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Role of melatonin in modulation of oxidative stress induced by delta-aminolevulinic acid in adult male albino rats.

El-Sawi, MR, Badawy, ME and El-Gharieb, NM.
Zoology Department, Faculty of Science, Mansoura University.

Abstract

Backgrounds: Delta-aminolevulinic acid (ALA) is a heme precursor that accumulates in acute intermittent porphyria (AIP) due to enzymatic deficiencies in the heme biosynthetic pathway, its accumulation has been associated with several symptoms because it works as an endogenous source of reactive oxygen species, which can exert oxidative damage to cell structures. The present work was designed to examine the ability of melatonin, a well known antioxidant and a free radical scavenger secreted from the pineal gland, to revert ALA-promoted damage in brain, liver and kidney of rats.

Results: The present data demonstrated that chronically ALA-treated rats (40 mg / kg body wt day after day for 14 days) exhibited very highly significant increases in malondialdehyde (MDA) and protein carbonyl (PC) whereas the level of glutathione (GSH) was significantly diminished in the tissue homogenates of all tested organs (brain, kidney & liver). Among antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH-Rd), glutathione-S-transferase (GST) activities were significantly diminished by ALA treatment. Intraperitoneal injection of melatonin (10 mg / kg body weight, every day for 14 consecutive days) significantly ameliorated all the tested parameters. Melatonin moderately increased SOD, CAT, GSH-Rd and GST activities, thereby counteracting the oxidative stress induced by ALA. Nevertheless, exogenous ALA caused a strong net rise in MDA and PC and a significant decrease in GSH when given together with ALA, melatonin antagonized these effects and largely protected the integrity of tissue structures.

From the present data, the protection of melatonin against ALA oxidative stress is obviously, so it is well recommended to use melatonin in patients suffering from symptoms related to ALA accumulation.

Key words: Delta-aminolevulinic acid, oxidative stress, melatonin, malondialdehyde, protein carbonyl, glutathione, superoxide dismutase, catalase, glutathione-reductase, glutathione-S-transferase.

Introduction

ALA is the first metabolite of the biosynthetic pathway of the heme group. It accumulates in the blood and other tissues; mainly the liver and brain of patients with hereditary porphyria (e.g., acute intermittent porphyria) and acquired porphyria (e.g., lead poisoning porphyria), where it triggers serious tissue and neurological damage (Bechara *et al.*, 2006).

ALA dehydratase (ALA-D) is an essential enzyme for all aerobic organisms because it participates in the biosynthetic pathway of tetrapyrrole molecules, which constitute prosthetic groups of physiologically important proteins such as hemoglobin and cytochromes (Jaffe *et al*., 1995 and Sassa, 1998). ALA-D is a sulfhydryl-containing enzyme; consequently, its activity is highly sensitive to the presence of prooxidant element, which can oxidize its -SH groups (Barbosa *et al*., 1998 and Soares *et al*., 2003). Inhibition of ALA-D activity leads to an accumulation of ALA which undergoes auto-oxidation inducing free radicals and in this way induced lipid peroxidation (Hermes-lima *et al*., 1991). AIP is an inherited disease, characterized by a prophobilinogen deaminase deficiency and, as a consequence, accumulation of ALA, primarily in the liver (Kappas *et al*., 1983).

Pervious studies have shown, both *in vitro* and *in vivo* that ALA by it self is a pro-oxidant (Monteiro *et al*., 1989 and Neal

et al., 1997). ALA is moreover able to mobilize iron from firritin and so is assumed to promote its auto-oxidation (Demasi *et al*., 1996). Over production and accumulation of ALA, as it occurs in AIP, can be the origin of an endogenous source of ROS, which can then exert their oxidative damage to cell structure (Princ *et al*., 1997). This precursor of heme rapidly undergoes enolization and subsequently aerobic oxidation at pH 7.0- 8.0 with the formation of reactive oxygen species such as hydrogen peroxide (H_2O_2) and free radicals including the superoxide anion radical ($O_2^{-\cdot}$) and hydroxyl radical ($OH^{\cdot}$), as well as the ALA enoyl radical ($ALA^{\cdot}$) (Monteiro *et al.*, 1989). Accumulation of ALA and porphobilinogen (PBG) in cellular fluid and tissues of ALA patients has long been known to be closely related with clinical symptoms (Gorchein, 1984).

Melatonin is a ubiquitously direct acting free radical scavenger and an indirect antioxidant (Pablos *et al*., 1997; Reiter *et al*., 2000). While being highly efficient in detoxifying the devastatingly reactive hydroxyl radical ($^{\cdot}OH$) (Tan *et al*., 1993; Qi *et al*., 2000), melatonin also directly interacts with single oxygen ($^1O_2$), peroxynitrite anion ($ONOO^-$) (El-Sawi, 2003) and nitric oxide ($NO^{\cdot}$) (Reiter *et al*., 1999). A single molecule of melatonin neutralizes two ($^{\cdot}OH$), the product of this interaction is cyclic 3-hydroxymelatonin (Tan *et al*., 1998). Melatonin was found to detoxify H_2O_2; the precursor of ($^{\cdot}OH$) (Tan *et al*., 2000). Melatonin exhibits several obvious advantages over classical antioxidants in terms of neuronal protection. First, melatonin possesses unique pharmacokinetic properties and is both lipophilic and, to a lesser degree, hydrophilic (Shida *et al*, 1994; Costa *et al*., 1997). Second, melatonin crosses the blood brain barriers with ease and distributes to all subcellular compartments including the membranes (Ceraulo *et al*., 1999), cytosol, mitochondria (Martin *et al*., 2000) and nucleus (Menendez-Pelaez *et al*., 1993). This makes melatonin available for on-site protection against attack by free radicals at multiple sites.

The aim of the present study was to examine the effects of melatonin on the activation of antioxidant enzymes and reduction of basic markers of oxidative stress MDA and PC induced by accumulation of ALA in an experimental model of porphyria.

Material and Methods

Chemicals

Melatonin and δ-aminolevulinic acid were purchased from Sigma Chemical Company, ST. Louis, MO, USA. .All other chemicals and reagents were of analytical grade.

Animals

Adult male albino rats weighing ~200 gram were kept under good ventilation, natural lighting (14 hrs light/10 hrs dark) and adequate stable diet. Food was withdrawn 18 hrs before starting the experiment, while water was available *ad libitum.*

Experimental protocol

The rats were divided randomly into four groups; six rats for each. The first group served as control and received an intraper-itoneal injection of physiological saline (0.9% NaCl). The second group was given intraperitoneal injections of melatonin (dissolved in a solution of 0.9% NaCl and ethanol; 20/1, vol/vol) at a dose of 10 mg/kg body weight every day for 14 consecutive days. The third group was given interperi-toneal injections of ALA dissolved in 0.9% NaCl at a dose of 40 mg/kg body weight of ALA day after day for 14 days. The fourth group was received intraperitoneal injections of melatonin at a dose of 10 mg/kg body weight every day for 14 consecutive days as well as intraperitoneal injections of ALA at a dose of 40 mg/kg body weight day after day for 14 days. All substances were dissolved in a final volume of 0.5 ml/injection.

Sampling and tissue extraction

At the end of the experimentation period, over night fasted rats were sacrificed using a sharp razor blade, Blood samples were collected in clean non-heparinized centrifuge tubes, then the tubes were let to stand for 15 min at 30°C after which the tubes were centrifuged at 3000 rpm for 15 min. Blood sera were carefully

separated. Aliquots of each sample are labeled and kept at -20°C for subsequent analysis.

Thereafter, brain, liver and kidney specimens were quickly removed, weighed and then homogenized in cold distilled water to form 10% (w/v) homogenate. After labeling the samples, they were kept at -20°C for later different biochemical determinations.

Methods

MDA was assayed by the method of Ohkawa *et al.* (1979) while PC was estimated using method of Smith *et al*. (1991). GSH was determined according to the method of Prins and Loose (1969). The activity of SOD was determined by using the method of Nishikimi *et al.* (1972). CAT activity was detected by the method of Bock *et al*. (1980). GST was assayed according to the method of Habig *et al.* (1974). GSH-Rd was estimated according to the method of Beutler (1975). Finally, tissue protein was determined according to the method of Bradford 1976.

Statistical analysis

The data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's method for pair wise multiple comparisons. Results are expressed as the mean ± S.E. for six measures. Significance was considered at a level of $P < 0.05$.

Results

Administration of ALA increased lipid peroxidation (LPO) levels as indicated by the increase in MDA in brain, kidney and liver homogenates versus those of control or melatonin-injected rats (Table 1). These increases were significant ($p<0.001$). When melatonin was given with ALA it significantly reduced ($p<0.001$) MDA levels in brain and liver homogenates almost to the level of control rats, whereas MDA level in kidney homogenate still significantly higher ($p<0.5$) than that of the control rats.

As shown in table (2), intraperitoneal injection of ALA showed significant increases ($p<0.001$) in PC concentration in brain, kidney and liver when compared with control rats. On the other hand, cotreatment of melatonin with ALA significantly improved the concentration of PC when compared with control rats. However, the level still significantly higher (PC) than that of control rats.

The results related to the changes in GSH content in rat brain, kidney and liver are presented in table (3). In case of rats injected with ALA; GSH levels were significantly diminished ($p<0.001$) than those of control or melatonin-injected rats. When melatonin was given to animals injected with ALA, the decrease in GSH levels was significantly improved.

In comparison to the control group, the activities of SOD, CAT, GST and GSH-Rd were significantly lowered ($P<0.001$) in ALA injected group (Tables 4, 5, 6 and 7). Whereas, administration of melatonin to rats injected with ALA was shown to significantly improve the activities of SOD, CAT, GST to be near the normal level while the reduction in GSH-Rd activities in both brain and liver homogenates was found to be ameliorated to reach the control level. Conversely, in the kidney, the reduction in GSH-Rd activity did not improve completely. Additionally, there were no significant effects for all tested parameters in melatonin administration on the animals of the control group.

Table (1): Brain, kidney and liver malondialdehyde (MDA) level (nM/mg wet tissue) of different rat groups.

Parameter	Control	Melatonin	ALA	ALA + Melatonin
Brain	193.9 ±0.73	193.7 ±1.14	528.1 ±1.04***	201.3 ±3.87°°°
Kidney	141.2 ±0.76	139..8 ±0.83	421.3 ±0.83***	151.7 ±4.57* °°°
Liver	117..9 ±3.57	117.8 ±3.42	433.5 ±1.90***	128..8 ±1.54°°°

Values are mean of 6 animals ± SE; values with asterisk (s) are statistically
(* = significant at P<0.05, *** = significant at P<0.001) different from control or melatonin-injected rats. Whereas, values with circles are statistically
(°°° = significant at P<0.001) different from ALA-injected rats.

Table (2): Brain, kidney and liver protein carbonyl (PC) content (µM/g wet tissue) of different rat groups.

Parameter	Control	Melatonin	ALA	ALA + Melatonin
Brain	3.73 ±0.15	3.60 ±0.15	6.91 ±0.16***	4.45 ±0.14** °°°
Kidney	3.75 ±0.15	3.36 ±0.14	7.60 ±0.24***	4.80 ±0.15** °°°
Liver	1.28 ±0.02	1.03 ±0.02	6.83 ±0.33***	2.00 ±0.06** °°°

Values are mean of 6 animals ± SE; values with asterisks are statistically
(** = significant at P<0.01, *** = significant at P<0.001) different from control or melatonin injected group. Whereas, values with circles are statistically
(°°° = significant at P<0.001) different from ALA-injected rats.

Table (3): Brain, kidney and liver glutathione (GSH) content (mg GSH/g wet tissue) of different rat groups.

Parameter	Control	Melatonin	ALA	ALA + Melatonin
Brain	1.63 ±0.14	1.68 ±0.10	0.49 ±0.02***	1.18 ±0.06* °°°
Kidney	0.35 ±0.02	0.36 ±0.02	0.06 ±0.007***	0.25 ±0.003* °°°
Liver	1.23 ±0.02	1.33 ±0.03	0.39 ±0.01***	0.99 ±0.02* °°°

Values are mean of 6 animals ± SE; values with asterisk (s) are statistically
(* = significant at P<0.05, *** = significant at P<0.001) different from control or melatonin-injected rats. Whereas, values with circles are statistically
(°°° = significant at P<0.001) different from ALA-injected rats.

Table (4): Brain, kidney and liver Superoxide dismutase (SOD) activity (U/g wet tissue) of different rat groups.

Parameter	Control	Melatonin	ALA	ALA + Melatonin
Brain	142.8 ±0.90	146.9 ±1.52	98.2 ±1.73***	133.1 ±3.38* °°°
Kidney	166.6 ±0.91	170.4 ±1.21	121 ±1.18***	155.1 ±4.31* °°°
Liver	176 ±1.83	177.1 ±1.96	121.2 ±1.22***	164.1 ±4.09* °°°

Values are mean of 6 animals ± SE; values with asterisk (s) are statistically
(* = significant at P<0.05, *** = significant at P<0.001) different from control or melatonin-injected rats. Whereas, values with circles are statistically
(°°° = significant at P<0.001) different from ALA-injected rats.

Table (5): Brain, kidney and liver catalase (CAT) activity (µM H_2O_2/Sec/g wet tissue) of different rat groups.

Parameter	Control	Melatonin	ALA	ALA + Melatonin
Brain	35.0 ±1.85	36.5 ±2.00	7.48 ±1.31***	7.48 ±1.76* °°°
Kidney	41.8 ±0.51	42.5 ±0.66	11.91 ±0.60***	36.1 ±0.66* °°°
Liver	36.1 ±0.18	37.8 ±0.24	9.85 ±0.68***	31.6 ±0.57* °°°

Values are mean of 6 animals ± SE; values with asterisk (s) are statistically (* = significant at P<0.05, *** = significant at P<0.001) different from control or melatonin-injected rats. Whereas, values with circles are statistically (°°° = significant at P<0.001) different from ALA-injected rats.

Table (6): Brain, kidney and liver glutathion-S-Transferase (GST) activity (µM/g wet tissue) of different rat groups.

Parameter	Control	Melatonin	ALA	ALA + Melatonin
Brain	5.00 ±0.29	5.21 ±0.25	2.05 ±0.31***	3.93 ±0.23** °°°
Kidney	4.95 ±0.19	5.21 ±0.08	2.05 ±0.22***	3.70 ±0.53* °°
Liver	5.83 ±0.23	6.15 ±0.19	1.76 ±0.13***	4.50 ±0.39** °°°

Values are mean of 6 animals ± SE; values with asterisk (s) are statistically (* = significant at P<0.05, ** = significant at P<0.01, *** = significant at P<0.001) different from control or melatonin-injected rats. Whereas, values with circles are statistically (°° = significant at P<0.01, °°° = significant at P<0.001) different from ALA-injected rats.

Table (7): Brain, kidney and liver glutathione reductase (GSH-Rd) (U/mg protein) of different rat groups.

Parameter	Control	Melatonin	ALA	ALA + Melatonin
Brain	38.7 ±0.98	40.9 ±1.28	24.95 ±2.37***	36.7 ±1.47°°°
Kidney	34.4 ±1.15	36.8 ±1.49	21.41 ±1.94***	29.9 ±1.02** °°°
Liver	38.7 ±0.98	40.9 ±1.28	24.95 ±2.37***	36.7 ±1.47°°°

Values are mean of 6 animals ± SE; values with asterisks are statistically (** = significant at P<0.01, *** = significant at P<0.001) different from control. Whereas, values with circles are statistically (°°° = significant at P<0.001) different from ALA-injected rats.

Discussion

Injection of ALA usually results in a blood concentration of approximately 20-30 µg/ml after 30 min which is maintained for up to 24 hrs and is similar in magnitude to the level observed in patients with prophyrias (Mc Gillion *et al.*, 1975). After a single injection of ALA, a rapid uptake by a variety of tissues is observed with the highest concentration occurring in the liver. Thus, multiple injections with ALA at a dose of 40 mg/kg body weight, as applied in the current study, are believed to mimic the conditions of AIP or the other disturbances related to ALA accumulation (Karbownik *et al.*, 2000)

The present results showed that multiple injections of ALA to rats caused significant increases (P<0.001) in MDA and PC levels compared to control or melatonin-injected rats. Similar changes were already observed in different experimental situations designed by Karbownik *et al*. (2000); Weis *et al*. (2003); Noriega *et al*. (2003) for MDA as well as Lelli *et al*. (2005) for MDA and PC. They concluded that MDA is a sufficiently precise marker of oxidative stress and they attributed this increase in MDA due to the nature of ALA as aprooxidant which when produced in excess or accumulated, it works as an endo-genous source of toxic oxygen derivatives, such as $OH^{\cdot}$, $O_2^{\cdot -}$ and H_2O_2 (Monteiro *et al.*, 1989 and Hermes-Lima *et al.*, 1991).

Additionally, administration of melato-nin concomitantly with ALA led to a signifi-cant decrease in MDA formation as well as a decrease in PC. These results are in agreement with that obtained by Carneiro and Reiter (1998), they demonstrated that melatonin administration significantly decreased LPO in liver and kidney of rats. And also with other findings obtained by many authors worked on different experimental models. For MDA, Princ *et al.* (1998) in rat cerebellum and Juknat *et al.* (2003) in mouse astrocytes. Tan *et al.* (1994) explained how melatonin greatly inhi-bits MDA formation. Melatonin can scavenge free radicals and/or inhibit their formation, and additionally, melatonin per se can act by up-regulating endogenous antioxidant defenses. The protective effect of melatonin against oxidative damage induced by ALA treatment, as indicated by significant increases in the levels of MDA and PC, may relate to its lipophilic and hydrophilic properties (Reiter, 1998), its ability to localize in a superficial position in lipid bilayers near the polar heads of phospholipids (Ceraulo *et al.*, 1999), its preventive actions against protein damage (Tesoriere *et al.*, 1999) and to its ability to scavenge the initiating agents of LPO (Reiter *et al.*, 2000).

Conversely, GSH content was signific-antly diminished in ALA injected-rats versus control rats. This finding was in agreement with these obtained by Neal *et al.* (1996) and Noriega *et al*. (2003). Meister (1994) reported that the GSH works as an antioxidant, and he attributed this property to the presence of numerous –SH groups, which can react with the free radicals and products of lipid peroxidation such as lipid peroxides and aldehydes protecting in this way against development of oxidative stress (Tandon *et al.*, 2002). Thus, it can supposed that the reduction in GSH after ALA injection might result from the utilization of –SH groups to scavenge free radicals formed (Jurczuk *et al.*, 2006). Administration of melatonin was found to improve significantly GSH content. Urata *et al.* (1999) attributed this to the ability of melatonin to stimulate the synthesis of GSH.

In ALA injected rats significant reductions ($P<0.001$) in the activities of SOD, CAT, GST and GSH-Rd were observed. These results are in accordance with that obtained by Pereira *et al.* (1992) and Demasi *et al.* (1996) for SOD; Tomas-Zapico *et al.* (2002) for SOD and GSH-Rd; Noriega *et al.* (2003) for SOD, CAT and glutathione peroxidase (GSH-Px), they attributed this presumably due to the vulnerability of their active centers to free radicals.

On the other hand, administration of melatonin with ALA was found to preserve SOD, CAT, GST and GSH-Rd activities arou-nd the normal values obtained in control group. Beside its ability to scavenge free radi-cals, melatonin stimulates a host of antiox-idative enzymes including SOD, GSH-Px and GSH-Rd, thereby further reduction in the oxidative state of cells has been achieved (Pablos *et al.*, 1997; Reiter *et al*., 2000). SOD is considered as a major antioxidative enzyme, because it dismutases the superoxide anion ($O_2^{\cdot-}$) radical to hydrogen peroxide (H_2O_2) and reduces the formation of peroxynitrite. Antolin *et al.* (1996) indicated that melatonin increased the tissue mRNA levels for both manganese and copper SOD levels in animals treated with melatonin. Melatonin stimulates the activity of GSH-Px, which metabolizes H_2O_2 to water. In this process, GSH gets oxidized and forms oxidized glutathione (GSSG). The GSSG is reduced back to GSH in the presence of the enzyme GSH-Rd, the activity of which is also stimulated by melatonin (Pablos *et al*., 1997). Melatonin was found also to stimulate the activity of CAT, which is also involved in reducing the H_2O_2 and thus reduce the generation of hydroxyl radicals (Montilla *et al*., 1997).

Conclusion

Multiple injections of ALA to rats leads to significant increase in MDA and an increase in PC in brain, kidney and liver tissue homogenates, while significant decreases in GSH level and SOD, CAT, GST and GSH-Rd activities were registered which provide the evidence of oxidative stress.

Melatonin has a prophylactic effect against oxidative stress incorporated with ALA accumulation. Thus, the administration of melatonin might be beneficial for AIP therapy.

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دور الميلاتونين في تعديل الضغط التاكسدى المحدث باستخدام حمض الأمينوليفيولينك في ذكور الجرذان البيض البالغة.

ممدوح رشاد فرج الصاوي، محيى الدين بدوى، نيرمين محمد الغريب
قسم علم الحيوان، كلية العلوم، جامعة المنصورة

يعتبر حمض الأمينوليفيولينك المادة المنشأة للهيم، والذي يتراكم في البرفريا الحادة المتقطعة (الفرفرين) نتيجة للنقص الانزيمى في سلسلة التخليق الحيوي للهيم، حيث أن تراكمه يؤدى إلى ظهور العديد من الأعراض المرضية، وذلك لأنه يعمل كمصدر داخلي لإطلاق أصناف عديدة من الأكسجين المتفاعل والتي تسبب ضرر تأكسدي للتراكيب الخلوية المختلفة.

وقد صممت هذه الدراسة لفحص مقدرة الميلاتونين- وهو هرمون يعمل كمضاد للأكسدة وقانص للشوارد الحرة و يفرز من الغدة النخامية- كعامل وقائى من الأضرار الناجمة عن الضغط التأكسدى المحدث باستخدام حمض الأمينوليفيولينيك، حيث قسم 24 جرذا عشوائيا الى 4 مجموعات كالتالى:

مجموعة طبيعية ضابطة.

مجموعة معاملة بالميلاتونين بجرعة قدرها 10 مج/كجم من وزن الجسم في التجويف البريتونى لمدة 14 يوما متتاليا.

مجموعة معاملة بجرعات مزمنة من حمض الأمينوليفيولينيك قدرها 40مج/كجم من وزن الجسم في التجويف البيريتونى، يوم بعد يوم لمدة 14 يوما.

مجموعة معاملة بالميلاتونين (10 مج/كجم من وزن الجسم) في التجويف البريتونى لمدة 14 يوما متتاليا وأيضا بجرعات مزمنة من حمض الأمينوليفيولينيك (40مج/كجم من وزن الجسم) في التجويف البيريتونى، يوم بعد يوم لمدة 14 يوما.

ولقد خلصت الدراسة الحالية على الأتي:-

أولا: في مجموعة الجرذان المعاملة بجرعات مزمنة من حمض الأمينوليفيولينيك- 40مج/كجم من وزن الجسم في التجويف البيريتونى، يوم بعد يوم لمدة 14 يوما فقد لوحظ الأتي:-

أ- زاد محتوى المالونيل ثنائي الألدهيد (MDA) وكذلك البروتين كربونيل (PC) زيادة ذات دلالة احصائية، بينما انخفض مستوى الجلوتاثيون (GSH) انخفاضا ذو دلالة احصائية في مطحون نسيج المخ والكلية والكبد.

ب- انخفض نشاط الإنزيمات المضادة للأكسدة مثل فوق الأكسيد المحول (SOD) ، الكاتاليز (CAT)، الجلوتاثيون المختزل (GSH-Rd) والجلوتاثيون الناقل (GST) انخفاضا ذو دلالة احصائية.

ثانيا: في مجموعة الجرذان المعاملة بالميلاتونين بجرعة قدرها 10 مج/كجم من وزن الجسم أيضا في التجويف البريتونى لمدة 14 يوما متتاليا وبالتزامن مع حقن حمض الأمينوليفيولينك كما في المجموعة السابقة، فقد لوحظ تحسنا ذو دلالة احصائية في كل المعايير المقيسة حيث وصل بعضها إلى المستوى الطبيعي.

وعلى ذلك ومن خلال مقدرة الميلاتونين الواضحة على الحماية من الضغط التأكسدى الناجم عن الحقن بحمض الأمينوليفيولينك توصى هذه الدراسة باستخدام الميلاتونين كعلاج مساعد للمرضى الذين يعانون من أعراض نتيجة لتراكم حمض الأمينوليفيولينيك.