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# Neurotoxic effects of thimerosal at vaccines doses on the encephalon and development in 7 days-old hamsters

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Abstract

Objectives**:** To determine if thimerosal administration in amounts equivalent to vaccines content produces neurotoxic effects on the encephalon in postnatal hamsters and on experimentation animal's development. Design: Experimental, prospective, bioetapic study. Setting: San Fernando Faculty of Medicine, Universidad Nacional Mayor de San Marcos. Biological material: Seven days-old hamsters. Material: We divided 45 postnatal hamsters in three groups: group A (n=15), group B (n=15) and group C (n=15). We administered three intramuscular equivalent doses of sucrose and thimerosal in 20 µL of saline to groups B and C, respectively, on birth-days 7 (0,227 µg), 9 (0,216 µg) and 11 (0,220 µg). Group A received only 20 µL of saline solution. Main outcome measures: Body weight, encephalon weight, height (skull-caudal length), and encephalon histopathological alterations. Results: Anova and student t tests showed statistical significance in favor of low body weight, low encephalon weight, and smaller stature in group C with respect to groups A and B hamsters (*p<0,0001*). $X^2$ statistical significance in relation to the presence of hystopathological alterations in group C was also obtained (*p<0,0001*). We observed greater relative risk of encephalic alterations in group C. Conclusions: The administration of thimerosal in equivalent doses to vaccines content was associated with low corporal weight, low encephalon weight, and smaller stature in postnatal hamsters. Neurotoxic effects were also produced at encephalic level: at hippocampus *(regions CA1, CA3 and DG),* cerebral cortex, and cerebellum (Purkinje cells and granulose cells); with decrease in neuronal density, neuronal necrosis, axonal demyelinization, and gliosis. In addition, risk increase in developing any of these alterations was high just in the animal group receiving thimerosal.

Key Words: Vaccines; thimerosal; mesocricetuss; ethylmercury compounds; mercury poisoning, nervous system.

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## INTRODUCTION

Vaccination impact on public health during the XX century has been enormous. Definitely, vaccines have been one of the most effective methods for preventing disease, disability and death, as it helps to reduce and control health care costs, thus becoming an essential weapon in the modern society against infectious diseases [(1)]. However, it has aroused great controversy that has called into question the safety and reliability of some vaccines because of the side effects that could be caused by the preservative thimerosal (*tiomersal*) that it contains, which, after being introduced to the body, it dissociates in ethylmercury (ethylHg), a neurotoxic derivative organic mercurial [(2,3)].

In recent years, there have been several studies that reported an extraordinary increase of children's neurodevelpmental disorders [(4-17)]. At the same time, a growing minority of medical doctors and scientists around the world declared seriously question about the use of thimerosal in childhood immunization, in connection with neurodevelopmental diseases such as autism spectrum disorders (ASD) and other cognitive problems [(12,14,15, 17)]. Because of that, several institutions have been handed important statements related to this topic [(17-26)].

Seven major retrospective epidemiological population–based studies have been conducted in the United States of America (US), which has evaluated the association between thimerosal, pediatrics vaccines content and ASD. Of these, six investigations found such a causal relationship [(27-32)], including a recent meta-analysis study type [(32)], while one finally came to the conclusion that it can neither accept or reject these hypothesis [(33)]. Other epidemiological studies, which have took place outside the US, has not found an apparent association [(34-39)].

Kravchenko et al, working with cultured human cells, documented that thimerosal was detrimental, not only because of its major toxic effects, but also because it was capable of changing cellular properties, concluding that its use in medical biological preparations, particularly those intended for children, was inadmissible [(40)]. After this publication, soviet countries removed thimerosal from vaccines for their children in the early 80's. Several other studies end up in similar conclusions, noting the inconvenience of thimerosal in vaccines because of its potential to induce allergic responses [(41,42)], its poor antiseptic effectiveness [(43)] and / or its degradation in neurotoxic substances [(44)].

Despite this multiple investigations, the US Food and Drug Administration (FDA) have never taken preference of thimerosal-free vaccines. In fact, although since 1999 the US medical societies and health authorities decided the urgent and expeditious withdrawal of this preservative in their shots [(22,23)], such a process did not take place until 2003. This has led to severe criticism by the US Congress itself [(17)].

In Peru, the authorities of the Ministry of Health (Minsa) continue using vaccines with high thimerosal content (whose multidose submission form is, to date, used in some health establishments), noting that it has no side effects [(45)]. This generated a serious challenge by the public, part of the medical community and a number of non-governmental organizations. This debate requires that begin to shed light on the investigations and take concrete steps on this issue.

Facing this situation, we have decided to develop this study, which will determine the possible neurotoxic effects generated after thimerosal administration at vaccine's doses (especially the alterations to be reflected at brain level) in an animal experimentation model, and the possible influences that this substance may have on the development and growth of the experimental animals, because, for ethical reasons, this substance can not be tested in humans.

## METHODS

We conducted a two-phase experimental longitudinal prospective study, which came with a qualitative-

quantitative approach. The study population consisted of postnatal Gold-Syrian hamsters (*Mesocricetus auratus*), which had to meet the following criteria:

• Inclusion criteria: postnatal Gold-Syrian hamsters <7 days old, whose weight and height (skull-caudal length), on the seventh day were 8,5 ± 0,5 g, and 4,5 ± 0,5 cm, respectively, without prior thimerosal (or other any mercury) exposure. Hamsters with anomalies and / or certain diseases before the study were excluded.
• Exclusion criteria: postnatal hamsters suffering some serious injuries and / or death during the experimentation period, and hamsters that fail to comply with the full content management system.

The first phase of the study, that last 45 days, consisted in the acquisition, upbringing, and subsequent mating of adult hamsters. In the second phase, we proceeded to select the sample by a conglomeration of random probability sampling (litter), which brings together 45 postnatal hamsters, which were distributed in three groups: group A (n = 15), group B (n = 15) and group C (n = 15). To determine the power and sample size, we consider $\alpha = 0,05$ and $\beta = 0,10$.

Three equivalent doses of sucrose and thimerosal were administered intramuscularly to groups B and C, respectively, on day 7 (0,227 µg / dose), day 9 (0,216 µg / dose) and day 11 (0,220 µg / dose) of birth, diluted in 20 µL of saline. On the same dates, group A received only 3 intramuscular doses of 20 µL of saline. Calculation of the dose was performed according the body weight and the amount of thimerosal that a child was exposure since birth up to his 6 first months of life, imitating the US Vaccination scheme of the year 2001 [46]. The implementation timing of substances was based on the analogy between human and murine development of the central nervous system and the immune system, including the neuronal migration and immunological tolerance stages [47-49], as shown in Table 1. For substances administration, Nº 2 ultrafine insulin-type syringes were used.

On day 25 after birth (estimated time of thimerosal metabolism) [50], animal weight and height were evaluated; then, we proceeded to its slaughter and removal of brains, which after being weighted and cut, were immediately fix on 10% formalin. Specific cut-off points were determined for the microscopic hystopathological study of the hippocampus, cerebral cortex, and cerebellum. There were two main tracks to the study of the hippocampus: a hemisphere was cut parasagittaly to 0,8 mm from the median line that separates both cerebral hemispheres; in the other, a coronal cut was made 10 mm after the *bregma* point and perpendicular to the face-axis flow. For sample processing and microscopic study, a number of special stainings were used (hematoxylin-eosin, Pollak's tricromic, Gomori's tricromic, luxol fast blue and silver), for the likely presence of histopathological lesions can be determined more efficiently. All the histopathological samples were evaluated by the same specialist in pathology, who was unaware of the membership of each study group. For data collection, we used special cards where body weight, brain weight, height, and histopathology changes at brain level variables were included.

Table 1. Substances administration scheme.

| | **HUMAN AGE** (months) | | |
|---|---|---|---|
| | **2** | **4** | **6** |
| **Equivalent age in a postnatal hamster** [¥] | Day 7 | Day 9 | Day 11 |
| **Thimerosal's content on vaccines (µg)** [δ] | 125 | 125 | 125 |
| **Average weight of a human child (Kg)** | 4,4 | 5,8 | 6,8 |
| **Thimerosal's doses (µg/Kg)** | 28,4 | 21,6 | 18,4 |
| **Group A** | Saline (NaCl al 9‰) | | |
| **Group B Sucrose's doses for administration (µg/dose)** | Saline + sucrose | | |
| | 0,227 | 0,216 | 0,220 |
| **Grupo C Thimerosal's doses for administration (µg/dose)** | Saline + thimerosal | | |
| | 0,227 | 0,216 | 0,220 |
| **Total administrated volume (µL/dose)** | 20 | 20 | 20 |

[¥] According to studies made in murines [47-49].
[δ] According to the US Vaccination scheme 2001 [46].

The testing hypotheses were:

• H1: the administration of thimerosal, in amounts equivalent to vaccines content, produce neurotoxic effects at brain level in post-natal hamsters, as well as restriction in their growth and development.
• H2: among the histopathology, changes at brain level could be: reduction of neuronal density, neuronal necrosis, axonal demyelinization, and gliosis; mostly.

The statistical processing was performed as follows:

• Analysis of quantitative variables: before the application of the tests, we check if the data showed a normal distribution by using the Anderson-Darling, Kolmogorov-Smirnov and Ryan-Joiner tests. Then the F test (one-way Anova) was used to compare the means of body weights, brain weights, and heights of the different study groups ($\alpha$ = 0,01). Also, the Student T test for independent samples was used in order to compare the means of body weights, brain weights, and heights between groups, and establish an order of relation ($\alpha$ = 0,01).
• Analysis of qualitative variables: $X^2$ (chi-square) test was used to establish the degree of homogeneity among the study groups, in terms of histopathological alterations ($\alpha$ = 0,01). In addition, the incidence of brain abnormalities between groups B and C were comparatively studied to establish the degree of association with the previous thimerosal exposure. The following statistic criteria were used: relative risk (RR), absolute risk reduction (difference of risk, ARR), relative risk reduction (RRR) and the number of hamsters needed to produce some damage (NNT). All processing and data analysis were done in the SPSS 13.0 program and Minitab ® 15.1.0.0, for Windows.

## RESULTS

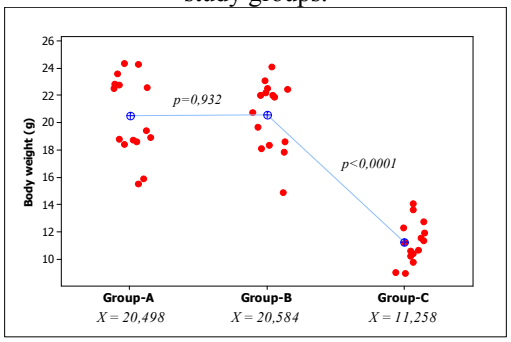
It was found that the mean body weight of group A was 20,5 g (*s*=2,93), 20,6 g in group B (*s*=2,53) and 11,3 g in group C (*s*=1,52). The maximum values of body weight were found in groups A and B (24,3 and 24,1 g, respectively), which were significantly higher than the maximum body weight of group C (14,1 g). Moreover, the lower body weight in groups A and B (15,5 and 14,9 g, respectively), were also higher compared with the values of group C, even more than the latter's higher value. The minimum value of body weight in group C was 8,9 g, very inferior to the rest obtained. In the variance analysis (Anova), it was found that the difference between means and variances in the study groups was significant (F [5,12] = 74,63, *p<0,0001*). Similar values were observed in groups A and B, with no significant statistical difference (t [2,467] = -0,09, *p=0,932*); but, both differed significantly from group C (t [2,467] = 10,84, *p<0,0001*) (Figure 1).

The brain mean weight in group A was 0,96 g (*s*= 0,077), 0,97 g in group B (*s*=0,086), and 0,76 g (*s*= 0,082) in group C. The maximum values of brain weight were as follows: group A 1,07 g, group B 1,11 g, and group C 0,83 g. As with body weight, it was found that the difference between means and variances in the study groups was significant (F [5,12] = 33,24, *p<0,0001*). For groups A and B we observed similar values, without statistically significant differences (t [2,467] = -0,25, *p=0,804*); but, both differed significantly from group C (t [2,467] = 7,14, *p<0,0001*) (Figure 2).

The mean height in group A was 8,71 cm (*s*= 0,61), 8,69 cm in group B (*s*= 0,54), and 7,50 cm (*s*= 0,54) in group C. The difference between means and variances in the study groups was significant (F [5,12] = 22,91, *p*<0,0001). About the height, it was found that the values of groups A and B were similar, without significant differences between them (t [2,467] = 0,06, *p=0,950*). There were differences with group C, which showed the lowest height values; these values were below average gained in the first two groups (t [2,467] = 5,77, *p<0,0001*) (Figure 3).

In the hippocampus' hystopathological study (*CA1, CA3 and DG regions*), a significant difference in terms of homogeneity was observed; in other words, the study groups showed differences in

**Figure 1.** Body weight values comparison by study groups.

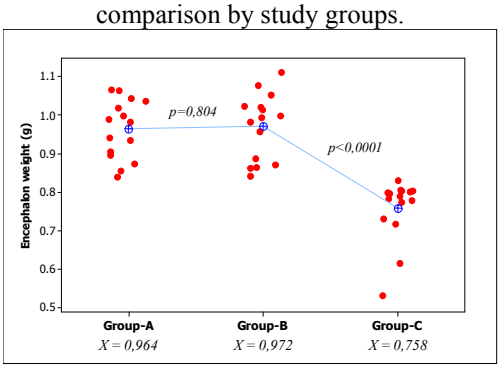



the hystopathological variables, including reduced neuronal density, neuronal necrosis, axonal demyelinization, and gliosis ($X^2$ [9,21] = 36,600, $X^2$ [9,21] = 22,200, $X^2$ [9,21] = 17,206, and $X^2$ [9,21] = 25,797, respectively, *p<0,0001*) (Figure 4). In some histologic samples from group C neuronal swelling was observed, which was not significant. The values obtained from the descriptive analysis provided a lower incidence of alterations in groups A and B, in comparison with group C, as can be seen when the RR was observed for all the hystopathological variables at this level; there was 14, 12, 6, and 6,5 times more risk of reduced neuronal density, neuronal necrosis, axonal demyelinization, and gliosis, respectively, in the case of history of thimerosal exposure, according to the scheme administration used.

In the cerebral cortex, a significant difference with respect to the assumption of homogeneity was observed between the study groups as well; the presence of reduced neuronal density, neuronal necrosis, axonal demyelinization, and gliosis was observed ($X^2$ [9,21] = 22,200, $X^2$ [9,21] = 26,250, $X^2$ [9,21] = 24,231, and $X^2$ [9,21 = 16,200], *p<0,0001*, respectively) (Figure 5). The calculated RR revealed cortical alterations 12, 7,5, 7, and 5,5 times higher risk of reduced neuronal density, neuronal necrosis, axonal demyelinization, and gliosis, respectively, in the case of history of earlier thimerosal exposure, according to the administration used regime.

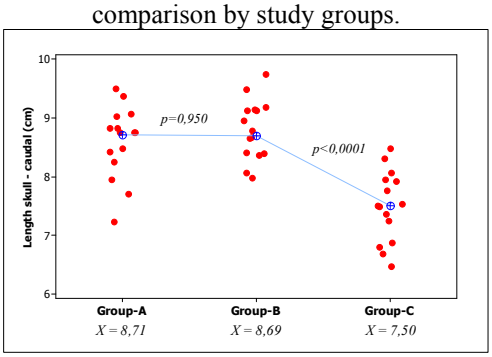
At cerebellum (molecular layer, Purkinje cells, and granulose cells), the existence of significant differences became clear to test the hypothesis that the various study groups differ with respect to the hystopathological alterations. The

**Figure 2.** Encephalon weight values comparison by study groups.




reduction, axonal demyelinization, and necrosis of the Purkinje and granulose cells, in addition to gliosis in all layers, were found significant ($X^2$ [9,21] = 16,200, $X^2$ [9,21] = 33,362, $X^2$ = [9,21] 10,556, and $X^2$ [9,21] = 24,231, respectively, *p<0,0001*) (Figure 6). The RR for the alterations in the cerebellum of samples from group C showed levels that existed 5,5, 7 and 3,6 times more risk of reduction, axonal demyelinization, and necrosis of the Purkinje cells and granulose cells, respectively; also, the risk of gliosis was 7 times higher in this group.

**Figure 3.** Height (Length skull-caudal) values comparison by study groups.




## DISCUSSION

The comparison of the mean values of body weight, brain weight, and height between groups A and B were observed similar, that justifies the assumption that the two groups did not differ significantly in terms of their level of development, despite the fact that they receive different substances, that due to their safe nature surely only fulfill the role of control groups,

with regard to group C. Moreover, group C had the lowest values of these parameters, thus sustaining the hypothesis of a delay in the development and growth in this group; however, we must remember that, initially, body weight and height has been established as criteria included in the study (weight and height at the 7th day of life of 8,5 ± 0,5 g, and 4,5 ± 0,5 cm, respectively). Therefore, we conclude that the stimulus because of this delay in the development and growth of hamsters in group C was after their first week of birth and, in the case of this experiment, is attributed to the effects of thimerosal in the animal organism, because the fact that all the other variables (food, conditions of captivity, and external stimuli) were identical in the three study groups. Although the exact mechanisms can not be detailed, the high degree of significance of the statistical evidence supports this hypothesis.

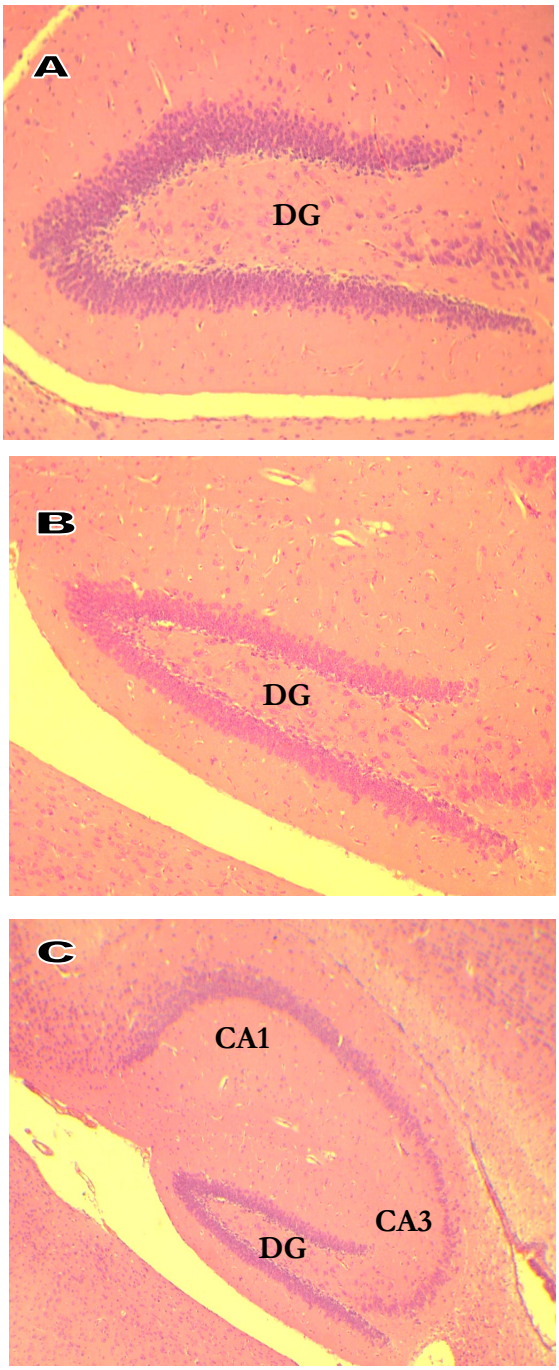
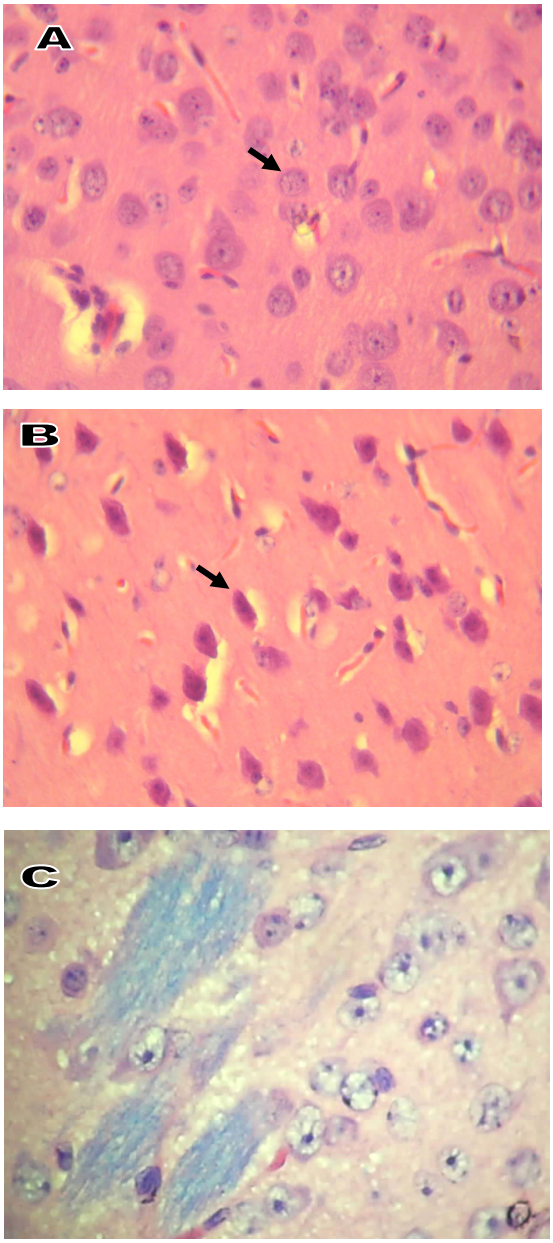



**Figure 4.** Hippocampus: A. Dentate Gyrus section (DG) (H-E x100); B. Reduced neuronal density in DG (H-E x100); C. Reduced neuronal density in CA3 and DG in comparison with CA1 (H-E x50).

Other studies have shown similar results to ours. Hornig et al [51] used mice particularly vulnerable to immunological disorders, whose brains were still developing, and exposes them to vaccines with thimerosal (at the equivalent doses to the weight and age of the human immunization's schedules, the same as those used by us), finding the presence of several abnormalities, such as growth retardation, reduced locomotion, inadequate response to novelty, significant anomalies in the architecture of brain areas related to emotion and cognition, disruption of hippocampus' cells, and changes in intracerebral glutamate carriers and receptors. Burbacher et al [52] conducted a comparative study of the brain and systemic distribution of total mercury (Hg) and inorganic Hg after intramuscular administration of thimerosal (at vaccine doses and the same timeline as human vaccines) and oral methylmercury (methylHg) in infant monkeys. They documented that, after injections with thimerosal, the peak Hg blood levels increased significantly, coinciding with the results found in human newborns by Stajich et al [53]; also realized that its mean blood life was approximately 8,6 days, similar to Pichichero et al report in post vaccinated children [54]. However, the total brain / blood Hg ratio and the intracerebral inorganic Hg were higher in the group receiving thimerosal, and concluded that Hg blood levels are not good indicators of the risk of adverse neurological effects caused by the preservative, because despite that Hg does




(H-E x400); B. Pyramid neurone's necrosis. (H-E x400); C. Axonal demyelinization (luxol fast blue x400).

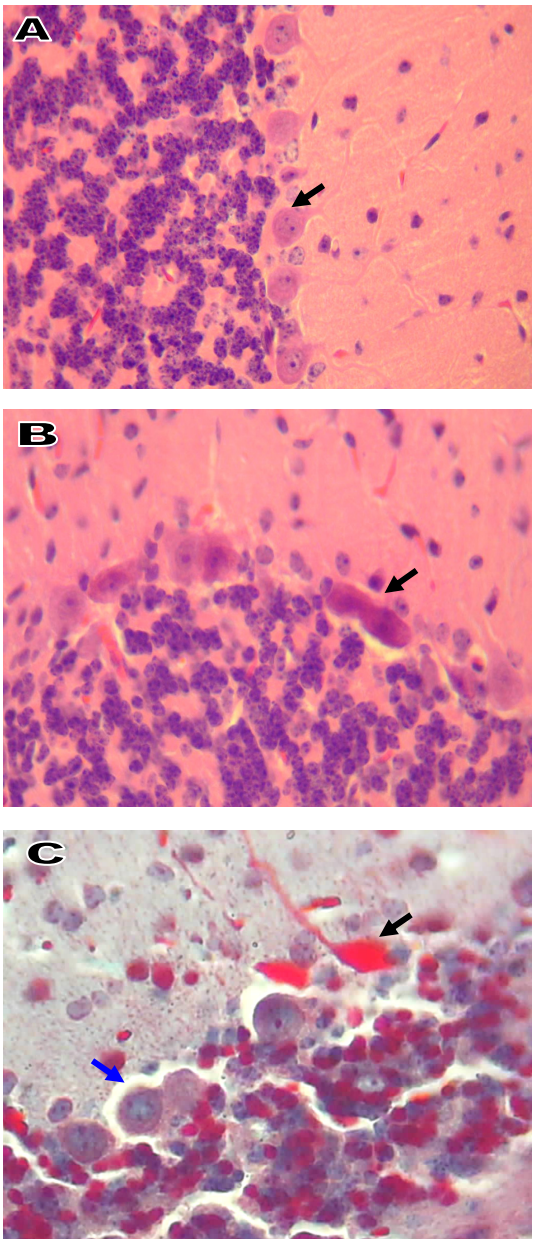



Figure 6. Cerebellum: A. Purkinje cells (H-E x400); B. Purkinje cells' necrosis (H-E x400); C. Purkinje cells' necrosis (black arrow) and normal (blue arrow) (Gomori's tricromic x400).

not accumulate in the blood, deposits of this heavy metal in the child's brain could happen; so the data of thimerosal safety, based only on blood clearance, is not valid [52]. Qvarnstrom et al also quantified the distribution of body methylHg, ethylHg, and inorganic Hg after their oral administration to murines, determining that thimerosal from ethylHg comes quickly to animals organs (kidneys, liver, lymph nodes) in an increasing fashion during periods of exposure, which can later be demoted to inorganic Hg [55]. In the same way, when the authors comparatively studied the ethylHg and inorganic Hg distribution applied by injection (imitating dose per weight and the chronology of human vaccination, as in this study), results showed that levels of mercury were higher in the liver and kidneys of animals exposed to inorganic Hg, while in the group of animals exposed to ethylHg, Hg concentrations were higher in the blood and the brain [56]. On the other hand, Harry et al have come to the conclusion that the

distribution of Hg among body tissues, after its intramuscular administration, it is not comparable to the results obtained after its oral presentation, and, furthermore, that methylHg does not seem to be a good model of comparison with compounds containing ethylHg [57]. Zareba et al, in a study about to be published in the *J Toxicol Appl*, have reported similar results in newborns mice, and pointed out that, after its intramuscular administration on postnatal day 10, ethylHg showed significant differences in its pharmacokinetics and tissue distribution in comparison with methylHg: higher proportion of inorganic Hg at the brain, of organic Hg at the kidneys, and the largest concentration of the two mercurial forms at the liver, compared with an equivalent dose of methylHg exposure.

Several other authors have reported substantial amounts of thimerosal in blood and central nervous systems of animals were the preservative has been tested, establishing its passage through the blood-brain barrier [58-59]. Studies in rodents have shown that this substance is highly unstable, dissociating in the body in ethylHg and thiosalicylate; ethylHg is able to move through cell membranes, and then intracellularly, will become in inorganic Hg, the most toxic mercurial form, which accumulates preferentially in the brain and kidneys [60]. Gasset et al observed a higher rate of fetal death when thimerosal was applied topically in pregnant rats [58]. Itoi et al also found abortive properties when this substance was applied in the conjunctiva of pregnant rabbits; additionally, these authors reported the occurrence of congenital malformations only in the animals groups exposed to thimerosal [61]. Digar et al documented mortality rates four times higher after thimerosal application in the yolk sac of chicken eggs [62]. Batts et al observed a reduction in the fertility of sheep, for which found that thimerosal produce toxicity on the ciliary function [63]. Goncharuk et al also showed a decline in fertility and higher mortality in rats exposed to the inhalation of ethylHg compounds [64]. Kahn et al observe ovarian atrophy and reduced survival of mice exposed to inorganic Hg [65].

Goth et al have recently reported that thimerosal is highly toxic to the immune system, and that alters the properties or causes the death of mice dendritic cells *in vitro* at nanomolar doses. It is worth noting that these cells act as presenters of antigens and stimulate immunity through the activation of T lymphocytes [66]. Lately, similar studies have found that thimerosal inhibits secretion of multiple inflammatory cytokines (tumor necrosis factor alpha (TNF), interleukin (IL) 6, and IL 12p70) along dendritic cells, prompting the increase of humoral immunity (TH2), and the inhibition of cellular immunity (TH1). These effects were mediated by the depletion of intracellular glutathione in the dendritic cells, and it was observed the correction of the latter, with the exogenous application of the substance [67]. Ueha-Ishibashi et al assessed the cytotoxic action of thimerosal over rat's thymic lymphocytes *in vitro*, noting that micromolar concentrations of the preservative (3 – 30 μM) depolarized cell membranes increasing intracellular calcium levels in a dose-dependent fashion; thimerosal also caused the loss of integrity of cell membranes, oxidative damage and cells' apoptosis [68].

Our research showed very significant differences, in terms of increase incidence of serious injuries at brain hystopathological level, in the group of animals exposed to thimerosal in all the statistical tools employed compared to the control groups. The previous pattern was observed for all the hystopathological variables (low neuronal density, neuronal necrosis, axonal demyelinization, and gliosis), and in all the brain regions studied (hippocampus, cerebral cortex, and cerebellum), as shows in Table 2.

These results are consistent with recent studies on the metabolism of thimerosal. This substance is a preservative used in some vaccines and biological agents in concentrations ranging from 0,003 to 0,01%. It contains 49,6% of Hg by weight. In saline solutions, its dissociates to ethylHg and thiosalycilic chloride acid [69];

Table 2. Statistical criteria by hystopathological variables between groups B and C.

| | STUDY REGION | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | HIPPOCAMPUS | | | CEREBRAL CORTEX | | | CEREBELLUM | | |
| VARIABLES | RR | RRA (%) | NNT | RR | RRA (%) | NNT | RR | RRA (%) | NNT |
| **Reduced neuronal density** | 14 | 87 | 1,15 | 12 | 73 | 1,36 | 5,5 | 60 | 1,67 |
| **Neuronal necrosis** | 12 | 73 | 1,36 | 7,5 | 87 | 1,15 | 7 | 80 | 1,25 |
| **Axonal demyelinization** | 6 | 67 | 1,50 | 7 | 80 | 1,25 | 3,67 | 53 | 1,88 |
| **Gliosis** | 6,5 | 75 | 1,36 | 5,5 | 60 | 1,67 | 7 | 80 | 1,25 |

RR: Relative risk.
RRA: Absolute risk reduction (risks difference).
NNT: Number of hamsters needed to treat to produce some type of damage.

once untied, ethylHg has a high affinity for sulfhydryls (SH) radicals found in some antioxidant enzymes, such as glutathione or metallothioneins (proteins produced in the liver), enzymes that have a limit union to heavy metals (saturation), leaving the upper ethylHg free (70-72). This enables us to ensure that an increase of thimerosal exposure could exceed the limit of saturation of natural antioxidants. As a result, the upper ethylHg would join other important SH groups of structural and / or functional proteins.

Moreover, it has been recently described the importance of intracytoplasmatic calcium channels of neurons, cerebellum cells, and the brain capillary endothelial cells (the same that makes up the blood-brain barrier) in mice, in the neurogenesis progression and neuronal differentiation process [73,74]. Precisely these channels' activation is mediated by the coordinated oxidation of SH radicals, which thimerosal exerts a powerful modulating effect increasing the calcium intracytoplasmatic concentrations; in this way, it has been noted that the latter can alter the physiology of the nerve cells, thanks to its impact on the state of oxido-reduction, conducing to functional disorders, glutathione depletion and increased oxidative stress [75-79].

Also, the high liposolubility of thimerosal allow its easily pass through the blood-brain barrier, causing irreversible damage on nerve cells. For example, it has also been found that Hg can alter cell's number and division; in this way, can affect neural development, causing alterations in cell proliferation and local neuropathological effects, which have been linked to specific conduct deficits [80].

Numerous biomolecular studies describe thimerosal ability to cause neurological disorders that entails as the causative agent of various children neurodevelopment diseases [81]. Haley et al [70] have demonstrated thimerosal neurotoxicity because of the enormous power of penetration its has in fatty tissues, such as the central nervous system, documenting human neuronal necrosis *in vitro*, after exposing them to thimerosal nanomolar solutions. Other human nerve cells studies also demonstrated that nanomolar to micromolar thimerosal concentrations are able to induce neuronal death, neurodegeneration, cell membranes damage, and DNA alterations, after just few hours of exposure [71, 72, 82--84]. Lately, it has been shown that tiny amounts of thimerosal

are equally capable of the critical disrupt of the interneuronal communication channels and the biochemical events necessary for the proper neurological development in humans [85,86]. In addition, it has been documented that thimerosal is toxic over neurotubules, interferes with brain antioxidant enzymes, damages DNA repair enzymes, interferes with mitochondrial energy production, block the glutamate recaptation brain proteins, has the ability to join neuronal DNA and can disrupt the cell membrane functions of nerve [87-89]. Leong et al demonstrated that exposure to nanomolar concentrations of Hg, altered the structure of the neuronal membrane and the growth of cell lines [90]; such findings have been replicated when using thimerosal [91-93]. James et al [72] have shown that thimerosal induces oxidative stress and apoptosis of neurons, astrocytes, and human T cells through the activation of mitochondrial metabolism forms of cell death. The latter explains the observed alterations in neuronal structure, which would result in a decrease of neuronal population; as a result, which is associated in the clinic, such as attention deficit disorder, psychomotor development alterations, language delays, behavior problems, loss of vision and hearing, seizures, autism, etc [94-96].

Recently, several investigations have shown that thimerosal is also capable of severely damaging methylation, a critic metabolic pathway to promote the normal neurological development [72,97-99]. It has been found that exposure to heavy metals, such as Hg, lead, and aluminum, may cause neurological diseases in humans because they are able to interfere with methylation and reduce various developmental factors, such as neurotropic growth factor, insulin-like growth factor type 1 and brain-derived neurotropic factor, all of which are essential for the proper development and survival of the nervous system [100, 101].

Thimerosal has also been associated with profound effects on the immune system. Havarinasab et al have studied immunity disorders after oral administration of organic and inorganic Hg compounds in mice. These authors have described that, if it is true that all the mercurial compounds have immunosuppressive effects, both methylHg as ethylHg show greater immunodepressant properties in comparison with inorganic Hg. However, in susceptible mice strains, Hg administration produces a transient immunocompromised state, from 1 to 3 weeks of duration, followed by a second phase of immunostimulation because of the activation of polyclonal B lymphocytes, in which appears antinucleolar antibodies, antifibrillar nuclear antibodies, systemic deposit of autoimmune complex, glomerulonephritis, serum increases of immunoglobulin (Ig) E, IgG 1, IgG 2a, IL 2, IL 4, IL-15, and interferon gamma, hypergammaglobulinemia, and splenomegaly, condition called "*mercury induced autoimmunity syndrome*" [102-106]. These authors reported that, at equimolar doses, methylHg has the lowest immunostimulants, autoimmunogenic, and autoimmune complex effect generator properties; while ethylHg have effects comparable to those of inorganic Hg, being responsible for serious injury mediated by autoimmune mechanisms and a high mortality [102].

While the harmful dose of thimerosal in humans (and particularly in infants and young children) has not been clarified yet officially, it is known that the mercurial component that contains (ethylHg), which its adverse effects are attributed, can produce neurotoxicity as is shown in the multiple investigations above. Due to, still to date, there is no definitive data available that compares the toxicity of ethylHg with methylHg, another mercurial agent of recognized neurological toxicity, widely studied through the years; the FDA consider both as equivalents at the risk evaluation, so they grouped this two compounds under the label of "*organic mercurials*", determining that its maximum permissible dose of exposure is 0,4 micrograms *per* kilogram of body weight *per* day (µg/kg/day) [107]. It is been pointed that, toxicity provoked by organic mercurials depends of the specific compound type, the entry way, dose, time, and age of exposure [47, 108].

The most relevant effects of thimerosal's ethylHg exposure are associated with the higher vulnerability of fetal and children brains, because the chronic exposure to organic Hg is especially toxic for an immature nervous system, producing alterations in its structural development (cortical and cerebellar neurons focal necrosis, axonal demyelinization, etc.), and functional development (interference in the cortical and subcortical neuronal layers migration process) [109-112], which were confirmed in our study.

It is to point out that, one of the most consistent neurological abnormalities found in *post-mortem* studies, and in the imaging of autistic people's brain studies, is a significant loss of Purkinje cells and atrophy at cerebellar level [113-116]. Numerous animals studies have shown that these cells are especially vulnerable to different aggressions, in which heavy metals exposure (Hg, lead, arsenic, cadmium and bismuth) is highlighted [117-123], damaging its glutamate recaptation receptors. The excessive neuronal stimulation associated to increased intracranial glutamate levels augments the production of oxygen reactive species, which also induce oxidative stress, cytotoxicity, and neuronal damage [124, 125], reason why it has been postulated that, the exposure to heavy metals in early stages of life, can start this chain of events that, finally, conduces to neuronal death [126]. It is convenient to point out that, the most ambitious and recent genetic study of the ASD did not found evidence that could support the hypothesis that these diseases were hereditary, but it found certain link with genes related to neurexins, which are precisely the responsible of the glutamate-mediated synaptogenesis [127].

Also, other recent studies have shown evidence of gliosis associated to Purkinje cell loss and a strong diffuse brain neuroinflammatory process in children with autism [128-131]. Precisely, the persistence of inorganic Hg in the experimentation animal brains, after being exposed to methylHg, thimerosal or inorganic Hg, has been associated with a significant microglia cells increase, astrocyte number's decrease, and compromise of Purkinje cells [52,132-134]. Finally, diverse neuropathological studies have shown abnormalities in the cytoarchitecture organization of the cerebral cortex and subcortical structures in patients with autism, suggesting that such defects in the neuronal maturation and cortical organization can be responsible for the neurological problems seen in this disease [128,135 -137].

All these evidence have conduced to recent review articles, which have associated the hystopathological findings at encephalon level, oxidative stress, lipidic peroxidation, and the glutathione deficiency seen in autism and other child's neurodevelopment diseases, with heavy metals exposure, concluding that the accumulation of the latter can happen in children whose detoxification capacity is limited or it is found compromised. The heavy metals can then reach critical levels that conduce to oxidative stress, decompensation, and neurological damage, resulting in a decrease of nervous cells and loss of developmental skills previously acquired [81,138-142].

In conclusion, thimerosal exposure, in quantities equivalent to those of human vaccines, reduced the body weight, encephalon weight, and height of postnatal hamsters in a significant way; in this way, it produced a lesser development and growth delay. Also, it produced severe neurotoxic effects at encephalon level expressing hystopathological alterations at hippocampus, cerebral cortex, and cerebellum levels. It is to remind that these conclusions can only be applied to the administration scheme previously detailed (dose, concentration, dilution, entry way, and application interval). Among the hystopathological alterations found at hippocampus level (CA1, CA3 and DG regions), the cerebral cortex (occipital, parietal and frontal lobes), and cerebellum (Purkinje cells and granulose cells), a reduction of neuronal density, neuronal necrosis, axonal demyelinization, and gliosis was distinguished. Also, the risk to present some of these alterations was very high only in the group of postnatal hamsters

exposed to thimerosal.

Due to the vast gaps in knowledge of thimerosal's pharmacokinetics and pharmacodynamics, as its toxic properties over the nervous and immune systems, it is required to make more studies of quantitative characters in animal models as soon as possible. Nevertheless, while it is true, it is very difficult to extrapolate these findings to other animal experimentation groups and over human beings, our results, as the multiple scientific evidence recently published about thimerosal, clearly indicates the toxic nature of this substance, at the same dose and the same chronology as human immunizations; therefore we suggest the employment of alternative preservatives in vaccines, especially those intended to pregnant women, neonates, and small children based in the prevention and precaution principles of all medical interventions.

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# TABLES AND FIGURES

**Table 1.** Substances administration scheme.

| | **NEONATAL AGE** (months) | | |
|---|---|---|---|
| | **2** | **4** | **6** |
| **Equivalent age in a postnatal hamster** ¥ | Day 7 | Day 9 | Day 11 |
| **Thimerosal's content on vaccines** (µg) δ | 125 | 125 | 125 |
| **Average weight of a human child** (Kg) | 4,4 | 5,8 | 6,8 |
| **Thimerosal's doses** (µg/Kg) | 28,4 | 21,6 | 18,4 |
| **Group A** | **Saline** (NaCl 9‰) | | |
| **Group B** | **Saline + sucrose** | | |
| **Sucrose's doses for administration** (µg/dose) | 0,227 | 0,216 | 0,220 |
| **Group C** | **Saline + thimerosal** | | |
| **Thimerosal's doses for administration** (µg/dose) | 0,227 | 0,216 | 0,220 |
| **Total administrated volume** (µL/dose) | 20 | 20 | 20 |

¥ *According to studies made in murines* [47-49].
δ *According to the US Vaccination scheme. 2001* [46].

**Table 2.** Statistical criteria by hystopathological variables between groups B and C.

| | STUDY REGION | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | HIPPOCAMPUS | | | CEREBRAL CORTEX | | | CEREBELLUM | | |
| **Variable** | **RR** | **ARR (%)** | **NNT** | **RR** | **ARR (%)** | **NNT** | **RR** | **ARR (%)** | **NNT** |
| Reduced neuronal density | 14 | 87 | 1,15 | 12 | 73 | 1,36 | 5.5 | 60 | 1,67 |
| Neuronal necrosis | 12 | 73 | 1,36 | 7.5 | 87 | 1,15 | 7 | 80 | 1,25 |
| Axonal demyelinization | 6 | 67 | 1,5 | 7 | 80 | 1,25 | 3.67 | 53 | 1,88 |
| Gliosis | 6.5 | 75 | 1,36 | 5.5 | 60 | 1,67 | 7 | 80 | 1,25 |

RR: Relative risk.
ARR: Absolute risk reduction (risks difference).
NNT: Number of hamsters needed to treat to produce some type of damage.

**Figure 1.** Body weight values comparison by study groups.

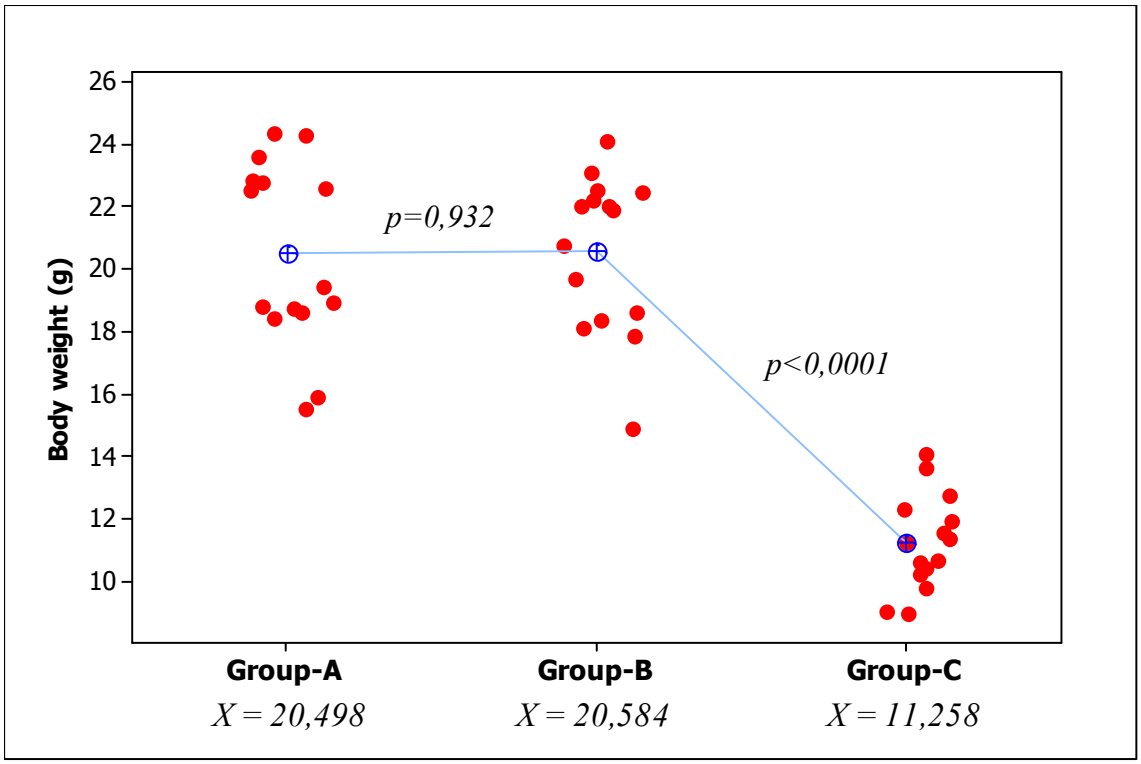

**Figure 2.** Encephalon weight values comparison by study groups.

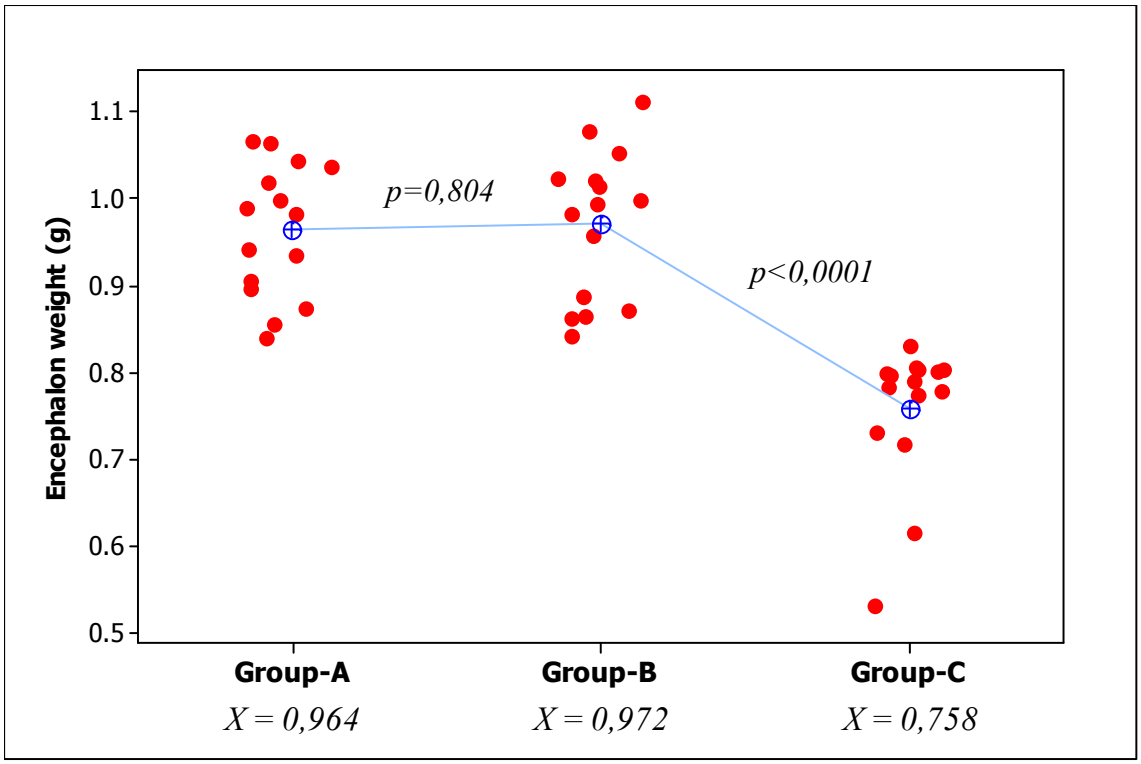

**Figure 3.** Height (Length skull-caudal) values comparison by study groups.

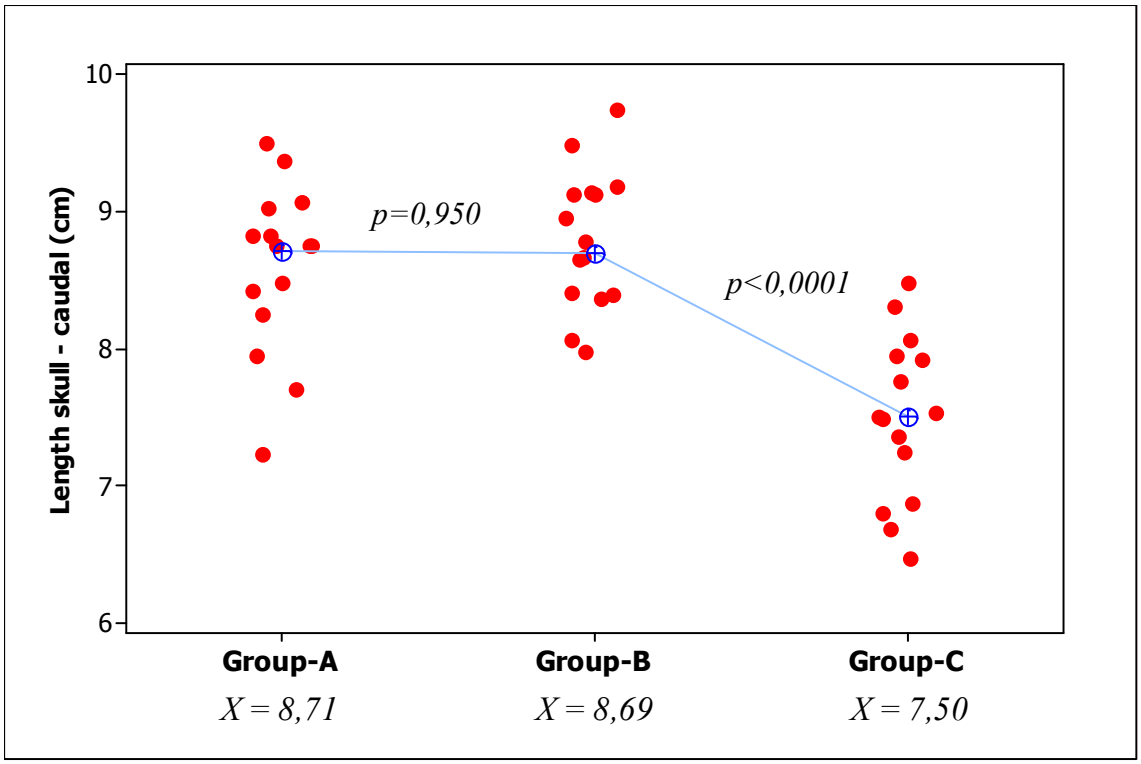

**Figure 4. Hippocampus:** A. Dentate Gyrus section (DG) (H-E x100); B. Reduced neuronal density in DG (H-E x100); C. Reduced neuronal density in CA3 and DG in comparison with CA1 (H-E x50).

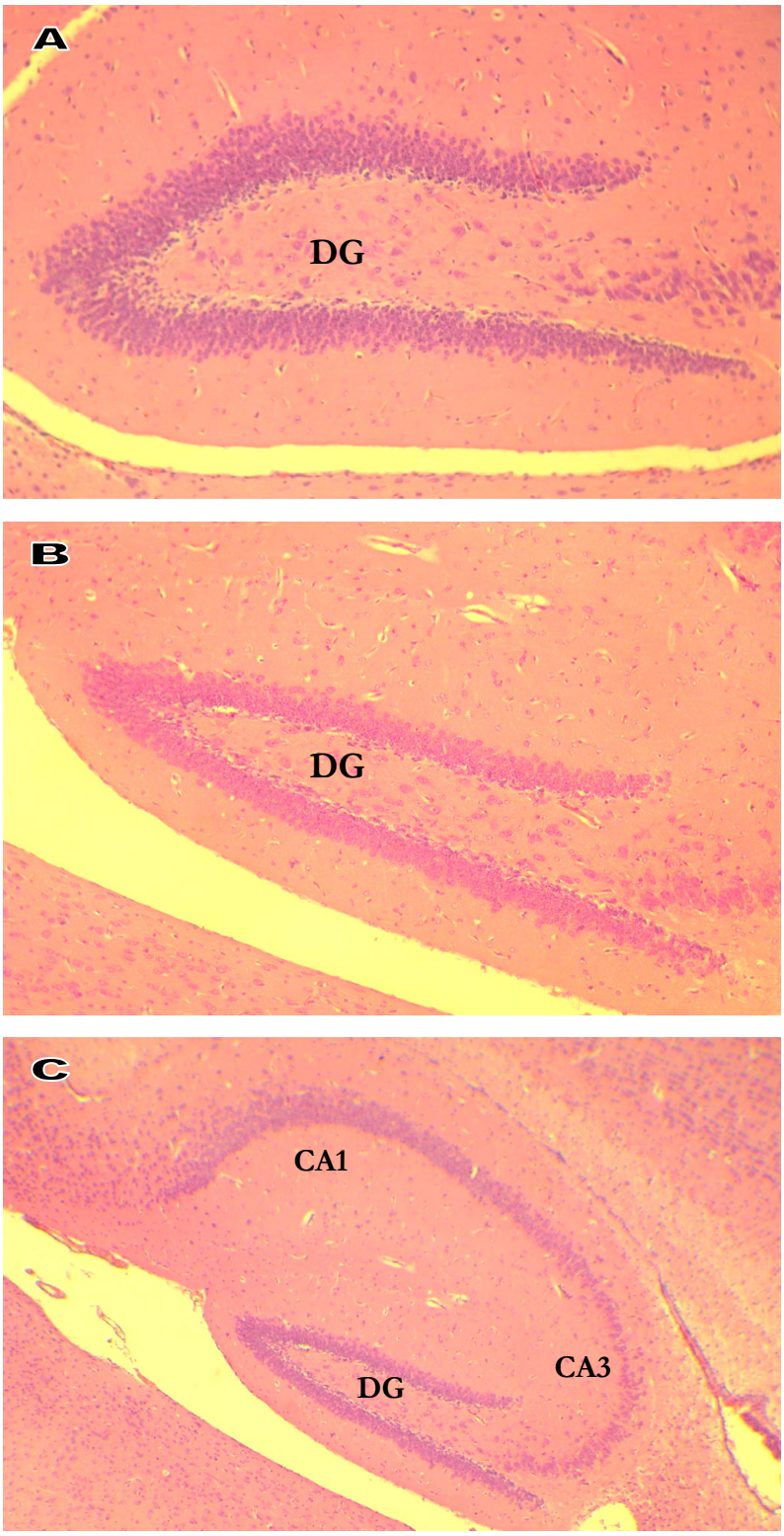

**Figure 5. Cerebral Cortex:** A. Pyramid neurons. (H-E x400); B. Pyramid neurone's necrosis. (H-E x400); C. Axonal demyelinization (luxol fast blue x400).

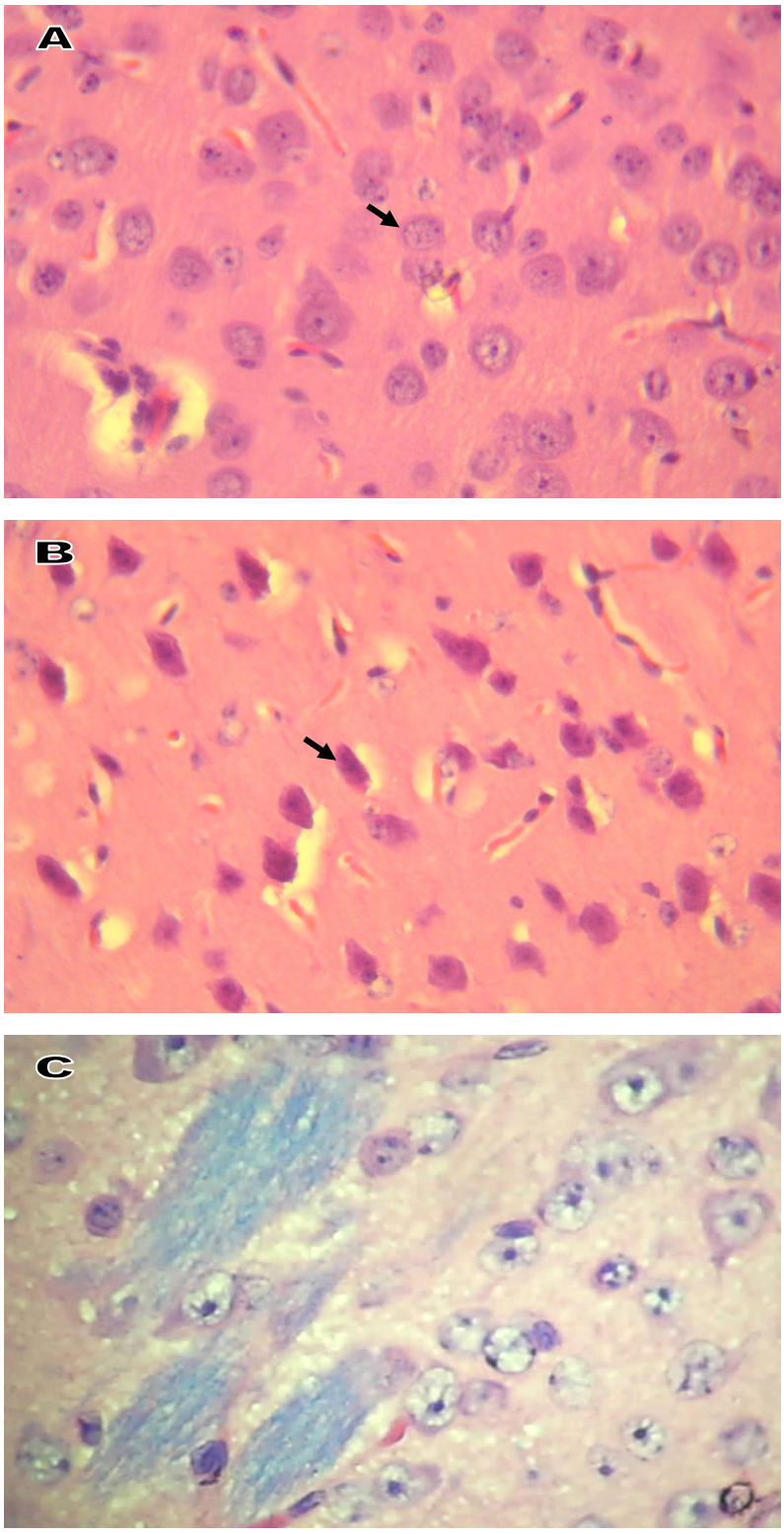

**Figure 6. Cerebellum:** A. Purkinje cells (H-E x400); B. Purkinje cells' necrosis (H-E x400); C. Purkinje cells' necrosis (black arrow) and normal (blue arrow) (Gomori's tricromic x400).