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ORIGINAL PAPER

CURCUMIN ABATES FORMALDEHYDE-INDUCED NEUROTOXICITY VIA NO PATHWAY AND THE CHANGE OF MINERALS (CALCIUM, IRON, ZINC, COPPER, MAGNESIUM) IN BRAIN TISSUE*

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Abstract

Oxidative stress has been defined as an imbalance between higher cellular levels of reactive oxygen species (ROS). If ROS are not controlled by enzymatic and non-enzymatic antioxidants, they can cause oxidative injury. Brains are protected by antioxidants from nitro-oxidative and peroxidative damage. The antioxidant enzymes are superoxide dismutase and catalase, which contain zinc (Zn) and copper (Cu) as cofactors. Also, trace elements have important effects on brain development and function. In this study, we aimed to investigate the effect of curcumin administration on the exchange of nitric oxide (NO) and on the calcium (Ca), iron (Fe), Zn, Cu, and magnesium (Mg) levels in brain tissue. Animals (a total of 30 adult Wistar albino rats, 4-6 months old) were randomly divided into three groups (n = 10): control, formaldehyde-exposed, and treated daily with curcumin after formaldehyde exposure (100 mg kg^{-1}). At the end of the experimental period (the 14th day), NO levels were measured by ELISA. Ca, Fe, Zn, Cu, and Mg levels in whole-brain tissues were determined by Atomic Absorption Spectrophotometry in all groups. NO and Mg levels were increased and Cu and Ca levels were decreased in the group treated with curcumin when compared with the formaldehyde-only group. These changes were not statistically significant ($P > 0.05$). However, Fe levels were significantly reduced and Zn levels were significantly increased ($P < 0.05$). In conclusion, the administration of curcumin as an antioxidant may be a factor in regulating the mineral balance of the brain in conditions of oxidative stress caused by the application of FA. Curcumin may play a role in reducing FA-induced cellular damage, and may contribute to the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's.

Keywords: curcumin, formaldehyde, calcium, iron, zinc, copper, magnesium, nitric oxide.

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INTRODUCTION

Oxidative stress is defined as an imbalance between higher cellular levels of reactive oxygen species (ROS), e.g. superoxide radical, hydrogen peroxide and nitric oxide (NO). If ROS are not controlled by enzymatic and non-enzymatic antioxidants, they can cause oxidative injury. The antioxidant enzymes are superoxide dismutase and catalase, which contain zinc (Zn) and copper (Cu) as cofactors. The brain is extremely susceptible to oxidative damage induced by these ROS, because it generates very high levels of ROS due to its very high aerobic metabolism and blood perfusion, and because it has relatively poor enzymatic antioxidant defense (Naziroğlu 2007). The brain contains polyunsaturated fatty acids (PUFAs), which can readily be peroxidized. Brains are protected by antioxidants from nitro-oxidative and peroxidative damage.

Formaldehyde (FA) is a biological compound widely used in the medical industry, and it naturally exists in the body fluids and cells of organisms. ROS formation increases as a result of the interaction of FA with the DNA and lipids in most tissues. This causes lipid peroxidation, oxidative stress and damage. Neuronal damage is an important contributor to the cause of oxidative stress, through hypoperfusion. As a result of the cytotoxic and genotoxic effects of FA, cancers have been reported in humans and in the animals used in experiments (International Agency for Research on Cancer 2006). People exposed to FA have been observed to lose consciousness, a condition associated with changes in neurofilament proteins and with hippocampal neuron demyelination.

For the development and function of the brain, trace elements such as zinc (Zn), manganese (Mg) and iron (Fe) are necessary. The homeostasis of trace elements is also important for function in the brain and for the prevention of brain disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease, Hallervorden-Spatz disease, and Friedreich's ataxia. These have been reported to be caused by neuronal degeneration and cell death (Shoham, Youdim 2000).

Magnesium (Mg) was found to be the third most common element in the body and is more abundant than calcium (Ca) and phosphorus (P). The most important tasks of Mg are its roles in protein and fat synthesis, manipulation of ATP, and nerve and blood vessel stabilization. By regulating the entrance of Ca into the cell, Mg plays a role of a natural Ca antagonist. Mg deficiency increases plasma levels of NO, one of the free radicals. The application of intracellular Mg reduces NO production by nitric oxide synthase (NOS) inhibition. NO is involved with brain neurotransmitter compounds, as demonstrated in experimental studies on animals, and in intracellular communication activity, especially with a role in learning and memory (Schumann, Madison 1991). NO is quick to react with oxygen, superoxide radicals, and transition metals such as Fe and Cu in order to contain its unpaired

electrons. The amounts of NO's quick reactions have been reported to be increased in many pathological conditions, including cancer, and may be a contribution to neurotoxicity (Ambs et al. 1997).

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione-diferuloyl methane] is a natural substance obtained from turmeric (*Curcuma longa* L. *rhizomes*) and has been used for many years in the treatment of inflammatory diseases, and it has nontoxic effects in cases of overdose. Curcumin is known to be effective as an anti-oxidant, anti-tumorigenic, antimicrobial, antihyperlipidemic and anti-inflammatory substance. Curcumin is also said to offer protection from neurodegenerative conditions such as Alzheimer's disease and multiple sclerosis (Cole et al. 2007). Curcumin is a Fe chelator and has the ability to connect with Fe, Cu, and free radicals.

It has not been studied whether curcumin modifies the alterations in elements and NO levels in the brain cortex in FA-exposed rats. Hence, we aimed to evaluate the trace element and NO values to determine whether there would be a protective effect by curcumin in FA-exposed rats.

MATERIAL AND METHODS

Animal experiments

All experimental procedures were performed according to the guidelines for the ethical treatment of experimental animals, and an ethical approval for the study was received from the Laboratory Animals Local Ethical Committee of Ondokuz Mayis University (HADYEK/139), Samsun. Thirty male Wistar albino rats (200-250 g each) were housed under standard laboratory conditions, maintained at 22°C and on a 12/12-h light/dark cycle. All animals were allowed free access to standard chow and ad-lib freshwater from a water-supply system. The diet (metabolic energy 2650 kcal kg^{-1}) consisted of dry matter (at least 88%), raw protein (at least 24%), raw cellulose (at most 7%), raw ash (at most 8%), raw fat (at least 6%) and salt (at most %1). Same management conditions were applied for all experimental groups during the experimental period.

The rats were divided into three groups (n = 10). Group I (control, group) comprised control animals that received intraperitoneal injections of saline. Group II (FA group) animals received an intraperitoneal injection of FA at a dose of 9 mg kg^{-1} body mass every other day (Zhou et al. 2006). The rats in Group III were given curcumin 100 mg kg^{-1} daily via intragastric gavage, along with injections of FA (Sintara et al. 2012). At the end of the 2-week experimental period, the brains were removed after decapitation.

Brain tissue sample preparation

The animals were euthanized under general anesthesia and tissues were removed, dissected at 4°C, and freeze-clamped as described above after 14 days of trial. The whole-brain tissues were homogenized in phosphate buffered saline with pH of 7.4 (10 ml g^{-1} tissue) using a homogenizer, and then cooled on ice. The brain homogenates were centrifuged at 10,000 g for 10 min, and later with subsequent ultra-centrifugation of the supernatant solution at 100,000 g for 15 min at 4°C, and they were then stored at –80°C until assay (Cernak et al. 2001).

Determination of nitric oxide in the brain tissue

The level of NO in the brain was measured using a commercial colorimetric ELISA kit (Nitric Oxide Assay Colorimetric Kit, Cayman Chemical Company, Ann Arbor, USA) according to the manufacturer's instructions, on the supernatant produced from brain tissues previously described by Gren et al. (1982).

Element assay

Evaluation of brain-tissue Ca, Fe, Zn, Cu, and Mg content was carried out by Atomic Absorption Spectrophotometry (AAS). The previously frozen tissue samples (–80°C) of all groups were weighed to 0.5 g. They were placed in a microwave wet combustion system (1600 W, 50%, 210°C, 10 h), and 7 ml of nitric acid and 3 ml of ultra-pure water were added. The results were read by AAS with hollow cathodes by dilution with ultra-pure water and attaching the mineral-specific lamps (Ca 422 nm, Fe 285 nm, Mg 285 nm, Cu 324 nm, Zn 213 nm). The samples and standards for specific minerals, concentrated and diluted, were measured with AAS, using ultra-pure water as a blind solution. The calibration curve was drawn by giving an order standard for the prepared study, and a 0.995-1.000 confidence interval and a 99.5% calibration coefficient (CV) were achieved. The diluted brain samples were read in order. After reading 5-10 samples, the device was calibrated using blind and standards. The reading of the samples was then continued. The values reflected on the computer monitor, using the automatic calculations of the device connected to the computer, were taken as output, and normal values were found by multiplying with the dilution coefficient.

Statistical analysis

Statistical data were described by mean ± standard error of means (SE). One-way analysis of variance was performed to analyze all of the brain groups. The Duncan's multiple-range test was used to evaluate whether there were significant differences in NO, Fe, Cu, Ca, Mg, and Zn concentrations in all of the rat brains. The relationships among NO, Fe, Cu, Ca, Mg, and Zn concentrations were evaluated by the Pearson's correlation analysis.

RESULTS AND DISCUSSION

The rats were separated into three groups. The control animals (Group I) were injected intraperitoneally with saline. Group II rats were injected intraperitoneally with FA 37% at a dose of 9 mg kg^{-1} body mass every other day. Group III rats were given curcumin 100 mg kg^{-1} daily via intragastric gavage with injections of FA. The NO, Ca, Fe, Zn, Cu, and Mg levels in the brain tissues of the three groups are shown in Table 1.

Table 1

Levels of nitric oxide (NO), iron(Fe), copper (Cu), calcium(Ca), magnesium (Mg) and zinc (Zn) in brain tissue of rat among groups

Parameters (mM L^{-1}) and groups	Mean±SE	Min	Max	*P* value	Ranks
NO					
Control	20.020±10.66	12.400	27.650		14.800
Formaldehyde	16.590±6.35	12.040	21.140	0.302	13.500
Formaldehyde and curcumin	23.920 ±12.98	14.630	33.200		18.200
Ca					
Control	3.876±2.367	2.056	5.697		16.330
Formaldehyde	4.473±2.715	2.531	6.415	0.220	18.600
Formaldehyde and curcumin	2.572±2.149	1.035	4.109		10.200
Fe					
Control	0.097±0.017^{a}	0.083	0.110		7.440
Formaldehyde	0.172±0.022^{b}	0.157	0.188	0.000	24.500
Formaldehyde and curcumin	0.112±0.018^{a}	0.099	0.125		12.300
Cu					
Control	0.041±0.004	0.038	0.044		12.060
Formaldehyde	0.045±0.007	0.039	0.050	0.426	17.650
Formaldehyde and curcumin	0.043±0.005	0.039	0.047		15.000
Mg					17.220
Control	4.077±1.73^{a}	2.744	5.409		10.100
Formaldehyde	2.960±0.201^{b}	2.816	3.103	0.068	17.900
Formaldehyde and curcumin	3.286±0.525^{ab}	2.910	3.661		
Zn					
Control	0.052±0.011^{ab}	0.043	0.061		15.110
Formaldehyde	0.048±0.005^{a}	0.044	0.052	0.061	11.150
Formaldehyde and curcumin	0.064±0.022^{b}	0.048	0.080		18.750

^{a}, ^{b} – the difference between the values bearing the different letters in the same row is statistically significant ($P < 0.05$)

While the NO and Mg levels of the curcumin-treated group increased slightly when compared with the FA-only group, their Cu and Ca levels decreased. These changes were not statistically significant ($P > 0.05$). Also, the Fe levels in the curcumin-treated group were significantly reduced, but their Zn levels were significantly increased ($P < 0.05$).

A significant positive correlation was observed between the Mg and Fe levels ($r = 0.857$), Mg and Zn levels ($r = 0.909$), and Fe and Zn levels ($r = 0.681$) in the FA-only group.

A negative correlation was determined between the amount of Ca in the FA-only group and the amount of Fe in the curcumin-treated group ($r = -0.678$*); and between the amount of Mg in the FA-only group and the amount of Fe in the curcumin-treated group ($r = -0.637$). In the curcumin -treated group, NO and Zn ($r = 0.844$) and Ca ($r = 0.838$) levels were significantly positively correlated. Also, the levels of Ca and Zn ($r = 0.751$*) and of Mg and Zn ($r = 0.698$*) were observed to have a significant positive correlation. The correlations among the groups are contained in Table 2.

Table 2

The correlation relationships of minerals between experimental groups

Examined parameters						
	NO	Ca	Mg	Fe	Zn	Cu
NO	1.000	0.205	0.140	-0.164	0.409*	-0.123
Ca		1.000	0.162	0.347	0.153	-0.096
Mg			1.000	-0.248	0.042	-0.170
Fe				1.000	-0.060	0.277
Zn					1.000	0.003
Cu						1.000

* $P < 0.05$

Cu, Fe, Zn, and Mg are the most essential metals, which act as cofactors. They catalyze various biological reactions. Any disturbance in their homeostasis results in destructive effects through the generation of free radicals, with subsequent lipid peroxidation, oxidative stress, demyelination, and denudation of axons (Dhillon et al. 2011). The imbalance between higher cellular levels of ROS, e.g. superoxide radicals, hydrogen peroxide and NO, is defined as oxidative stress (Naziroğlu 2007). The brain is particularly susceptible to oxidative stress compared with other organs because it utilizes the highest amount of oxygen. The brain contains high concentrations of polyunsaturated fatty acids that are prone to lipid peroxidation. It is also rich in Fe, which can catalyzes hydroxyl radical formation.

The generation of ROS by FA consumption increases oxidative stress in the mouse brain, lung and liver (Matsuoka et al. 2010). In our previous study for evaluating oxidative stress between formalin treated and curcumin treated rats, we found that oxidative stress caused by formaldehyde exposure

had been reduced with the application of curcumin (Ciftci et al. 2015). The antioxidant enzymes are superoxide dismutase and catalase, which contain Zn and Cu as cofactors. Brains are protected by antioxidants from nitro--oxidative and peroxidative damage (Naziroğlu 2011). In this study, the aim was to investigate the effect on NO levels and trace minerals, such as Fe, Zn, Ca, Mg and Cu, with the application of FA in the brain, and to investigate the role of curcumin treatment.

Fe is an essential element for the function of all cells, including the central nervous system, and it is one of the most abundant metals in the body. Fe is required for brain functions such as myelination, neurotransmitter synthesis, NO metabolism, and other brain biochemical activities (Hidalgo, Nunez 2007). In our study, we identified increased levels of Fe in the group with FA-only application, and reduced levels of Fe in the group treated with curcumin ($P < 0.05$). However, body Fe accumulation may give rise, in time, to neurodegenerative and aging processes; in cells, the rise to the labile or reactive Fe pool is probably involved in the induction of oxidative damage to vital cellular components. The concentration of Fe in the brain increases with age, and is relatively higher in the brains of subjects with neurodegenerative diseases, such as Parkinson's (Kristinsson et al. 2012).

Cu is an essential element that is important for reinforcement of biological functions. Cu is bound to enzymes or proteins, including cytochrome *c* oxidase, lysyl oxidase, ceruloplasmin, and superoxide dismutase, under normal circumstances (Rucker et al. 1998). We determined that when exposed to FA, Cu levels increased in the brain. However, when treated with curcumin, Cu levels decreased, but this decrease was defined as statistically not important ($P > 0.05$). In a previous study, researchers determined that the amount of Fe and Cu decreases with curcumin treatment (Huang et al. 2011). This occurs with reduced lipid peroxidation as a result of the strong metal binding and free-radical-capture properties of curcumin. There is information suggesting that curcumin protects cells from oxidative stress by playing an important role in brain and central nervous system functions, and in the antioxidant system (Nair et al. 2015), where curcumin is not connected with Zn metal (Baum-Ng 2004). Thus, Zn provides a positive contribution into the antioxidant system by slightly increasing when it is exposed to curcumin ($P < 0.05$). Zn is responsible for the protection of the blood-brain barrier against the oxidative stress of free radicals, and is essential for the metabolism and synthesis of coenzymes that mediate biogenic amine synthesis. Zn may play a role in intercellular signaling in the nervous system, as a neurotransmitter. Thus, a high Zn level prevents the development of neurological disorders and is essential in maintaining homeostasis within normal brain function.

The radical effect is emphasized by NO, which is needed for the realization of many physiological functions, both as a contributor to antioxidant defense and in the case of over-production. One of the important roles of NO

is to act as a novel biological messenger (Bredt, Snyder 1994). NO is an unconventional neurotransmitter or neuromodulator in the central nervous system. It also plays a role in the development and function of the brain, and in neurotoxicity, secretion and in animal learning. NO deposits in the brain are associated with Alzheimer's disease (Koprowski et al. 1993), multiple sclerosis (Goodwin 1995) and ischemia and stroke (Bagasra et al. 1995). The most important toxic effect of NO is created by highly toxic peroxynitrite, which is composed by the reaction of superoxides to form NO. Studies have reported this to be caused by necrosis (Mitrovic et al. 1995) membrane lipid peroxidation, and inhibition of cellular respiration, with single-strand breaks in the DNA of peroxynitrite (Brown et al. 1995). This affects energy conservation mechanisms and the oxidative post-translation modification of protein, and ultimately causes neuronal cell death. In our study, we saw a decrease in the level of NO with the application of FA. This situation is considered to arise from the transformation of peroxynitrite into a form that is a toxic oxidant and much more effective than NO. When treated with curcumin, NO levels increased again ($P > 0.05$). Curcumin's neuroprotective effect is caused by increasing NO release via the increased blood flow. In addition to exerting a tonic dilator influence on cerebral circulation, basal release of NO may protect the cerebral endothelium by inhibiting aggregation of platelets and leukocytes. In studies, high levels of NO, similar to oxygen, have been reported to lead to damage connected to transition metals, such as Fe, which help protein structures in the cell by entering the cell and causing the release of free Fe into the environment (Smith et al. 2006). In parallel with our study, using human subjects and rats, there have been reported increases in plasma (Neiman, Benthin 1997) and decreases of NO levels in the brain due to alcohol intake (Kurban, Mehmetoğlu 2008). An increase in NO levels was reportedly caused by the increase of free radical production in the brains of rats exposed to electromagnetic fields of 900 MHZ and 1800 MHZ (Ciftci et al. 2012).

In our study, we identified a decrease in the level of NO in the brain when exposed to FA, increase in the amount of accumulated Ca, and then its reduction with curcumin application ($P > 0.05$). It was also determined that there was a decreased amount of Mg as an antagonist to Ca when exposed to FA in the brain, but it increased again when curcumin was administered. Low levels of Mg and high levels of Ca reportedly result in neuron damage by causing free-radical generation, lipid peroxidation and protein destruction (Siesjo 1988). The lack of Mg ions leads to the formation of more radicals by stimulating the synthesis of NO. In parallel with our study, rats with a decreased (Heath, Vink 1996) level of Mg caused by acute alcohol exposure (Altura et al. 1995) have been reported to suffer brain damage, while a high level of Mg leads to neuroprotection in the brain. Abnormal increases in intracellular Ca^{2+} trigger intracellular responses. This increase in Ca levels leads to neuronal death by playing a central role in the cell death process (e.g., ischemia, hypoglycemia, hypoxia or other trauma). Increasing the Ca^{2+}

amount induces more production of NO by activating the NOS enzyme. The combination of the superoxide anion and NO gives rise to peroxynitrite, which in turn decomposes into OH and NO, free radicals that are highly reactive and might be the final common mediators of NO toxicity. In parallel with our study, the event of an injury activates endothelial NO synthesis. The cell membranes are induced to produce endothelial NO products and this is caused by increased exogenous Ca^{2+} ionophores in the brain (Stein, Vannucci 1988, Faraci, Brian 1994). In the case of neuronal injury and in the event of an increase in NO and free radicals, the cell's Ca level was increased with extracellular glutamate. The activation of extracellular glutamate is the most important way that cell receptors have reportedly been activated (Dawson et al. 1996).

CONCLUSIONS

In conclusion, the administration of curcumin as an antioxidant may be a factor in regulating the mineral balance of the brain in conditions of oxidative stress caused by the application of FA. Curcumin may play a role in reducing FA-induced cellular damage, and may contribute to the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's.

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