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RESEARCH ARTICLE

ANTI-PLASMODIAL EFFECT OF NICOTINAMIDE- VITAMIN B3 AGAINST CHLOROQUINE RESISTANT MALARIA IN AN IN VIVO MODEL

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Abstract

Nicotinamide (NICO), an amide derivative of nicotinic acid, used in high doses for four decades, has shown antimicrobial and potential anti-malarial activity *in vitro*. However, its antiplasmodial effects in drug-resistant *in vivo* models were previously unexplored.Our study explored the antimalarial impact of Nicotinamide (NICO) in chloroquine-resistant malaria mouse models. We administered NICO at doses from 1.82 mg/kg to 4.55 mg/kg body weight, beginning 72 hours post-infection and continuing until 7th day. On the 8th day, all animals were euthanized, and blood and organs were collected for experiments.When administered alone, NICO reduced parasitemia by 88%, which further reduced to 95.27% when combined with chloroquine (65 mg/kg body weight). *Plasmodium*-specific 18S rRNA analysis via RT-PCR in liver confirmed reduced parasitemia with Nicotinamide aloneand in combination with chloroquine. Liver pathology scores significantly improved with the high NICO dose (4.55 mg/kg body weight, $p=0.012$) and its combination with chloroquine (65 mg/kg body weight, $p<0.0001$) compared to untreated chloroquine-resistant mice. Intracellular Reactive Oxidative Species (ROS) levels in liver lymphocytes and serum Nitric Oxide (NO) levels were notably reduced with combination therapy ($p<0.001$, $p<0.0001$, respectively). Our study demonstrates NICO's antimalarial potential alone and its synergistic effect with chloroquine, offering promise against chloroquine-resistant malaria.

Introduction:-

Nicotinamide, an active form of Vitamin B3, serves as a precursor for NAD^+ and $NADP^+$ synthesis (Braidy N et al., 2019). It has been used for treating conditions like pellagra, osteoarthritis, and inflammation (Jonas et al., 1996;Braidy N et al., 2019). Additionally, nicotinamide exhibits antimicrobial activity against various pathogens, including *Mycobacterium tuberculosis*, HIV (Murray et al., 2003), *Plasmodium falciparum*, and *Leishmaniain vitro* (Sereno et al., 2005). Its inhibitory effects extend to sirtuindeacetylases, particularly SIRT1, which regulates

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diverse biological processes, and SIR2 in *P. falciparum*, known for its histone deacetylase and ADP ribosyltransferase activities (Tcherniuk et al., 2017).

The emergence and widespread prevalence of multidrug resistance in *Plasmodium* infection pose significant obstacles to effectively controlling the disease through chemotherapy. The scientific community has put forward two strategies to address this issue.

Firstly, there is the pursuit of new chemotherapeutic agents, as proposed by Olliaro& Wirth in 1996 focusing on creating drugs with novel mechanisms of action to combat resistance. Secondly, there is the concept of reversing chloroquine resistance using compounds with lower intrinsic antimalarial activity, as suggested by Martin et al. in 1987. Given that chloroquine was historically one of the most successful antimalarial drugs, the idea is to augment its effectiveness with adjuncts or modulators, potentially rendering it useful again in regions where it is currently ineffective due to resistance.

Nicotinamide inhibits *Plasmodium* growth alone and in combination with drugs like chloroquine, artemether, and pyrimethamine (Tcherniuk et al., 2017), particularly targeting *Plasmodium falciparum* during the ring stage in an *in vitro* model. In this study, we investigated Nicotinamide's antimalarial potential and its synergy with chloroquine against chloroquine-resistant malaria using a mouse model. Our results demonstrate that the combination therapy of Nicotinamide and chloroquine exhibits lethal activity against chloroquine-resistant *Plasmodium berghei*withAmodiaquine and Rifampicinserving as reference treatments to validate the efficacy of the combination therapy. This research underscores Nicotinamide's promise in combination therapies for combating drug-resistant malaria, particularly in targeting specific *Plasmodium* life cycle stages.

Materials and Methods:-

Chemicals-

Chloroquinediphosphate (CQ), Rifampicin (Rif), and Amodiaquine (A) were procured from TCI (Tokyo Chemical Industry, Tokyo, Japan), while Nicotinamide (NICO) was obtained from MP Biomedicals (Santa Ana, California). All drugs and chemicals utilized in the study were of analytical grade and sourced from authorized suppliers.

Drug preparation-

All the drugs were extrapolated for *in vivo* studies in mice as recommended for human usage and prepared fresh corresponding to the weight of the mice every day. The study used Chloroquine (CQ) at 65 mg/kg body weight, three Nicotinamide (NICO) doses (1.82 mg/kg, 3.64 mg/kg, 4.55 mg/kg body weight),Rifampicin (Rif) (15 mg/kg body weight) and Amodiaquine (A) (10mg/kg body weight) (A+Rif as positive control) (Badejo et al., 2014)and Normal saline (NS; negative control). These were freshly prepared and given orally daily for four days, commencing 72 hours post-infection (pi), until the 7th day.

Mice-

Balb/c and Swiss mice, both male and female, aged 6-8 weeks, weighing 22-25g were procured from the Central Animal Facility at the Postgraduate Institute of Medical Education and Research. They were housed under standard laboratory conditions: controlled temperature (25°C ± 0°C), 12-hour light/dark cycles, and free access to food and water. The study adhered to ethical guidelines outlined in the Animal Ethics Procedures and CPCSEA Guidelines and was approved by the Institutional Animal Ethics Committee (Reference No. 90/91/IAEC/631).

Parasite strains-

Chloroquine-resistant (CQR) and Chloroquine-sensitive (CQS) *Plasmodium berghei* (*Pb*) NK65 strains were sourced from the National Institute of Malaria Research (NIMR), New Delhi. Balb/c mice were used for experiments, while Swiss Albino mice were employed for maintaining the malaria strains through weekly serial passages. Infection was induced by intraperitoneal (ip) injection of $1x10^6$ parasitized RBCs (pRBCs) (Manhas P et al., 2023). Parasitemia percentage (PP) was calculated by Giemsa-stained thin blood smear counting, following the formula by Kabiru et al. (2013).

PP = (Total no. of pRBCs/Total no. of RBCs) X 100

Animal grouping-

A total of 38 Balb/c mice were used to validate the murine model for CQR and CQS *P. berghei*NK65 strains (see Supplemental Fig. S1).

Determination of the most effective dose of Nicotinamide-

To determine the most effective test drug dose for reducing parasitemia, 48 mice were divided into four groups (as shown in Fig. 1). These mice were infected with $1X10^6$pRBCs carrying either the CQR or CQS *P. berghei* strain. After 72 hours pi, NICO, CQ, and NS were administered to different subgroups. Parasitemia was assessed at various time points, and the dose with the greatest reduction in parasitemia was selected for further combinational therapy studies with CQ in the murine malaria model.

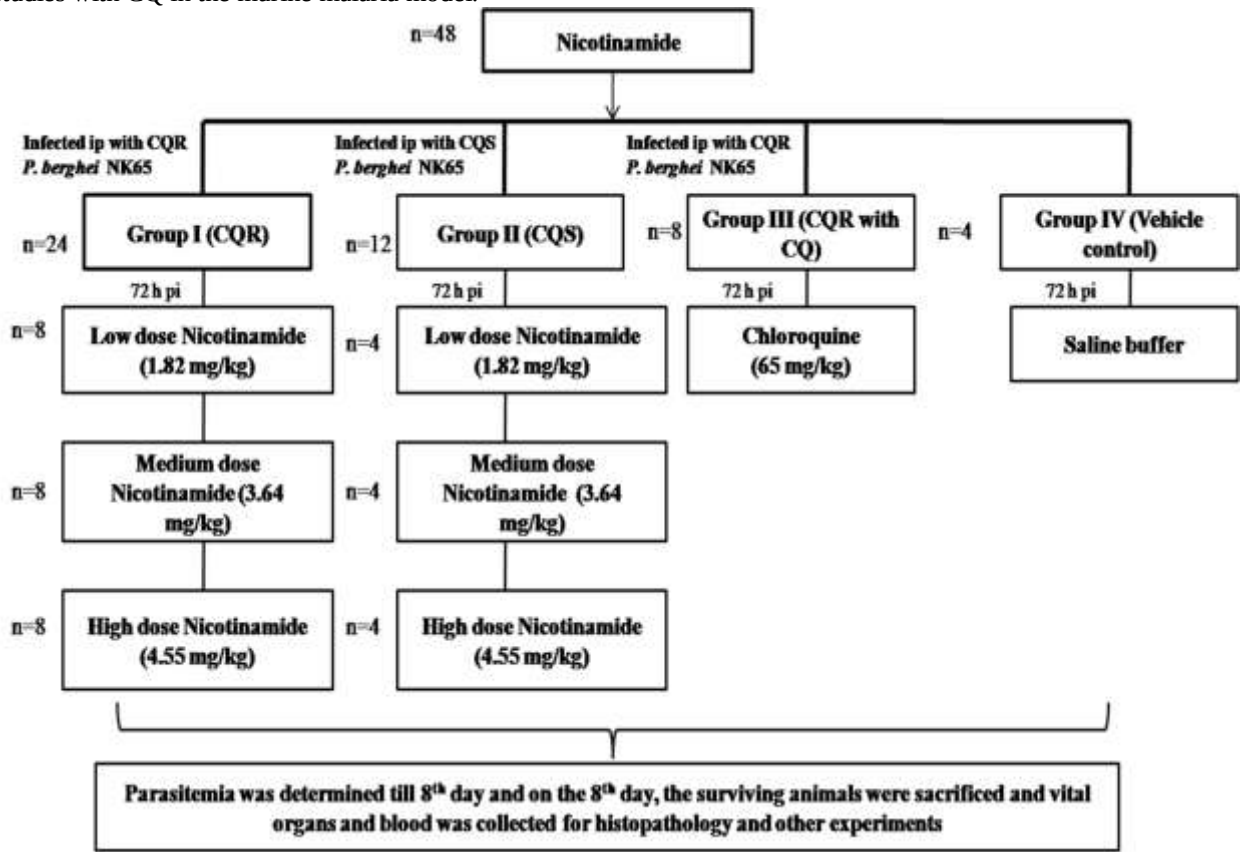

Fig. 1:- Flow chart of the allocation of mice infected with *Pb*NK65 and treated with three different doses of Nicotinamide for selection of dose of nicotinamide for combination therapy with chloroquine.

Combination therapy of Nicotinamide and Chloroquine-

A total of 52 mice were utilized for combination therapy, categorized into three groups: the vehicle group, which received NS (n=8); drug control group, consisting of CQR*Pb*NK65 treated with A+Rif (n=8), CQR*Pb*NK65 treated with CQ (n=8), and CQS*Pb*NK65 treated with CQ (n=8); test drug group consisting of 8 mice in CQR*Pb*NK65 treated with CQ and 4 mice in CQS*Pb*NK65 treated with CQ and 8 mice in CQR*Pb*NK65 treated with CQ for survival study (Fig. 2).

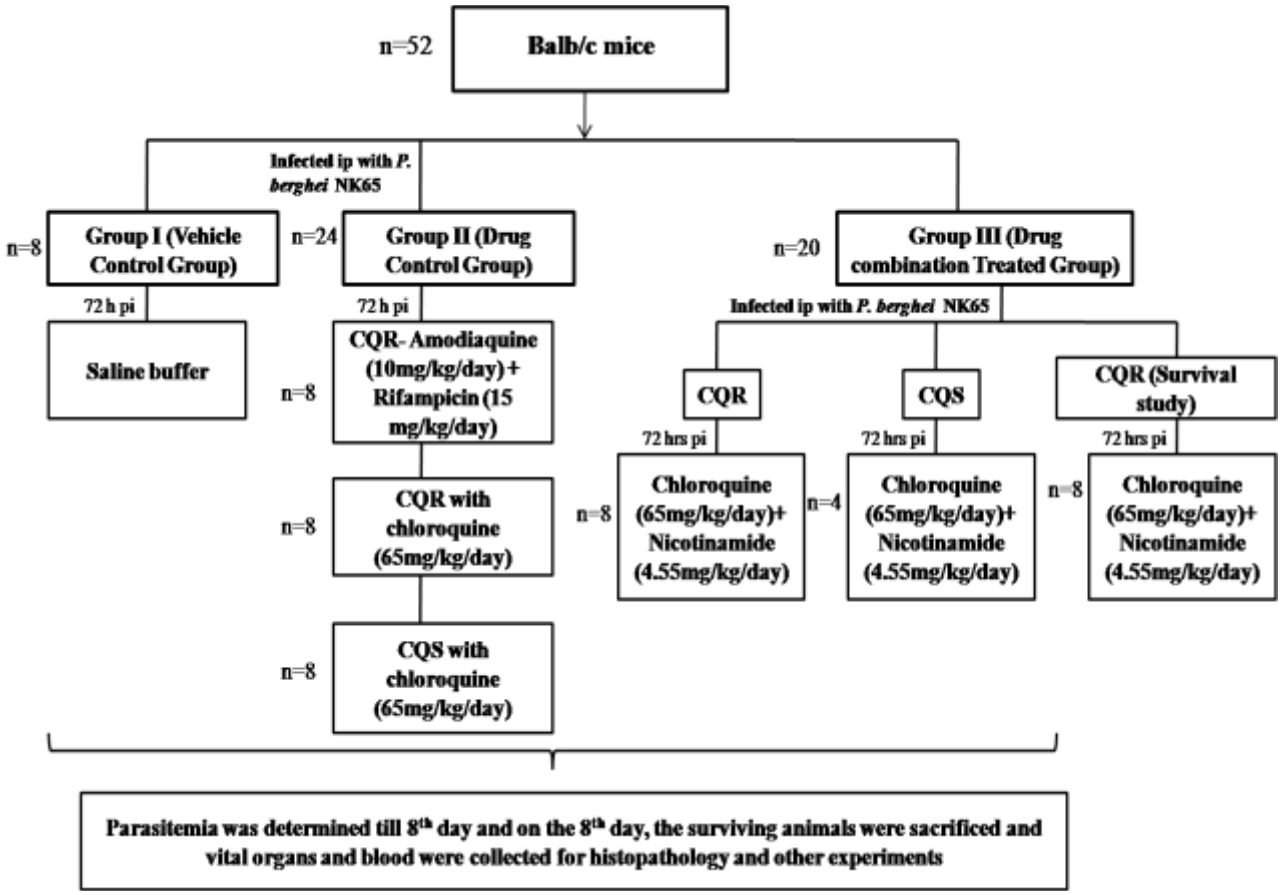

Fig. 2:- Flow chart of the allocation of mice infected with *Pb*NK65 and treated with combination of Chloroquine and Nicotinamide.

Determination of percentage growth inhibition-

The test drug combination group, comprising CQR*Pb*NK65 treated with CQ+NICO (n=8), CQS*Pb*NK65 with CQ+NICO (n=4), and CQR*Pb*NK65 treated with CQ+NICO for a 60-day survival study (n=8) (Fig. 2).

The percentage growth inhibition was determined on the 8th day by the following formula to estimate the curative effect of the administered drugs (Adetutuet al., 2016):

Decrease in Parasitemia (%) = {(APC – APT)/APC} X 100

Where APC= Average percentage parasitemia in the control group; APT= Average percentage parasitemia in the test group.

Monitoring mean survival time for the curative study-

The mean survival time (MST) for eachgroup was determined by recording the days from parasite inoculation to each mouse's death in the curative group over 60 days. MST was calculated using the formula (Birru et al., 2017):

MST = (Total Survival Time of All Mice in a Group) / (Total Number of Mice in the Group)

Blood and tissue collection-

At the experiment's end, surviving animals were euthanized under anesthesia (ketamine 80 mg/kg, xylazine 20 mg/kg body weight). Blood was collected via cardiac punctureand perfusion was done with normal saline. Vital organs (liver, spleen, brain, kidney, lungs) were harvested, washed in sterile PBS, and preserved in 10% buffered formalin for histological analysis. Part of the liver was stored in normal saline for mononuclear cell (MNC) isolation by flow cytometry, and another portion was kept in RNA later at -80°C for mRNA study.

Blood samples were allowed to clot for 30-60 minutes at room temperature and then centrifuged at 3500g for 10 minutes at 4°C to obtain serum. The serum was stored at -20°C. For liver and kidney function assessment, 150-200 µl of serum was set aside. An additional 200 µl of serum was preserved for Nitric oxide (NO) level measurement. Serum samples were pooled when the collected blood volume was insufficient.

Quantitative RT (qRT)-PCR for Plasmodium-specific 18S ribosomal RNA (rRNA) expression study-
RNA isolation and cDNA synthesis-
The drugs impact was assessed by quantifying the parasite load in the liver through *Plasmodium*specific18S rRNA RT-PCR. Liver tissue (50-100 mg) was stored in RNA stabilizing agent (Qiagen, Germany) after animal sacrifice for RNA extraction using the modified TRIzol method (Husse et al., 2019).

Next, 1 µg of isolated RNA was converted into cDNA using the RevertAidTM First strand cDNA synthesis kit (ThermoScientificTM, Lithuania) and specific primers (see Supplemental Table S1, adapted from Gonçalves et al., 2007, and Saita et al., 2014). The synthesized cDNA samples were stored at -20°C until RT-PCR analysis.

RT-PCR-
The protocol used cycling parameters detailed in Supplemental Table S2 to amplify the 18S rRNAgene, with samples run in duplicate. Target gene quantification relied on determining the Ct (Threshold value).

For relative quantitation of target gene expression, the comparative Ct method (ΔΔCt) was employed. The RT-PCR software calculated Ct values for the target gene, endogenous controls, and the reference sample. HPRT, the reference gene, was used as a normalizer in this analysis.

Histopathological Assessment
Histology studies were conducted at the Department of Biotechnology & Experimental Medicine, PGIMER, Chandigarh. On the 8th day, vital organs (liver, spleen, brain, kidney, lungs) were aseptically collected from the experimental groups.

Tissues were fixed in 10% neutral buffered formalin, processed routinely, and stained with Hematoxylin& Eosin to assess organ pathology, as per Basir et al.'s 2012 method. Microphotographs of relevant sections were taken with a light microscope at 20X and 40X (EVOS, Life Technologies, USA). Malaria-specific pathological features in different organs were graded on a scale from absent/negative (0) to severe/abundant/marked (4) (Sinha et al., 2022).

Biochemical parameters-
The assessment of hepatic and renal function involved determining the serum levels of various markers, including Alkaline Phosphatase (ALP), Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), bilirubin concentration, as well as urea and creatinine levels (Shittu et al., 2020). This was accomplished using an automated Analyzer (SIEMENS ADVIA® Chemistry 1200, USA).

Determination of Reactive Oxidative Species (ROS)-
ROS production was studied in lymphocytes (mononuclear cells) from the livers of mice in various groups, sacrificed on the 8th day post-infection. The procedure involved mixing 250 µl of the cell suspension with 3 µl of DCFH-DA dye (Cayman, Ann Arbor, Michigan), followed by 20-minute incubation at 37°C. After centrifugation at 1600 rpm for 5 minutes, the supernatant was removed, and the cells were washed twice in 1X PBS to remove excess dye(Aranda et al., 2013). Finally, the Mean Fluorescence Intensity (MFI) of the cells was measured at 488 nm using the BD FACS CantoTM II instrument.

Determination of Reactive Nitrogen Species (RNS)-
Nitrite levels were estimated in the serum using Griess reagentfrom Sigma-Aldrich, USA (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% napthylaminedihydrochloric acid in water). For this, 100 µl of Griess reagent was mixed with 100µl of the serum sample and incubated at RT for 5 min and Absorbance was determined at 540 nm by micro colorimetric assay (Sinha et al., 2022). The nitrite concentration (expressed in mg/ml) was estimated with reference graph generated using a standard solution of sodium nitrite.

Statistical analysis-
GraphPad PRISM (v.7.00) was used for data analysis. The statistical analysis of inter-group differences of various *in vivo* parameters was determined by unpaired Student's t-test. The data were checked for normality. Comparison between more than two groups was done by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Data were expressed as Mean±SD or Mean±SEM and P-value <0.05 was determined as significant.

Results & Discussion:-

The emergence and spread of multidrug resistance in *Plasmodium* infection remain foremost obstacle and a pending problem for successful chemotherapeutic control of disease. Thus, the development of drugs capable of reversing chloroquine resistance or enhancing its antimalarial properties represents a promising step toward controlling malaria transmission.

Assessment of Mean Parasitemia, Growth Inhibition and Survival Rate-
Nicotinamide, an amide form of Vitamin B3, is obtained by synthesis within the body, through dietary sources, or supplements (Surjana et al., 2010). In recent years, it has garnered attention for its positive effects in various disease conditions, although its role in malaria remains underexplored. In this study, we investigated its antimalarial activity in Chloroquine-resistant malaria, utilizing three different doses ranging from 1.82 mg/kg to 4.55 mg/kg body weight.

Our findings revealed that NICO exhibited the highest antimalarial activity at both low (1.82 mg/kg body weight) and high (4.55 mg/kg body weight) doses when administered alone. However, it had the least effect at the medium dose (3.64 mg/kg body weight) in Chloroquine-resistant infected mice, in comparison of untreated mice (Fig. 3a,3b). The high dose (4.55 mg/kg body weight) resulted in 88% growth inhibition (Fig. 3c) and 100% survival rate (Fig. 3d).These results suggest a hormetic U-shaped dose response, where the effect reverses at higher concentrations. This phenomenon can be explained by the presence of two types of receptors for vitamins: a small number of high-affinity receptors activated at low doses and a large number of low-affinity receptors activated at high doses (Hayes 2008, Calabrese et al., 2010).

In chloroquine-sensitive malaria, both the low dose (1.82 mg/kg body weight) and medium dose (3.64 mg/kg body weight) exhibited similar effects, with increasing drug concentration leading to decreased maximum parasitemia. A high dose of 4.55 mg/kg body weight significantly reduced parasitemia to around 60% (Fig. 3b). Previous studies have also shown that higher doses of NICO inhibit the growth of CQS*P. falciparumin vitro* in a dose-dependent manner. It also blocks the transition of ring stages to trophozoite and acts by regulating Sir2 (Prusty et al., 2008, Bielitza et al., 2015).Thus the high dose of Nicotinamide was selected for combination with CQ for combination therapy.

Combination therapy of Chloroquine and Nicotinamide-
The combination therapy of CQ (65 mg/kg body weight) and HNICO (4.55 mg/kg body weight) significantly reduced parasitemia to 2.83% in the CQR infected group, compared to CQR with (p=0.0495) and without CQ treatment (p=<0.0001). This indicates a synergistic effect of the combination therapy. The anti-plasmodial response was similar to that of the A+Rif combination. In mice infected with CQS parasites and treated with the combination of CQ and HNICO, parasitemia was almost negligible (Fig. 4a). Consequently, the combination inhibited the parasite by 95.27%, and all the animals were alive by the 8th day (Fig. 4b, 4c).

In CQS *P. falciparum*, the combination of CQ and NICO induced a 50% antiparasitic effect at 0.47±0.18mM (Tcherniuk et al., 2017). This synergistic effect may be attributed to the inhibition of heme polymerization by CQ and the simultaneous inhibition of Sir2 function by Nicotinamide.

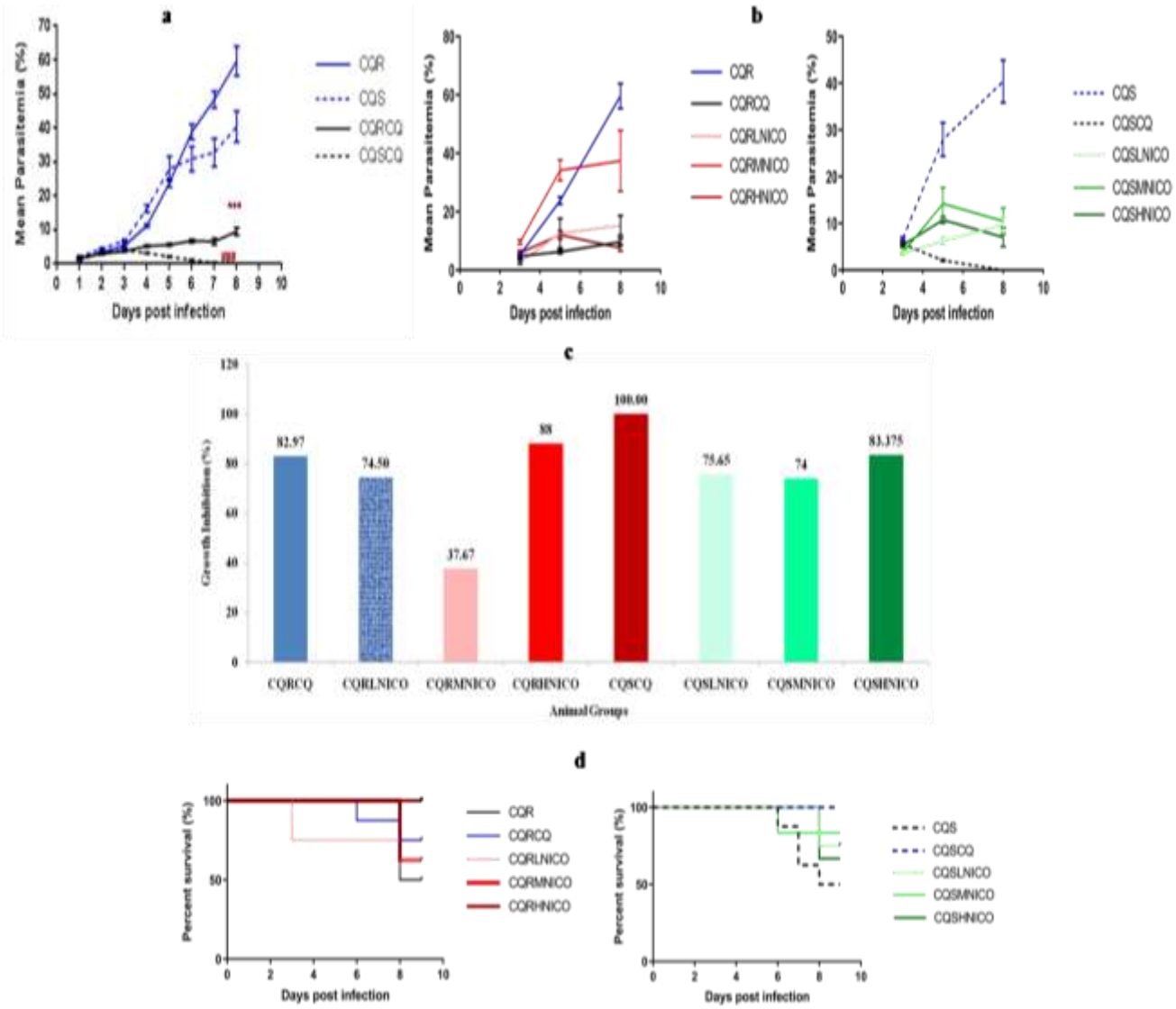

Fig. 3(a) Comparison of Mean parasitemia (%) in mice infected with CQR and CQS *Pb*NK65 at day 8. **(b)** Evaluation of Mean Parasitemia (%) on day 8 in Mice Infected with *Pb*NK65 and subjected to different Nicotinamide dosing regimens. **(c)** Percentage growth inhibition of parasitemia was evaluated on the 8th d in infected mice blood on treatment with different doses of Nicotinamide. **(d)** Kaplan-Meier survival curves for CQR and CQS *Pb*NK65 infected mice treated with different doses of Nicotinamide. CQ- Chloroquine, CQR- Chloroquine resistant strain; CQS- Chloroquine sensitive strain; CQRCQ- Chloroquine resistant *P. b*strain treated with chloroquine (65mg/kg); CQSCQ- Chloroquine sensitive *P. b* strain treated with chloroquine (65mg/kg), LNICO- Low dose (1.82mg/kg); MNICO- medium dose (3.64mg/kg); HNICO- High dose (4.55mg/kg). Data are expressed as Mean±SD or Mean±SEM. Significant difference represented by asterisk ***= p<0.001(CQR vs CQRCQ), ###= p<0.001(CQS vs CQSCQ).

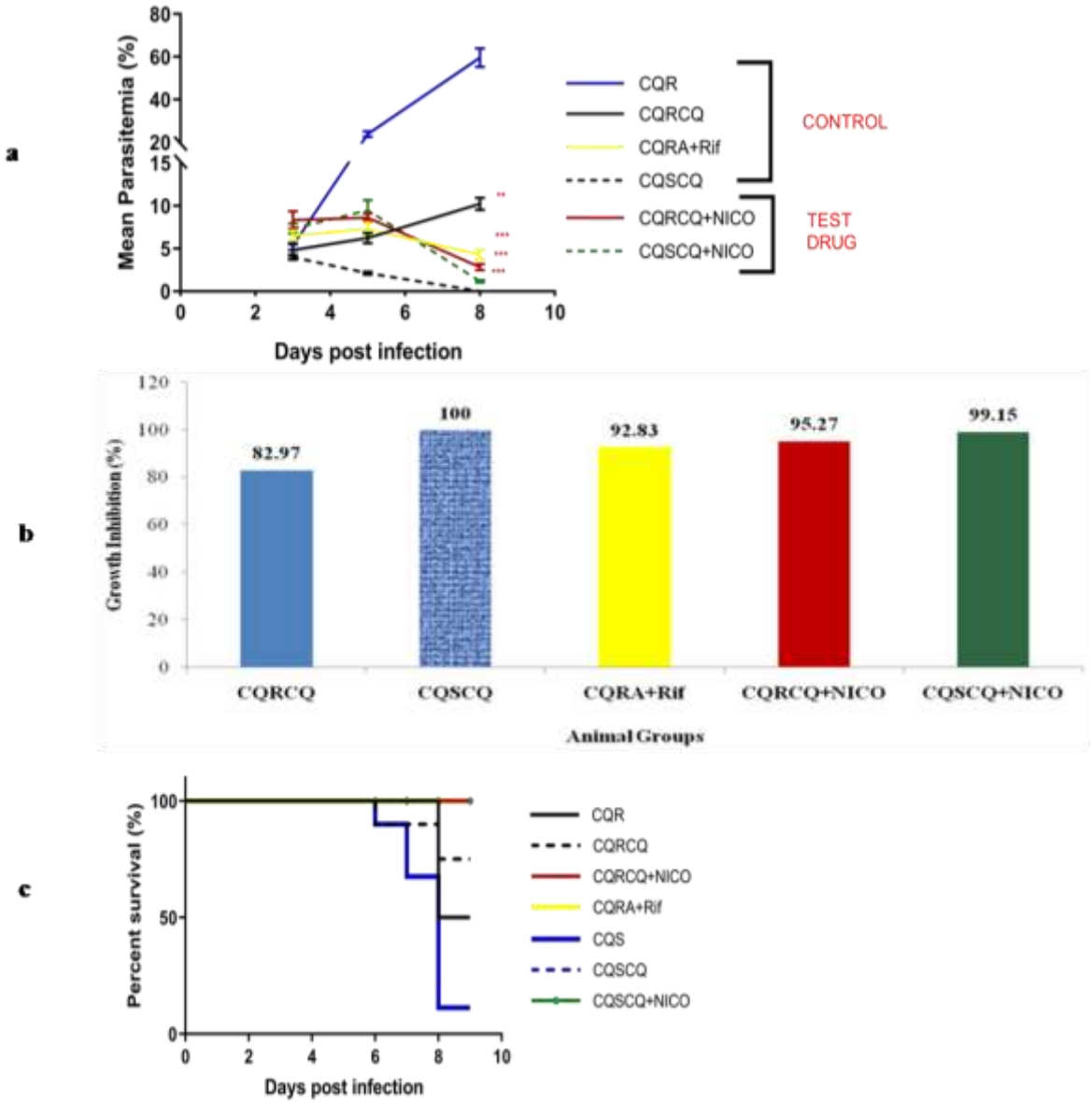

Fig. 4(a) Mean Parasitemia (%) in mice infected with *Pb*NK65 and treated with combination of Chloroquine and Nicotinamide till 8th day. **(b)**Percentage growth inhibition of parasitemia on treatment with combination of chloroquine with high dose of Nicotinamide. **(c)** Kaplan-Meier survival curves for mice infected with *Pb*NK65 and treated with combination of Chloroquine and Nicotinamide till 8th day.CQ- Chloroquine, CQR- Chloroquine resistant strain; CQS- Chloroquine sensitive strain; NICO- Nicotinamide; CQ+NICO– combination of Chloroquine(65 mg/kg) and Nicotinamide(4.55 mg/kg); A+Rif- combination of Amodiaquine(10 mg/kg) and Rifampicin (15 mg/kg); VC- Vehicle group (NS).Data are expressed as Mean±SD or Mean±SEMand significant difference represented by asterisk ***= p<0.001, **=p<0.01, *=p <0.05.

Survival study-

Mean parasitemia (%) in mice infected with the CQR *P.b* CQ+NICO initially increased until the 5th day. Afterwards, it decreased until the 8th day, and then increased again until the 10th day before decreasing once more. By the 33rd day, 5 mice had succumbed to the infection, but 3 mice achieved 0% parasitemia by the 45th day, showing no recurrence until the 60th day. The Mean Survival Time for this group of animals was 35±21.38 days (Fig. 5).

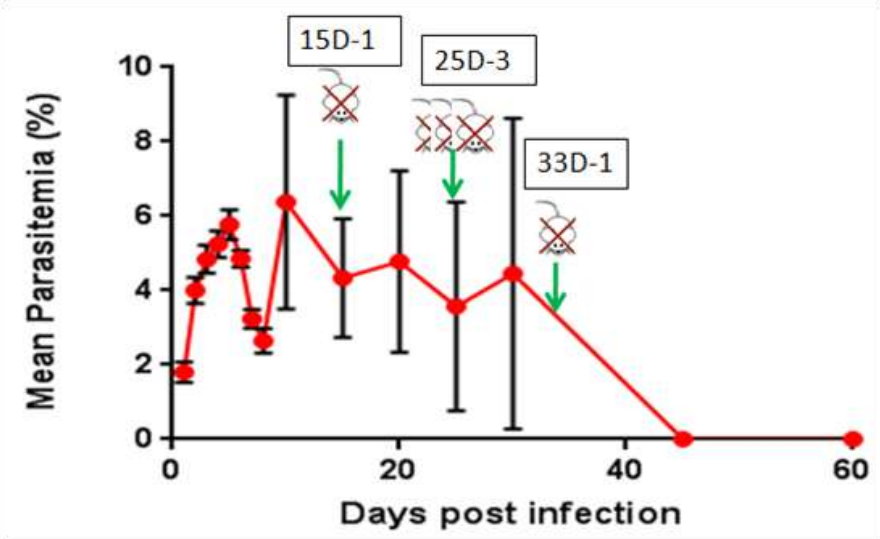

Fig. 5:- Mean parasitemia (%) of the mice infected with CQR *Pb*NK65 and treated with the combination of Chloroquine and Nicotinamide till 8th d and then observed for survival for 60 days. D- day of mouse death

Expression & Quantification of Parasitemia in the liver by Real time PCR-

As malaria progresses, the parasite hides in organs after evading the spleen's defenses. To assess the impact of antimalarial drugs on these hidden parasites, we use Real-Time PCR in the murine liver. This method helps determine the efficacy of drugs with potential antimalarial properties. In our study, we used *Plasmodium*-specific 18S ribosomal RNA (rRNA) expression to measure plasmodial RNA in mice infected with both CQR and CQS *Pb* NK65 strains. Treating the mice with different Nicotinamide doses (LNICO, MNICO, HNICO) significantly reduced 18S rRNA expression ($p<0.0001$) in both CQR and CQS groups (Fig. 6a). Furthermore, combining HNICO with CQ led to a further reduction in 18S RNA expression ($p<0.001$) in both groups (Fig. 6b), suggesting a potential synergistic effect of the combination therapy.

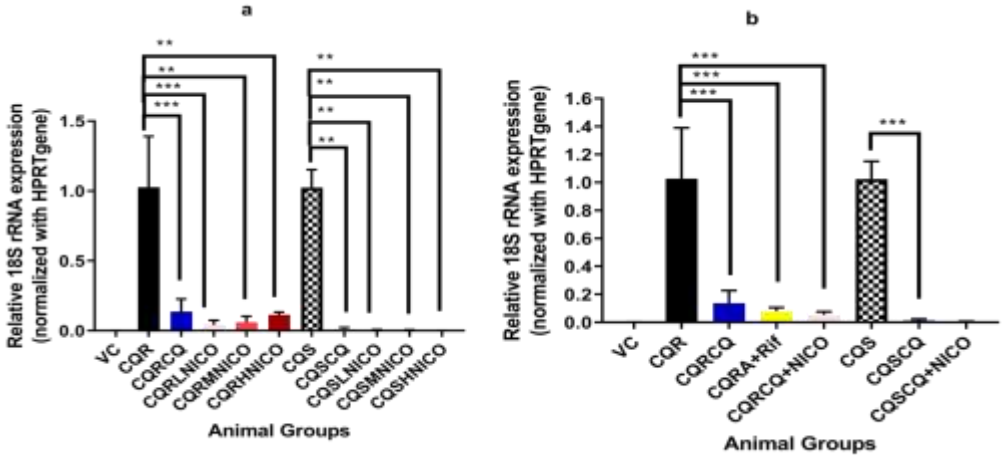

Fig. 6:-Relative fold change expression of 18S rRNA in liver of mice infected *P. berghei*NK65 and treated with (a) different doses of Nicotinamide (b) combination of Chloroquine and Nicotinamide. CQ- Chloroquine; CQR- Chloroquine resistant strain; CQS- Chloroquine sensitive strain; LNICO- Low dose (1.82mg/kg); MNICO- Medium dose (3.64mg/kg); HNICO- High dose(1.82mg/kg); VC-Vehicle group (NS); CQ+NICO- combination of Chloroquine (65 mg/kg) and Nicotinamide (4.55 mg/kg); A+Rif- combination of Amodaquine (10 mg/kg) and Rifampicin (15 mg/kg). Results were analyzed using one way ANOVA followed by Bonferroni multiple comparison test. Error bars represented as standard error and significant difference represented by asterisk ***= $p<0.001$, **=$p<0.01$, *=$p<0.05$.

The potential explanation for the enhanced combined effect of these drugs lies in the way they mutually influence each other's metabolism. Nicotinamide is fully absorbed and undergoes metabolism in the liver, primarily by various cytochrome 450 enzymes. Notably, it inhibits enzymes like CYP2D6, CYP3A4, and CYP2E1 (Gaudineau and Auclair 2004) and is converted into NAD^+. Importantly, chloroquine is metabolized by CYP3A4 (Rendic et al., 2020) and is also a substrate for this enzyme. Consequently, they can interfere with each other's metabolism, potentially affecting their efficacy.

Histomorphological changes in the vital organs and correlation with biochemical parameters

Malaria can have various clinical presentations, ranging from asymptomatic to severe illness, with associated pathological changes. In the liver, LNICO (1.82 mg/kg body weight) and HNICO (4.88 mg/kg body weight) treatments significantly reduced pathology scores compared to untreated groups infected with the CQR strain ($p=0.0023$ and $p=0.012$, respectively). In the CQS group, chloroquine treatment also reduced damage significantly ($p=0.0004$) (Fig. 7a). Notably, when combining CQ (65 mg/kg) with HNICO (4.88 mg/kg), pathology scores decreased even further ($p<0.0001$), resulting in minimal to mild effects compared to severe changes in the untreated group (Fig. 8a).

Treatment with different doses of Nicotinamide resulted in minimal to mild pigment deposition in the liver, contrasting with abundant pigment observed in the untreated group. However, when CQ+NICO combination therapy was administered, there was an absence of pigment (Fig. 9a).

Malaria infection can lead to multi-organ failure, with malarial hepatopathy being a well-known condition. This hepatopathy is associated with the activation of Kupffer cells, which phagocytose malarial pigment or infected red blood cells (Chua et al., 2021). Jaundice resulting from malaria can lead to histopathological changes, including damaged hepatocytes, liver cell congestion, inflammatory infiltrates, cholestasis, hemozoin deposition (Wu et al., 2021; Kochar et al., 2003), and Kupffer cell hyperplasia in malaria patients (Viriyavejakul et al., 2014).

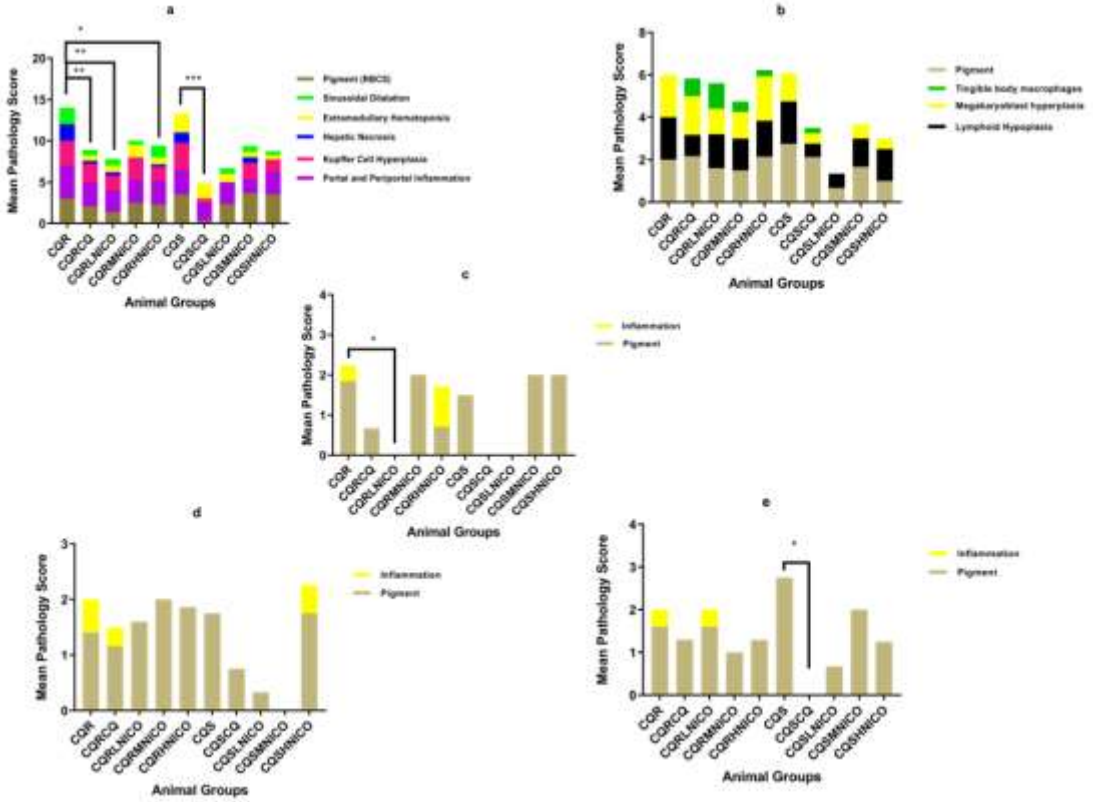

Fig. 7:- Malaria pathology score in various organs of mice infected with *P. berghei*NK65 and treated with different doses of Nicotinamide (a)liver (b)spleen (c)brain (d)kidney (e)lungs. CQ- Chloroquine; CQR- Chloroquine resistant strain; CQS- Chloroquine sensitive strain; LNICO- Low dose (1.82mg/kg); MNICO – Medium dose (3.64mg/kg); HNICO- High dose (4.55mg/kg); VC-Vehicle group (NS).Results were analysed using one way ANOVA followed

by Bonferroni multiple comparison test. Error bars represent standard error and significant difference represented by asterisk ***= p<0.001, **=p<0.01, *=p<0.05.

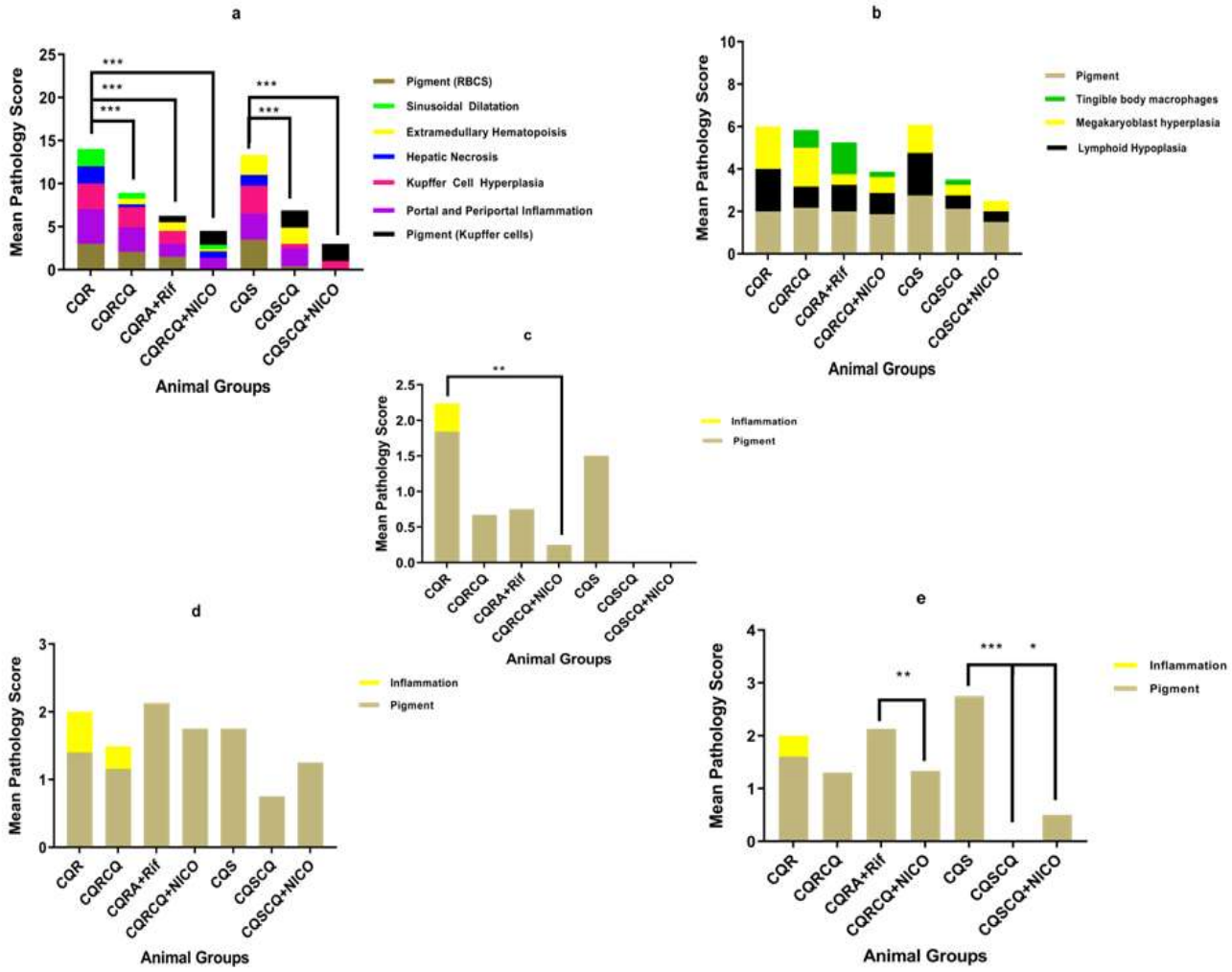

Fig 8:- Malaria pathology score in various organs of mice infected with *P. berghei*NK65 and treated with combination of CQ and Nicotinamide (a)liver (b)spleen (c)brain (d)kidney (e)lungs. CQ- Chloroquine; CQR- Chloroquine resistant strain; CQS- Chloroquine sensitive strain; LNICO- Low dose (1.82mg/kg); MNICO- Medium dose (3.64mg/kg); HNICO- High dose (4.55mg/kg); VC-Vehicle group (NS); CQ+NICO- combination of Chloroquine (65 mg/kg) and Nicotinamide (4.55 mg/kg); A+Rif- combination of Amodaquine (10 mg/kg) and Rifampicin (15 mg/kg).Results were analyzed using one way ANOVA followed by Bonferroni multiple comparison test. Error bars represent standard error (SEM). Significant difference represented by asterisk ***= p< 0.001, **=p<0.01, *=p<0.05.

In brain, there was a significant change in the histology on treatment with low dose of Nicotinamide (1.82 mg/kg body weight) (Fig. 7c), but in combination study the Chloroquine was combined with high dose of Nicotinamide that reduced the pathology significantly as compare to the untreated group, both in CQR infected (p=0.0075) and CQS infected group (<0.0001) (Fig. 8c).

In other organs, like spleen, kidney and lungs; although the histological effects were reduced in treated groups alone (Fig. 7b, 7d, 7e respectively) and in combination with NICO in both CQR and CQS infected mice but the differences were not statistically significant (Fig. 8b, 8d, 8e respectively). The representative images of various organs showing malaria pigment (Fig. 9).

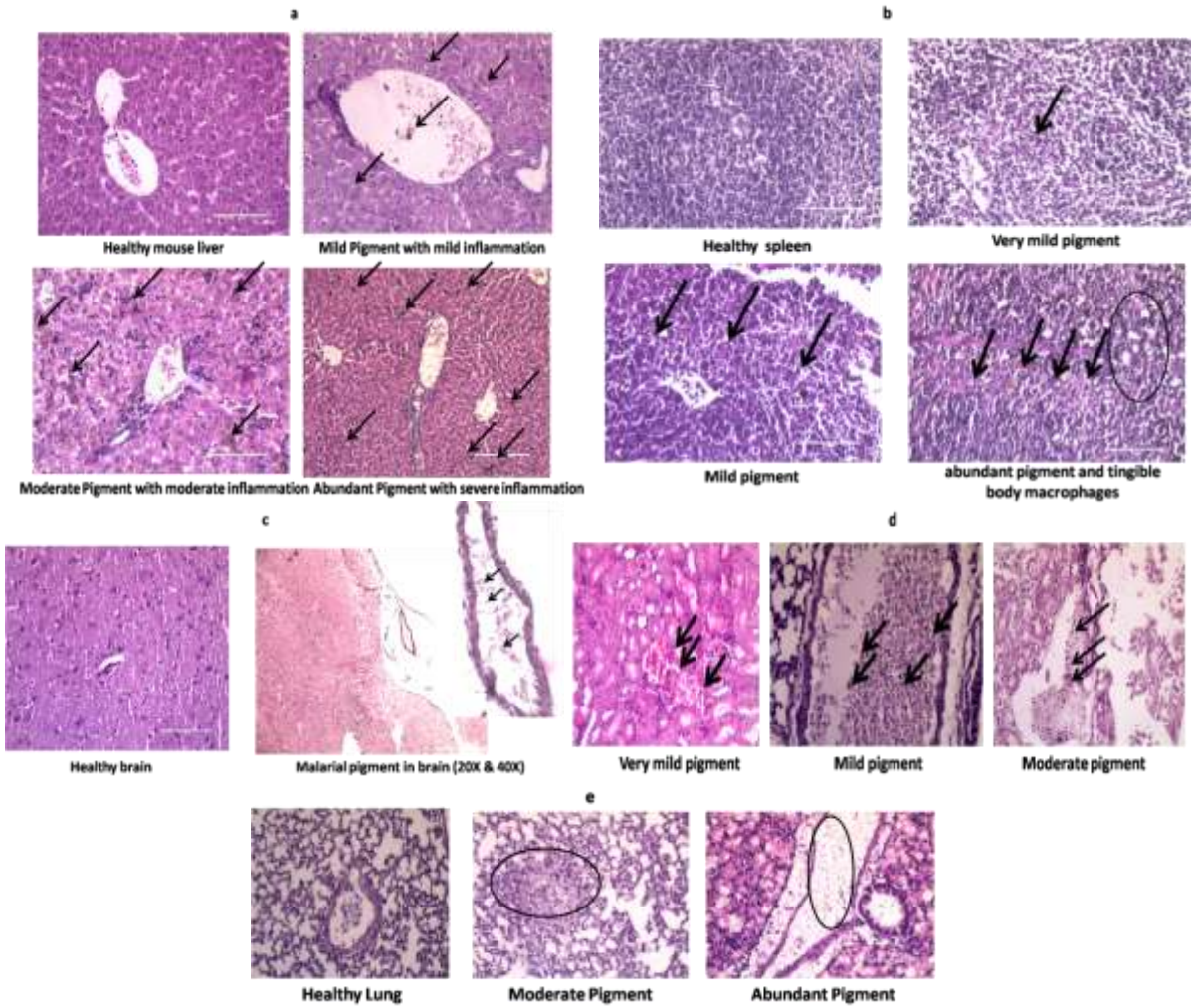

Fig. 9:- Representative images illustrating the histological impacts of rodent malaria across various organs (a)liver, (b)spleen, (c)brain, (d)kidney, (e)lungs.

Effect of drugs on Liver and Kidney Function tests-

Malaria-induced liver damage often elevates liver function enzymes (ALT, AST, bilirubin) significantly, surpassing three times the normal values. This is linked to hepatic oxidative stress (Tirkey et al., 2005; Hafiz et al., 2016). In our study, different Nicotinamide doses (LNICO-1.82 mg/kg, MNICO-3.64 mg/kg, HNICO-4.55 mg/kg body weight) had varied effects: LNICO normalized liver and kidney function enzymes, MNICO increased them, and HNICO showed minimal change (Table 1a). Combining CQ (65 mg/kg body weight) with HNICO (4.55 mg/kg body weight) normalized enzyme levels (Table 1b). Studies in mice infected with *Pb*NK65 have revealed that malaria-induced liver dysfunction raises enzyme levels and induces oxidative stress due to hemozoin deposition, along with the production of pro-inflammatory and anti-inflammatory cytokines (Taramelli et al., 2000; Schwarzer et al., 2015; Gomes et al., 2022).

Table 1:- LFT & RFT levels in serum of *Plasmodium berghei* infected mice treated with (a) different doses of Nicotinamide (b) combination of Chloroquine and Nicotinamide.

a

Animal group	Urea (mg/dl)	Creatinine (mg/dl)	SGOT (AST)	SGPT (ALT)	ALP (U/L)	Bilirubin (mg/dl)
VC	40±4.2	0.14±4.2	338.5±19.3	32.04±6.7	150.35	0

CQR	58.66±27.61	0.34±0.05 (3)	1328±85.3 (4)	433.67±44.8 (12)	103.66±16.2	0.16
CQRCQ	42±7.2	0.31±0. 11	498.66±12	132.3± 0 (4)	145.3±53	0.1
CQRLNICO	49.33±9.07	0.45±0.21	549.14±102.99	113.74±40.80 (3.5)	84.67±3.5	0
CQSLNICO	40±2.8	0.15±0	233.62±25.2	57.335±5.32	146±8.48	0
CQRMNICO	95.5±13.43	0.66+0.06 (5)	1605.9±313.79 (5)	441.5±12.29 (13)	221.5±6.36	0
CQSMNICO	62.67±25.48	0.12±0.09	664.36±469.12	166.22±104.61 (5)	168±28.16	0.03±0.03
CQRHNICO	42.33±4.04	0.67±0.31 (5)	717.7±388.6	192.2±209.43 (6)	161.6±52.3	0
CQSHNICO	126±89.77 (3)	0.15±0.07	1372.72±458.03 (4)	202.69±6.4 (6)	238±6.55	0.14±0.03

CQR- Chloroquine resistant, CQS- Chloroquine sensitive, LNICO- Low dose of Nicotinamide (1.82mg/kg body weight); MNICO-medium dose of Nicotinamide (3.64mg/kg body weight); HNICO- High dose of Nicotinamide (4.55mg/kg body weight); VC- Vehicle control (NS);Data are presented as Mean±SD. Blue color- fold change from 3-10 times in bracket; yellow color- fold change >10 times.

b

Animal group	**Urea (mg/dL)**	**Creatinine (mg/dl)**	**SGOT (AST)**	**SGPT (ALT)**	**ALP (U/L)**	**Bilirubin (mg/dl)**
VC	52.8±3.9	0.18±0.014	157.34±56.99	52.5±10.5	210.8±0.013	0.06±0.03
CQRCQ	70.88±17.4	0.35±0.16	578.38±191.5 (3)	96.63±27.4	474.63±114.4	0.06±0.013
CQSCQ	36.83±4.7	0.07±0.03	306.5±107.6	70±76.4	210.83±50.4	0.07±0.04
CQRA+Rif	39.67±7.68	0.2±0.06	306.67±68.3	63±27.2	222.5±55.07	0.06±0.017
CQRCQ+NICO	45.44±10.5	0.19±0.08	288.57±169.6	49±22.6	188±72.2	0.06±0.04
CQSCQ+NICO	42.33±10.4	0.17±0.05	249±33.77	45.33±19.65	202.33±33.08	0.04±0.04

CQ- Chloroquine, CQR- Chloroquine resistant, CQS- Chloroquine sensitive, NICO- Nicotinamide; LNICO- Low dose (1.82 mg/kg body weight); MNICO-medium dose (3.64 mg/kg body weight); HNICO- High dose (4.55 mg/kg body weight); CQ+NICO- Combination of CQ (65 mg/kg body weight) with Nicotinamide (4.55mg/kg body weight); A+Rif- Combination of Amodiaquine (10 mg/kg) + Rifampicin (15 mg/kg), VC- Vehicle control (NS); Data are presented as Mean±SD. Blue color- fold change from 3-10 times in bracket; yellow color- fold change >10 times.

Estimation of the ROS in liver-

Ongoing research is focused on understanding the complex interplay between ROS, the immune response, and the malaria parasite. Researchers are exploring how ROS production by both host cells and parasites influences the course of infection, disease severity, and potential therapeutictargets. ROS production is not limited to RBCs. Immune cells, such as neutrophils and macrophages, also produce ROS as part of their response to infection. These ROS can contribute to the control and elimination of malaria parasites. Malaria infection can alter the peripheral blood lymphocyte profile, with the extent and type of variation depending on the host's immune system (Antwi-Baffour et al., 2018; Matsumoto et al., 2000). Studies suggest that intracellular reactive oxygen species (ROS) produced by T cells play a role in T cell, activation-induced apoptosis (Peng et al., 2021). Mature RBCs possess

antioxidant properties, promoting T cell survival and expansion by reducing oxidative stress in activated T cells and inhibiting T cell apoptosis, especially in the absence of monocytes (Fonseca et al., 2001; Garner et al., 2021). Therefore, the level of lymphocyte ROS can indirectly indicate the presence of RBCs with antioxidant properties, signifying malarial infection in the system. Furthermore, malaria treatment drugs like chloroquine and artemisinin can act as sources of oxidation, generating free radicals (Pandey et al., 2001;Vasquez et al., 2021). Thus, ROS production by lymphocytes during malaria and its treatment is influenced by various factors.

Being the inaugural exploration of its kind, this study signifies a pioneering initiative directed towards unraveling the intricate dynamics of ROS production in T lymphocytes within the malaria-infected liver. The mean fluorescent intensity levels of ROS in lymphocytes were found after adjusting the gating for lymphocytes (see Supplemental material, Fig. S2). MFI was found to be the lowest with the LNICO (1.82 mg/kg body weight) (~1000) and peaked at the MNICO (3.64 mg/kg body weight) (~11000). Notably, these levels were significantly lower ($p<0.01$) in comparison to both CQR and CQS infected mice, as illustrated in Fig.10a. These findings align with the biochemical parameters and histological observations in the liver across all three Nicotinamide dose groups.

The levels of hemozoin in the NICO-treated groups corresponded closely with the ROS levels, indicating that lymphocytes were activated due to hemozoin-induced liver injury in both CQR and CQS infected groups. It's worth noting that the higher ROS levels observed with MNICO, even exceeding those in the CQR untreated control group, could be attributed to the accumulation of Nicotinamide, potentially due to receptor saturation or the presence of its major metabolite, 2PY (N-methyl-2-pyridone-5-carboxamide), known as a uremic toxin (Lenglet et al., 2016), which tends to accumulate in tissues, including blood (Rutkowski et al., 2008), and may contribute to tissue injury.

The ROS levels then decreased to around 5000, possibly due to the lower affinity of a larger number of receptors, as previously described, with the high dose of Nicotinamide (4.55 mg/kg body weight). It's important to note that while Nicotinamide is generally regarded as an antioxidant, at high doses, it may carry mild or infrequent toxic potential (Knip et al., 2000; Hwang et al., 2020). Moreover, it may not solely suppress ROS generation, and it could even enhance the response of $CD8^+$ T cells in infection-induced pathology, as demonstrated by Choi et al. in 2015.

Combining CQ (65 mg/kg body weight) with HNICO (4.55 mg/kg body weight) significantly lowered ROS levels compared to untreated CQR ($p<0.001$) and CQS ($p<0.01$) groups, as seen in Fig. 10b. This is likely due to the combined effect of CQ's heme-binding property, enhanced by NICO, and Nicotinamide-induced T cell activation, resulting in reduced oxidative stress. The representative fluorescence images of ROS in liver lymphocytes are provided for different Nicotinamide doses (Supplemental Fig. S3.1) and the CQ-NICO combination therapy (Supplemental Fig. S3.2). These findings align with the reduced liver pathology and the restoration of normal liver and renal function test (LFT & RFT) levels following CQ+NICO therapy in both CQR and CQS infected animals.

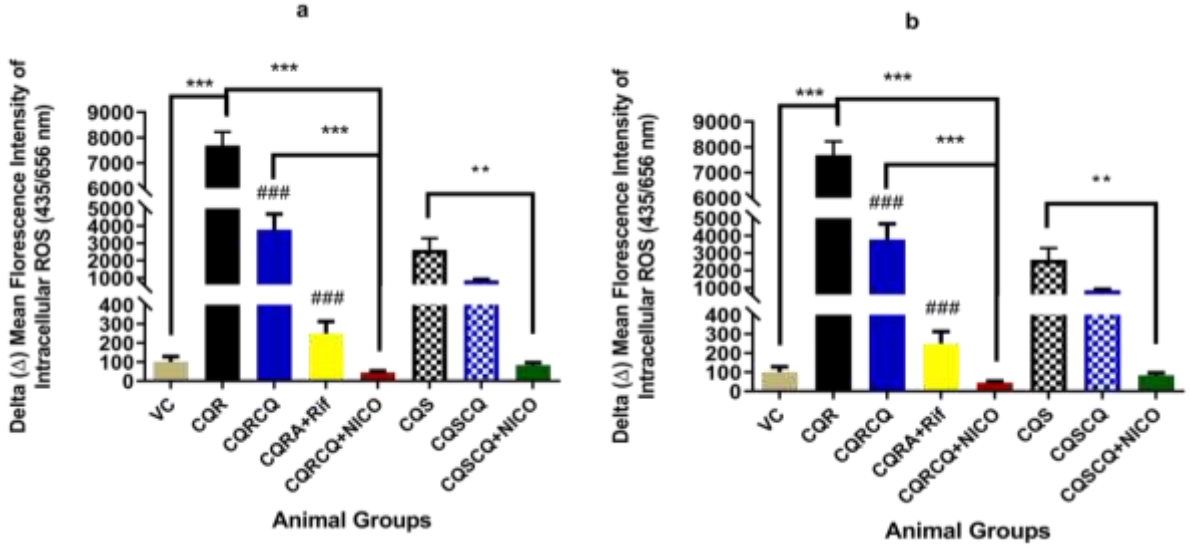

Fig. 10:-Delta (Δ) Mean Florescence Intensity of Intracellular ROS (435/656 nm) in mice liver infected with *Pb*NK 65 and treated with a) different doses of Nicotinamide (b) combination of CQ+NICO. CQR- chloroquine resistant strain; CQS- Chloroquine sensitive; CQ- Chloroquine treated; LNICO- Low dose(1.82 mg/kg); MNICO- Medium

dose (3.64 mg/kg); HNICO- High dose (4.55 mg/kg), CQ+NICO- combination of Chloroquine (65 mg/kg) and Nicotinamide (4.55 mg/kg); A+Rif- combination of Amodiaquine (10mg/kg) and Rifampicin (15 mg/kg); VC- Vehicle group (NS).Results were analysed using one way ANOVA followed by Bonferroni multiple comparison test. Error bars represent standard deviation (SD). Significant difference represented by asterisk ***= p< 0.001, **=p<0.01, *=p<0.05.

Estimation of RNS in serum-

In the context of oxidative stress in malaria, a critical element to consider is the presence of the free radical species, Nitric oxide (NO) (Pabón et al., 2003; Vasquez et al., 2021). Malaria infection triggers a cytokine cascade, resulting in the release of nitrogen and oxygen radicals with potential antiplasmodial properties. NO is generated by a group of enzymes called NO synthases (NOS), which convert L-arginine into L-citrulline. The role of NO in malaria infection is still a subject of debate in scientific literature. Some studies suggest that cerebral malaria may be linked to low NO bioavailability (Gramaglia et al., 2006; Bangirana et al, 2018), while others propose that increased NO levels lead to parasite death (Favre et al., 1997; Dzodzomenyo et al., 2018).Moreover, NO's effects can be seen as either beneficial (Perkins et al., 1999; Chiwakata et al., 2000) or harmful (Weiss et al., 1998), with the overproduction of NO possibly having pathogenic effects (Gomes et al., 2022).

In the present study, NO levels decreased with all Nicotinamide doses- LNICO (1.82 mg/kg body weight), MNICO (3.64 mg/kg body weight), and HNICO (4.55 mg/kg body weight). Notably, in the CQR infected group, a significant decrease was observed with MNICO (3.64 mg/kg body weight), reducing NO levels to 4.1 µg/ml compared to 12.16 µg/ml in untreated mice (p=0.0497). In the CQS infected group, NO levels significantly increased with MNICO (3.64 mg/kg body weight, p<0.001) compared to the untreated group and CQS HNICO (4.55 mg/kg) and CQSCQ (65 mg/kg body weight) (p<0.0001) (Fig. 11a). These findings align with previous studies showing increased NO production during malaria infection (Kremsner et al., 1996; Gomes et al., 2022).

There was a significant decrease in NO levels with the combination of CQ+NICO (p<0.0001), reducing NO levels to 6.26 µg/ml compared to the untreated CQR-infected group. However, in the CQS-infected group, NO levels decreased non-significantly (Fig. 11b). In the context of CQ+NICO combination therapy, a significant difference was observed compared to the untreated group but not significantly less than the CQ-treated group, reinforcing the idea that NO production depends on disease severity and immunity against malaria, which is influenced by the antimalarial properties of the drug. Nicotinamide, known for its antioxidant properties (Zingarelli et al., 1996), is recognized for inhibiting iNOS synthase activity or reducing NO production. This experiment suggests the potential pathogenic role of NO, similar to the effects of NOS inhibitors. While the drugs reduced parasitemia, further investigations are needed to definitively confirm the role of NO in pathology and mortality in this study.

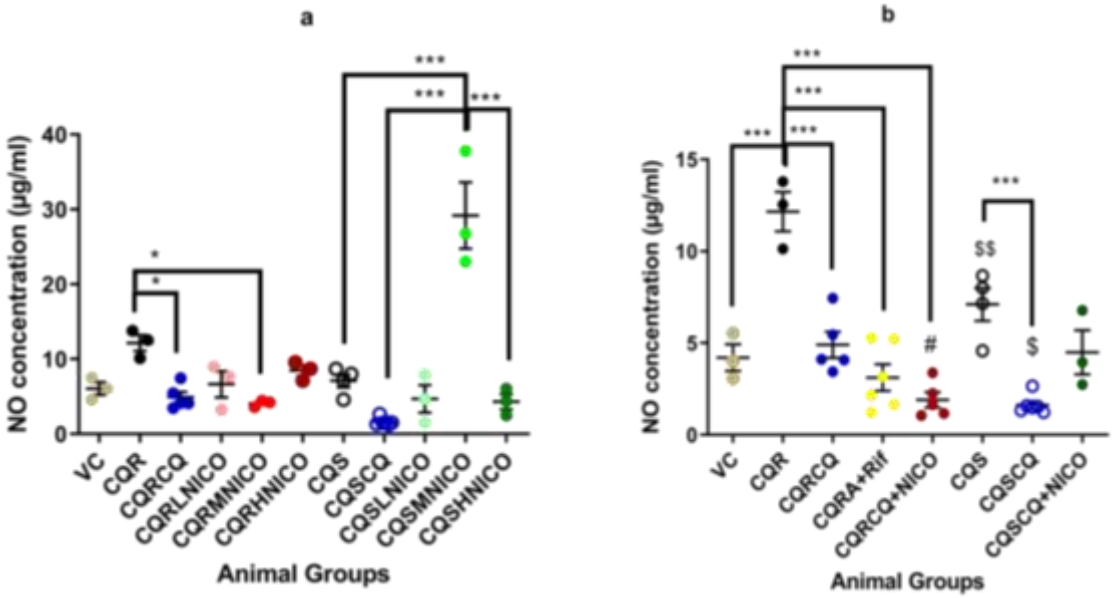

Fig. 11:-Nitric Oxide production in serum of mice infected with *Pb*NK65 and treated with a) different doses of Nicotinamide b) combination of Chloroquine and Nicotinamide. CQR- chloroquine resistant; CQS-

Chloroquinesensitive; CQ- Chloroquine treated; LNICO- Low dose(1.82 mg/kg body weight); MNICO- Medium dose (3.64 mg/kg body weight); HNICO- High dose (4.55 mg/kg body weight), CQ+NICO- combination of Chloroquine (65 mg/kg body weight) and Nicotinamide (4.55 mg/kg body weight); A+Rif- combination of Amodiaquine (10mg/kg body weight) and Rifampicin (15 mg/kg body weight); VC- Vehicle group (NS).Results were analyzed using one way ANOVA followed by Bonferroni multiple comparison test. Error bars represent standard deviation. Significant difference represented by asterisk ***= $p< 0.001$, **=$p<0.01$, *=$p<0.05$.

Conclusion:-

Our findings provide compelling evidence that Nicotinamide holds potential as more than just a vitamin supplement; it can also serve as a valuable anti-malarial agent or an enhancer of anti-plasmodial activity, particularly when used in combination with chloroquine to combat chloroquine-resistant malaria. The observed synergistic effect between Nicotinamide and chloroquine is particularly promising. This suggests that Nicotinamide not only possesses intrinsic anti-malarial properties but also enhances the efficacy of chloroquine, even in the context of resistant strains of malaria. This finding has significant implications for the treatment of malaria, especially in regions where chloroquine resistance is a prevalent issue.

While our study provides valuable insights, several limitations warrant consideration. First, we did not employ specialized staining or molecular techniques to distinguish between live and dead parasites. Second, the blood films were prepared just before euthanizing the animals, making it challenging to determine parasite viability. Furthermore, we recommend future research to explore a wider range of Nicotinamide (NICO) and Chloroquine (CQ) combinations for a more comprehensive understanding of dose-response relationships. A pharmacokinetic study with various time points could further enhance dosing schedule optimization and drug interaction insights. Additionally, expanding ROS and RNS analysis to vital organs like the spleen and brain is essential, given the multi-organ impact of malaria.

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Declarations

- **Ethical Approval (applicable for both human and/ or animal studies. Ethical committees, InternalReview Boards and guidelines followed must be named-** The study was approved by InstitutionalAnimal Ethics Committee (Ref. No. 90/91/IAEC/631) dated 21.09.2018. All mice were handled inaccordance with good animal practice according to the Animal Ethics Procedures and CPCSEA Guidelines (section 2.3)
- **Additional headings with statements on consent to participate and consent to publish are also required)-** Not applicable
- **Competing interests (always applicable and includes interests of a financial or personal nature)**-None
- **Authors contributions (Please ensure that all authors are individually mentioned with their full names)-** Conceptualization- HarpreetKaur, SumeetaKhurana, BikashMedhi; Study Supervision- SumeetaKhurana, BikashMedhi, AbhishekMewara; Experimentation- HarpreetKaur; Histopathology analysis- HarpeetKaur, Alka Bhatia; Data curation- HarpreetKaur; Draft Preparation- HarpreetKaur; Revision and editing- HarpreetKaur, SumeetaKhurana, RakeshSehgal
- **Funding (it should be provided in the Declarations, separate from the Acknowledgements)-** This Research work did not receive any specific grant from any funding agency.
- **Availability of data and materials (a statement on how any datasets used can be accessed)**-It will be made available on demand.

APPENDIX A. Supplementary Data

References:-

1. Adetutu, A.; Olorunnisola, O.S.; Owoade, A.O.; Adegbola, P. Inhibition of in vivo growth of *Plasmodium berghei* by Launaeataraxacifolia and Amaranthusviridis in mice. Malaria research and treatment. 2016, 2016.
2. Antwi-Baffour, S.; Kyeremeh, R.; Buabeng, D.; Adjei, J.K.; Aryeh, C. et al. Correlation of malaria parasitaemia with peripheral blood monocyte to lymphocyte ratio as indicator of susceptibility to severe malaria in Ghanaian children. Malaria journal. 2018, 17(1), 1-9.

3. Aranda, A.; Sequedo, L.; Tolosa, L.; Quintas, G.; Burello, E.; Castell, J.V.; Gombau, L. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: a quantitative method for oxidative stress assessment of nanoparticle-treated cells. Toxicology *in vitro*. 2013, 27(2), 954-963.
4. Badejo, J.A.; Abiodun, O.O.; Akinola, O.; Happi, C.T.; Sowunmi, A.; Gbotosho, G.O. Interaction between rifampicin, amodiaquine and artemether in mice infected with chloroquine resistant *Plasmodium berghei*. Malaria journal. 2014, 13(1), 1-6. https://doi.org/10.1186/1475-2875-13-299.
5. Bangirana, P.; Conroy, A.L.; Opoka, R.O.; Hawkes, M.T.; Hermann, L.; Miller, C.; Namasopo, S.; Liles, W.C.; John, C.C.; Kain, K.C. Inhaled nitric oxide and cognition in pediatric severe malaria: A randomized double-blind placebo controlled trial. PLoS One. 2018,13(1), e0191550.
6. Bielitza, M.; Belorgey, D.; Ehrhardt, K.; Johann, L.; Lanfranchi, D.A.; Gallo, V.; Schwarzer, E.; Mohring, F.; Jortzik, E.; Williams, D.L.; Becker, K. Antimalarial NADPH-consuming redox-cyclers as superior glucose-6-phosphate dehydrogenase deficiency copycats. Antioxidants & Redox Signaling. 2015, 22(15), 1337-1351.
7. Birru, E.M.; Geta, M.; Gurmu, A.E. Antiplasmodial activity of Indigoferaspicata root extract against *Plasmodium berghei* infection in mice. Malaria journal. 2017, 16(1), 7. https://doi.org/10.1186/s12936-017-1853-5.
8. Braidy, N.; Berg, J.; Clement, J.; Khorshidi, F.; Poljak, A.; Jayasena, T.; Sachdev, P. Role of nicotinamide adenine dinucleotide and related precursors as therapeutic targets for age-related degenerative diseases: rationale, biochemistry, pharmacokinetics, and outcomes. Antioxidants & redox signaling. 2019, 30(2), 251-294. https://doi.org.10.1089/ars.2017.7269.
9. Calabrese, V.; Cornelius, C.; Dinkova-Kostova, A.T.; Calabrese, E.J; Mattson, M.P. Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. Antioxidants & redox signaling. 2010, 13(11), 1763-1811. https://doi.org/10.1089/ars.2009.3074.
10. Chiwakata, C.B.; Hemmer, C.J.; Dietrich, M. High levels of inducible nitric oxide synthase mRNA are associated with increased monocyte counts in blood and have a beneficial role in *Plasmodium falciparum* malaria. Infection and immunity. 2000, 68(1), 394-399. https://doi.org/10.1128/IAI.68.1.394-399.2000.
11. Choi, H.J.; Jang, S.Y.; Hwang, E.S. High-dose nicotinamide suppresses ROS generation and augments population expansion during $CD8^+$ T cell activation. Molecules and Cells. 2015, 38(10), 918. https://doi.org/10.14348/molcells.2015.0168.
12. Chua, C.L.L.; Ng, I.M.J.; Yap, B.J.M.; Teo, A. Factors influencing phagocytosis of malaria parasites: the story so far. Malaria journal. 2021, 20, 1-15. https://doi.org/10.1186/s12936-021-03849-1
13. Dzodzomenyo, M.; Ghansah, A.; Ensaw, N.; Dovie, B.; Bimi, L.; Quansah, R.; Gyan, B.A.; Gyakobo, M.; Amoani, B. Inducible nitric oxide synthase 2 promoter polymorphism and malaria disease severity in children in Southern Ghana. PloS one. 2018, 13(8), e0202218.
14. Favre, N.; Ryffel, B.; Bordmann, G.; Rudin, W. The course of *Plasmodium chabaudichabaudi* infections in interferon-gamma receptor deficient mice. Parasite immunology. 1997, 19(8), 375-383. https://doi.org/10.1046/j.1365-3024.1997.d01-227.x.
15. Fonseca, A.M.; Porto, G.; Uchida, K.; Arosa, F.A. Red blood cells inhibit activation-induced cell death and oxidative stress in human peripheral blood T lymphocytes. Blood, The Journal of the American Society of Hematology. 2001, 97(10), 3152-3160. https://doi.org/10.1182/blood.v97.10.3152.
16. Gerner, M.C.; Bileck, A.; Janker, L.; Ziegler, L.S.; Öhlinger, T.; Raeven, P.; Müllner, E.W.; Salzer, U.; Gerner, C.; Schmetterer, K.G.; Baron, D.M. Packed red blood cells inhibit T-cell activation via ROS-dependent signaling pathways. Journal of Biological Chemistry. 2021, 296. https://doi.org/10.1016/j.jbc.2021.100487.
17. Gomes, A.R.Q.; Cunha, N.; Varela, E.L.P.; Brígido, H.P.C.; Vale, V.V. et al. Oxidative stress in malaria: potential benefits of antioxidant therapy. International Journal of Molecular Sciences. 2022, 23(11), 5949. https://doi.org/10.3390/ijms23115949.
18. Gonçalves, L.A.; Vigário, A.M.; Penha-Gonçalves, C. Improved isolation of murine hepatocytes for *in vitro* malaria liver stage studies. Malaria journal. 2007, 6, 1-8. https://doi.org/10.1186/1475-2875-6-169.
19. Gramaglia, I.; Sobolewski, P.; Meays, D.; Contreras, R.; Nolan, J.P.; Frangos, J.A.; Intaglietta, M.; Van Der Heyde, H.C. Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. Nature medicine. 2006, 12(12), 1417-1422. https://doi.org/10.1038/nm1499.
20. Hafiz, T.A.; Mubaraki, M.A.; Al-Quraishy, S.; Dkhil, M.A. The potential role of *Punicagranatum* treatment on murine malaria-induced hepatic injury and oxidative stress. Parasitology research. 2016, 115, 1427-1433. https://doi.org/10.1007/s00436-015-4876-2.
21. Hayes, D.P. Adverse effects of nutritional inadequacy and excess: a hormetic model. The American journal of clinical nutrition. 2008, 88(2), 578S-581S. https://doi.org/10.1093/ajcn/88.2.578S.
22. Husse; Jana; Leliavski; Alexei, et al. RNA isolation using TRIzol® Reagent from mice tissues. 2019.

https://doi.org/10.13140/RG.2.2.32087.62882.
23. Hwang, E.S.; Song, S.B. Possible adverse effects of high-dose nicotinamide: mechanisms and safety assessment. Biomolecules. 2020, 10(5), 687. https://doi.org/10.3390/biom10050687.
24. Jonas, W. B.; C. P. Rapoza; W. F. Blair. The effect of niacinamide on osteoarthritis: a pilot study. Inflammation Research.1996, 45, 330-334. https://doi.org/10.1007/BF02252945.
25. Kabiru, A.Y.; Abdulkadir, A.; Gbodi, T.A.; Bello, U.M.; Makun, H.A.; Amah, D.J.; Ogbadoyi, E.O. Evaluation of Haematological Changes in *Plasmodium berghei*-infected mice administered with aqueous extract of Phyllantusamarus. Pakistan journal of biological sciences:PJBS. 2013. https://doi.org/10.3923/pjbs.2013.510.516.
26. Knip, M.; Douek, I.F.; Moore, W.P.T.; Gillmor, H.A.; McLean, A.E.M.; Bingley, P.J.; Gale, E.A.M.; ENDIT Group. Safety of high-dose nicotinamide: a review. Diabetologia. 2000, 43, 1337-1345. https://doi.org/10.1007/s001250051536.
27. Kochar, D.K.; Agarwal, P.; Kochar, S.K.; Jain, R.; Rawat, N. et al. Hepatocyte dysfunction and hepatic encephalopathy in *Plasmodium falciparum* malaria. Qjm. 2003, 96(7), 505-512. https://doi.org/10.1093/qjmed/hcg091.
28. Kremsner, P.G.; Greve, B.; Lell, B.; Luckner, D.; Schmid, D. Malarial anaemia in African children associated with high oxygen-radical production. The Lancet. 2000, 355(9197), 40-41.
29. Lenglet, A.; Liabeuf, S.; Bodeau, S.; Louvet, L.; Mary, A.; Boullier, A.; Lemaire-Hurtel, A.S.; Jonet, A.; Sonnet, P.; Kamel, S.; Massy, Z.A. N-methyl-2-pyridone-5-carboxamide (2PY)—major metabolite of nicotinamide: an update on an old uremic toxin. Toxins. 2016. 8(11), 339. https://doi.org/10.3390/toxins8110339.
30. Manhas, P.L.; Sharma, M.; Mewara, A.; Sachdeva, M.U.; Sehgal, R.; Malhotra, P. Dynamics of *Plasmodium berghei* NK-65 parasitaemia and $CD3^+$ $CD4^+$ $CD25^+$ Fox-$p3^+$ T-regulatory cells in experimentally induced malaria during early, mid, and late-pregnancy in BALB/c mice. Indian Journal of Microbiology. 2023, 63(3), 380-385.
31. Martin, S.K.; Oduola, A.M.; Milhous, W.K. Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science.1987, 235(4791), 899-901. https://doi.org/10.1126/science.3544220.
32. Matsumoto, J.; Kawai, S.; Terao, K.; Kirinoki, M.; Yasutomi, Y.; Aikawa, M.; Matsuda, H. Malaria infection induces rapid elevation of the soluble Fas ligand level in serum and subsequent T lymphocytopenia: possible factors responsible for the differences in susceptibility of two species of Macaca monkeys to *Plasmodium coatney*iinfection. Infection and immunity. 2000, 68(3), 1183-1188. https://doi.org/10.1128/iai.68.3.1183-1188.2000
33. Olliaro, P.; Cattani, J.; Wirth, D. Malaria, the submerged disease. Jama. 1996, 275(3), 230-233. http://doi:10.1001/jama.1996.03530270070034.
34. Pandey, A.V.; Bisht, H.; Babbarwal, V.K.; Srivastava, J.; Pandey, K.C.; Chauhan, V.S. Mechanism of malarial haem detoxification inhibition by chloroquine. Biochemical Journal. 2001, 355(2), 333-338. https://doi.org/10.1042/0264-6021:3550333.
35. Peng, H.Y.; Lucavs, J.; Ballard, D.; Das, J.K.; Kumar, A.; Wang, L.; Ren, Y.; Xiong, X.; Song, J. Metabolic reprogramming and reactive oxygen species in T cell immunity. Frontiers in immunology. 2021,12, 652687. https://doi.org/10.3389/fimmu.2021.652687.
36. Perkins, D.J.; Kremsner, P.G.; Schmid, D.; Misukonis, M.A.; Kelly, M.A.; Weinberg, J.B. Blood mononuclear cell nitric oxide production and plasma cytokine levels in healthy Gabonese children with prior mild or severe malaria. Infection and immunity. 1999, 67(9), 4977-4981. https://doi.org/10.1128/IAI.67.9.4977-4981.1999.
37. Prusty, D.; Mehra, P.; Srivastava, S.; Shivange, A.V.; Gupta, A.; Roy, N.; Dhar, S.K. Nicotinamide inhibits *Plasmodium falciparum* Sir2 activity *in vitro* and parasite growth. FEMS microbiology letters. 2008, 282(2), 266-272. https://doi.org/10.1111/j.1574-6968.2008.01135.x.
38. Rendic, S.; Guengerich, F.P. Metabolism and interactions of chloroquine and hydroxychloroquine with human cytochrome P450 enzymes and drug transporters. Current drug metabolism. 2020, 21(14),1127-1135. https://doi.org/10.2174/1389200221999201208211537.
39. Saita, S.; Shirane, M.; Ishitani, T.; Shimizu, N.; Nakayama, K.I. Role of the ANKMY2-FKBP38 axis in regulation of the Sonic hedgehog (Shh) signaling pathway. Journal of Biological Chemistry. 2014, 289(37), 25639-25654. https://doi.org/10.1074/jbc.M114.558635.
40. Schwarzer, E.; Arese, P.; Skorokhod, O.A. Role of the lipoperoxidation product 4-hydroxynonenal in the pathogenesis of severe malaria anemia and malaria immunodepression. Oxidative medicine and cellular longevity. 2015, 2015. https://doi.org/10.1155/2015/638416.
41. Shittu, O.; Opeyemi, O.A.; Salawu, M.K.; Ashiru, A.A.; Medaiyese, S.A. et al. Alterations in histological, biochemical and hematological parameters in *Plasmodium berghei* NK-65 infected balb/c mice treated with

Brideliaferruginea stem bark extract. Journal of Complementary and Integrative Medicine. 2020, 18(1), 93-105. https://doi.org/10.1515/jcim-2018-0219

42. Sinha, S.; Medhi, B.; Radotra, B.D.; Batovska, D.I.; Markova, N.; Bhalla, A.; Sehgal, R.. Antimalarial and immunomodulatory potential of chalcone derivatives in experimental model of malaria. BMC Complementary Medicine and Therapies. 2022, 22(1), 1-14. https://doi.org/10.1186/s12906-022-03777-w.
43. Surjana, D.; Halliday, G.M.; Damian, D.L. Role of nicotinamide in DNA damage, mutagenesis, and DNA repair. Journal of nucleic acids. 2010, 2010. https://doi.org/10.4061/2010/157591
44. Taramelli, D.; Recalcati, S.; Basilico, N.; Olliaro, P.; Cairo, G. Macrophage preconditioning with synthetic malaria pigment reduces cytokine production via heme iron-dependent oxidative stress. Laboratory Investigation. 2000, 80(12), 781-1788. https://doi.org/10.1038/labinvest.3780189.
45. Tcherniuk, S.O.; Chesnokova, O.; Oleinikov, I.V.; Oleinikov, A.V. Nicotinamide inhibits the growth of *P. falciparum* and enhances the antimalarial effect of artemisinin, chloroquine and pyrimethamine. Molecular and biochemical parasitology. 2017, 216, 14-20. https://doi.org/10.1016/j.molbiopara.2017.06.004.
46. Tirkey, N.; Pilkhwal, S.; Kuhad, A.; Chopra, K. Hesperidin, a citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. BMC pharmacology. 2005, 5, 1-8. https://doi.org/10.1186/1471-2210-5-2.
47. Vasquez, M.; Zuniga, M.; Rodriguez, A. Oxidative stress and pathogenesis in malaria. Frontiers in cellular and infection microbiology. 2021, 11, 68182.
48. Viriyavejakul, P.; Khachonsaksumet, V.; Punsawad, C. Liver changes in severe *Plasmodium falciparum* malaria: histopathology, apoptosis and nuclear factor kappa B expression. Malaria journal. 2014, 13(1), 1-10. https://doi.org/10.1186/1475-2875-13-106.
49. Weiss, G.; Thuma, P.E.; Biemba, G.; Mabeza, G.; Werner, E.R.; Gordeuk, V.R. Cerebrospinal fluid levels of biopterin, nitric oxide metabolites, and immune activation markers and the clinical course of human cerebral malaria. Journal of Infectious Diseases. 1998, 177(4), 1064-1068. https://doi.org/10.1086/515229.
50. Wu, Y.; Huang, S.; Xiao, S.; He, J.; Lu, F. Impact of galectin-receptor interactions on liver pathology during the erythrocytic stage of *Plasmodium berghei* malaria. Frontiers in Immunology. 2021, 12, 758052. https://doi.org/10.3389/fimmu.2021.758052.
51. Zingarelli, B.; Salzman, A.L.; Szabó, C. Protective effects of nicotinamide against nitric oxide-mediated delayed vascular failure in endotoxic shock: potential involvement of polyADPribosylsynthetase. Shock (Augusta, Ga.). 1996, 5(4), 258-264. https://doi.org/10.1097/00024382-199604000-00005.