• Research Article

Curcumin alters motor coordination but not total number of Purkinje cells in the cerebellum of adolescent male Wistar rats

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OBJECTIVE: The present study aimed at investigating the effects of curcumin on the motor coordination and the estimate of the total number of cerebellar Purkinje cells of adolescent Wistar rats exposed to ethanol.

METHODS: The total of 21 male Wistar rats aged 37 d old were divided into three groups, namely ethanol, ethanol-curcumin, and control groups. The ethanol group received 1.5 g/kg ethanol injected intraperitoneally and water given per oral; the ethanol-curcumin group received 1.5 g/kg ethanol injected intraperitoneally and curcumin extract given per oral; the control group received saline injection and oral water. The treatment was carried out daily for one month, after which the motor coordination performance of the rats was examined using revolving drum apparatus at test days 1, 8, and 15. The rats were finally sacrificed and the cerebellum of the rats was further processed for stereological analysis. The estimate of the total number of Purkinje cells was calculated using physical fractionator method.

RESULTS: The ethanol-curcumin group performed better than both ethanol and control groups in the motor coordination ability at day 8 of testing ($P< 0.01$). No Purkinje cell loss was observed as a result of one month intraperitoneal injection of ethanol.

CONCLUSION: Curcumin may exert beneficial effects on the motor coordination of adolescent rats exposed to ethanol via undetermined hormetic mechanisms.

KEYWORDS: curcumin; Purkinje cells; antioxidants; rats, Wistar

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1 Introduction

Epidemiological studies have revealed that males and adolescent consumed more ethanol or ethyl alcohol and therefore were more prone to ethanol intoxication than females and any other age groups[1,2]. Ethanol has been reported to cause dysfunction of many organs and systems of the body, including the nervous system[3,4]. In rat models, it has been reported that ethanol decreased the number of certain brain cells such as pyramidal and granule cells in the hippocampus[5-8], as well as Purkinje and granule cells in the cerebellum[9,10].

Ethanol is eliminated from the body via several metabolic pathways. Generally ethanol is metabolized through oxidative using alcohol dehydrogenase, cytochrom P450 (CYP450), and catalase enzymes and non-oxidative pathways. In the brain, ethanol is oxidized by CYP2E1

(an isozyme of CYP450) into acetaldehyde, which is subsequently metabolized into acetate and nicotinamide adenine dinucleotide-hydrogen by aldehyde dehydrogenase 2 enzyme. It has been suggested that both acetaldehyde and acetate cause the impairment of the functioning of the central nervous system in rats, including motor coordination[11,12].

Rhizome of tumeric (*Curcuma longa*) has long been used as food additive and traditional medicine throughout Asian countries[13]. Among the major compositions of the rhizome of tumeric which is suggested to have therapeutic effects, is curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione). Curcumin has been documented to exert antioxidant, anti-inflammatory, antiproliferative, and anticarcinogenic effects[14]. In the brain, it has been reported that curcumin could suppress the progress of ageing[13], Alzheimer's disease[15,16], and several neurotoxic substance-induced oxidative stresses[17-19], possibly through the inhibition of oxidative damage of brain cells. Moreover, curcumin improved 3-nitropropionic acid-induced motor and memory deficits[17]. It remains uncertain, however, whether curcumin could protect the brain, including the cerebellum, from ethanol-induced cell loss and motor performance disturbance. We have therefore examined the possible preventive effects of curcumin on the ethanol-induced motor incoordination and Purkinje cells deficits in the cerebellum of adolescent male Wistar rats.

2 Materials and methods

2.1 Animals and grouping

Twenty-one male Wistar rats aged one month old (body weight 100 g) were obtained from the animal breeding house of the Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. Rats are considered adolescent when they are approximately 30 to 50 post-natal days of age[20,21]. The rats were divided into three groups, namely, control group, ethanol group, and ethanol-curcumin group. The control group was given water per oral and injected intraperitoneally with normal saline. The ethanol group was treated with water per oral and injected intraperitoneally with 15% ethanol in normal saline as described in other studies[22,23]. The amount of ethanol given per rat was 1.5 g/kg body weight. The dose used in this study was made lower than that of the other studies[22,23] due to high mortality rate of the rats in response to higher doses. The ethanol-curcumin group was treated with curcumin extract (50 mg/kg body weight) per oral and injected with 15% ethanol in normal saline as that given to the ethanol group.

2.2 Plant extracts

The curcumin extract was curcumin synthetic obtained from the Curcumin Research Centre, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. All of the treatments were carried out for 30 d. In order to avoid alcohol withdrawal syndrome, the amount of ethanol given to the ethanol and ethanol-curcumin groups was reduced into half daily, and finally down to zero, from day 31 to day 34. The rats were then left for 3 d before the revolving drum tests were carried out. During the experiment, the rats were fed with pellets (Japfa Comfeed, Indonesia) consisting of protein 19% to 20%, fat 4% to 8%, crude fiber 3% to 5%, ash 5% to 7%, calcium 0.9% to 1.2%, and total phosphorous 0.7% to 0.9%. Water was given *ad libitum* throughout the experiment. The experimental procedure was approved by the Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada, Indonesia (approval number: KE/FK/418/EC).

2.3 Revolving drum test

The protocol of the revolving drum test was based on previous studies[24,25] with a slight modification, and was performed 3 d subsequent to the last treatment. No test of motor coordination was carried out prior to the treatment. The experimental apparatus consisted of a cylinder with two side-walls attached to the boundary of the cylinder. A box containing clothes was placed 80 cm below the revolving drum apparatus, therefore when rats fell off the drum they landed softly in this box.

The rat was placed on top of the running surface of the stationary revolving drum. It was left to stay there for 1 min to allow the rat to habituate with the tool. The rat was taken off the running surface. The revolving drum was turned on to rotate at 16 rotations per minute. The rat was placed back on top of the running surface of the drum, facing the direction opposite to direction of rotation of the revolving drum. In order to maintain this position the rat was required to walk forward on the revolving drum. A stopwatch was started at the time when the rat was placed on top of the running surface. The number of falls of the rat during three consecutive minutes was recorded. The stopwatch was stopped every time the rat fell, and started again once the rat was placed back on the running surface. This procedure was undertaken on test days 1, 8, and 15, with no trial in between. In order to ensure that the rats were habituated to the task performed, and hence, they might start at similar baseline of performance, the rats were trained to walk on the running surface of the apparatus for six consecutive days, 10 min maximum for each trial, prior to the real tests.

2.4 Tissue preparation

One day after the last behavioural tests, all rats were sacrificed. They were anaesthetised with Ketalar (50 mg/mL ketamine hydrochloride and 0.1 mg/mL phemerol chloride; PT Kalbe Farma, Jakarta, Indonesia) and ether (Asia Lab, Yogyakarta, Indonesia). Trans-cardiac perfusion was carried out through the left ventricle,

first with a small amount of isotonic saline followed immediately with a fixative consisting of 400 mL of 4% formaldehyde. The forebrains (defined here as the cerebral hemisphere minus the olfactory bulb) and the cerebellum of the rats were removed, weighed separately, and immersed in the same fixative mixture for a further 72 h.

2.5 Total number estimation of the cerebellar Purkinje cells

The cerebellar tissues of rats were cut according to the multistage fractionator technique as described in previous studies[9,26]. At stage 1, each cerebellar tissue was cut parasagittally into slices with a thickness of approximately 2 mm. A number between 1 and 2 was selected by lottery. If number 2 was chosen, every second slice was taken for further fractionation. The sampling fraction of the first stage was 2 (f_1=2). Similar procedure was repeated at stages 2 and 3 when the cerebellar slices were cut into smaller pieces. The sampling fractions of these stages were f_2 = 3 and f_3 = 3, respectively.

The selected slices from stage 3 were then dehydrated in graded concentration of ethanol solution, cleared with toluene, and infiltrated with parafin. The tissue was subsequently embedded in parafin blocks. The tissue was cut using Microm HM 310 microtome with the section thickness of 6 μm. One of every 20 sections (f_4 = 20) was selected by lottery, mounted on slides, and stained with toluidine blue. The sections were examined under Olympus light microscope with a ×40 magnification of the objective lens. The number of Purkinje cells (n) was counted from these sections. The total number of Purkinje cells for each cerebellum (N) was then calculated using the following formula[9, 26]: $N = f_1 \times f_2 \times f_3 \times f_4 \times n$.

2.6 Statistical analyses

The raw data of the number of fall during revolving drum tests were transformed using the formula: $X(t)=\sqrt{(x+0.5)}$, where x=number of fall recorded in each trial and $X(t)$ = transformed value. This transformation was carried out in order to avoid mathematical complications arising out of some values being zero[25].

Prior to the statistical analyses, all of the data (the square root data, the body weights, the total number estimate of Purkinje cells, and the cerebral and cerebellar weights), which were expressed in numerical form, were tested for their normality (using one-sample Kolmogorov Smirnov test) and variance (using Levene test), in order to determine the types of statistical tests, whether parametric or non-parametric. The non-parametric test used was Kruskal-Wallis test, followed with post-hoc analysis using Mann-Whitney U test whenever appropriate. The parametric test used was one-way analysis of variance, followed with post-hoc Tukey test whenever necessary. All statistical analyses were done on SPSS version 12 at a significance level of 0.05.

3 Results

3.1 Revolving drum test

The square root transformation data of the number of falls of rats during the revolving drum test are shown in Table 1. The Kruskal-Wallis test on these data demonstrated that a significant difference between groups occurred in the number of fall at day 8 of testing (P< 0.01), but not at day 1 or day 15 of testing. Post-hoc analysis using Mann-Whitney U test showed that there was a significantly lower number of falls of the ethanol-curcumin group than both the control group and ethanol group at day 8 of testing (P< 0.01).

3.2 Body weights

The data on body weights in the three-day revolving drum tests are shown in Table 2. One-way analysis of variance of these data showed a significant difference between groups (P<0.01) in the body weights of the rats in the three day revolving drum tests. Post-hoc analysis using Tukey test of the data showed that the body weight of ethanol-curcumin group was significantly lower than that of control group at day 15 of testing (P<0.05).

3.3 Total number of Purkinje cells and brain weights

The Purkinje cells counted were those having nucleoli visible under the microscope (Figure 1). The data on the total number estimate of Purkinje cells and cerebral and cerebellar weights are shown in Table 3. The Kruskal-Wallis test on these data showed no significant difference between groups in the total number estimate of Purkinje cells. One-way analysis of variance of these data also revealed no significant difference between groups in the cerebral weights. In addition, the Kruskal-Wallis test

Table 1 The square root transformation data of the number of falls of the three groups of rats in the revolving drum tests

(Mean ± standard error of mean)

Group	n	Number of falls		
		Day 1	Day 8	Day 15
Control	7	4.26±0.97	4.99±0.23	4.87±0.51
Ethanol	7	4.57±0.90	6.50±0.68**	5.30±1.17
Ethanol-curcumin	7	3.58±0.51	2.53±0.43** △△	2.78±0.70

**P< 0.01, *vs* control group; △△ P< 0.01, *vs* ethanol group.

Table 2 Body weights of the three groups of rats in the revolving drum tests

(Mean ± standard error of mean, g)

Group	*n*	Body weight		
		Day 1	Day 8	Day 15
Control	7	233.57±11.06	250.00±10.24	265.71±12.84
Ethanol	7	221.43±12.71	229.29±13.29	238.57±15.65*
Ethanol-curcumin	7	195.00±3.93	205.71±5.05	212.14±6.35*

*$P< 0.05$, *vs* control group.

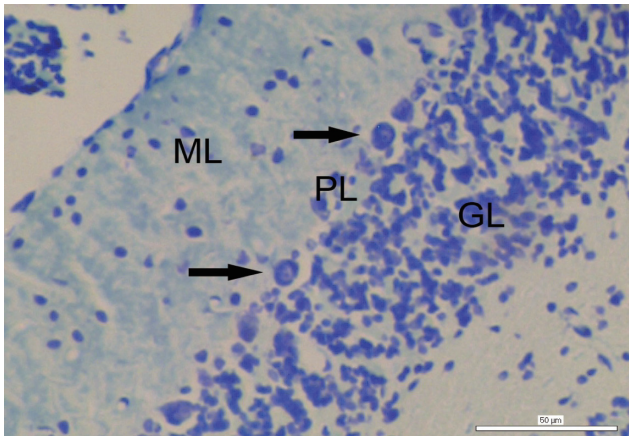

Figure 1 The micrograph of the three layers of cerebellum (Light microscopy, ×100)

The Purkinje cells counted were those having nucleoli clearly visible (as indicated by arrows). ML: molecular layer; PL: Purkinje cell layer; GL: granule cell layer.

Table 3 The total number estimate of the Purkinje cells and the cerebral and cerebellar weights of the three groups of rats

(Mean ± standard error of mean)

Group	*n*	Total number of Purkinje cells ($\times 10^3$)	Cerebral weight (mg)	Cerebellar weight (mg)
Control	7	322.20±71.76	1 381.99±29.79	372.83±34.43
Ethanol	7	308.42±46.82	1 381.87±27.26	321.10±13.03
Ethanol-curcumin	7	300.34±30.71	1 319.69±39.93	312.57±11.77

on these data showed no significant difference between groups in the cerebellar weights of the rats.

4 Discussion

The main finding of our present study was that one month intraperitoneal injection of ethanol accompanied with the administration of oral curcumin extract caused better performance in motor coordination task compared to both the control and the ethanol groups at day 8 of testing. In fact there was a tendency of lower number of falls of the ethanol-curcumin group compared to the other two groups throughout the three days of testing (Table 1). Given the nature of revolving drum test, animals with lower body weights might take advantage over those with heavier body weights. Indeed, the body weights of the ethanol-curcumin group remained the lowest throughout the three days of testing, and significantly lower than the control group at day 15 of testing (Table 2). However, this may not be the reason for the better motor performance of the ethanol-curcumin group compared to the other two groups, since the number of falls of the ethanol-curcumin group was significantly lower at day 8 of testing, but not at day 15 of testing. In addition, the ethanol group, which was consistently lower in body weight compared to the control group throughout the experiment, tended to demonstrate higher number of falls than the control group (Tables 1 and 2). The exact reason for the slimmer body of the ethanol-curcumin group compared to the other two groups is currently uncertain. Several studies, however, reported that curcumin may prevent fat deposition and hence, obesity, through several mechanisms, including inhibition of angiogenesis and modification of inflammation processes[27]. It is known that new formation of vascularisation promotes the growth of adipose tissue. Curcumin may suppress this formation through the down-regulation of several angiogenesis-promoting factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and other factors[27]. Curcumin may also suppress inflammatory processes related to

adipogenesis, as indicated by its effects on various inflammatory agents, including cyclooxygenase-2, VEGF, as well as other pro-inflammatory cytokines[28].

Curcumin has been shown to counter the dysfunctioning of cerebellum due to a number of neurotoxic insults such as aluminium[29], lead[30], and pentylenetetrazol[31]; degenerative process such as ageing[13]; or pathological conditions such as streptozotocin-induced diabetes[32,33]. It is traditionally believed that the neuroprotective effects of curcumin are attributed to the potentiation of antioxidative activity of curcumin[34]. Indeed, several oxidative stress markers in the cerebellum were altered upon the administration of curcumin, such as the normalization of the activities of mitochondrial complexes (complexes I, II, and IV)[29], and the decrease of methane dicarboxylic aldehyde, catalase, and glutathione S-transferase levels[31]. Other studies reported the decrease of lipid peroxidation and lipofuscin content, the increase of superoxide, catalase, and glutathione peroxidase enzyme activities, and reduced glutathione levels, as a result of curcumin administration[13,30]. However, more recently, it has been suggested that such beneficial effects of phytopharmaceuticals, including curcumin, was due to their hormetic effects rather than simply direct anti-oxidant activities[34,35]. "Hormesis" is generally defined as beneficial effects resulting from the response of an organism to a repeated low-intensity of an otherwise harmful stressor[36]. Low-dose curcumin may not be effective to stimulate reactive oxygen species-scavenging activities. Curcumin may actually activate a nuclear transcription factor (NRF)-2 which subsequently translocates into nucleus. NRF-2 then binds to antioxidant response element in genes which encode anti-oxidant enzymes. NRF-2 also targets gene that encodes heme oxygenase-1, a cytoprotective protein against different types of stress[34]. If this hormetic pathway holds responsible for the beneficial effects of curcumin, this may explain the unusual better performance of ethanol-curcumin group compared to the control group. However, it seems that to date no available studies report on the presence of hormetic characteristics on the effects of curcumin, for example, the presence of the typically biphasic responses to curcumin at different doses (beneficial at lower dose and toxic at higher dose).

Ethanol has also been considered to exert some beneficially hormetic effects on the behaviour of rodents at low doses[37,38]. It has been reported that low-dose ethanol facilitates social behaviour of adolescent rats, but not mature rats[37]. Some studies have demonstrated the stimulation of spontaneous locomotor activity as a result of low-dose ethanol administration[38]. However, our study may not lend support to the notion that such hormetic mechanism was at work, since the mean number of falls of the ethanol group consistently tended to be higher than the other two groups during the experiment throughout the three days of testing (Table 1). This is in spite of the absence of any significant deficit of the number of Purkinje cells in the cerebellum of the ethanol group (Table 3). Ethanol might already start disrupting the motor functions of the rats although no obvious loss of the number of Purkinje cells was observed.

Purkinje cells of rats appear to be susceptible to ethanol exposure at certain age. A number of studies on the effects of ethanol on cerebellum which administered ethanol at very early age[9,39-45] revealed that the temporal window of vulnerability of Purkinje cells of rats peaks at post-natal days 4 to 6[46]. Other studies showed that long-term exposure of ethanol to 12 months old rats caused pathological changes of Purkinje cells, as characterized with terminal dendritic segments elongation[47], synaptic loss[48], reticulum endoplasmic dilation[49] and degenerating bodies formation[50]. White *et al*[51] reported that adolescent rats seem typically more persistent to motor impairment due to alcohol insult compared to older rats. Taken together, these data may suggest that adolescent period may be relatively resistant to ethanol-induced motor dysfunction, and probably, Purkinje cell loss compared to any other age. However, further studies are required to confirm or refute this notion.

In conclusion, our study showed that curcumin extract may exert beneficial effects on motor coordination functions of rats exposed to ethanol at adolescent period. It is possible that such effects may result from yet unknown hormetic mechanisms, either through its sole action or through its interaction with ethanol. To our knowledge, no studies provide detailed analysis on such mechanisms. Further research on the precise mechanisms of such effects is therefore warranted.

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6 Competing interests

The authors declare that they have no competing interests.

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