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CHLOROGENIC ACID PREVENTS ALCOHOL-INDUCED BRAIN DAMAGE IN NEONATAL RAT

Abstract

The present investigation evaluates the neuroprotective effect of chlorogenic acid (CA) in alcohol-induced brain damage in neonatal rats. Ethanol (12 % v/v, 5 g/kg) was administered orally in the wistar rat pups on postnatal days (PD) 7-9. Chlorogenic acid (100 and 200 mg/kg, p.o.) was administered continuously from PD 6 to 28. Cognitive function was estimated by Morris water maze (MWM) test. However, activity of acetylcholinesterase, inflammatory mediators, parameters of oxidative stress and activity of caspase-3 enzyme was estimated in the tissue homogenate of cerebral cortex and hippocampus of ethanol-exposed pups. It has been observed that treatment with CA attenuates the altered cognitive function in ethanol-exposed pups. There was a significant decrease in the activity of acetylcholinesterase in the CA treated group compared to the negative control group. However, treatment with CA significantly ameliorates the increased oxidative stress and concentration of inflammatory mediators in the brain tissues of ethanol-exposed pups. Activity of caspase-3 enzyme was also found significantly decreased in the CA treated group compared to the negative control group. The present study concludes that CA attenuates the neuronal damage induced in alcohol exposed neonatal rat by decreasing the apoptosis of neuronal cells.

Keywords

Chlorogenic acid • Neuroprotective • Alcohol • Neonates • Apoptosis

Introduction

In pregnancy, chronic consumption of alcohol produces adverse effects on the brain of the foetus, such as abnormalities in social and executive functioning, motor skill, defect in memory, learning and general intelligence [1]. Foetal alcohol spectrum disorder (FASD) is a broad terminology in which prenatal exposure to alcohol results in growth retardation and neurological defects [2]. Literature data suggest that a huge burden of cost is required to care the patient suffering from FASD-associated mental retardation [3]. Exposure to alcohol alters the development of brain by interfering in cellular, neurochemical and molecular events in the foetus [4]. Several regions in the brain are susceptible to alcohol, such as cerebellum, hippocampus and the neocortex. These areas are associated with behavioural deficit [5]. Fate of alcohol suggest that it is metabolised into acetaldehyde first, which leads to generate the free radicals and it also decreases the

expressions of antioxidant enzymes [6]. Literature indicates that oxidative stress induced due to alcohol consumption damages neuronal cells by activating necrosis and apoptosis pathways [7, 8]. Proper management of FASD requires different strategy and, in the recent time, alternative medicine has shown potential for the management of it.

Chlorogenic acid is a phenolic compound present in several herbal plants, such as blueberries, apple and coffee viz [9]. Reported studies suggest that CA inhibits the growth of bacteria and reduces the chances of chemical-induced cancers [10, 11]. In addition to it, CA has shown an anti-inflammatory activity by inhibiting the inflammatory cytokines, such as TNF- α , IFN- γ , IL-1 β and NF- κ B [12-14]. However, it has also shown to possess a strong antioxidant property by reducing the generation of reactive oxygen species. Thus, an effort was made to evaluate the neuroprotective effect of CA on alcohol-induced brain damage in neonatal rat.

Material and method

Animals

Male Wistar rat 5 days old pups were procured from shanghai medical college, China. All the animals were housed under controlled conditions as specified by guidelines. All experimental procedures were approved by Institutional animal ethical committee of Ankang central hospital, China (ACH/IAEC/2016/12) and the given study followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use.

Experiment

All the pups were separated into four different groups, such as control group which received distilled water, negative control group received ethanol at a dose of 5g/kg by intragastric intubation on postnatal days (PD) 7, 8, and 9 and, chlorogenic acid treated group received

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CA (100 and 200 mg/kg) with ethanol from PD 6 to 28. Morris water maze was used for the estimation of cognitive function in between PD 24-28 day.

Behaviour study *Morris water maze test*

Cognitive function in all the pups was assessed by MWM test between PD 24 and PD 28. Circular tank with 60 cm of height and 180 cm of diameter was used in the study. A platform was placed in the tank 2 cm below the water level having the height 38 cm and 12.5 cm diameter which was visible to pups. Tank was divided into four different quadrants and a different symbol was placed in the room to differentiate the different quadrants of the tank. Training was given to all the pups for 4 days continuously. In each session, 4 trials were given at a interval of 30 sec in which animals were placed in all quadrants. The pups were kept as such for the duration of 20 sec on the platform after they climbed on it. The time taken by the pups to reach the platform was considered as escape latency. However, the time spent in the target quadrant was estimated by removing the stage from the tank and the pups were allowed to search the platform.

Tissue preparation

All the animals were sacrificed by cervical dislocation and brain from all the pups were isolated. Hippocampus and cerebral cortex were separated out from each brain. Isolated tissues were incubated for half an hour with 1 ml of buffer which contains detergent solution (1%) and 1 mM DTT. Thereafter, tissues were centrifuged at 4 °C at 10000 rpm for the duration of 10 min and supernatant (cytoplasmic fraction) was stored at 4 °C after separating it out. Nuclear lysis buffer (100 ml) was used to resuspend the nuclear pellet. Suspension was incubated for the duration of half an hour at 4 °C after stirring the sample vigorously and microcentrifuge was used to centrifuge the sample at 4 °C for the duration of 10 min at 14000 rpm. Supernatant i.e. nuclear fraction was poured in to centrifuges tube and stored at 4 °C. These separated fractions were used for the biochemical estimation.

Estimation of biochemical parameter *Assessment of activity of acetylcholinesterase enzyme*

Activity of acetylcholinesterase enzyme was estimated in the cytoplasmic fraction of hippocampus and cerebral cortex as per the previously described method. Results were calculated using molar extinction coefficient of chromophore ($1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and expressed as percentage of control.

Estimation of markers of oxidative stress

Levels of lipid peroxidation (LPO) were determined as per the previously reported method in the cytosolic fraction of brain tissues. Amount of malondialdehyde (MDA) was estimated at 532 nm. Activity of superoxide dismutase (SOD) was determined in the cytosolic fraction of brain tissue by using riboflavin sensitized method. The alteration in absorbance was observed for 4 min at a wavelength of 460 nm. Activity of catalase (CAT) enzyme was observed in the cytosolic fraction of brain tissues depending upon the ability of catalase to oxidize H_2O_2 . The alteration in the level of absorbance was determined for the duration of 3 min at 1 min interval at 240 nm. Level of nitrite was determined using Greiss reagent in the cytosolic fraction of brain tissues. The absorbance was estimated at a wavelength of 546 nm.

Estimation of cytokines

Enzyme-linked immunosorbent assay ELISA kit was used for the determination of cytokines (IL1 β and TNF α) in the cytosolic fraction of brain tissues of alcohol-induced brain damage in neonatal rats. The procedure was used as per the instruction given in the individual kit.

Estimation of NF-kB p65 unit

Levels of NF-kB p65 were estimated by using ELISA kit in the nuclear fraction of brain tissue. ELISA plate reader was used for the detection of change in the color at 405 nm.

Estimation of activity of Caspase-3

Caspase-specific peptide was added to the tissue homogenate for the estimation of activity of protease enzyme. These peptides were conjugated with p-nitroaniline which

acts as an indicator. Activity of caspase-3 enzyme was depending on the cleavage of p-nitroaniline by caspase-3 and the change in the colour was detected at 405 nm due to release of p-nitroaniline from the substrate.

Statistical analysis

All data were expressed as means \pm SD (n = 6). The statistical analysis was performed using one-way ANOVA. Post-hoc comparison of means was carried out by Dunnett's post hoc test (Gradpad prism 6.1., CA, USA) multiple comparisons. The level of statistical significance was set at $P < 0.05$.

Result

Effect of chlorogenic acid on the cognitive function

Effect of chlorogenic acid on the cognitive function in alcohol-induced brain damage in neonatal rat was shown in Fig. 1. There was significant increase in the escape latency and decrease in the time spent at the target quadrant in negative control group of rats compared to the control group. However, treatment with CA significantly decreases the escape latency and increases the time spent at the target quadrant compared to the negative control group.

Effect of chlorogenic acid on acetylcholinesterase enzyme

Effect of chlorogenic acid on acetylcholinesterase in the brain tissues, such as cerebral cortex and hippocampus, of alcohol-induced brain damage in neonatal rat was shown in Fig. 2. It was observed that the activity of acetylcholinesterase enzyme was found to be significantly enhanced in the cytosolic fraction of cortex and hippocampus of negative control group of rats compared to control group. There was a significant decrease in the activity of acetylcholinesterase enzyme in the cytosolic fraction of cortex and hippocampus tissue of CA treated groups compared to the negative control group.

Effect of chlorogenic acid on oxidative stress parameters

Effect of chlorogenic acid on the parameters of oxidative stress in the cerebral cortex and

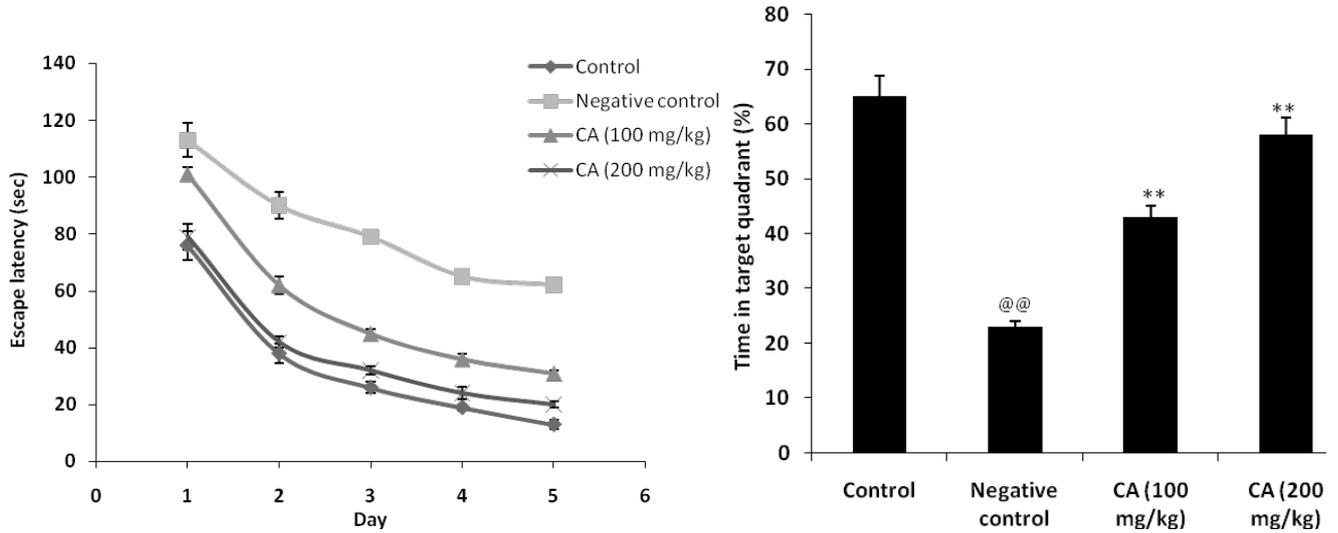


Fig. 1. Effect of chlorogenic acid on the cognitive function in alcohol-induced brain damage in neonatal rat. Values are expressed as Means \pm SD (n = 6), ^{@@}p < 0.01 Vs control group, ^{**}p < 0.01 vs negative control group.

hippocampus tissues of alcohol-induced brain injury in neonatal rat model was shown in Table 1. There was significant increase in the levels of MDA and nitrite in the cytosolic fraction of the brain tissue (cerebral cortex and hippocampus) in the negative control group compared to the control group. Moreover, activity of SOD and CAT was found to be significantly decreased in the brain tissues of the negative control group compared to the control group of rats. Treatment with CA significantly decreased the levels of MDA and nitrite and increases the activity of SOD and CAT in the cytosolic fraction of the brain tissue (cerebral cortex and hippocampus) compared to the negative control group of rats.

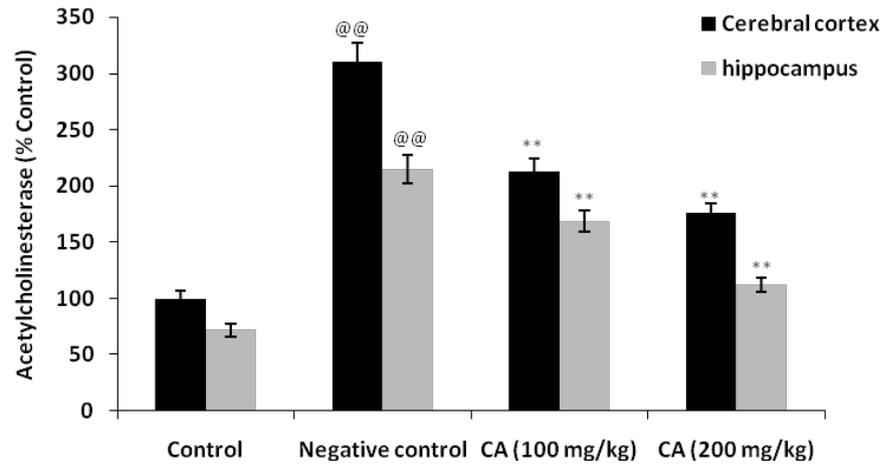


Fig. 2. Effect of chlorogenic acid on acetylcholinesterase in the brain tissues, such as cerebral cortex and hippocampus, of alcohol-induced brain damage in neonatal rat. Values are expressed as Means \pm SD (n = 6), ^{@@}p < 0.01 vs control group, ^{**}p < 0.01 vs negative control group.

Table 1. Effect of chlorogenic acid on the parameters of oxidative stress in the cerebral cortex and hippocampus tissues of alcohol-induced brain injury in neonatal rat model.

Group	Cerebral cortex				Hippocampus			
	MDA	SOD	CAT	Nitrite	MDA	SOD	CAT	Nitrite
Control	1.82 \pm 0.14	6.32 \pm 0.82	4.79 \pm 0.73	174.6 \pm 11.2	1.93 \pm 0.16	4.26 \pm 0.58	3.27 \pm 0.33	102.6 \pm 6.4
Negative Control	6.56 \pm 0.52 ^{@@}	0.58 \pm 0.02 ^{@@}	0.72 \pm 0.18 ^{@@}	482.8 \pm 23.5 ^{@@}	5.17 \pm 0.48 ^{@@}	0.19 \pm 0.03 ^{@@}	0.52 \pm 0.17 ^{@@}	315.8 \pm 18.2 ^{@@}
CA (100 mg/kg)	4.12 \pm 0.28 ^{**}	1.93 \pm 0.11 ^{**}	1.65 \pm 0.32 ^{**}	351.7 \pm 17.3 ^{**}	4.38 \pm 0.32 ^{**}	1.23 \pm 0.37 ^{**}	1.14 \pm 0.21 ^{**}	271.9 \pm 12.1 ^{**}
CA (200 mg/kg)	3.86 \pm 0.19 ^{**}	2.65 \pm 0.35 ^{**}	2.91 \pm 0.46 ^{**}	286.8 \pm 8.6 ^{**}	3.11 \pm 0.23 ^{**}	1.96 \pm 0.48 ^{**}	1.84 \pm 0.32 ^{**}	235.5 \pm 9.5 ^{**}

Values are expressed as Means \pm SD (n = 6), ^{@@}p < 0.01 vs control group, ^{**}p < 0.01 vs negative control group

Effect of chlorogenic acid on cytokines

Effect of chlorogenic acid on inflammatory cytokines, such as TNF- α and IL-1 β in the brain tissues, including the cerebral cortex and hippocampus, of alcohol-induced brain damage in neonatal rat was shown in Fig. 3. It was observed that levels of TNF- α and IL-1 β were significantly increased in the brain tissues of the negative control group of rats compared to the control group. However, altered levels of TNF- α and IL-1 β were attenuated in the CA treated group of rats compared to the negative control group in a dose dependent manner.

Effect of chlorogenic acid on NF- κ B

Effect of chlorogenic acid on NF- κ B in the brain tissues, such as cerebral cortex and hippocampus of alcohol induced brain damage in neonatal rat was shown in Fig. 4. There was a significant increase in the levels of p65 of NF- κ B in the nuclear fraction of brain tissue (cerebral cortex and hippocampus) in the negative control group of rats compared to the control group. It was observed that the levels of p65 of NF- κ B were significantly decreased in a dose dependent manner in CA treated group of rats compared to the negative control group.

Effect of chlorogenic acid on the activity of caspase

Effect of chlorogenic acid on the activity of caspase-3 enzyme in the brain tissues, such as cerebral cortex and hippocampus, of alcohol-induced brain damage in neonatal rat was shown in Fig. 5. Activity of caspase3 enzyme was found to be significantly enhanced in the brain tissue of the negative control group compared to the control group of rats. However, treatment with CA significantly reduces the activity of caspase-3 enzyme in the brain tissues of alcohol-induced brain damage in neonatal rat compared to the negative control group.

Discussion

Chronic consumption of alcohol by mother at the period of pregnancy leads to altered neurological function in the offspring [2]. Several reports reveal that exposure of CNS depressant drugs and alcohol in rats in the third

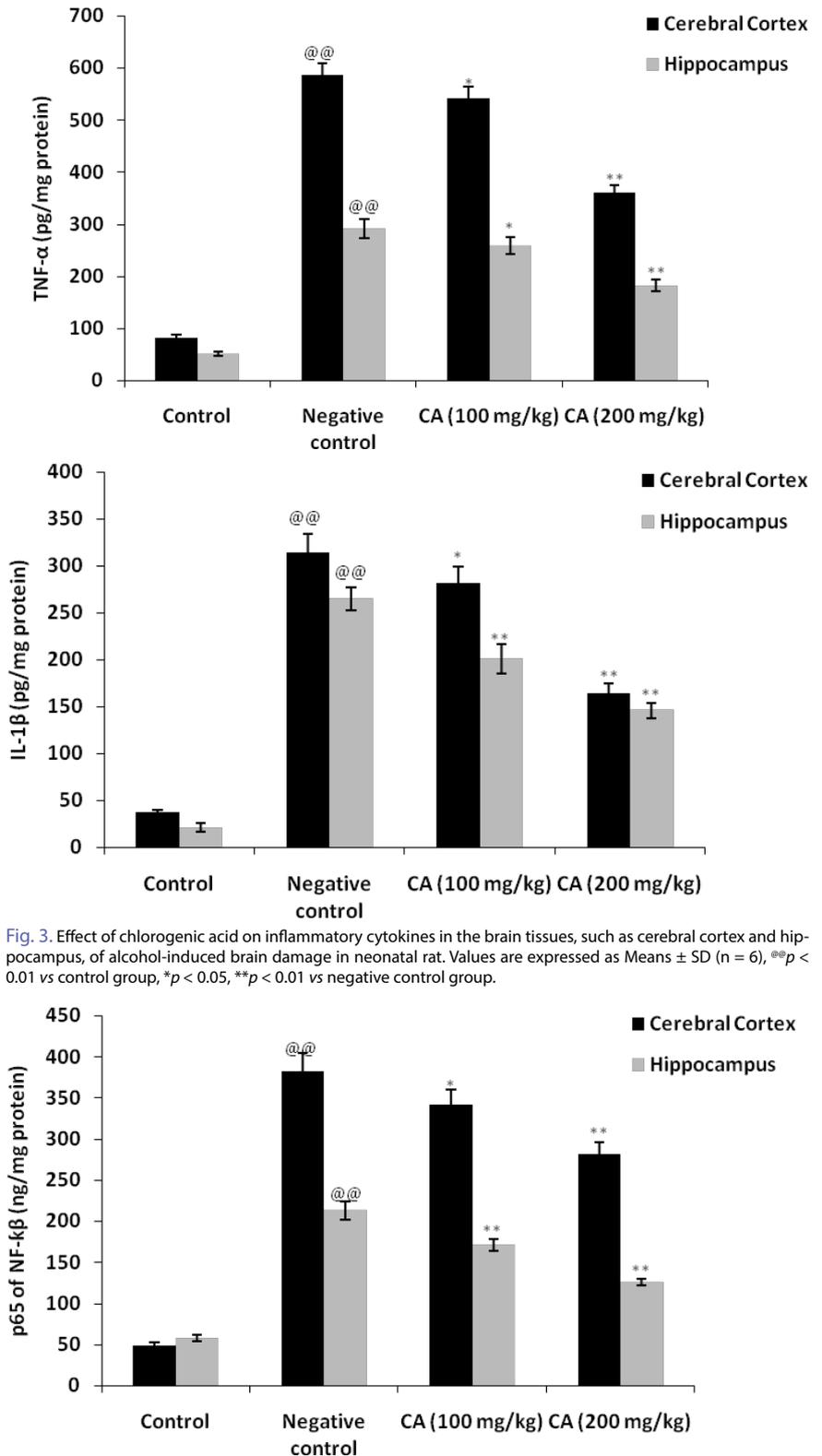


Fig. 3. Effect of chlorogenic acid on inflammatory cytokines in the brain tissues, such as cerebral cortex and hippocampus, of alcohol-induced brain damage in neonatal rat. Values are expressed as Means \pm SD (n = 6), @@p < 0.01 vs control group, *p < 0.05, **p < 0.01 vs negative control group.

Fig. 4. Effect of chlorogenic acid on NF- κ B in the brain tissues, such as cerebral cortex and hippocampus, of alcohol-induced brain damage in neonatal rat. Values are expressed as Means \pm SD (n = 6), @@p < 0.01 vs control group, *p < 0.05, **p < 0.01 vs negative control group.

trimester or first few weeks of postnatal period induces the apoptosis of neuronal cells [15]. Due to which brain function get altered in these rats after the maturation. In this study, alcohol was given to rats between PD 7 and 9, showing a significant alteration in the cognitive function in rats.

Literature data reveal that antioxidant drugs have the potential to attenuate the cognitive functions, such as learning and memory in several chronic conditions too [16]. Data of our study represent that treatment with CA significantly improves the cognitive function in alcohol-induced brain injury in neonatal rats.

Apoptosis in neuronal cells has been reported to be attenuated by acetylcholine as it stimulates the muscarinic receptor and thereby enhances the proliferation glial cells [17]. Exposure to alcohol decreases the neuronal cells which leads to disturbance of cognitive function [18]. Result of our study shows that treatment with CA decreases the activity of acetylcholinesterase in the brain cells. Our study supports these findings. Moreover, consumption of alcohol chronically stimulates the inflammation by enhancing the inflammatory cytokines in the brain cells [19]. In this investigation, elevated levels of TNF- α and IL-1 β were attenuated with CA treatment

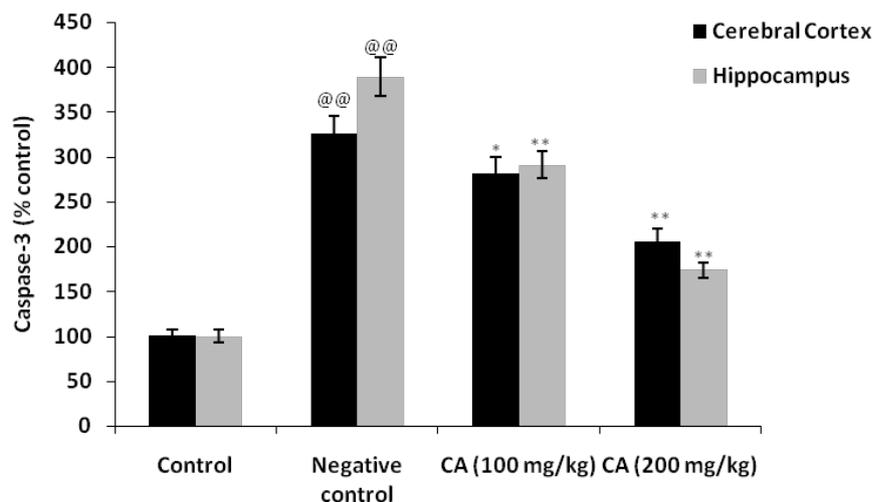


Fig. 5. Effect of chlorogenic acid on the activity of caspase-3 enzyme in the brain tissues, such as cerebral cortex and hippocampus, of alcohol-induced brain damage in neonatal rat. Values are expressed as Means \pm SD (n = 6), ^{@@}p < 0.01 vs control group, ^{*}p < 0.05, ^{**}p < 0.01 vs negative control group.

in alcohol-induced brain injury neonatal rat model. In addition to it, levels of NF- κ B and activity of caspase-3 enzyme were found to be increased in the brain tissues of alcohol treated rats, thereby activating the pathway of apoptosis and damaging neuronal cells. Literature suggested that chronic exposure of alcohol generates oxidative stress, which in turn activates NF- κ B, responsible for the fragmentation of DNA in the neuronal cells [20]. CA treated group showed significant decrease in the levels of NF- κ B and activity of

caspase-3 and, thus, having protective effects on neuronal cells.

Conclusion

The present study concludes that CA attenuates the neuronal damage induced in alcohol exposed neonatal rat by decreasing the apoptosis of neuronal cells. Our data demonstrates that apoptosis was decreased in the CA treated rats on the basis of its anti-inflammatory and antioxidant property.

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