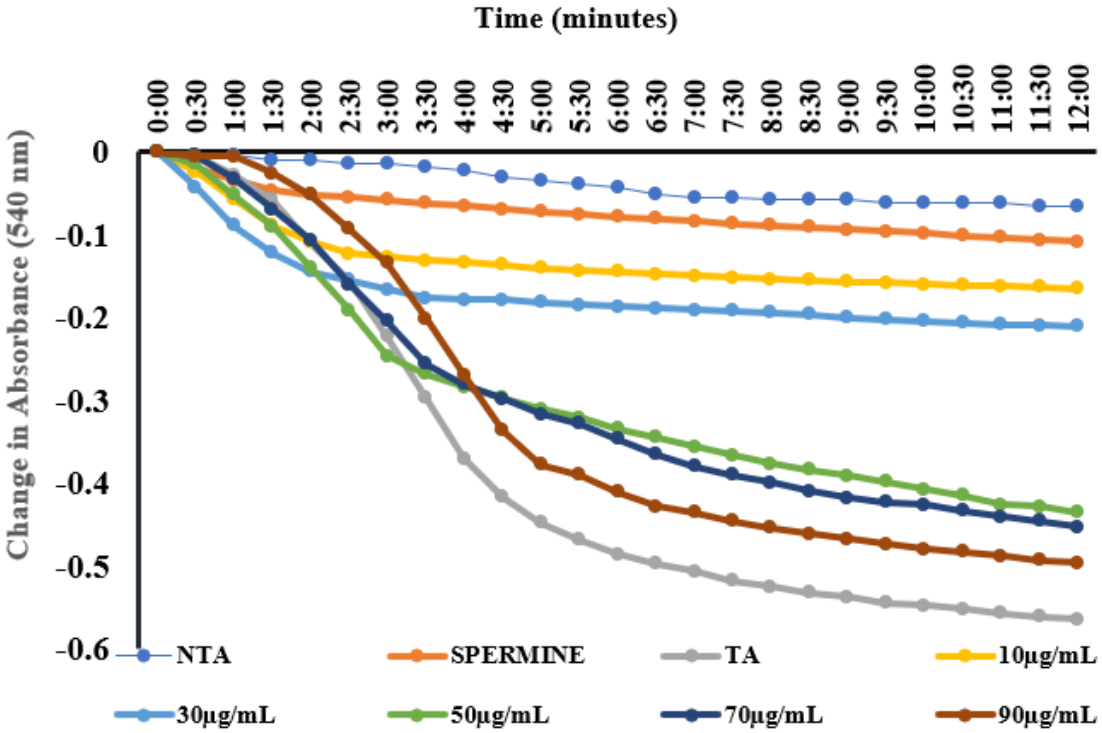

Figure 2:
Representative profile showing the effects of varying concentrations of cefixime on mPT pore

RESULTS

Figure 1 shows the change in absorbance of mitochondria in the presence of rotenone respiring on succinate over a period of twelve minutes. As revealed from the graph, there was no change in absorbance. Nevertheless, addition of exogenous calcium (Triggering Agent) caused large amplitude mPT pore opening which was significantly reversed by spermine (a standard inhibitor of mPT pore opening).

The representative profile in Figure 2 shows the effect of varying concentrations of cefixime on mPT pore opening. At concentrations 10, 30, 50, 70 and 90µg/ml, there was induction of mPT pore opening by 2.5, 3.2, 6.7, 7.0 and 7.6 folds, respectively, when compared to the NTA

Effect of varying concentrations of cefixime on cytochrome c release was depicted in Figure 3. There was a concentration-dependent release of cytochrome c and the effect was statistically significant starting from 30µg/ml, when compared to the NTA. A similar trend of result was observed on its effect on mitochondrial ATPase activity. There was also a concentration-dependent increase in the mitochondrial ATPase activity as depicted by Figure 4. In addition to the in vitro study, the effect of oral administration of cefixime on mPT pore was investigated.

Figure 5 shows that the mitochondria isolated from the control rat were intact as the mPT pore was evidently opened by exogenous calcium and significantly inhibited by spermine**.**

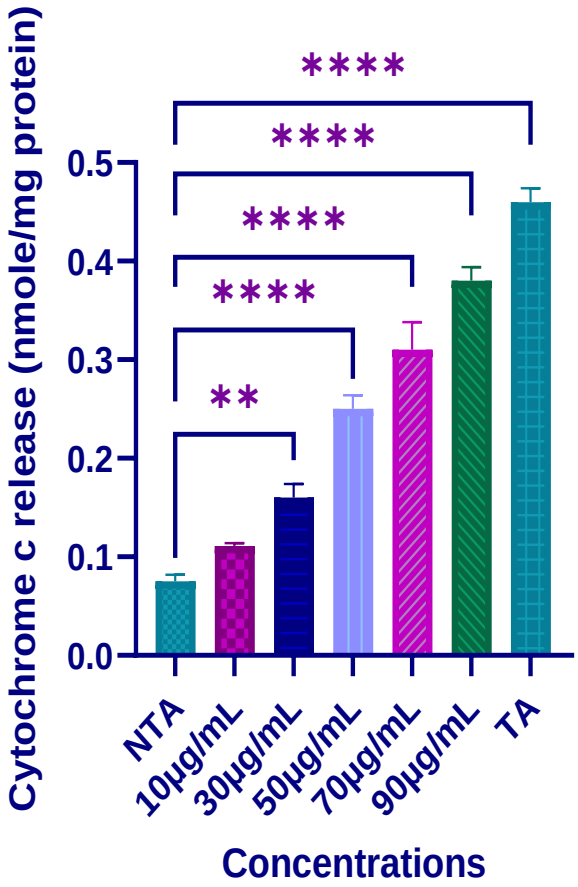

Figure 3:
Effect of varying concentrations of cefixime on cytochrome c release.
TA: triggering agent (calcium). **p < 0.01, ****p < 0.0001 compared to NTA

Oral administration of cefixime also caused induction of mPT pore opening in a dose-dependent manner. Doses 10, 20, 30 and 40 mg/kg elicited induction folds of 3.4, 4.3, 4.9 and 6.3 folds, respectively, when compared to the control (Figure 6).

The effect of cefixime on mATPase was activity was measured using the released inorganic phosphate as an index. As revealed in Figure 7, there was dose-dependent enhancement of mATPase activity highly significant from 30mg/kg.

The effect of cefixime on lipid peroxidation was investigated by measuring the concentrations of MDA formed using rat liver mitochondrial membrane as the lipid-rich media. Its effect on LPO as depicted in Figure 8 showed dose-dependent generation of lipid peroxidation.

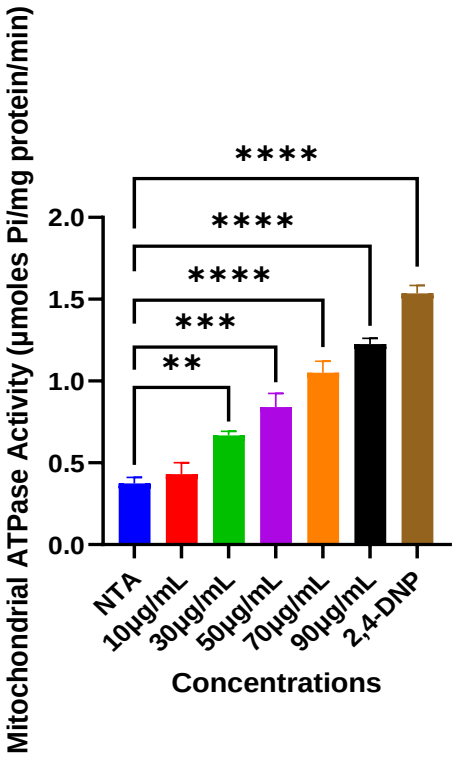

Figure 4:
Effect of varying concentrations of cefixime on mitochondrial ATPase activity.
**p < 0.01, ***p < 0.001, ****p < 0.0001 compared to the NTA

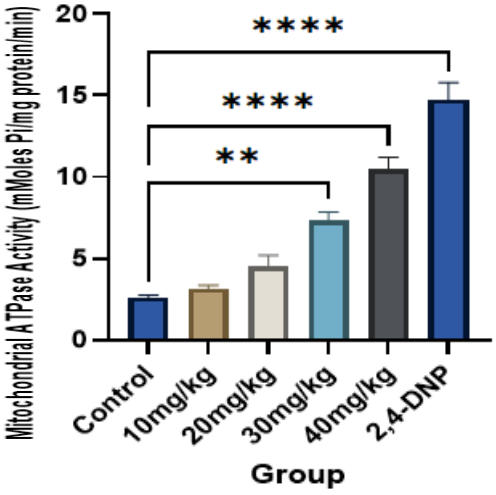

Figure 7:
Effect of oral administration of cefixime on mitochondrial ATPase.
**p < 0.01, ****p < 0.0001 compared to the control

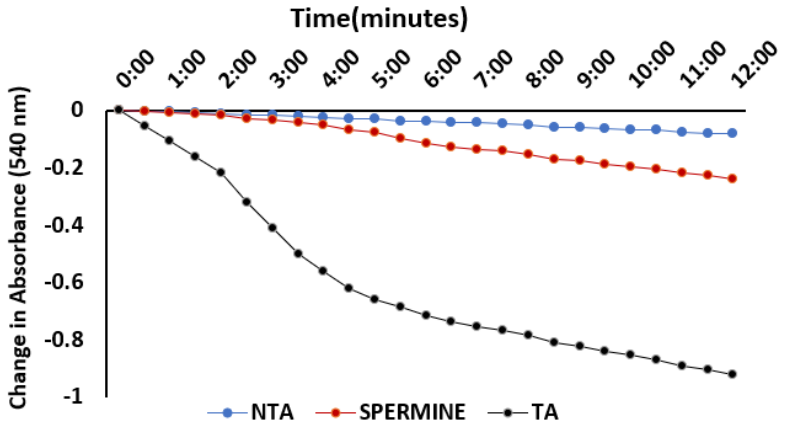

Figure 5:
Determination of intactness for the *in vivo* mPT experiment

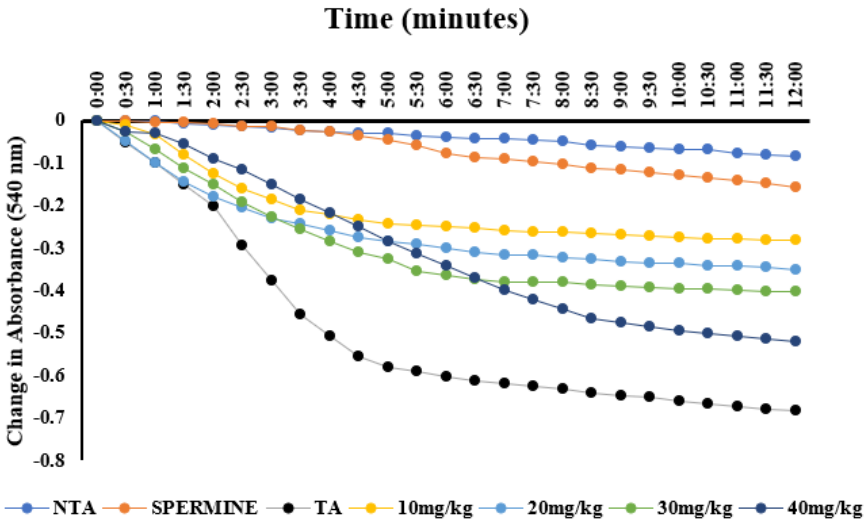

Figure 6:
Representative profile showing the effects of oral administration of cefixime on mPT pore

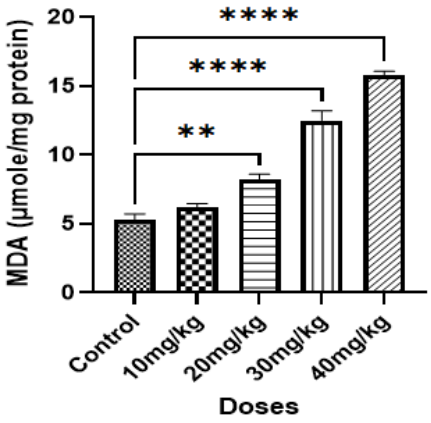

Figure 8:
Effect of oral administration of cefixime on MDA level.
***p < 0.01, ****p < 0.0001 compared to the control*

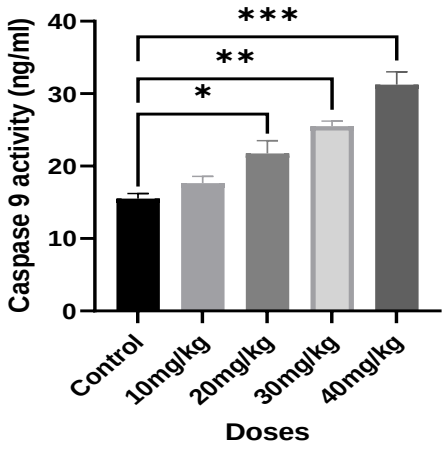

Figure 9:
Effects of oral administration of cefixime on caspase 9 activity.
*p < 0.05 **p < 0.01, ***p < 0.001, compared to the control.

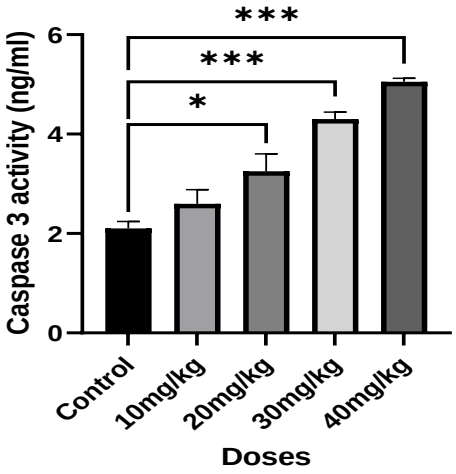

Figure 10:
Effects of oral administration of cefixime on caspase 3 activity.
*p < 0.05, **p < 0.01, ***p < 0.001 compared to the control.

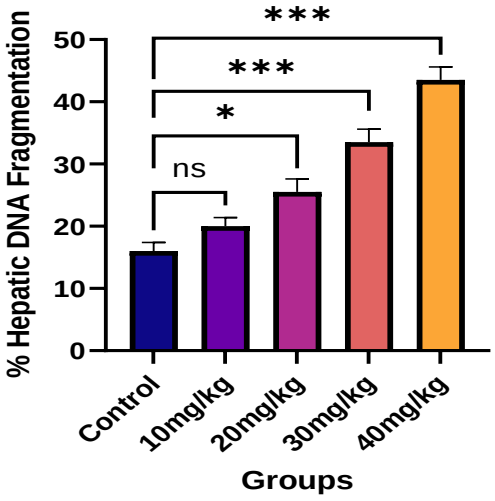

Figure 11:
Effects of oral administration of cefixime on hepatic DNA fragmentation.
*p < 0.05, ***p < 0.001 compared to the control

Figures 9 and 10 show the effect of cefixime on caspases 9 and 3 levels. There was significant increase in the levels of caspases 9 and 3 when compared to the control.

The result on hepatic DNA fragmentation (Fig. 11) also followed similar trend as cefixime caused induction of DNA fragmentation in a dose-dependent fashion relative to the control.

DISCUSSION

Targeting mitochondrial dynamics as a strategy for the treatment of mitochondrial disorders has received wide spread attention (Suliman and Piantadosi, 2016). The potentially dual physiological and pathological role of the mPT pore has attracted attention in terms of drug discovery (Andries *et al*., 2005). The mPT pore now serves as a pharmacological target in the development of drugs that are relevant in situations of dysregulated apoptosis (Giampaolo *et al*., 2021). The present study investigated the effect of cefixime, a third-generation cephalosporin antibiotic, on mitochondrial-mediated cell death via mPT pore. The suitability of the mitochondria used in this study was first ascertained.

The calcium-induced pore opening and its reversal by spermine (standard inhibitor of mPT pore opening) indicated that the mitochondria used in this study were intact ab initio, not compromised and suitable for use. The induction of mPT pore opening by varying concentrations of cefixime suggests that the drug interacted with some mitochondrial components to induce pore opening. This suggests that cefixime is an inducer of mPT pore opening. Various studies have reported that when the pore opens, cytochrome c is released and the cellular bioenergetics is impaired as the ATP is being hydrolyzed (Bauer and Murphy, 2020). The release of cytochrome c and enhancement of mitochondrial ATPase activity by varying concentrations of cefixime suggests that the mPT pore did open which resulted to cytochrome c release and eventual ATP hydrolysis. Oral administration of cefixime also showed similar results on induction of pore opening and ATPase activity. This also suggests that cefixime was available at the target site to induce the opening of the pore and ATPase activity. The increase in induction folds as well as ATPase activity with doses suggests increase in bioavailability of cefixime at the target site. The opening of the mPT pore causes the release of cytochrome c into the cytosol which mediates the organization of the apoptosome and then the activation of the caspase cascade (Kantrow and Piantadosi, 1997; Kroemer *et al*., 2007). The increase in the levels of caspases 9 and 3 suggests that cefixime activated caspase-dependent apoptotic cell death (Schwabe and Luedde, 2018; Bock and Tait, 2020). One of the hallmark of apoptosis is nuclear DNA fragmentation by a specific nuclease called caspase-activated DNase (CAD). The increase in caspase 3 level caused the activation of CAD which lead to specific cleavage of DNA into fragments. Our results on hepatic DNA fragmentation suggest that the oral administration of cefixime effected increase in caspases levels, which lead to the activation of DNase, causing the subsequent cleavage of nuclear DNA into fragments as evidenced with increase in

percentage DNA fragmentation when compared with the control.

The results from this study show that cefixime induces mitochondrial-mediated cell death via mitochondrial permeability transition pore opening. Its anticancer property should be further explored.

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