Research article

Zinc intake during pregnancy increases the proliferation at ventricular zone of the newborn brain

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Neurogenesis involves cell proliferation, cell cycle arrest, differentiation, migration and the natural developmental death of the neural precursors. These processes are highly co-ordinated and governed by cell-cycle genes and neural transcription factors. Zn plays a crucial role as a functional and structural component of enzymes and transcription factors and components of the intracellular signaling pathway associated with the regulation of cell proliferation. The influence of additional Zn intake during pregnancy on the neuronal proliferation at ventricular zone of the developing fetus has been studied. Pups delivered by the group of mice provided with drinking water with 4.0 mM Zn supplement throughout pregnancy contained an increased number of proliferating neurons in the ventricular zone at P0 compared to those delivered by the mice provided with drinking water without any Zn supplement. This finding provides direct evidence to support the notion that maternal Zn levels influence the development of the nervous system of the offspring.

Keywords: BrdU, central nervous system, cortex, embryonic development, neuron

Introduction

Zinc (Zn) is an essential trace element present in all organs, tissues, fluids, and secretions of the body and it is widely distributed in the central nervous system. Zn plays a crucial role as a functional and structural component of enzymes and transcription factors[1,2] and components of the intracellular signaling pathway associated with the regulation of cell proliferation.[3] Postmitotic neurons and glial-like cells (oligodendrocytes and astrocytes) are generated from neuroepithelial stem cells in a process known as neurogenesis that involves cell proliferation, cell-cycle arrest, differentiation, migration and the natural developmental death of the neural precursors.[4,5] These processes are highly co-ordinated, interactive and governed by cell-cycle genes and neural transcription factors which control the correct positional identity of the neural cells from the stem/progenitor cells.[6–10]

A growing number of studies have shown the influence of maternal Zn on fetal growth and development.[11–13] The current study has shown the influence of additional Zn intake during pregnancy on neuronal proliferation of the developing fetus. For this, pregnant mice were given oral Zn supplement with their drinking water during the period of gestation.

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Materials and methods

Animals

Female, ICR-strain mice were used throughout the study (aged, 6–7 weeks; average body weight, 20–25 g). The day of mating was confirmed by the presence of vaginal plug and defined as embryonic (E) day 0. All animals were housed and used following the appropriate guidelines.

Zn administration and bromodeoxyuridine injection

Mice (n = 3) were provided with drinking water (deionized water) supplemented with 4.0 mM Zn (as $ZnSO_4$; Fisher Scientific, Loughborough, UK) from E0 (Zn group). A parallel group of mice (n = 3) were given the drinking water without any Zn supplement (control group). The pregnant mice were given a single intraperitoneal bromodeoxyuridine (BrdU) injection (2 ml/100 g body weight) between 09.00–10.00 am on E18 where usually delivery took place on E19.

Tissue processing

On the day of delivery (P0) between 09.00–10.00 am one randomly selected newborn pup from each mother was sacrificed by cold shock and brain was processed for BrdU immunohistochemistry. The average number of pups from the control group was 15 and that from the Zn group was 16. Briefly, brains were fixed overnight in 4% paraformaldehyde (Merck; Darmstadt, Germany) in phosphate buffered saline (PBS) at 4ºC followed by dehydration using the HM300 tissue processor (Microm; Systerm, Bellusco MI, Italy) in a series (70%, 85%, 95%, 100%) of ethanol steps, cleared in xylene (Clear Rite 3, Richard Allan Scientific, MI, USA) and finally embedded in paraffin using Tissue Embedding Centre, AP280 (Microm) to prepare tissue sections (7 μm thickness) in coronal plane on silinized glass slides.

BrdU immunohistochemistry

In situ BrdU immunohistochemistry was essentially performed according to the manufacturer's guidelines (BrdU detection kits; BD Biosciences, Pharmigen, SD, USA). Briefly, the sections were deparaffinized in xylene (3 times for 10 min each) then transferred to an ethanol series (twice in 100%, 90%, 70% ethanol) followed by washing (3 times in PBS for 5 min each). Sections were incubated in 3% H_2O_2 (Merck) for 10 min to block endogenous peroxidase activity followed by washing. Sections were then incubated with biotinylated anti-BrdU antibody (1:10) in the supplied diluent buffer followed by washing and incubation with ready-to-use streptavidin/horseradish peroxidase (HRP) for 30 min at room temperature. Detection of the presence of the complex of biotinylated anti-BrdU and avidin-HRP was performed using DAB as substrate which was allowed to react for 5 min or less until the desired colour intensity developed. The slides were then rinsed in running, sterile, distilled water (3 times for 2 min each) and counterstained with haematoxylin (Microm; Walldorf, Germany) for 30–60 s, followed by rinsing in water. Dehydration was performed using a series of ethanol (70%, 90%, 100%, and 100%) for 5 min each. Then the sections were cleared in three changes of xylene and were covered with a coverslip with mounting medium for image analysis.

Image analysis and quantification

Sections were digitized using a digital camera (Nikon E4500) mounted on an Olympus BX 41 microscope. The brightness and scale of each image were standardized. BrdU-positive cells are observed as having a dark-brown nuclear area.

Results

To investigate the influence of maternal Zn on neuronal proliferation of the developing fetus, a group of pregnant mice were provided with additional 4.0 mM $ZnSO_4$ in the drinking water. BrdU was injected intraperitoneally to the pregnant mice on the day before delivery usually at E18. Proliferating neurons were identified by immunohistochemistry using anti-BrdU. The number of proliferating neurons was markedly increased in the ventricular zone of pups delivered by mice supplied with additional 4.0 mM Zn in their drinking water (Fig. 1B) compared to the number of proliferating neurons in the ventricular zone of pups delivered by mice given no additional Zn (Fig. 1A).

Discussion

Almost all neurons are generated by early postnatal life and are generally not replaced with new ones.[14,15] In brain, ventricular zone is the major site of proliferation and presumably produces all the cell types. However, a second proliferative zone namely subventricular zone also contributes large numbers of neurons to the developing cortex.[16] In most brain regions, the generation of neurons is normally restricted to a discrete developmental period with exceptions for the regions such as hippocampus,

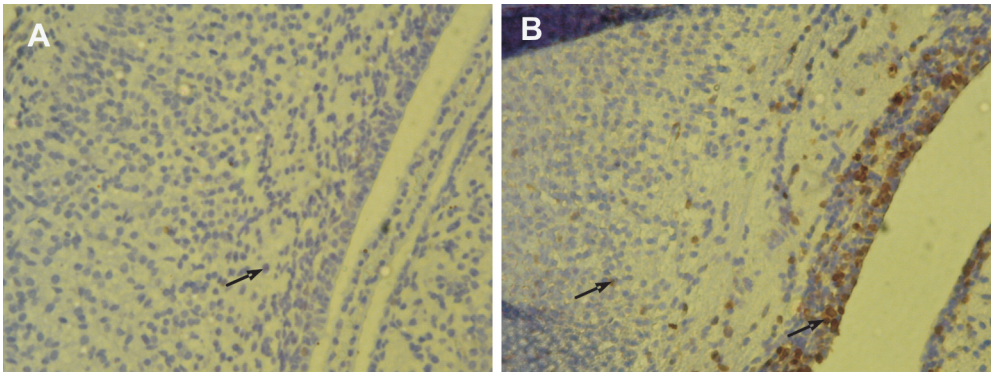

Figure 1 **Additional Zn during pregnancy increases the neuronal proliferation at the ventricular zone of the newborn brain. Proliferating neurons (brown in color shown with arrow) are identified using immunohistochemical detection of BrdU incorporation. Representative photomicrograph of selected region of neocortex of coronal sections are shown with proliferating neurons (arrow) derived from P0 brains delivered by (A) mice given no additional Zn and (B) mice given additional Zn (4.0 mM) in drinking water.**

dentate gyrus and the subventricular zone of several species.[17–25] In addition, granule neurons are generated throughout life from a population of continuously dividing progenitor cells residing in the subgranular zone of the dentate gyrus in the rodent brain.[23,26] Neurogenesis has also been demonstrated in the normal adult human brain.[27]

Zn participates in the regulation of cell proliferation in several ways. It is essential to enzyme systems that influence cell division and proliferation.[1,2,28,29] During DNA synthesis, Zn affects thymidine kinase, activity of which increases dramatically during the G_1 and early S phases of the cell cycle. Zn also influences hormonal regulation of cell division. Insulin-like growth factor-I and the pituitary growth hormone axis is responsive to Zn status. Circulating growth hormone concentrations are decreased by Zn deficiency in rats that causes failure of pituitary to secrete growth hormone.[30,31] However, Zn deficiency was not observed as a risk factor for low birth weight.[12]

In the current study, we have investigated neuronal proliferation at a later stage of embryonic development (*i.e.* E18) in mice. At this stage, proliferation at ventricular zone is generally minimal. Therefore, as expected, we have observed only few proliferating neurons in the ventricular zone of the brains obtained from the pups delivered by the control group (Fig. 1A). However, the number of proliferating neurons markedly increased in the ventricular zone of the brain obtained from the Zn-treated group (Fig. 1B). This shows that an oral Zn supplement to the mother during pregnancy can increase neuronal proliferation in the developing neocortex of the embryo. This observation is consistent with the report that Zn supplementation of Zn-deficient rats enhances lymphocyte proliferation and IFN-γ responses in their pups.[32] Zn deficiency was found to increase the expression of Zn transporters in brain, which facilitates increased brain Zn uptake and results in the conservation of brain Zn during Zn deficiency.[33] Involvement of the Zn transporter in Zn uptake in brain also might contribute to the neuronal proliferation that we have observed.

In neuronal cells, Zn deficiency induces oxidative stress, alters the normal structure and dynamics of the cytoskeleton, affects several transcription factors and results in decreased cell proliferation and increased apoptosis. These closely associated events affect neuronal function and critical developmental events (neuronal proliferation, differentiation, plasticity and survival) when Zn availability decreases.[34] Zn was reported to be structurally important for reelin, a large secreted protein, implicated in the cortical development of the mammalian brain. A 2.0-Å crystal structure from the fifth and sixth reelin repeats fragment revealed the presence of Zn^{2+} bound to them.[35] However, regular consumption of more than the recommended intake can have adverse effects.[29]

In the current study, we have provided 4.0 mM Zn supplement in the drinking water. T he total amount of water intake by the Zn-treated group did not differ significantly compared to that of the control group. The total number of pups delivered by the Zn group also did not vary significantly from that of the control group. No immediate, noticeable changes were observed of any toxic effect of Zn consumption during pregnancy which could be implicated in embryonic brain development. The long-term effect of maternal Zn consumption during pregnancy on offspring could be answered with further investigation.

To that end, we are currently investigating the number and distribution of apoptotic neurons and expression of Zn-inducible stress-related proteins like metallothionein. However, this finding is expected to raise basic questions on the fate of the additional neurons, in terms of distribution and function, in response to maternal Zn supplementation. In addition, the current study supports the importance of Zn supplementation to Zn-deficient mothers for proper brain development in neonates.[13,36]

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