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Abstract: The objective was to determine the effect of combined antituberculosis (anti-TB) drug therapy and an antioxidant, melatonin, on the free radical and organic acid profiles in an experimental rat model. A combined anti-TB drug, Rifater, consisting of 12.0 mg rifampicin, 0.8 mg isoniazid, and 23.0 mg pyrazinamide and 18.56 μg melatonin/kg body weight per day (corresponding to average physiological human intake) were orally administered to Sprague–Dawley rats. Hydroxyl radical production was monitored by quantifying 2,3-dihydroxybenzoic acid produced after intraperitonial sodium salicylate injections. Organic acid extractions and gas chromatography-coupled mass spectrometry analyses were performed on collected urine samples. The results show hydroxyl radicals ($P = 0.0019$) and organic acids (P-value range: 0.037 to < 0.001), characteristic of a multiple acyl-CoA dehydrogenase defect (MADD), were elevated with Rifater treatment and these elevations were significantly lowered with melatonin pretreatment (P-value range: 0.031 to < 0.001), probably because of its inherent antioxidant activity. We conclude that hydroxyl radical production and an increased organic acid profile induced by anti-TB medication indicates inhibition of the electron transport chain. We also conclude that free radicals leading to clinical symptoms associated with an MADD metabolic profile induced by anti-TB treatment could be alleviated by melatonin intervention.

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Introduction

Treatment of pulmonary tuberculosis (TB) consists of two phases, i.e. an intensive phase of treatment with four anti-TB drugs (2 months) and then a maintenance phase for the rest of the treatment period (4 months). TB treatment is carried out using a combined therapy of anti-TB drugs which include isoniazid, rifampicin, ethambutol and pyrazinamide with less risk for development of drug resistance. Various combinations of anti-TB drugs, such as Rifater, are commercially available as a single administration, which constitutes a fixed combination of isoniazid, rifampicin and pyrazinamide and this three-drug combination is widely used in TB treatment. However, while these agents are effective antibiotics, TB patients treated with rifampicin, pyrazinamide, and isoniazid can experience hepatotoxicity resulting in a discontinuation of treatment in these patients [1,2]. Anti-TB chemotherapy can also be associated with a number of other side-effects which include myocardial damage, respiratory complications [3], hypoglycaemia [4], neurological complications [5–7], and acidosis [6].

At present, very little is known about the metabolism of anti-TB chemotherapeutics. It is however well known that the catalase enzyme of *Mycobacterium tuberculosis* is responsible for the conversion of isoniazid to its active bactericidal equivalent isonicotinic acid hydrazide as well as acetylisoniazid [8]. Superoxide is formed during isoniazid oxidation and is thought to be involved in the activation process [9]. Further isoniazid metabolites that have been detected in humans and rats include isonicotinic acid, isonicotinylglycine [10], acetylhydrazine, and diacetylhydrazine [11]. This can also take place via host-specific enzymes, such as N-acetyltransferase [12]. Rifampicin is already in its active state at the stage of administration and can be converted into two inactive products, Rip-Ma 3-formyl-23-[*O*-(alpha-D-ribofuranosyl)] and Rip-Mb 23-[*O*-(alpha-D-ribofuranosyl)] by mycobacteria [13]. A number of rifampicin metabolites have also been identified in human plasma, urine and saliva [14, 15]. Pyrazinamide in turn is metabolized into a number of metabolites which include pyrazinoic acid, 5-hydroxypyrazinoic acid, 5-hydroxypyrazinamide [16] and pyrazinuric acid [17]. Although a number of drug metabolites have been identified, their effects in vivo have not yet been fully established.

The safety of anti-TB treatment is presently under scrutiny and various drug combinations and treatment times have been suggested to alleviate the burden of toxic side-effects [2]. Here we show that the anti-TB drug combination, Rifater, increases the oxidative stress in an animal model and affects the organic acid profiles. We also show that the application of the antioxidant, melatonin,

alleviates this oxidative stress and negates the associated increased organic acid profiles.

Materials and methods

Melatonin administration

Melatonin (Sigma, St Louis, MO, USA) was administered to mildly anaesthetized experimental animals by gastric gavage in a saline suspension with commercially available maize meal. Each animal was given 18.56 μg melatonin/kg body weight (corresponding to reported physiological human intake) [18]. The maximum volume per gavage was 1.0 mL for a 500–600 g animal.

Sodium salicylate

Radical assessment was carried out as previously described [19]. In brief, sodium salicylate (Sigma) was administered to all experimental animals under mild general anaesthesia at a dose of 20 mg/kg body weight. Salicylate spontaneously binds hydroxyl radicals ($^{\bullet}$OH) as they are generated to form 2,3- and 2,5-dihydroxybenzoic acid (DHBA) which is excreted [20] and can be quantified via gas chromatography-coupled mass spectrometry (GC-MS). Ingelman-Sundberg et al. [21] however determined that cytochrome P450 catalyses the formation of 2,5-DHBA, but not 2,3-DHBA. Hence, the measurement of 2,3-DHBA provides a method for monitoring the formation of $^{\bullet}$OH [21].

In vivo study

Thirty normal adult (> 500 g) Sprague–Dawley rats were obtained from the central breeding facility of the University of Stellenbosch and housed in standard laboratory rodent cages in a thermally controlled environment with free access to water and standard rodent diet. All procedures were carried out with the necessary ethical approval, according to the Helsinki Protocol and MRC and NIH regulations for the treatment of laboratory animals. Rats received Rifater FC (Aventis, Paris, France) orally in a dose representing 12.0 mg rifampicin, 0.8 mg isoniazid, 23.0 mg pyrazinamide per 1 kg body weight, and 18.56 μg melatonin/kg body weight via gastric gavage under general anaesthesia (described above). Experimental animals were fasted 4 hr prior to intraperitonial injection of 20 mg/kg body weight sodium salicylate. Table 1 gives a layout of animal groups for various experimental procedures for 2,3-DHBA determinations. For organic acid determination, the same procedure was followed in the absence of salicylic acid. The animals were then transferred to standard metabolic cages for 24 hr with ad libidum access to water only. Urine samples were collected at 6-hr intervals in a 1.0 M hydrochloric acid solution on ice and then stored at −80°C for analysis using GC-MS.

Creatinine determinations

Creatinine determinations were made using the methodology described by Chalmers and Lawson [22]. Creatinine determination on each of the urine samples was completed prior to organic acid extraction. Creatinine values are used to normalize results obtained from different sources [23] and the values (in mg%) indicate the amount of urine to be used, the amount of internal standard to be added, as well as the volumes of bis-trimethylsilyl-trifloroacetamide (BSTFA) and trimethy-chlorosilane (TMCS) to be used for derivatization. The creatinine values were determined spectrophotometrically by a standard procedure using the Technicon RA 100 analyser system (Technicon Instruments, Tarrytown, NY, USA).

Organic acid extractions

An amount of urine, as determined from the creatinine value, was acidified with 5 N HCL (Merck, Darmstadt, Germany) to a pH $<$ 2. The internal standard (3-phenylbutyric acid, Sigma) was added as a volume of 25 μmol/mg creatinine, followed by the addition of 6 mL of ethylacetate (Sigma) to the mixture. The solution was shaken for 10 min and centrifuged at 710 g at room temperature for 1 min. The organic phase (top phase) was removed with a Pasteur pipette and placed into a second large teflon culture tube. Diethylether (Sigma) (3 mL) was added to the aqueous phase and the solution was shaken for 10 min and centrifuged for 1 min as before. The organic phase was again removed and added to the first organic phase. An excess of anhydrous Na_2SO_4 (approximately 2 mg) (Sigma) was added to remove any

Table 1. Grouping of experimental rats in various treatment protocols

Group	Animal numbers	Treatment
1 (control group)	1–6	Days 1–6: saline; day 7: 20 mg/kg sodium salicylate administered prior to urine collection
2	7–12	Days 1–6: Rifater FC; day 7: Rifater FC and 20 mg/kg sodium salicylate administered prior to urine collection
3	13–18	Days 1–7: 18.56 μg/kg melatonin; day 8–14: Rifater FC and 18.56 μg/kg melatonin; day 15: 18.56 μg/kg melatonin, Rifater FC and 20 mg/kg sodium salicylate administered prior to urine collection
4	19–24	Days 1–6: saline; day 7: once off Rifater FC and 20 mg/kg sodium salicylate administered prior to urine collection
5	25–30	Days 1–6: saline; day 7: once off Rifater FC, 18.56 μg/kg melatonin and 20 mg/kg sodium salicylate administered prior to urine collection

remaining water from the sample. The tube was briefly vortexed and centrifuged for 5 min as before. The organic phase was then decanted from the pellet into a small Teflon-lined culture tube and dried under nitrogen.

Derivatization and analysis by GC-MS

The dry organic acid extract was derivatized at 70°C for 30 min with 22.6 μL BSTFA/μmol creatinine (Sigma) and 4.5 μL TMCS/μmol creatinine (Sigma). Of this, 0.2 μL was injected into the GC-MS.

The GC-MS analyses were carried out using an Agilent 6890 GC ported to a 5973 mass selective detector (Palo Alto, CA, USA). For the acquisition of an electron ionization mass spectrum, an ion source temperature of 200°C and electron energy of 70 eV was used. The gas chromatograph was equipped with an SE-30 capillary column, a split/splitless injection piece (250°C) and a direct GC-MS coupling (260°C). The splitless injection (0.6 μL) was used during the MS analysis. Helium (1 mL/min) was used as the carrier gas. An oven temperature of 60°C, isometric for 2 min, was used as an initial temperature after which a rise of 4°C/min was continued until a temperature of 120°C was reached. This was followed by a temperature increase of 10°C/min until a final temperature of 280°C was reached. This temperature was then maintained for a further 4 min.

Data processing

Statistical analyses were carried out using one-way ANOVA of the area under the curve (AUC) of the various treatment groups. Tukey post-hoc comparisons were made to determine statistical difference. All P-values < 0.05 were considered statistically significant.

Results

Our aim was to evaluate the contribution of the Rifater FC combinational anti-TB drug formulation to the $^{\bullet}$OH and organic acid profiles in an animal model, as well as to test the effects that melatonin would have on these. $^{\bullet}$OH produced by Rifater in the presence and absence of melatonin were monitored by measuring the production of 2,3-DHBA. Table 2 and Fig. 1 indicate that administration of anti-TB drugs 7 days prior to salicylate administration and urine collection (group 2) resulted in a marked increase in 2,3-DHBA ($P = 0.0019$).

Preconditioning the animal model with 18.56 μg/kg melatonin for 7 days prior to a 7-day Rifater/melatonin co-administration (i.e. a total of 14 days prior to salicylate administration and urine collection: group 3) resulted in a significant reduction ($P = 0.038$) below that of the control levels (group 1) of the free radical burst observed when Rifater is administered alone ($P < 0.001$). A once off Rifater–melatonin co-treatment (group 5) prior to sodium salicylate injection did not result in any significant changes when compared with that of a Rifater single treatment as for experimental animal group 4. Both groups 4 and 5 followed the same trend as the 7-day Rifater treatment group (results not shown).

Table 2. Statistical comparison of organic acid values between experimental animal groups

	MAUC	S.E.M.	P-values
2,3-DHBA			
Control	1.12(a,c)	0.23	a = 0.019*
Rifater	1.99(a,b)	0.24	b < 0.00l*
Rifater + melatonin	0.34(b,c)	0.14	c = 0.038*
Butyrylglycine			
Control	22.60(a, c)	4.17	a = 0.001*
Rifater	82.39(a,b)	6.09	b = 0.031*
Rifater + melatonin	45.87(b,c)	2.85	c = 0.212
Ethylmalonic acid			
Control	27.41(a,c)	3.82	a = 0.004*
Rifater	58.16(a,b)	8.24	b = 0.012*
Rifater + melatonin	31.155(b,c)	5.82	c = 0.864
2-Methylbutyrylglycine			
Control	3.2l(a, c)	1.68	a < 0001*
Rifater	19.13(a,b)	2.82	b < 0.001*
Rifater + melatonin	5.38(b,c)	1.43	c = 0.742
Suberic acid			
Control	1.97(a,c)	0.73	a = 0.037*
Rifater	6.77(b,c)	1.41	b = 0.889
Rifater + melatonin	2.79(b,c)	1.32	c = 0.888
Isovalerylglycine			
Control	6.88(a,c)	0.77	a = 0.001*
Rifater	24.62(a,b)	1.90	b = 0.002*
Rifater + melatonin	7.98(b,c)	2.15	c = 0.957

Mean values with lettering in common were compared, '*' indicates significant difference ($P < 0.05$).

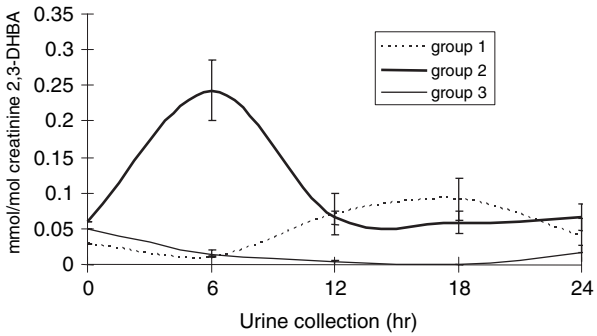

Fig. 1. Experimental animals were pretreated with Rifater and melatonin, after which urine was collected at different time points and the 2,3-dihydroxybenzoic acid quantified by GC-MS. Group 1 represents the controls with sodium salicylate alone; group 2 animals were pretreated with Rifater, and group 3 were pretreated with melatonin for 7 days followed by Rifater and melatonin for 7 days. Urine collections were made at 0–6, 6–12, 12–18, and 18–24 hr to obtain the 6, 12, 18 and 24 hr values, respectively. Each data point is calculated as an average of six values. Bars indicate the S.E.M.

Comparison of the organic acid profiles in the animals given the anti-TB drugs to those of the controls showed that the following metabolites occurred in significantly higher concentrations: isovalerylglycine, ethylmalonic acid, butyrylglycine, 2-methylbutyrylglycine and suberic acid (Fig. 2, Table 2).

This organic acid profile is characteristic of a multiple acyl-CoA dehydrogenase defect (MADD). Melatonin pretreatment prior to a 7-day Rifater/melatonin co-administration (i.e. a total of 14 days prior to salicylate administration and urine collection: group 3) resulted in a significant decrease in all the above-mentioned organic acids induced by the Rifater (except for suberic acid, $P = 0.889$) and consequently the organic acid concentration change between the control group and the melatonin pretreatment group became nonsignificant. The discrepancy in the suberic acid may be because of the higher S.E.M. for the melatonin pretreatment group. The raw data showed one animal in the group to have a higher suberic acid production than the others in the same group. This may be because of differences in metabolism, or an underlying infection. Exclusion of this animal from the group resulted in a significant P value of 0.03, when comparing the melatonin pretreatment group to the Rifater group.

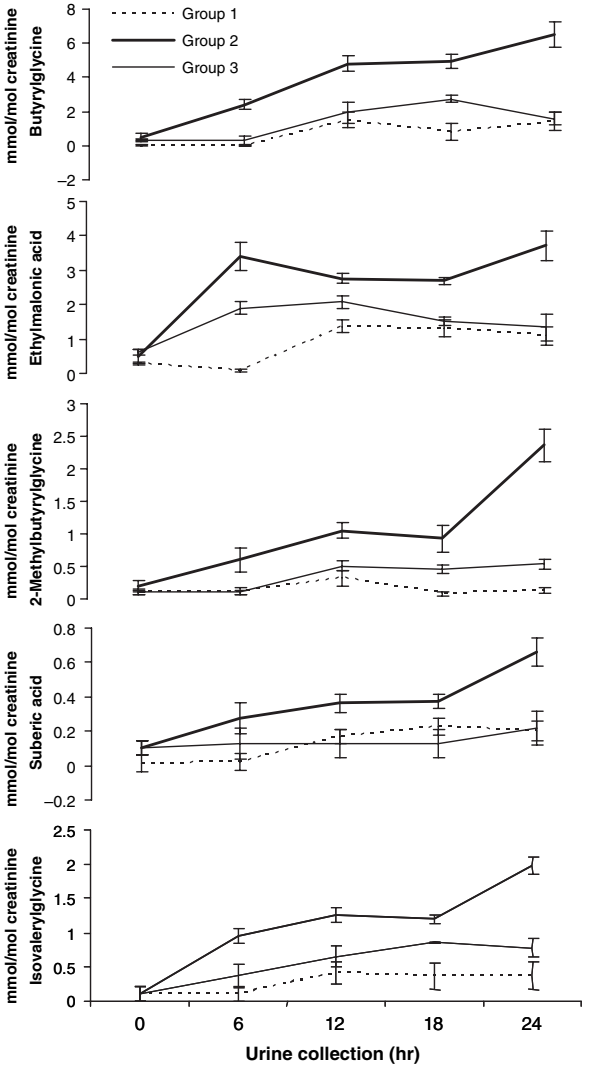

Fig. 2. Concentrations of organic acids in rats characteristic of an MADD profile, analysed by GCMS. The following treatment protocols are represented – group 1: distilled water administered at time 0; group 2: Rifater administered alone at time = 0 hr; group 3: pretreated with melatonin for 7 days followed by Rifater and melatonin for 7 days. Urine collections were made at 0–6, 6–12, 12–18, and 18–24 hr to obtain the 6, 12, 18, and 24 hr values, respectively. Each data point is calculated as an average of six values. Bars indicate the S.E.M.

Additionally, the Rifater-treated animals show a large increase in the concentrations of the MADD metabolites over a 24-hr period when compared with the control animals, which show little fluctuation between their beginning and end concentrations (Fig. 2). The average concentrations over the 24-hr period between the Rifater-treated and the control animals are statistically, significantly different ($P = 0.0065$) for isovalerylglycine, 2-methylbutyrylglycine, butyrylglycine, ethylmalonic acid, and suberic acid combined. The increase of the organic acids over 24 hr was also significant ($P = 0.0013$). Pretreatment of the experimental animals with melatonin gave significantly reduced organic acids over the 24-hr treatment period ($P = 0.048$) and, consequently, the organic acid concentration change between the control animals and the melatonin-treated animals was nonsignificant ($P = 0.083$). The significance of the increase of organic acids over 24 hr displayed the same tendency as for the average concentrations described above. Melatonin pretreatment significantly reduced the formation of the individual MADD metabolites induced when Rifater was administered alone ($P < 0.0001$).

Discussion

Although radical species may not initiate disorders such as cardiovascular disease [24, 25], they may influence disease progression significantly. In patients with pulmonary TB, inflammation-related oxidative stress has been implicated in the pathogenesis of lung fibrosis and dysfunction [26, 27]. This fibrosis is thought to be mediated by activated macrophages which are capable of releasing a variety of chemicals including oxygen free radicals [28, 29].

It is already known that isoniazid, rifampicin, and pyrazinamide, alone or in combination are responsible for oxidative stress and increased lipid peroxidation [30–33]. Isoniazid also exerts control over the electron transport chain in mycobateria at the level of NADH dehydrogenase [34]. Together these actions generate additional oxidative stress through the generation of superoxide [35], which is converted by superoxide dismutase (SOD) to hydrogen peroxide. In the presence of iron, hydrogen peroxide forms $^{\bullet}OH$ [35, 36]. Lipid peroxidation and the subsequent inhibition of the electron transport chain can lead to a secondary overflow of electrons and thus generate additional oxidative stress [35].

Our results clearly show that the increase in free radicals at 6 hr in the experimental animal model used was because of the administration of anti-TB drugs in the form of the combination therapeutic agent Rifater FC. It appears that animals could not effectively compensate for the increased $^{\bullet}OH$ production by endogenous antioxidant systems. TB-infected patients may experience a similar systemic radical increase after each daily intake of anti-TB therapy as the role of free radicals in TB patients was also demonstrated recently showing the total antioxidant

status in patients are lowered following anti-TB drug treatment [37].

Melatonin is well known as an efficient antioxidant and has been shown to alleviate the burden of oxidative stress by free radical scavenging in a magnitude of living systems [38–43]. Melatonin has no known toxicity [39] in persons taking it on a daily basis [44]. Melatonin is also known to reduce drug toxicity [45] and increase the efficacy of antibacterial drugs which include the anti-TB drug isoniazid [46]. Melatonin treatment was carried out at physiological dosages, daily for 7 days prior to initiation of the 7-day melatonin–Rifater co-administration followed by urine collection. At this concentration melatonin completely abolished the free radical burst induced by Rifater. This could be due to melatonin's free radical scavenging ability and/or its effect on upregulation of antioxidant enzymes such as SOD [47]. Apart from this, melatonin may further reduce the occurrence of these $^{\bullet}$OH induced by Rifater by reducing electron leakage at mitochondrial level [48]. Melatonin is reported to stabilize mitochondrial inner membranes by interaction with the lipid bilayers and in so doing improve electron transport chain activity [48]. However, melatonin did not have any free radical reducing effect in a single co-administration dose of melatonin and Rifater (group 5) and the resulting free radical profile followed that of the 7-day Rifater treatment profile (group 2). It would appear that melatonin preconditioning prior to anti-TB therapy is effective in counteracting the subsequent free radical formation or that higher than physiological melatonin concentrations are necessary for once-off treatments.

A second observation following treatment with Rifater was the overall significant increase in concentration of certain organic acids over the 24-hr experimental period. The organic acids analysed fit a profile for MADD, an autosomal recessive inborn error of metabolism [49] associated with a defect on either the electron transport flavoprotein (ETF) or the ETF dehydrogenase enzyme [50–52]. The electron transport inhibition and the consequent $^{\bullet}$OH and abnormal organic acid profile associated with MADD may be involved in the side-effects which individuals experience with TB treatment.

Treatment of MADD usually involves L-carnitine supplementation [53–55], which, similar to melatonin, has antioxidant properties [56, 57]. Melatonin, at the experimental concentration used, lowered the overall organic acid concentration significantly to levels where the elevated organic acid levels relative to the control became nonsignificant. This lowered organic acid profile may be ascribed to the direct action of melatonin preventing electron leakage from the mitochondria and preserving the integrity of mitochondrial inner membrane [58]. In addition, melatonin has a high redox potential and either accepts or donates electrons, thereby promoting electron flow [58].

The increased $^{\bullet}$OH and organic acids associated with an MADD metabolic profile suggests Rifater's involvement in the inhibition of the electron transport chain. This is further substantiated by melatonin's ameliorative effect on the above-mentioned side-effects induced by Rifater. The organic aciduria profiles suggest that the elevation of toxic organic acids should be addressed in TB patient treatment, as this could manifest a secondary disease situation which could be alleviated by dietary intervention. Whether oxidative stress is a primary factor in the pathogenesis of TB needs to be established, but it may contribute considerably to the pathology of the disease. Monitoring oxidative stress is an important step towards protecting against the damaging effect of free radicals and allows subsequent changes in diets and, if necessary, the administration of antioxidant supplementation.

Acknowledgements

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