

AN ANIMAL MODEL TO ASSESS THE EFFECTS OF HYDROXYUREA EXPOSURE SUGGESTS THAT THE ADMINISTRATION OF THIS AGENT TO PREGNANT WOMEN AND YOUNG INFANTS MAY NOT BE AS SAFE AS WE THOUGHT

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Abstract

The cytostatic agent hydroxyurea (HU) has proven to be beneficial for a variety of conditions in the disciplines of oncology, hematology, infectious disease and dermatology. It disrupts the S-phase of the cell cycle by inhibiting the ribonucleotide reductase enzyme, thus blocking the transformation of ribonucleotides into deoxyribonucleotides, a rate limiting step in DNA synthesis. HU is listed as an essential medicine by the World Health Organization. Several studies have indicated that HU is well tolerated and safe in pregnant women and very young pediatric patients. To our knowledge, only a few controlled studies about the adverse effects of HU therapy have been done in humans. Despite this, the prevalence of central nervous system abnormalities, including ischemic lesions and stenosis have been reported. This review will summarize and present the effects of HU-exposure on the prenatal and perinatal development of the rat cerebellar cortex and deep cerebellar nuclei neurons. Our results call for the necessity to better understand HU effects and define the administration of this drug to gestating women and young pediatric patients.

Keywords: hydroxyurea; cerebellum; neuron; immunohistochemistry; electron microscopy; cell death; apoptosis

1. Introduction

Hydroxyurea (HU) is an inhibitor of the ribonucleotide reductase enzyme. It impairs DNA synthesis in a wide variety of cells and organisms, including *Saccharomyces cerevisiae* [1]. This antimetabolite has provided therapeutic benefits in the treatment of neoplastic diseases and hemopathies, and it has emerged as is an important option for many pregnant women and pediatric patients with sickle-cell anemia [2]. Estimates indicate that approximately 250,000 children are born annually with this hematological disorder worldwide [3]. Several case reports suggest that HU may have minimal or no major adverse effects on the development of the human fetus and in very young children [4, 5]. However, the Center for the Evaluation of Risks to Human Reproduction is concerned that HU may increase the risk for congenital anomalies or developmental abnormalities in fetuses after exposure of pregnant women [6]. Moreover, anemia and central nervous system abnormalities have been reported in young pediatric patients treated with HU [7].

Previous research has revealed that the administration of this teratogenic agent to embryo rodents generates the apoptosis of neuroepithelial cells in the fetal telencephalon [8-9]. In the perinatal life, on the other hand, cerebellar external granule cells (EGL) depletion and ectopic location of granule cells (GCs) due to the HU-exposure have been reported [10-11]. Despite these results, the influence of this agent on the development of rat cerebellum has not been completely elucidated. The cellular response to damage induced by HU has received little attention. We will show here that the results of our research have important implications for the administration of HU to pregnant mothers and infants.

This review is focused on the development of the rat cerebellum following the treatment with a single dose of the cytotoxic agent HU. Two procedures were followed.

In the first one, rats were exposed to HU *in utero* and sacrificed at regular intervals from 5 to 35 h after drug administration or in the adulthood. In the second one, animals were treated with HU in the perinatal life and killed at appropriate times ranging from 6 to 48h after treatment administration or in the adulthood.

2. Hydroxiurea: an overview

HU is a water soluble, low molecular weight and non-alkylating compound (chemical formula $\text{CH}_4\text{N}_2\text{O}_2$) [12] that was first synthesized in Germany almost 150 years ago by Dresler and Stein in a series of experiments attempting to extract derivatives from urea [13]. At first, in 1928, it was observed that HU induces leukopenia, anemia macrocytosis and death. In the late 1950s, this drug underwent further preclinical testing and was noted to have significant activity against LI210 leukemia cells and various solid tumors [14]. The clinical use of HU as an anti-tumor agent began in the 1960s [15,16]. The therapeutic spectrum of this medicine has been expanded for the treatment of patients with chronic myeloid leukemia, essential thrombocytosis, polycythaemia vera and sickle-cell anemia [12, 17]. HU is also used for the management of dermatological conditions including psoriasis [18] and HIV infection [19]. HU is currently listed as an "essential medicine" by the World Health Organization [20].

3. Mechanisms of action of the hydroxyurea

The effect of the HU is cell-cycle specific. The drug acts in the S phase, causing an arrest of proliferating cell populations in the G_1/S phase of the cell cycle [12, 14]. To this day, the mechanism of action of HU remains incompletely understood [21]. Previous studies have indicated that this agent cleaves the DNA molecule directly [22].

On the other hand, Weinlich and Fritsch [23] showed that HU alters thymidine incorporation during DNA replication, which results in the inhibition of this process, thereby impairing cell proliferation. Moreover, this agent affects the DNA by fragmenting the metaphase chromosomes [24]. An alternative mechanism that has been proposed suggests that HU may kill the cells via the generation of oxidative stress [25].

The ribonucleoside reductase is a multisubunit enzyme responsible for the reduction of ribonucleotides to their corresponding deoxyribonucleotides, which are the building blocks for DNA replication [26]. HU inactivates this enzyme by quenching the tyrosyl free radical required for enzyme activity, and a spontaneous regeneration of the active enzyme occurs when the HU is removed [26-27]. Inhibition of the class I form of ribonucleotide reductase blocks the transformation of ribonucleotides into deoxyribonucleotides, depleting the intracellular deoxynucleotide triphosphate pool and halting DNA synthesis. This mechanism of action causes DNA replication fork stalling and leads to the formation of strand breaks [28], all without interfering with the synthesis of ribonucleic acids or proteins [15-26].

4. Teratogenic effects of hydroxyurea

Clinical experience with HU has been accumulated for the past 25 years. The bulk of the current evidence suggests that this antimetabolite is a well-tolerated medication and efficacious for many gestating women and pediatric patients with hematological diseases [29]. Several studies have revealed that HU may have minimal or no effects on developing human fetuses [30-31] and very young children [4]. Despite that, the Center for the Evaluation of Risks to Human Reproduction is concerned that HU may increase the risk for congenital anomalies or growth abnormalities in fetuses after exposure of

pregnant women [6, 32]. Anemia and central nervous system abnormalities (ischemic lesions and stenoses) have been reported in pediatric patients [7].

There are many reports indicating that HU is a potent mammalian teratogenic agent. When administered to pregnant dams, it induces in the offsprings a myriad of effects such as the loss of mesenchymal cells in the lungs [33], alterations in the craniofacial tissues [34], and malformations in the hindlimbs, tail and neural tube, which seem to be mediated by the activation of p38 mitogen-activated protein kinase pathways [35]. HU also causes microencephaly, hydrocephalus [36] and apoptosis, mediated by the tumor protein p53, in the neuroepithelial cells of the mouse fetal telencephalon [8,9]. On the other hand, rodents exposed to this hydroxylated derivative of urea during the perinatal life present cell depletion in the cerebellar EGL and ectopic location of GCs [10-11].

5. Justifying the choice of the cerebellum as a model to assess the effects of hydroxyurea exposure

The rat cerebellum has been chosen as a model to analyze and interpret the toxic effects of HU-exposure on the development of the central nervous system for two reasons:

(I) The organization of neuron populations in the cerebellar cortex and the uniformity of synaptic circuits make this area a useful model to study the cellular and molecular mechanisms underlying neuronal development [37]. Cerebellar development proceeds with such precision that any perturbations in the central nervous system can be readily identified. This metencephalic region is composed of a limited number of neuronal phenotypes that are specifically integrated in a corticonuclear network and characterized by a distinctive morphology and molecular markers [38-40]. Many lines of evidence have indicated that cerebellar neurons are produced following strict

neurogenetic timetables [37]. Moreover, neuron generation is compartmentalized, with ventricular zone progenitors giving rise to GABAergic neurons and the rhombic lip precursors to glutamatergic cells [41-43]. During cerebellar development, GC precursors also arise from the rhombic lip, but they migrate tangentially to form a secondary matrix, the EGL [37].

(II) The cerebellum is highly vulnerable to insults, expression of mutant genes and intoxication [44-46]. In this context, it has been reported that several spontaneous murine mutations, including *lurcher*, *staggerer*, *reeler* and *weaver* cause a marked reduction in the volume and shape of the cerebellum due to the severe depletion of neurons [47-49]. In addition, it has also been indicated that the selective elimination of neuron precursors with X-irradiation [37], platinum compounds [50-51], impairment of the thyroid status [52], ethanol [53] and virus infection [54] produce an important reduction in size of the cerebellar cortex.

6. Embryonic effects of HU exposure: short-survival experiments

The development of the cerebellum consists of a series of sequential morphogenetic transformations that begin with the proliferation of the neuronal precursors and end with the myelination of axons of the fiber tracts. During the development of the cerebellar primordium, a specialized germinal matrix, the neuroepithelium gives rise to several types of neurons, including Purkinje cells (PCs), interneurons and deep cerebellar nuclei (DCN) neurons [55]. Previous research has indicated that the administration of HU to pregnant mice produces the apoptosis of neuroepithelial cells in the fetal telencephalon [8,9]. As the cerebellar neuroepithelium presents a cohort of asynchronous cycling cells in S-phase, the cellular death might be induced by neurotoxic factors, which would disrupt the proliferative activity. Here, we present an immunohistochemical and

ultrastructural study of the cell death of cerebellar neuroblasts following treatment with the fetotoxic compound HU. The data in this section are taken from our previously published results [56].

Pregnant rats were treated with a single dose of saline or HU (600 mg/kg i.p) on embryonic days (E)13, 14 or 15 and their progeny sacrificed at regular intervals from 5 to 35 h after drug administration. The quantification of several parameters such as the density of pyknotic, mitotic and PCNA-reactive cells, denotes that the administration of HU disrupts the proliferative behavior of neural progenitors in the cerebellar neuroepithelium and induces deleterious effects on this structure. Despite that, we have observed that some neuroepithelial cell precursors have the capacity to resume mitotic activity after being injured. These observations suggest that the degree of damage induced by HU and the extent of the subsequent repair process may influence the level of abnormalities in the cerebellum. As PCs and DCN neurons are the first cells that arise from the neuroepithelium [37, 42, 43] and the time-windows of HU exposure (from E13 to E15) correspond with the developmental timetables of these neurons [37, 57], we propose that several PCs and DCN neurons may have never produced.

Apoptosis is a specific type of cell death, a natural process that occurs during morphogenesis of the nervous system. Ultrastructural features of apoptosis involve condensation and margination of the chromatin, cell shrinkage, membrane blebbing and nuclear fragmentation [58]. In order to determine whether the HU-administration triggers apoptotic cell events in the cerebellar neuroepithelium, and if so, which type of cell death, TUNEL staining and transmission electron microscopy were used. Our microscopic observations will demonstrate that exposure of the embryonic cerebellum to HU induces apoptotic cell death in a large number of neuroblasts.

In the neuroepithelium, few apoptotic cells can be found during the normal development of the cerebellum. However, a progressive increase in the density of TUNEL-reactive cells was observed between 5-30h after HU administration (Figure 1a). The maximum density was found at 30 h. After that, values declined.

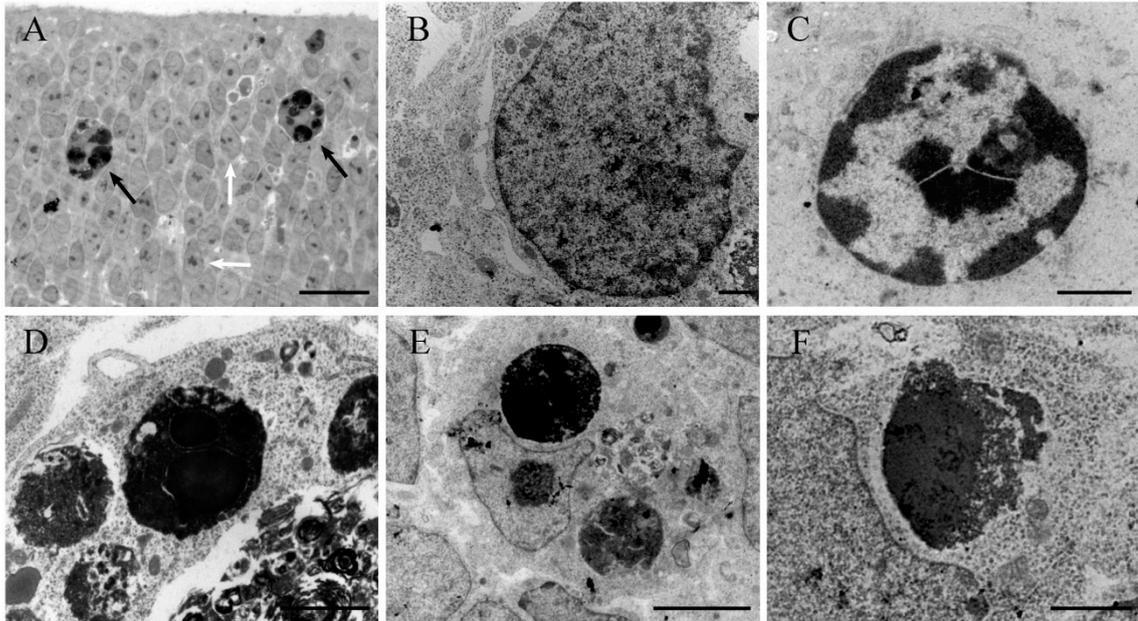


Figure 1. (A) Light microscope view of a thin plastic section from a rat cerebellum exposed to hydroxyurea in the prenatal life. The section presents among healthy neuroblasts (white arrows) and apoptotic profiles (black arrows) with dark spherical balls distributed within the cell. (B-F) Ultrastructural morphology of healthy (B) and apoptotic neurons (C-F) in the rat neuroepithelium following HU administration. (C) Early stage of apoptosis showing nuclear chromatin at the margin of the nucleus. (D-E) Late apoptotic stage showing typical clusters of apoptotic bodies. (F) Breakup of an apoptotic body and release of its contents into the cytoplasm. Scale bar: 20 μm (A), 1 μm (B and C), 2 μm (D and F), 5 μm (E).

At all time points, electron microscopy shows the presence of dying cells scattered throughout the neuroepithelium at different stages of apoptosis (Figure 1b-f). The earliest morphological signs of this type of cell death were observed in the nucleus. Chromatin condensation and segregation at the nuclear periphery were a typical initial characteristic. In some instances, chromatin compaction was associated with the convolution of the nuclear envelope, giving a star-like appearance. The mid-to-late apoptotic cell was characterized by the presence of several rounds and electron-dense

nuclear fragments, which were surrounded by cytoplasm but apparently were devoid of membranes. Following HU-treatment, numerous clusters of apoptotic bodies were visualized throughout the neuroepithelium, which exhibited an acute level of cytoplasmic degradation and fragmentation. Other ultrastructural changes included apoptotic cells engulfed by surrounding phagocytic cells, and dying cells with unusual apoptotic features such as a vacuolated cytoplasm, deteriorated organelles and a recognizable nucleus.

Two major points can be deduced from these results: (I) the administration of HU induces cell death by apoptosis on the cerebellar neuroepithelium, and (II) the use of HU would be a good model for studying the basic histological and ultrastructural features of cell apoptosis.

7. Embryonic effects of HU exposure: long-survival experiments

The adult cerebellum appears to be similarly organized across mammals. The cerebellar cortex is formed by three layers: the molecular layer (ML), whose neuronal components include stellate and basket neurons; the Purkinje cell layer, that contains PCs and candelabrum cells; and the granule layer, that consists of GCs, Golgi cells, unipolar brush cells and Lugaro cells [59-60]. The neural phenotypes that populate the cerebellum are generated following regular and precise timetables of neurogenesis [37]. Moreover, previous studies have demonstrated that, in normal rodents, cerebellar neurons are distributed according to neurogenetic gradients. This refers to the nonrandom spatial accumulation of neurons, according to age within and between neuronal populations [37]. Here, we show that a single administration of HU during the embryonic life modifies the regular cytoarchitecture of the cerebellum, and alters the

neurogenetic profiles and settled patterns of PCs and DNC neurons. The results of this section are taken from a previous published paper [49].

The pregnant dams were administered with a single injection of saline or HU (600 mg/kg i.p) on E12. After this treatment, they were injected several times with 6 mg of 5-bromo-2'-deoxyuridine (BrdU) in accordance with the procedure of Sekerkova et al. [61]. This marker was delivered following a progressively delayed labeling comprehensive procedure [37, 57] that consists of injecting pregnant dams in an overlapping series, in accordance with the following time-windows: E13-14, E14-15, E15-16.....E19-20. The offsprings were sacrificed at postnatal day (P)90 and several cerebellar features were quantified, per section, in each cerebellar cortex compartment (vermis, paravermis, and medial and lateral hemisphere), or alternatively in each deep nucleus (fastigial, interposed and dentate): (I) area of the cerebellum, (II) length of the cerebellar cortex, (III) ML area, (IV) PC number, (V) GC number, (VI) area of the IGL, (VII) area of the white matter, (VIII) the areas of the cerebellar nuclei, and (IX) number of DCN neurons.

Our results revealed no signs of toxicity in the pregnant dams after HU treatment. They gave birth as normal. Moreover, no sex differences were observed in the effect of the drug when the males and females were compared. Our data also indicated that HU-exposure does not compromise neither the cytoarchitecture of the cerebellar cortex nor the deep nuclei. However, it was observed that HU-administration contributes to an important cerebellum size reduction. This deficient growth occurred in each analyzed cerebellar compartment and deep nuclei. These results suggest that the effect of the HU exposure is toxicologically homogeneous throughout the mediolateral axis of the cerebellum.

To see whether the administration of HU alters the neurogenetic timetables of PCs and DCN neurons different sets of saline and treated rats were examined. Our results have revealed that, in both macroneurons, the entire span of neurogenesis, its pattern of peaks and valleys, and the peak production were different between rats administered with saline or HU. This occurs in each of the analyzed cortical compartments and deep nuclei. For example, in saline-injected rats, the neurogenesis of PCs and DCN neurons occurred between E12 to E15, with a PC production peak at E14 in each cortical compartment, as well as in each deep nucleus. In the HU-treated group, on the other hand, developmental timetables extended from E15 until E19 for PCs and DCN neurons, with a peak generation at E17 in each compartment of the cerebellar cortex as well as in each deep nucleus. These results indicate that E12 is a time of high susceptibility to insult. Moreover, it is shown here that the temporal sequence of PCs and DCN neuron production throughout the cerebellum was disturbed, suggesting that the neuroblasts that give rise to these macroneurons are highly susceptible to HU.

An important aspect during the developmental injury of the cerebellar cortex and the deep nuclei is ascertaining whether the spatial location of PCs and DCN neurons was modified due to HU-exposure. Our results revealed that in saline rats, neurons are settled following two neurogenetic gradients: (I) medial-to-lateral for the PCs; the vermis contains more late-generated neurons (younger neurons) than the lateral hemispheres, which in turn have more early-produced (older neurons), and (II) an opposite gradient for the DCN neurons was found (lateral-to-medial); the fastigial nucleus has more early-born neurons than the dentate nucleus, which in turn has more neurons that are late-produced. In HU-treated rats, on the other hand, the above-mentioned neurogenetic gradients were modified, indicating that the arrangement of cortico-nuclear connections in the cerebellum may be altered.

Two observations emerge from these experiments: (I) HU-exposure decreases the size of the cerebellar cortex and deep nuclei, and (II) this cytotoxic agent compromises the survival of PCs and DCN neurons, and disturbs the times of neuron origin and the neurogenetic gradients of these macroneurons.

8. Perinatal effects of HU exposure: short survival experiments

Early experiments have shown that HU-exposure in the perinatal life causes cell depletion in the EGL and malpositioning of GCs [10-11]. Despite these data, the effects of HU on the early postnatal development of the cerebellar cortex have not been completely elucidated. In this section, we characterize the type of cell death of EGL neuroblasts induced by HU-exposure in the early postnatal life. In addition to this, we also analyze the morphological and ultrastructural changes of Bergamann glial cells, and the microglial response after HU-treatment. These data are taken from previous published papers [62,63].

Groups of rats were injected with a single injection of saline or HU (2mg/g b.w) on P8 and sacrificed at different survival times from 6 to 24 hours, and on 48 and 72h after saline or HU-exposure. Following paraffin embedding and tissue processing, cell death was analyzed in the EGL during treatment, and its kinetic pattern was established. The dying neuroblasts exhibited characteristic apoptotic features in TUNEL labeling cerebellar sections (Figure 2a). To confirm the morphological features of cell death, light and electron microscopy was used.

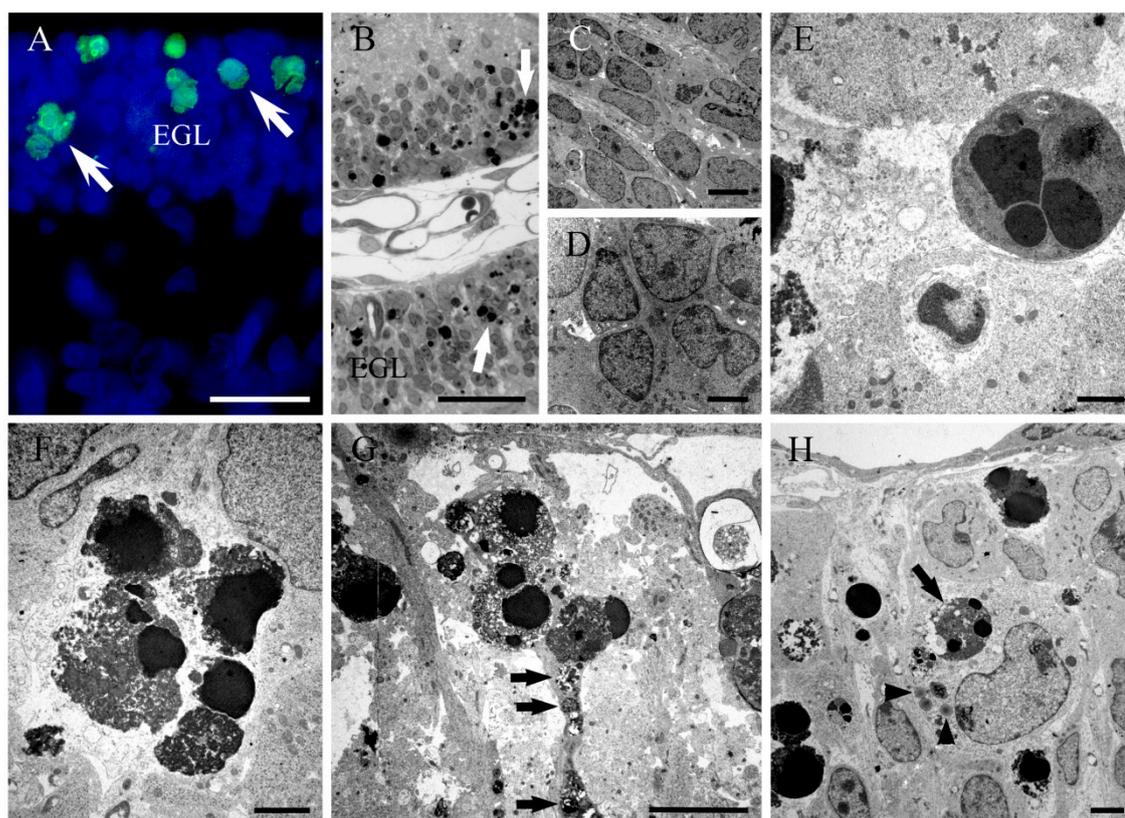


Figure 2. (A) TUNEL positive cells in the cerebellar external granular layer (white arrows) from a rat administered with hydroxyurea in the perinatal life. (B) Thin plastic section (0.5 μm) from a rat cerebellum following hydroxyurea treatment. The healthy neuroblasts have a homogeneous nucleoplasm contained within an intact nuclear membrane. The apoptotic cells (white arrows) present two or more spherical balls within the cell. (C-H) Electron micrographs of healthy (C-D) and apoptotic neurons (E-F) in the external granular layer after hydroxyurea exposure showing characteristic clusters of apoptotic bodies. (G) Bergmann glial processes containing electron-dense phagosomes (black arrow) in a hydroxyurea-treated animal. (H) Electron micrograph of an ameboid microglial cell engulfing an apoptotic body (black arrow) and the presence of several lipid droplets (head arrow). EGL: external granular layer. Scale bar: 20 μm (A), 25 μm (B), 5 μm (C and G), 2 μm (D, F, H), 1 μm (E).

Our results showed that, when plastic thin sections were studied with light microscopy, dying cell profiles were found (Figure 2b). The ultrastructural analysis showed the presence of neuroblasts at different stages of apoptosis (Figure 2c-f). The earliest signs of this type of cell death were the condensation of the chromatin and its segregation against the inner nuclear envelop. The mid-to-late apoptotic stage was characterized by the presence of several spherical and electron-dense nuclear fragments. Finally, numerous clusters of apoptotic bodies were visualized. From these experiments, it was deduced that HU-administration activates apoptotic cellular events, resulting in a

substantial depletion of cells. Activation of apoptosis has also been reported in the EGL after hyperoxia [64], X-ray exposure [65] and the administration of some treatments, such as lead [66], ethanol [67] and platinum compounds [51]. In this context, the experimental model of HU-induced apoptosis reported here could provide a good system to study the apoptotic mechanisms in the developing cerebellum.

We also analyzed the effect of HU on the viability and morphology of the Bergmann glial cells. After some immunohistochemical procedures, it was observed that HU decreases the number of Bergmann glial cells with respect to saline rats. Despite that, the typical palisade organization of this unipolar astrocyte was preserved. Moreover, our results also show the overexpression of the cytoskeletal protein vimentin and the formation of thicker immunoreactive glial processes, including the end-feet at the pial surface, in those surviving Bergmann glial cells [62]. The damage of radial glia may cause an alteration of the migratory pattern of GCs, Purkinje dendrite differentiation and development of synaptic ensheathment.

Our electron microscope analysis reveals that Bergmann glial processes present phagosomes containing apoptotic bodies and cell debris. This suggests that the surviving Bergmann glial cells can serve as facultative phagocytes. Moreover, our ultrastructural images indicate that dying cells at different stages of apoptosis were covered by laminar processes of Bergmann glia (Figure 2g). We propose that this tight relation may be isolating the degenerating cells in closed compartments in order to protect the undamaged neuroblasts against apoptosis. Some studies in *Drosophila* development have shown that apoptotic cells secrete Eiger, a TNF superfamily ligand, to induce apoptosis of healthy cells through the c-Jun N-terminal kinase pathway. In mammals, hair follicle cells undergo apoptosis through secretion of TNF- α by apoptotic cells [68]. It could be possible that the observed association between Bergmann glial

processes and apoptotic EGL cells is protecting the surrounding healthy cells from apoptotic signals such as these.

Microglia reside in the central nervous system, where they function as immune cells. Previous studies have indicated that these cells become activated in response to injury in order to maintain brain microenvironment homeostasis. Microglia activation plays an important role in the phagocytosis of dead cells or cellular debris [69]. As our studies have revealed that HU-exposure induces apoptosis in the developing cerebellum, we examined whether the damaged of EGL cells can lead to the activation of microglial cells. Tomato lectin histochemistry and transmission electron microscopy revealed that amoeboid microglial cells participate in the phagocytosis of injured neuroblasts in regions of the EGL with extensive cell death. Moreover, electron micrographs show activated microglia adjacent to injured EGL cells, and containing apoptotic figures and cellular debris (Figure 2h). This suggests that the signals produced by apoptotic cells may modify the dynamic behavior of microglia and trigger the recruitment and activation of glial cells to remove injured cells and repair the brain parenchyma.

In order to obtain more information about the effects of HU-exposure on the development of the EGL, groups of rats were administered with this pharmacological agent and examined at P5, P10 and P15. In each postnatal age, animals were sacrificed at appropriate times ranging from 6 to 48h after treatment administration. Studies were done in the cerebellar cortex lobe following the quadrapartite lobular division. Our results demonstrate that the vulnerability of EGL neuroblasts and Bergmann glia, just as the microglial activation, depends on the analyzed postnatal day, vermal lobe and survival time after drug exposure. Evidences presented here denote that the most important alterations occurred at P10, indicating that this is an age of high vulnerability to injury. It is also indicated here that EGL cells located in the anterior and central lobes

are the most susceptible to the action of the HU. Moreover, the time span from 6 to 24h is a time-window of high sensibility to this agent. We propose that these ages and regional differences in the EGL cells vulnerability to HU could be related to the different timetable of cortical maturation. Because the anterior and central lobes are late-maturing [37], they are more susceptible to HU-exposure. On the other hand, the posterior and inferior lobes are early-maturing [37] and therefore they are less vulnerable to HU.

9. Perinatal effects of HU exposure: long survival experiments

The structural and functional organization of the cerebellum is the end product of a complex developmental process. The cerebellar neurons, including PCs and GCs, are produced in overlapping waves and migrate to their final locations to achieve the final cerebellar structure and the organization of its circuits [37]. The disruption of this process may produce alterations in the arrangement of the cortical neurons. To our knowledge, no attempts have been made to determine the long-term effects of HU administration on the development and cytoarchitectonics of the cerebellar cortex.

In this section, we show that a single administration of HU in the early postnatal life alters the spatial location of PCs and GCs, and the arrangement of the PC dendritic tree. The data presented here were taken from a previously published paper [62]. In the study, groups of P9 rats were injected intraperitoneally with a single injection of saline or HU (2mg/g b.w). Then at P12, they were administered with BrdU (50mg/kg b.w; i.p). Animals were sacrificed at P45. Our results revealed that, in relation to saline, the HU condition disturbs the developmental program of the cerebellum, resulting in anomalies in the cortical cytoarchitecture. These included:

- (I) Ectopic placement of PCs and abnormalities in their morphology (Figure 3).

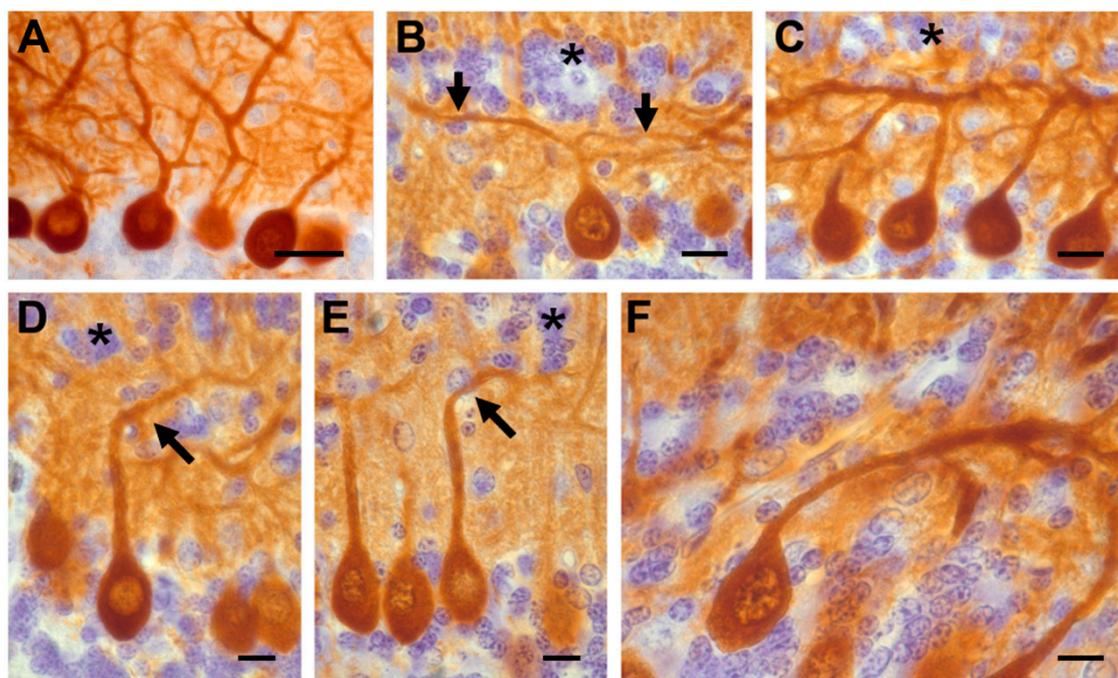


Figure 3. (A) Calbindin-reactive Purkinje cells in saline rats whose dendrites grew in the normal, upward direction. (B-F) Hydroxyurea-treated cerebella showing different patterns of dendritic arborization. Asterisks show ectopic interneurons and black arrows indicate the altered dendritic tree. Scale bar: 30 μm (A), 10 μm (B-F).

Examination of calbindin D-28k immunostained sections indicated that these macroneurons were piled 2-3 cell-thick. Some of these cells were found in the granular layer (GL). Moreover, in many PCs the following morphological abnormalities in the dendritic arborization were observed: (A) the ascending dendritic tree bifurcated in a T-shape manner, (B) the primary dendrite ascended obliquely to the pial surface, (C) the primary dendrite was oriented toward the GL, and (D) the neuronal body and dendritic tree were directed toward the white matter. Interestingly, the dendritic alterations of PCs were mainly encountered in regions where GC ectopia was presented.

(II) Malposition of GCs. In all cortical lobes, a large number of ectopic GCs forming a supernumerary layer were found in the ML. The position of the ectopic GCs presented four patterns: (A) small clusters of cells located near the cerebellar surface. These were observed in the lobules I and X as well as at the bottom of the fissures prima and secunda. (B) Arrangement in a monolayer just beneath the pia mater. This was seen in

the lobules II and III. (C) Imprecise aggregation in a thin strip oriented in parallel to the pial surface. This band is usually found in the upper ML. This pattern was found in the lobules IV, V and IX. (D) Occupying the middle-to-lower part of the ML (lobules VI to VIII) and parallel to the pial surface.

All these data indicate that the administration of HU in the early postnatal life causes anomalies in the cortical cytoarchitecture in the adulthood.

10. Conclusions

The current review describes and clarifies the effect of HU treatment on the development of the rat cerebellum. We have shown here that the immature cerebellum is highly vulnerable to HU exposure. A single injection of this agent *in utero* triggers apoptotic cell events in the cerebellar neuroepithelium, and alters the developmental timetables and the neurogenetic gradients of PCs and DCN neurons. Moreover, when administered in the perinatal life, HU produces the apoptotic elimination of EGL neuroblasts, and a reactive response of the Bergmann glial cells and microglia. Our results also reveal that HU-exposure decreases the size of the cerebellum and induces alterations of the cortical cytoarchitectonics, including dendritic alterations of PCs, which occur in parallel with the demise of GCs and ectopic location of these neurons. In the early postnatal period, the effect of HU-exposure depends on the analyzed postnatal day, vermal lobe and survival time after drug exposure. As the cerebellum is involved in several psychiatric and developmental disorders, including schizophrenia, autism spectrum disorder and attention deficit-hyperactivity disorder [70], we advise that extreme caution should be taken when administering HU to gestating women or infants as the effects of this agent on the cerebellum might persist throughout their offspring's lives. Further studies with laboratory animals receiving HU during the early prenatal life

are required before this agent can be promoted as safe for human fetuses and young children.

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Abbreviations

BrdU	5-bromo-2'-deoxyuridine
DCN	Deep cerebellar nuclei
E	Embryonic day
EGL	External granular layer
GCs	Granule cells
GL	Granular layer
HU	Hydroxyurea
ML	Molecular layer
P	Postnatal day
PCs	Purkinje cells

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