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Mitochondrial oxidative phosphorylation and dynamics in mouse liver infected with Plasmodium berghei and the modulatory effects of a novel compound purified from Phyllanthus amarus

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Research Article

Keywords: Bioenergetics, Mitochondrial biogenesis, Mitochondrial dynamics, Mitophagy, Natural products, Oxidative phosphorylation

Posted Date: August 11th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3210506/v1

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Abstract

Mitochondria occupy prominent position in cell metabolism, however, infection of the host by *Plasmodium* species causes their dysfunction and imbalance in homeostasis. Antimalarial orthodox drugs affect oxidative phosphorylation and mitochondrial dynamics in the host cell. In this study, we observed the influence of 1, 3, 16 trimethoxy-10 methyl-17-(pent-3-enyl)cyclopenta[α]phenanthrene (TMCP) purified from *Phyllanthus amarus* on oxidative phosphorylation and mitochondrial dynamics in *Plasmodium berghei*-infected mice by assessing the extent of mRNA of mitochondrial complexes and proteins responsible for mitochondrial biogenesis, fission and fusion. *P. berghei* decreases the expressions of complexes I to V while TMCPT at both doses (5 and 10 mg/kg) increased them. The PGC-1α, prohibitins 1 and 2 that *P. berghei* infection decreased in the infected control was increased significantly by TMCP (5 mg/kg). Increase in DRP-1 mediates mitochondrial fission in the infected mice treated with TMCP while significant expressions of OPA1 and mitofusin1 in mice treated with TMCP (5 mg/kg) elicit significant mitochondrial fusion. The TMCP (5 mg/kg) initiated PINK 1-dependent mitophagy while maximum FUNDC1 expression signified full modulation of hypoxia-induced autophagy. Furthermore, TMCP initiated up-regulation of prohibitins 1 and 2 relative to the infected control. The results obtained in this study indicates that TMCP prevents bioenergetic stress and modulate mitochondrial dynamics for effective mitophagy.

Introduction

Previous studies on mitochondria were majorly on ATP biosynthesis,cell death and other cellular metabolism thus portraying the organelles as the power house of the cell. However, several insults to the cells such as injury as a result of infection (for example *Plasmodium* infection) have explained various procedures like mitochondrial dynamics. Mitochondrial dynamics is one of the mitochondrial response to such events taking place in the cell that will ultimately affect the copy numbers of mitochondria via fission and fusion, biogenesis and mitophagy. Mitochondria undergo series of cycles in order to fuse (join together as one) or for fission (splitting) to take place. This events are adequately regulated by different types of events such as cellular energy requirement and pathophysiological stimuli and an array of mitochondrial and cytosolic proteins that are involved in mitochondrial dynamics. Liver cells are highly metabolic and active; because of this, they are one of the cells that are very rich in mitochondria. The pre-erythrocytic stage of *Plasmodium* infection occurs in the liver and the progression of infection in the liver tissue determines the erythrocytic stage of the infection. In the cause of liver infection, there are striking evidences that there is activated immune responses, perturbed metabolic processes and tissue remodeling in the livers of mice infected with *Plasmodium* species (Hu et al., (2023). Apart from *Plasmodium* infection, there are various injuries as a result of increased parasite overload that will definitely affect mitochondrial copy number and damage. This damage will necessitate the tissue repair and remodeling during which new mitochondria are synthesized and damaged ones are repaired or commuted to mitophagy. It follows therefore, that expressed RNAs in the liver will mainly be responsible for oxidative phosphorylation, mitochondrial biogenesis and dynamics. Antimalarial drugs causes liver

injury via free radical generation (Noorani et al., 2010). Mitochondrial biogenesis is a critical procedure for cellular regeneration and homeostasis. This procedure is highly essential after cellular injury as a result of infection and drug intake. The PGC-1α is a central mediator for this process and it also determines the effectiveness of other mediators such as nuclear respiratory factors 1 and 2 and mitochondrial transcription factor A (TFAM) (Wu et al., 1999; Jornayvaz and Shulman, 2010). The PGC-1α is able to perform this function via its association with a class of proteins called prohibitins. Prohibitins are conserved proteins that function in cell growth, proliferation and differentiation. They are expressed in the cytoplasm, nucleus and mitochondria and their location/ where they are found determines their functions ((Shi et al., 2018). In the mitochondria, prohibitins stabilizes mitochondrial structure, dynamics biogenesis and quality control. The energy supply of mitochondria is related to the expression of prohibitin 1(Signorile et al., 2019). Mitochondrial division is very important in order to maintain its copy number as cells divide, regulate cell death pathway and eliminate damaged mitochondria as part of quality control strategy. In this process, dynamin-related protein 1 (DRP 1) is the main component of mitochondrial division, recruited to the fission site by Fis 1 (Loson et al., 2013). Mitochondrial fusion is mediated by mitofusins 1 and 2 (for outer mitochondrial membrane) and optic atrophy 1 (OPA 1 for inner mitochondrial membrane). Mfn-1 and Mfn-2 can connect adjacent membranes through coiled-coil antiparallel homotypic (Mfn1-Mfn1) or heterotypic (Mfn1-Mfn2) dimers (Hoppins et al., 2011). Mitochondrial dynamics and oxidative phosphorylation are interconnected. Because mitochondrial fusion has been found to support increased oxidative phosphorylation (Yao et al., 2019; Punter et al., 2022). It is strongly believed also that *Plasmodium* infection causes bioenergetics stress in the host while intervention by treating the infection with different drugs may elicit multivariable effects. Mitochondrial dynamics in the mouse liver infected by Schistosoma mansoni has been reported (Chen et al., 2015). There is a very few report of such in *Plasmodium* infection and how drug intervention modulate this crucial process in *Plasmodium*-infected mice.

Given the fact that mitochondrial dynamics have been studied in other cell types, this study is also important in the liver because it has numerous number of mitochondria that are capable of influencing cellular function. This study therefore, focuses on the molecular mechanism of mitochondrial dynamics in the liver and the relevance of this to hepatocyte survival within the context of choice of drugs for malaria treatment and *Plasmodium*-mediated liver injury and oxidative phosphorylation. It further presented information on how TMCP, purified from *Phyllanthus amarus* modulate oxidative phosphorylation and mitochondrial dynamics in mice infected with resistant (ANKA) strain of *Plasmodium berghei*.

Materials and methods

Purification of the drug candidate

Purification of 1, 3, 16 trimethoxy-10 methyl-17-(pent-3-enyl)cyclopenta[α]phenanthrene (TMCP) was done via activity-guided assay using chromatographic method and it was characterized by using NMR spectroscopic techniques.

Infection of mice with resistant Plasmodium berghei

Ryle and Peters (1970) method of established infection was used in this study. Male mice (Swiss strain) and resistant (ANKA strain) of *Plasmodium berghei* were obtained from the Malaria Research Unit of the Institute of Advanced Medical Research and Training, College of Medicine, University of Ibadan, Nigeria. Twenty mice were infected via intraperitoneal injection of infected erythrocytes (1×10^7 inoculum size) and parasitemia was confirmed via microscopy after seventy-two hours. Another five mice that were not infected were used as the baseline (group I) and treated with the vehicle (10 percent v/v DMSO). The infected mice were divided (n = 5) into four groups and were treated as follows: group II (infected control) was treated with vehicle, group III was treated with amodiaquine-artesunate as the drug control while groups IV and V were treated with 5 and 10 mg/kg of TMCPT. After five consecutive days of oral and once daily treatment, mice were sacrificed and the liver tissue was obtained and stored in 70 µL trizol in Eppendorf tubes.

Isolation of mitochondria

Liver mitochondria were isolated using the method of Johnson and Lardy (1967). Liver was removed from sacrificed animal and washed in isolation buffer containing 210 mM Mannitol, 70 mM Sucrose, 5 mM Hepes-KOH (pH 7.4) and 1 mM EGTA, chopped into homogenisable pieces and further homogenized in a 10% suspension in the isolation buffer using a portable homogenizer (USA). The homogenate was centrifuged (300k Sigma, Germany) at 4°C twice at 260 g for 5 minutes each time. The supernatant was further centrifuged at 1500 g for 10 minutes to obtain the mitochondria as pellets. Mitochondria were washed twice at 1400 g each time for 10 minutes with washing buffer containing 210 mM Mannitol, 70 mM Sucrose, 5 mM Hepes-KOH (pH 7.4) and 0.5% BSA. Washed mitochondria were suspended using the suspension buffer containing 210 mM Mannitol, 70 mM Sucrose and 5 mM Hepes-KOH (pH 7.4), dispensed in aliquots into Eppendorff tubes and kept on ice until used. This procedure was repeated for mitochondria used for F_0F_1 ATPase analysis but 0.25 M sucrose was used as buffer.

Mitochondrial protein determination

The protein content of mitochondrial isolate was determined using the method of Lowry and his co-workers (1951). Mitochondria volume (10µl) were made up to 1ml in test tubes withdistilled water and 3 ml of a 100:1:1 mixture of 2 g Na_2CO_3 and 1% $CuSO_4.5H_2O$ in 0.1M NaOH was added to each test tube and the mixture was allowed to stand for 10 minutes after which 0.3 ml of a 5-fold dilution of 2N Folin reagent was added and the whole mixture was incubated at room temperature for 30 minutes, vortexed and absorbance read at 750 nm. Mitochondrial protein was estimated from a protein curve using bovine serum as standard.

Assay of mitochondrial permeability transition pore opening

The integrity and suitability of isolated mitochondria for mPT assays were confirmed using the method of Lapidus and Sokolove (1993). Mitochondrial volume equivalent to 0.4 mg/ml protein from the normal control group were pre-incubated in suspension buffer in the presence of 8 µM rotenone for 3.5 minutes

after which 5 mM succinate was added and the absorbance read for 12 minutes at 30 sec interval to ascertain the uncoupled status of mitochondria. To assess the response of isolated mitochondria to calcium, same mitochondrial protein was pre-incubated in suspension buffer in the presence of rotenone for 3 minutes after which 3 μM $CaCl_2$ was added. Thirty sec after, 5 mM sodium succinate was added and absorbance read. To assess the reversal effect of spermine, mitochondrial protein was pre-incubated in suspension buffer, rotenone and 4 mM spermine for 3 minutes after which 3 μM $CaCl_2$ was added. Thirty seconds after, the medium was energized with succinate and the absorbance read at 540 nm. Mitochondria from the normal control that have negligible change in absorbance in the absence of calcium, but with large amplitude swelling in the presence of calcium, whose amplitude swelling was significantly reversed by spermine were considered intact, uncoupled and suitable for further use (Lapidus and Sokolove, 1993). Equivalent mitochondrial protein from the treated groups was subjected to the same permeability transition under the same condition.

Mitochondrial F_0F_1 ATPase assay

Mitochondrial F_0F_1 ATPase activity was assessed using modified method of Lardy and Wellman (1953). Each test tube (in triplicate) contained 25 mM sucrose, 65 mM Tris-HCl (pH 7.4) and 0.5 mM KCl in a reaction volume of 1 ml. Mitochondria (0.5 mg/ ml protein from each group) were added to the designated tubes while mitochondria only, uncoupler and zero time tubes contained mitochondria isolated from the normal control group. Uncoupler (25 μM of 2,4-dinitrophenol) was added to the uncoupler tubes immediately after mitochondria were added and 1 ml of 10% sodium duodecylsulphate (SDS) was immediately added to the zero-time test tubes to stop the reaction. The ATP (1 mM) was added to sets of tubes designated ATP only, uncoupler, zero time, test groups and control while 'mitochondria only' did not contain ATP. These were transferred to the shaking water bath and incubated at 27ºC for 30 minutes; reaction was stopped with the addition of SDS, sequentially to the way ATP substrate was added. The reaction mixture was vortexed and 1 ml was withdrawn from each test tube. These were diluted further with 4 ml distilled water. One ml of acidified 1.25% ammonium molybdate and 1 ml of 9% freshly prepared ascorbic acid were added and the absorbance was read at 660 nm. Inorganic phosphate released was quantified using a phosphate standard curve.

Mitochondrial lipid peroxidation

Mitochondria (0.4 ml) were added to 1.6 ml of Tris-KCl and 0.5 ml of 30% TCA. Thereafter, 0.5 ml of 0.75% TBA was added and the test tubes were boiled at 80ºC for 45 min, cooled to room temperature and centrifuged at 3000 rpm for 10 min. The absorbance of the clear supernatant was read at 532 nm (Varshney and Kale 1990). TBARS level was calculated using its extinction co-efficient (Adam-vizi and Seregi 1982) of 0.156 $\mu M^{-1}cm^{-1}$.

Total RNA isolation

Total RNA was isolated from the liver samples following a method described by Omotuyi et al (2018). Briefly, tissues were homogenized in cold (4°C) TRI reagent (Zymo Research, USA, Cat:R2050-1-50, Lot:

ZRC186885). Total RNA was partitioned in chloroform (BDH Analytical Chemicals, Poole, England Cat: 10076-6B) following centrifugation at 15,000 rpm/15 min (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States). RNA from the clear supernatant was precipitated using equal volume of isopropanol (Burgoyne Urbidges & Co, India, Cat: 67-63-0). RNA pellet was rinsed twice in 70% ethanol (70 ml absolute ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y) in 30 ml of nuclease-free water (Inqaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ML)). The pellets were air-dried for 5 min and dissolved in RNA buffer (1 mM sodium citrate, p^H 6.4).

cDNA conversion:

Prior to cDNA conversion, total RNA quantity (concentration (µg/ml) = 40 * A_{260}) and quality (≧ 1.8) was assessed using the ratio of A_{260}/A_{280} (A = absorbance) read using spectrophotometer (Jen-way UV-VIS spectrophotometer model 6305, UK). DNA contamination was removed from RNA was removed following DNAse I treatment (NEB, Cat: M0303S) as specified by the manufacturer. 2 µl solution containing 100 ng DNA-free RNA was converted to cDNA using M-MuLV Reverse transcriptase Kit (NEB, Cat: M0253S) in 20 µl final volume (2 µl, N^9 random primer mix; 2 µl, 10X M-MuLV buffer; 1 µl, M-MuLV RT (200 U/µl); 2 µl, 10 mM dNTP; 0.2 µl, RNase Inhibitor (40 U/µl) and 10.8 µl nuclease-free water). The reaction proceeded at room temperature O/N. Inactivation of M-MuLV Reverse transcriptase was performed at 65°C/20 min.

PCR amplification and agarose gel electrophoresis.

PCR amplification for the determination of genes whose primers (Primer3 software) [3] are listed below (table 1.0) were done using the following protocol: PCR amplification was performed in a total of 25 µl volume reaction mixture containing 2 µl cDNA (10 ng), 2 µl primer (100 pmol) 12.5 µl Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB, Cat: M0486S) and 8.5 µl nuclease-free water. Initial denaturation at 95°C for 5 minutes was followed by 20 cycles of amplification (denaturation at 95°C for 30 seconds, annealing (see TM values for each primer pair on table 1.0) for 30 seconds and extension at 72°C for 60 seconds) and ending with final extension at 72°C for 10 minutes. In all experiments, negative controls were included where reaction mixture has no cDNA. The amplicons were resolved on 1.5% agarose gel (Cleaver Scientific Limited: Lot: 14170811) in Tris (RGT reagent, china, Lot: 20170605)-Borate (JHD chemicals, China, Lot 20141117)-EDTA buffer (pH 8.4).

Amplicon image processing

In-gel amplicon bands images captured on camera were processed on Keynote platform as previously reported (Omotuyi et al., 2020) and quantified using image-J software. All graphs were plotted as mean +/- SEM using graph-pad prism.

Primers:

S/N	Name	Forward Sequence	Reverse Sequence
1	Complex-I	GTACTCGCATCCTGGCGTTT	GGATGTGCTCAACAACCTGGA
2	Complex-II	AGA CAT CAG GTT TAC GGG GC	CTC TCG GTC CTT ACA GGT GC
3	Complex-III	TGCTTCTGCTGACGTACTGG	TATGGCGCACAAACAGAGGT
4	Opa1	CTGCAGGTCCCAAATTGGTT	TCTTTGTCTGACACCTTCCTGT
5	Complex-IV	CCT TGG ACG GCG GAA TG	TTC ACA ACA CTC CCA TGT GCT
6	Complex- V	GCCATTTTGTGCCAGTCGTC	CATTTTTGGAGACCAGTCCCG
7	DRP1	CCAGAGGAACTGGTGTGGTC	CCATTCTTCTGCTTCAACTCCATT
8	PGC – 1α	AACCCCTTCCAACCAGTGTG	TCCGATTGGTCGCTACACC
9	Prohibitin-1	GGTCAGAGTGAAAGCAGGTG	TCTGTGTCCAGCATCCACATT
10	Prohibitin-II	ATCCGTGTTCACCGTGGAAG	GGGGATCCTGAAGTGAAGGC
11	PINK-1	GAGGAGCAGACTCCCAGTTC	TTTCTGCCATTATGCACCTGGAC
12	Mitofusin-1	CCGGGGTGACCTTCGAGC	TCCAACACAAGGTTGACAAGA
13	Mitofusin-2	GGGAAGGTGAAGAAGCTTGG	CTTCACAGGTTGGGCATCGT
14	FUNDC1	ACCATAGACTTCCAACTCGGC	TCGCTTTCATAGTCTTGGGGA

Statistical analysis

Representative profiles of mitochondrial permeability transition pore kinetics was used after a minimum of three repeated assays that gave reproducible trend. Other data were expressed as mean ± standard deviation (SD). Experiments were repeated independently for at least three times. Data were analysed using one-way ANOVA followed by Tukey's test comparing each test column with the mean of other columns using Graph pad prism 7.0. Level of significance was set at $P < 0.05$.

Results

Plasmodium infection causes large amplitude increase in mitochondrial permeability transition pore opening, FoF1 ATPase activity and lipid peroxidation, reversed by TMCP

Response of mitochondria isolated from different sources to various treatments is presented in Fig. 1. Here, the absorbance of mitochondria isolated from uninfected mouse respiring on succinate was monitored for twelve minutes at thirty seconds interval in the absence of calcium that is, no triggering agent (NTA). There was no noticeable change in the absorbance. Thereafter, the same mitochondrial protein from this mouse respiring on succinate was subjected to response from exogenous calcium and a large amplitude swelling was observed. In the presence of spermine, the large amplitude opening of the mitochondrial pore was reversed. This confirms the integrity of mitochondria isolated under the same

condition. *Plasmodium* infection causes an irreversible opening of the mPT even in the absence of exogenous calcium as it could be seen in the large amplitude increase in changes in the absorbance measured at 540 nm over a period of twelve minutes at thirty seconds interval. Amodiaquine-artesunate and TMCP (at both doses) reversed the pore opening caused by *Plasmodium* infection (Fig. 1A). In Fig. 1B, *Plasmodium* infection causes a significant ($P < 0.0001$) increase in the activity of mitochondrial ATPase activity relative to other test groups. Amodiaquine-artesunate and TMCP significantly decreased the F_oF_1 ATPase activity of the mitochondrial enzyme. A dose-dependent increase in the effect of TMCP in reversing the ATPase activity of the enzyme was observed. Both amodiaquine-artesunate and TMCP decreased mitochondrial lipid peroxidation caused by *Plasmodium* infection and there was no significant difference in the preventive effect of the two drugs on peroxidation of mitochondrial lipids (Fig. 1C)

The TMCP increases oxidative phosphorylation via the upregulation of the mitochondrial complexes genes

Generally, Mitochondrial complexes control oxidative phosphorylation and they are critical to the fate of ATP yield through the electron transport system in the inner mitochondrial membrane. In Fig. 2A, *Plasmodium berghei* infection caused a significant ($P < 0.01$) decrease in the expression of NADH oxidoreductase (complex I) compared with the normal control while amodiaquine-artesunate further decreased ($P < 0.0001$) the expression of this gene. The TMCP resuscitated the NADH mRNA significantly relative to the infected control ($P < 0.05$) and the control drug ($P < 0.0001$). Amodiaquine-artesunate treatment lowered the expression of complex I relative to the normal control and infection by *Plasmodium berghei* without treatment (both at $P < 0.0001$). Figure 2B shows how amodiaquine-artesunate further decreased ($P < 0.0001$) the expression of complex II relative to the baseline. The effect of this drug on complex II was similar to what was obtained in complex I. TMCP at both doses increased ($P < 0.01$) the expression of complex II compared to the infected control. *Plasmodium berghei* infection silenced the expression of complex III when compared with the normal control ($P < 0.01$), amodiaquine-artesunate ($P < 0.001$), and TMCP ($P < 0.0001$) at 5 mg/kg. It was observed that the effect of TMCP on complex III decreased ($P < 0.001$) as the dose increased (Fig. 2C). Assay of complex IV is critical to the success of oxidative phosphorylation in the inner mitochondrial membrane because it is at this point that oxygen is reduced to water. In Fig. 2D, there was a decrease in the expression of this gene in the infected control ($P < 0.0001$), infected mice treated with amodiaquine-artesunate ($P < 0.001$), and infected mice treated with 10 mg/kg dose of TMCP ($P < 0.0001$) compared to the normal control. There was full restoration of the gene expression by the 5 mg/kg dose of TMCP and this was quite significant when compared with the normal control ($P < 0.05$), infected control ($P < 0.0001$) and infected mice treated with amodiaquine-artesunate ($P < 0.0001$). Therapeutic administration of amodiaquine-artesunate and both doses of TMCP increased the expressions of ATP synthase (complex V). Both amodiaquine-artesunate and TMCP (at both doses) increased ($P < 0.0001$ in both cases) the expression of complex V gene relative to the normal control and the infected control. The effect of TMCP was maximum at the highest dose (Fig. 2E).

Optic atrophy 1 (OPA 1) compliments Mitofusin genes to increase mitochondrial fusion while TMCP elicits the recruitment of DRP 1 than amodiaquine-artesunate in mitochondrial fission

Since the expression of *OPA 1* gene is highly necessary to maintain mitochondrial fusion, and the interaction of the gene products forms a complex with mitofusins1 and 2 to elicit a full fusion capacity, we assay for the extent of expression of these genes and discovered that *OPA 1* gene was most expressed in the mouse liver of the infected control compared with the base line ($P < 0.01$), infected mice treated with amodiaquine-artesunate ($P < 0.0001$), infected mice treated with TMCP at 5 mg/kg ($P < 0.01$) and 10 mg/kg ($P < 0.0001$). The expression of *OPA 1* gene was decreased in the infected mice treated with armodiaquine-artesunate and TMCP at 10 mg/kg (Fig. 3A). The therapeutic dose of armodiaquine-artesunate and TMCP at 5 and 10 mg/kg increased the expressions of mitofusin 1($P < 0.0001$ in each case) than in the infected control (Fig. 3B). However, TMCP increased this expression maximally at 5 mg/kg. While there was no significant difference in the expression of mitofusin 2 in both the normal and infected control, administration of amodiaquine –artesunate to infected mice increased the expression of mitofusin 2 than what was obtained in the infected control ($P < 0.01$), TMCP at 5 mg/kg ($P < 0.0001$) and 10 mg/kg ($P < 0.01$) as observed in Fig. 3C .

The TMCP initiates mitochondrial biogenesis and mitophagy than amodiaquine artesunate

To assess the effect of *Plasmodium* infection on mitochondrial biogenesis and mitophagy, we assayed for the key regulators of the two processes, *PGC-1α* and *PINK 1*. The results show that *Plasmodium* infection blunts the expressions of *PGC-1α* (Fig. 4A) and *PINK 1* (Fig. 4B). However, TMCP initiated over-expressions of these genes maximally at 5 mg/kg significantly higher than infected control ($P < 0.001$ in each case) and a higher expression ($P < 0.0001$) of *PGC-1α* and *PINK 1* ($P < 0.001$) than amodiaquine-artesunate. In both cases, TMCP (10 mg/kg) suppresses the expression of both *PINK 1* ($P < 0.01$) and *PGC-1α* ($P < 0.0001$) than amodiaquine-artesunate.

FUNDC1 and prohibitins 1 and 2 modulate oxidative phosphorylation and mitochondrial dynamics in TMCP-treated mice

FUNDC1 is a critical gene that recruits LC3 to the mitochondria to initiate hypoxia-induced mitophagy while prohibitin genes are active players in the modulation of several processes leading to mitochondrial homeostasis. In this study, it was observed that *Plasmodium* infection silenced the expression of *FUNDC1* relative to what obtains in the treated groups. Specifically, treatment of *Plasmodium* infection, using TMCP increased ($P < 0.0001$) the expression of *FUNDC1* gene maximally at the highest dose relative to the infected control (Fig. 5A). Furthermore, treatment of *Plasmodium berghei* infection with amodiaquine-artesunate treatment down-regulates *Prohibitin 1* gene relative to the infected control (Fig. 5B) and similar results were obtained for prohibitin 2 gene (Fig. 5C). Treatment of *Plasmodium*

infection with TMCP maximally up-regulates the mRNA expression of *prohibitins 1* and *2* ($P < 0.0001$, in each case) when compared with the standard drug (Figs. 4b and 4c). It was observed also that 5 mg/kg of TMCP caused an increase in the expressions of *prohibitins 1* and *2* relative to the highest dose.

Discussion

Malaria continues to be a treat to mankind in terms of health and socio-economic reasons. For some time, clearance of the parasite load has been the major achievement of malaria treatment in focus. However, since host mitochondria play prominent roles in the outcome of infection and disease progression (Luckhart and Riehle, 2020), this study explores how choice of drugs for treatment can influence the mitochondrial homeostasis and thus the outcome of the treatment on ATP-generating potentials and hydrolysis of the host mitochondria and its dynamics. In this study, *Plasmodium* infection causes irreversible opening of the mitochondrial permeability transition pore in the pathological version and this could result to loss of function and cell death. We have previously provided evidences that *Plasmodium* infection affects mitochondrial integrity via the opening of the mitochondrial permeability transition pore opening (Olanlokun et al. 2020, 2021a, 2021b, 2022). Again, the opening of the mitochondrial pore will affect the mitochondrial number and thus their homeostasis. The activation of F_oF_1 ATPase further causes ATP depletion via hydrolysis in infected hepatocytes and the reversal of this effect by TMCP helps to maintain cellular ATP content and their mobilisation for metabolic purposes. *Plasmodium* infection causes peroxidation of mitochondrial membrane lipids via the generation of free radicals that causes oxidative damage to the phospholipids (Nsiah et al., 2019). The mechanism by which TMCP prevents oxidative damage may be by the generation of protons that can reduce free radicals to harmless products such as molecular oxygen or water.

In this study, we have been able to show that *Plasmodium* infection down-regulate the mRNA expressions of mitochondrial complexes especially complexes II, III and IV. Curiously too, amodiaquine-artesunate down-regulate the mRNA expressions of these genes and concomitantly, the corresponding proteins. The implication of silencing the expression of complexes II, III and IV is indicative of the fact that the transfer of electrons to cytochrome oxidase (complex IV) will be impeded, thus leading to decrease in oxygen consumption. Again, glucose will not be fully oxidised and ATP generation will become impeded. The full oxidation of $FADH_2$ to generate electron transfer occurs at complex II. Therefore, silencing the mRNA expression of this complex may lead to the prevention of this fuel molecule to be fully oxidised, thus impairing cellular respiration. Previous report has reported that artesunate destabilises parasite membrane (Adebayo et al., 2020), it is not known if such destabilisation is extended to the host. An increase in the mRNA synthesis of complexes I and V indicated that although, amodiaquine-artesunate may not support ATP synthesis through $FADH_2$ complete oxidation of NADH did occur. Deleterious silencing of complexes II, III, IV and V mRNAs may indicate that indeed, bioenergetic stress occurs in *Plasmodium* infection and treatment of the disease via the administration of some drugs of choice. Therefore, infected cells will have to rely on glycolysis and substrate level phosphorylation for ATP synthesis.The TMCP appears to be a consistent drug candidates that maintains NADH and $FADH_2$

oxidation via oxidative phosphorylation. Previous study has shown that infection of the liver cells (by *Schistosoma mansoni*) can attenuate ATP generation in the liver; a side effect attenuated by TMCP administration (Chen et al., 2015). Results obtained in this study further show that TMCP influences the use of NADH and succinate for ATP generation. The safe, as well as therapeutic dose of TMCP appears to be at 5 mg/kg with respect to its influence on bioenergetics and mitochondrial dynamics. This is because this appears to be the dose that rightly modulates the components of oxidative phosphorylation and thus, ATP generation.

Mitochondria are very important organelles in eukaryotic organisms and are saddled with very important biological functions. At the same time, they are very dynamic and undergoes cycles of fusion and fission which are mediated by array of proteins such as mitofusin1, mitofusin 2, optic atrophy 1 (OPA1) and dynamin-related protein 1 (DRP1), respectively. The up-regulation of the *DRP 1* gene activates the synthesis of the protein which may correspondingly be activated via phosphorylation to enhance mitochondrial fission in the infected control. Mitochondrial fusion is a means by which their DNAs are preserved in which the wild type suppresses the mutant one in order to guide against further injury and at the same time increases oxidative phosphorylation (Youle et al., 2012; Hu et al., 2017). On the other hand, mitofusins 1 and 2 genes that controls mitochondrial fusion were oppositely activated in the same infected control; showing that both mitochondrial fusion and fission are oppositely activated. Our observation that the significant decrease in the expressions of mitofusins 1 and *2* genes in the infected control coupled with a significant increase in the expression of *DRP1* gene is an indication that *Plasmodium berghei* infection causes an imbalance in the mitochondrial dynamics thus making the mitochondria in the infected host susceptible to fission by increasing the expression of the genes that controls the components that increases mitochondrial fission. Our observation that there was an increase in the expression of *OPA 1*, a gene that controls the synthesis of the OPA 1 protein that fuses the inner mitochondrial membrane during fusion protein shows that there is the possibility of selective fusion of the inner and outer mitochondrial membrane. A previous study has shown that the inner and outer mitochondrial membranes can fuse separately, simultaneously or coordinately and thus their fusion machineries can function independently (Malka et al., 2005). Furthermore, increased mitochondrial inner membrane fusion may occur without necessarily influencing the outer membrane and vise versa (Mishra et al., 2014). Mitochondrial fusion and fission are differently mediated by amodiaquine-artesunate (AA) and TMCP in the sense that while AA increased mitochondrial fusion via an increase in the upregulation of *mitofusins 1* and *2* genes, with a concomitant down-regulation of the *DRP1* gene, TMCP mediated both fusion and fission when the two doses were considered. These show that different drugs can provoke mitochondrial dynamics in different ways (Malka et al., 2005).

Results obtained from this study further show that *Plasmodium berghei* infection alters mitochondrial biogenesis. *Plasmodium berghe*i infection silenced the expression of the *PGC−1α* and thus decreases mitochondrial biogenesis. Since mitochondria are the major synthetic machinery for ATP production in the cell, it follows therefore, that ATP generation could also be altered in the infected control via a down-regulation of *PGC−1α.*. it has been previously observed that expressions of isoforms of *PGC*, either α or β isoforms activates core mitochondrial biogenesis and function. It also increase oxidative metabolism

while a deletion or down-regulation of *PGC−1α* severely limit respiration in some tissues (Lai et al., 2008; Zechner et al., 2010; Rowe et al., 2013). Previous study has shown that *Plasmodium* infection disrupts mitochondrial biogenesis via the down-regulation of the *PGC−1α* (Yao et al., 2022). Coupled with its effects on the expressions of electron transport chain protein complexes, AA further down-regulated *PGC−1α* thus limiting ATP synthesis and cellular respiration in the course of malaria treatment. The observation that TMCP mediates ATP production via the up-regulations of mitochondrial complexes and *PGC−1α* further shows that this drug candidate, in addition to its antiplasmodial effects, can maintain ATP synthesis.

Mitophagy, a mitochondrial-specific form of autophagy, is a self-protective process by which damaged mitochondria are selectively degraded. *PINK 1*, a putative mitochondrial serine/threonine kinasegene protects the cells against apoptosis induced by oxidative stress. Malaria is a disease associated with oxidative damage (Atamna and Ginsburg, 1993) and treatment with some orthodox drugs that their mechanism of action is via free radical generation will further worsen the oxidant/antioxidant ration in the host (Barman et al., 2023). PINK 1 protein, a product of the *PINK 1* gene protect cells against stress induced apoptosis and PINK 1- deficient cells are susceptible to apoptosis (Wood-Kaczmar et al, 2008). In this study, *Plasmodium* infection caused the silencing of the *PINK 1* gene. Furthermore, there is correlation between down-regulation of mitochondrial complexes genes associated with silencing of *PINK 1* gene (Gautier et al., 2008) as observed in this study. It is interesting to note that The drug lead used in this study up-regulates *PINK1* gene than amodiaquine-artesunate. This could be because TMCP has functional groups that could make it serve as an antioxidant. Apart from this function, *PINK 1* is also central to the control of mitochondrial quality. Host mitochondria undergo various dysfunctional effect during *Plasmodium* invasion and as a result, deserves various means to mop up damaged mitochondria via mitophagy. The TMCP enhances mitophagy via an up-regulation of the *PINK1* gene. This occurs via the accumulation of PINK 1 protein on the outer membrane of damaged mitochondria to trigger selective mitophagy via PARKIN (Pickrell and Youle, 2015).

Mitochondria biogenesis is a critical component of mitochondrial quality control. The *PGC-1α* acts as a critical regulator of mitochondrial biogenesis via the transcriptional machinery to increase mitochondrial mass. During the pre-erythrocytic stage of *Plasmodium* infection, cell death in the liver decreases the mitochondrial mass vis-a-vis energy generation and imbalance in mitochondrial dynamics. To overcome this decrease in mitochondrial copy number, there is need for upregulation of *PGC-1α* and corresponding protein synthesis. In this study, *Plasmodium* infection silenced the expression of this gene and treatment with amodiaquine-artesunate could not resuscitate the expression of this gene to the same level of expression compared with the normal, un-infected control. The TMCP (5 mg/kg) up-regulates *PGC-1α* probably possibly by activating NRF1 and NRF2 which may in turn stimulate the synthesis of mitochondrial transcription factor A (TFAM) that subsequently mediates mtDNA replication and transcription. This could be via the PGC-1α-NRF1/2-TFAM pathway (Vina et al., 2009). The concormitant increase in the up-regulation of *PINK1* could be that *PGC-1α* is also an essential element in mitophagy regulation by mediating *PINK1* transcriptional activity by increasing the latter's upregulation thus

elevating the activation of the *PINK1/PARKIN* pathway thus mediating the degradation of damaged mitochondria as observed in previous studies (Ziviani and Whitworth, 2010; Zhang et al., 2019).

Through the destruction of red blood cells, *Plasmodium* infection induces hypoxia (Park et al., 2019). The FUNDC1 is an integral outer mitochondrial membrane gene and is a receptor for hypoxia-induced mitophagy (Liu et al., 2012); and because of this, we assayed for the expression of *FUNDC1*. Through this, it was further proved that indeed, infection of mouse with resistant *Plasmodium berghei* elicits hypoxia as a result of significant down-regulation of *FUNDC1*. Further to this, amodiaquine-artesunate up-regulates the expression of *FUNDC1*gene and further by the drug candidate in a dose-dependent manner. Our results show that TMCP enhances the upregulation of *FUNDC1* thus corroborating our results for the expressions of *PINK1* and *PGC-1α.*

Although, prohibitins 1 and 2 are ubiquitously present in the nucleus and cytosol, its localization also in the mitochondria suggests critical roles in mitochondrial bioenergetics, biogenesis and dynamics. The modulatory roles of these proteins in malarial disease suggest their critical role in the progression, treatment and outcome of the disease. Our findings that amodiaquine-artesuate and TMCP oppositely affects the expressions of prohibitins 1 and 2 indicates that both drugs can differently influence the outcome of the disease during chemotherapy. In this study, it was discovered that TMCP supports the upregulation of the prohibitins 1 and 2 as it did the mitochondrial complexes involved in oxidative phosphorylation. It could be that prohibitins modulate the stability and translation of the oxidative phosphorylation complexes in which the gene products of prohibitins serve as holdase or unfloldase chaperone preventing the oxidative phosphorylation proteins complexes from proteolysis (Nijtmans et al., 2000). Moreover, prohibitins are requires in the inner mitochondrial membrane for OPA1 stability thus, prohibitin may also regulate mitochondrial fusion.

In conclusion, this study shows that *Plasmodium* infection affects oxidative phosphorylation and determines the fate of the cell via mitochondrial dysfunction and homeostasis. Furthermore, more research has to be conducted on choice of drugs for the treatment of malaria. Effective drugs can also be optimised to enhance mitochondrial turnover in the host and also promote energy generation as a means to optimally overcome the disease.

Declarations

Ethical approval

All animals used for this experiment were handled in accordance with the rules and regulations for management of animals used in research as contained in the Guide for the Care and Use of Laboratory Animals (NRC, 1985). Furthermore, the research proposal was submitted for ethical consideration on 21 January, 2022 and after a review, this study was approved by the Animal use and Care Research Ethics of University of Ibadan, Nigeria and an Approval Number 018-0122/21 assigned to the study.

Competing interest

The authors declare that no competing interest exists.

Authors' contributions

JOO conceived the research idea, wrote the proposal, designed the experiment and performed assays, carried out statistical analysis and wrote the draft manuscript. COB handled the experimental animals, did the treatment and performed the assays. OOO made available the equipment and corrected the draft manuscript. All authors agreed to the submission of the manuscript to Journal of Bioenergetics and Biomembranes

Funding

The authors did not receive funding for this study. The research was funded by the authors.

Availability of data and materials

All data for this study are available in the manuscript.

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Figures

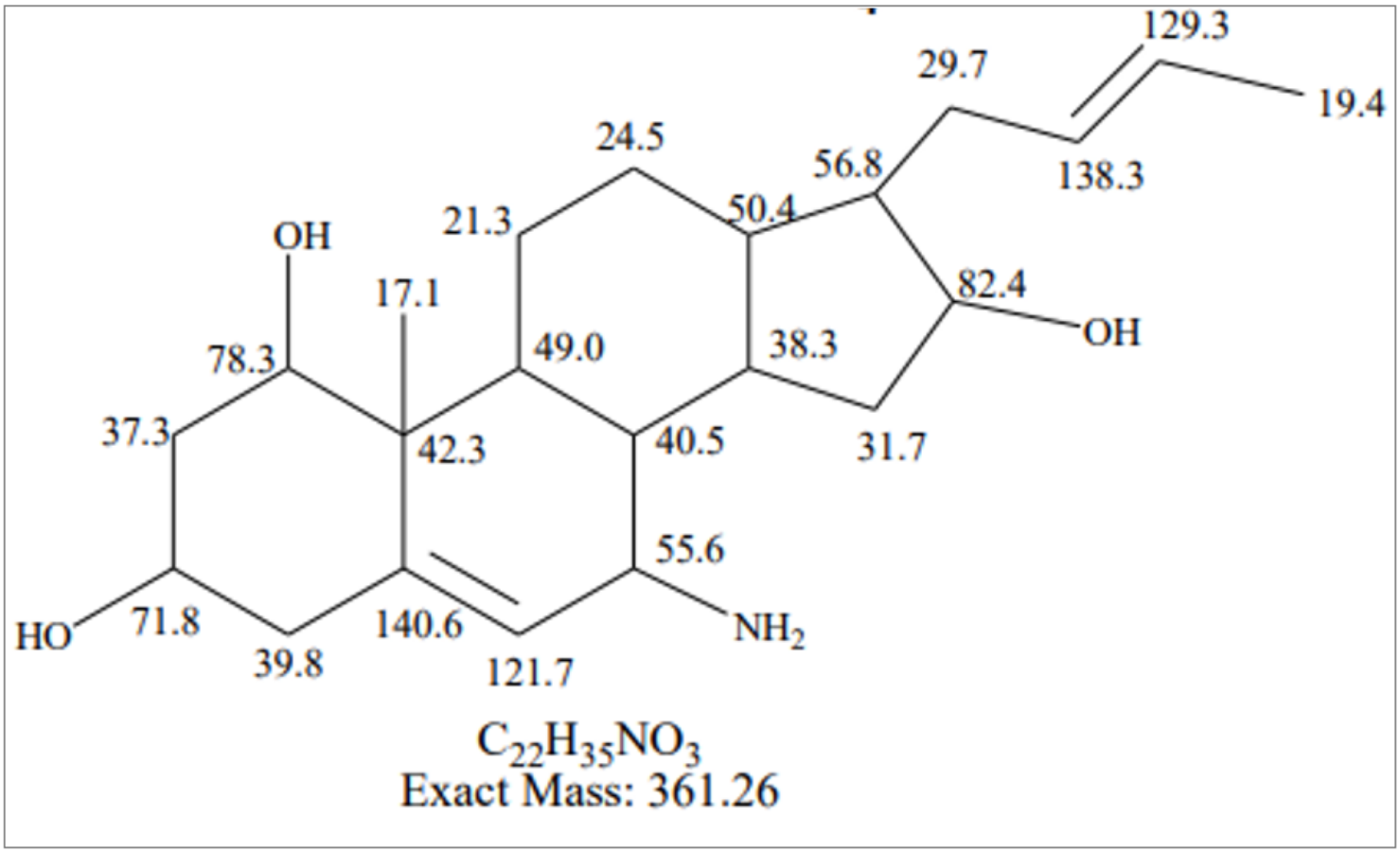


Figure 1

The structure of TMCP

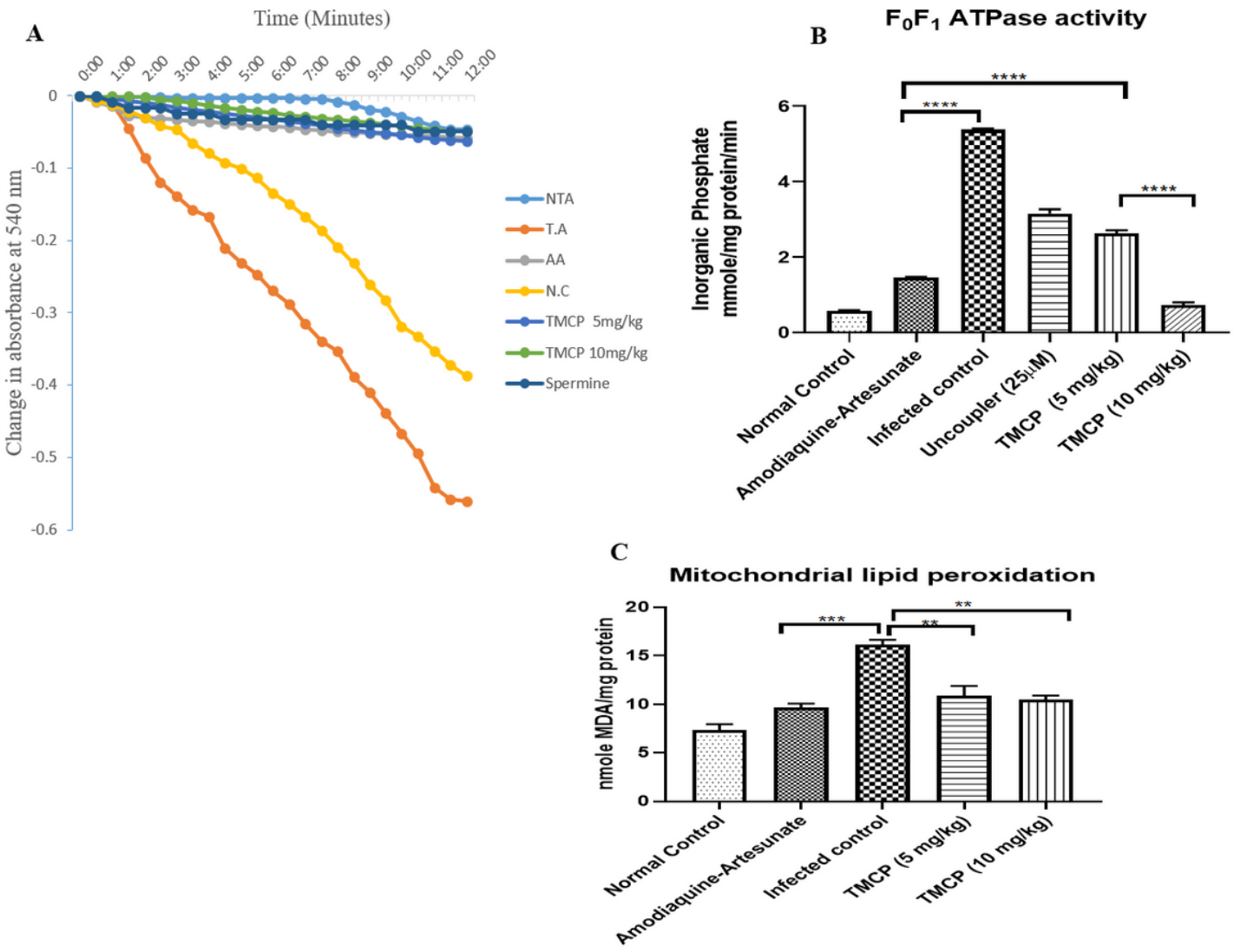


Figure 2

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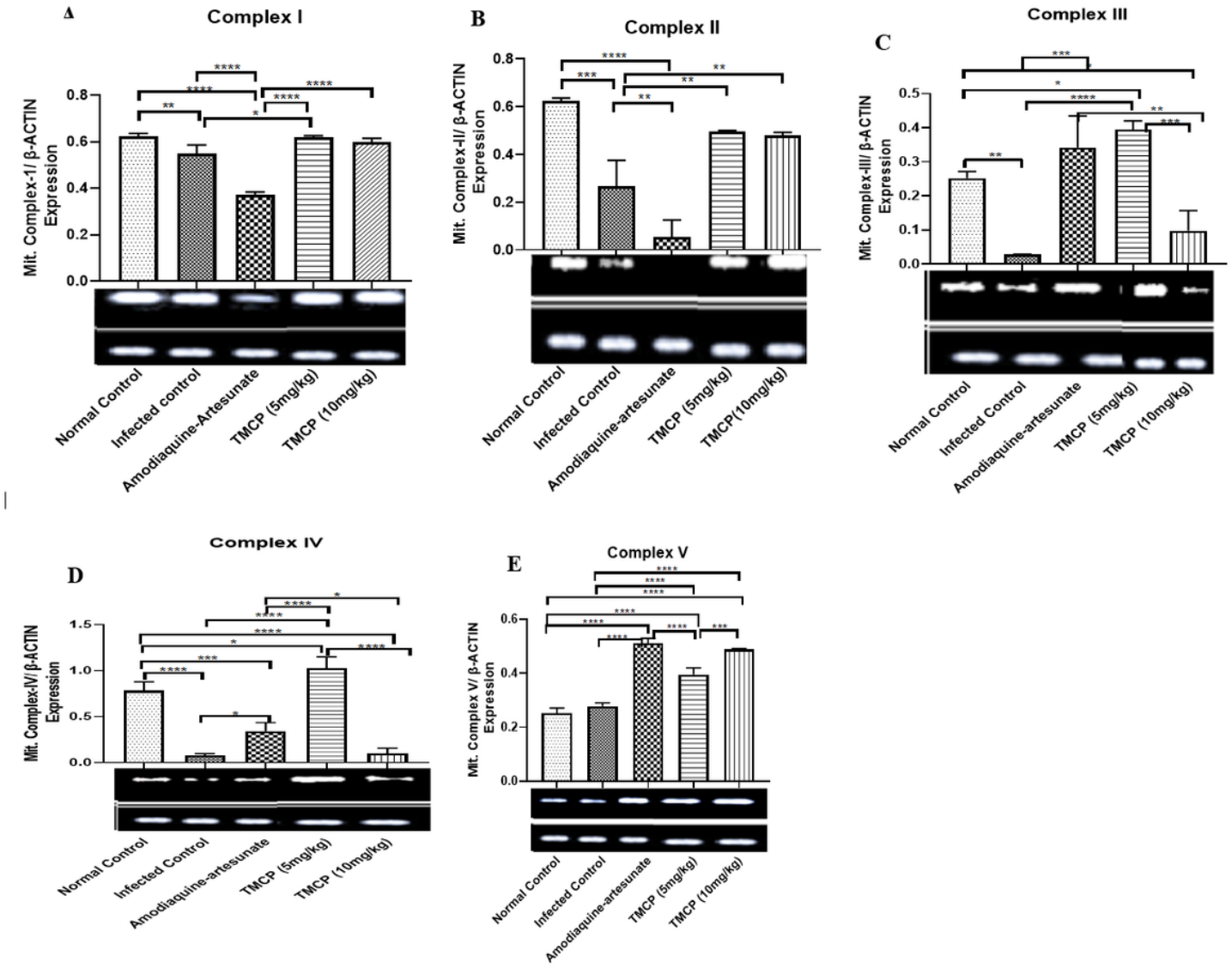


Figure 3

Fig. 2: *Plasmodium* infection affects the expression of the genes that codes for mitochondrial complexes and the modulatory effects of TMCP on the genes that codes for mitochondrial complexes I, II, III, IV and V (A to E). *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001 infected control vs other test groups.

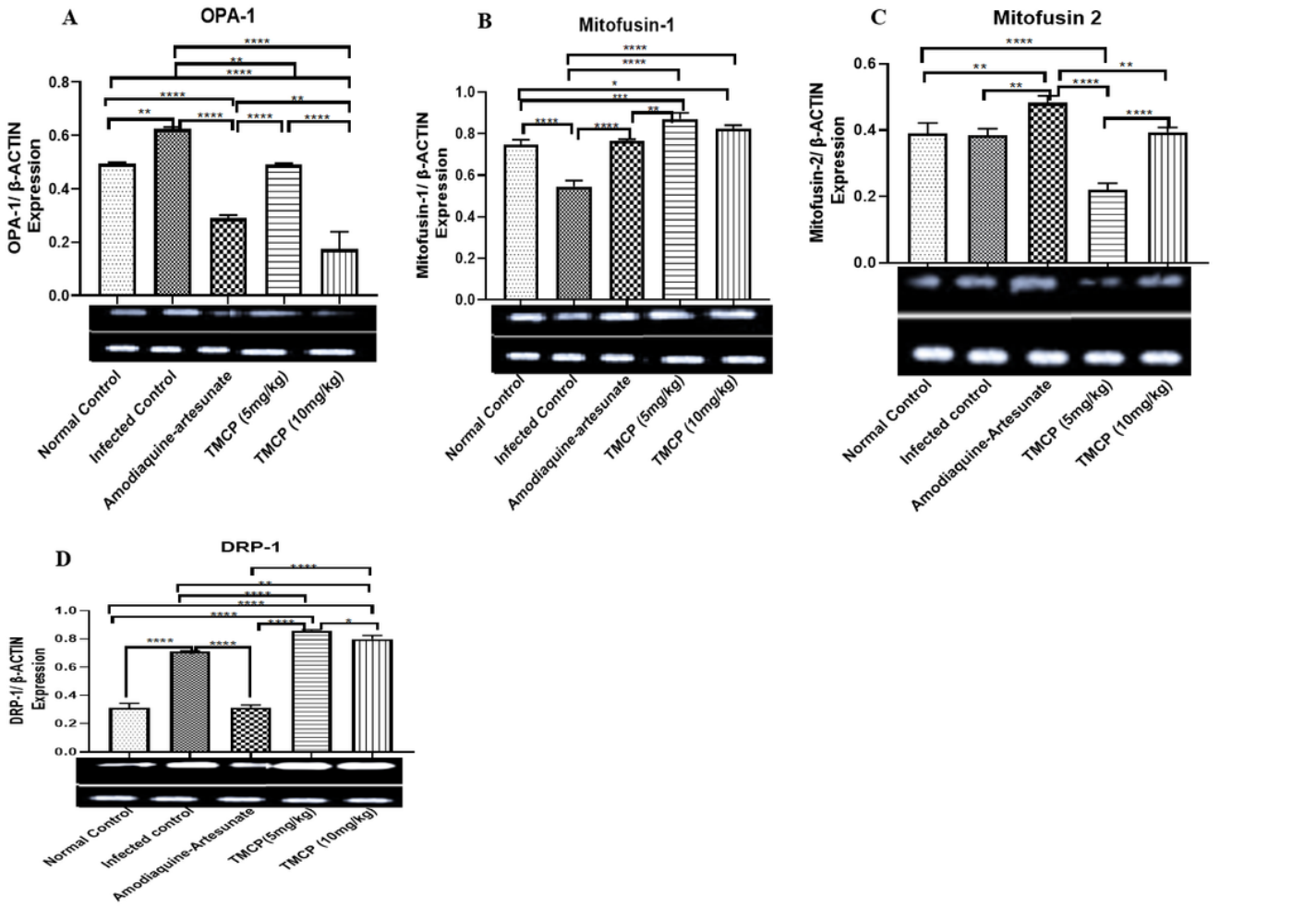


Figure 4

Figure 3: Modulatory effects of TMCP on mitochondrial fusion and fission. The effects of TMXP on Optic atrophy 1 (OPA 1) (A), Mitofusin 1 (B), Mitofusin 2 (C) and dynamin-related protein 1 (DRP 1) genes. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001 Infected control vs treated groups

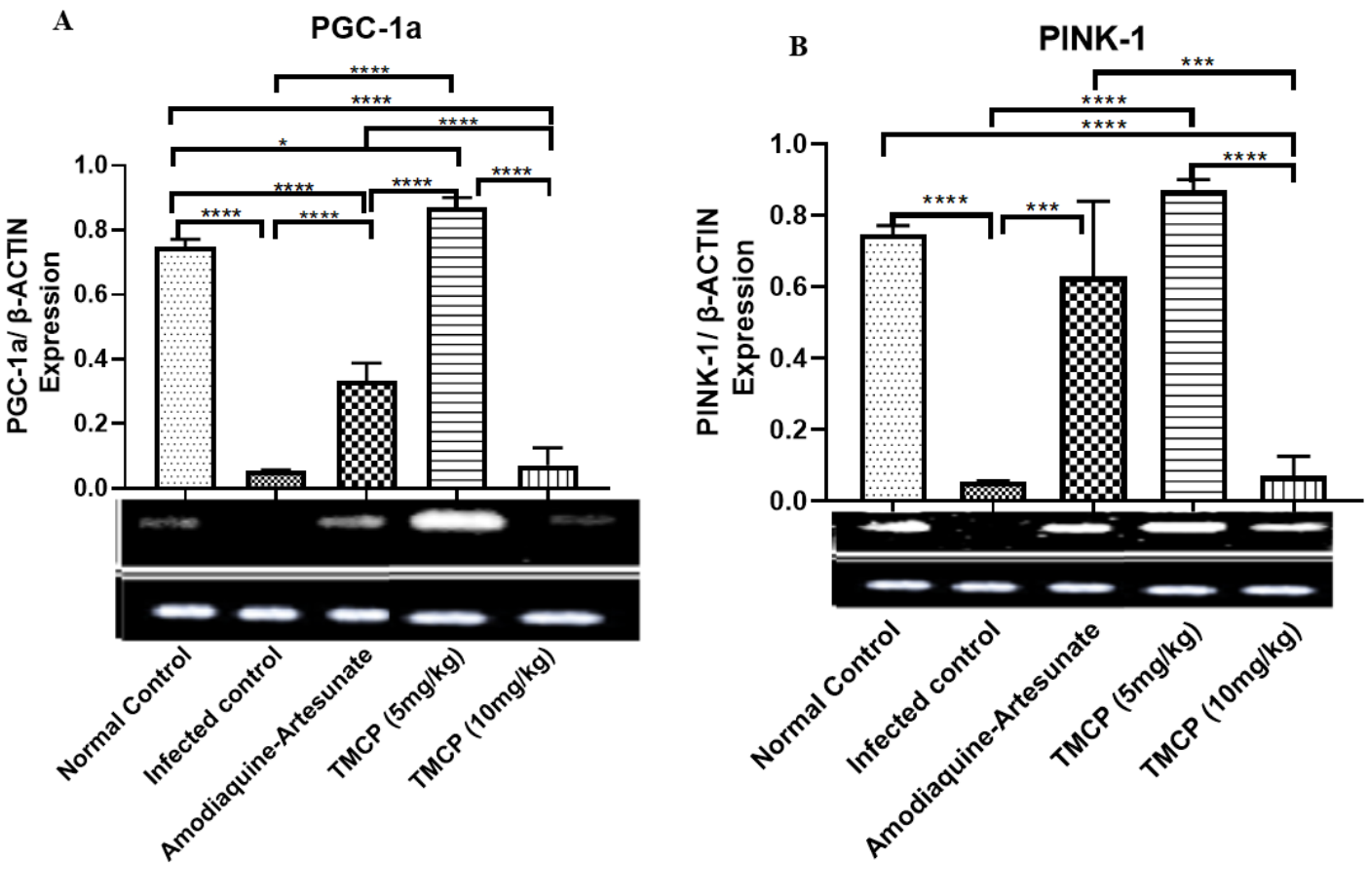


Figure 5

Figure 4: Mitochondrial biogenesis in mice infected with *Plasmodium berghei* (ANKA strain) treated with TMCP. Qunatitative assessment of mitochondrial biogenesis mediators: *PGC-1α* (A) and *PINK 1* (B) genes expression. *=P<0.05, ***=P<0.001, ****=P<0.0001 Infected control vs treated groups

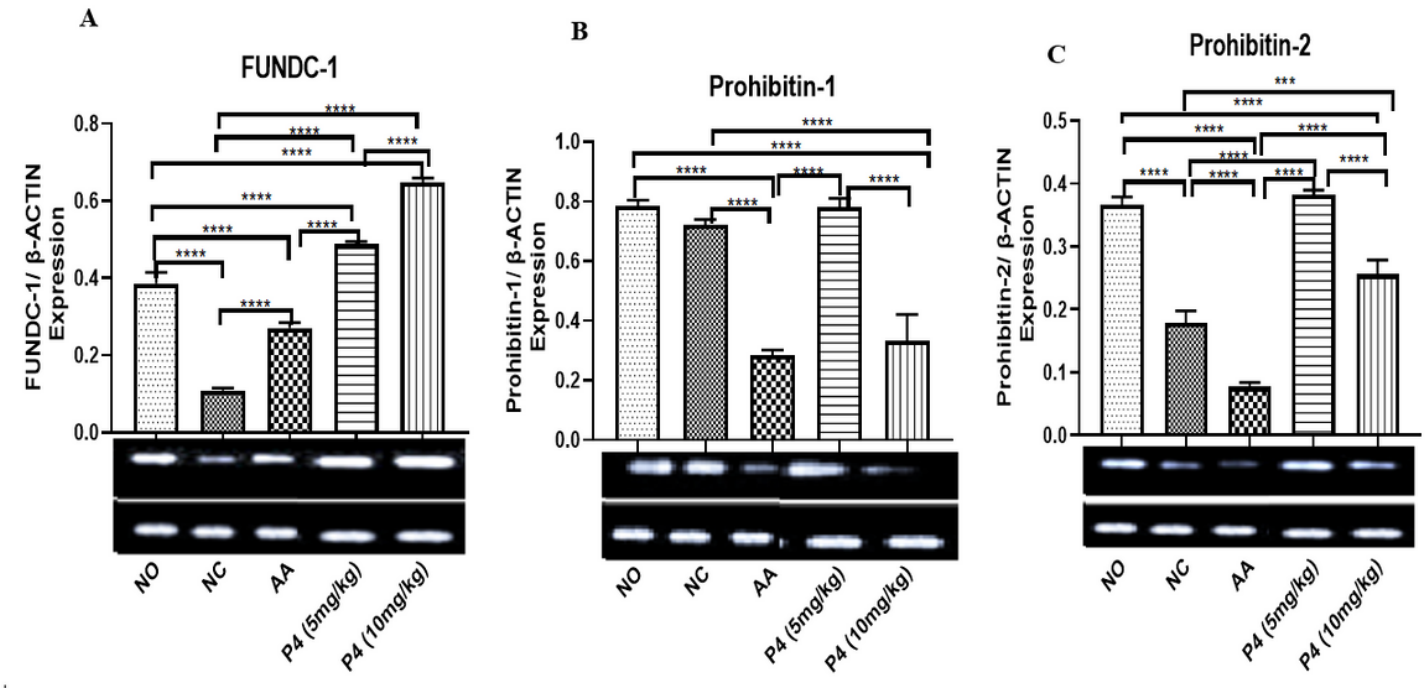


Figure 6

Figure 5: modulation of mitophagy through the mediating effects of FUNDC1 (A), prohibitin 1 (B) and prohibitin 2 (C). ***=P<0.001, ****=P<0.0001 Infected control vs treated groups