

A Morphometric Study on CA3 Hippocampal Field in Young Rats Following Maternal Administration of *Boswellia Serrata* Resin During Gestation

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Abstract

Objective

It has previously been shown that prenatal maternal administration of *Boswellia serrata* gum resin (Frankincense) improved learning and memory performance associated with an increase in the size of neuronal bodies in CA3 (Cornu Ammonis) of hippocampus. Continuing the previous work, a morphometric study was designed on CA3 field to examine precisely the effect of prenatal administration of frankincense on the structure of this region.

Materials and Methods

2 months-old male Wistar rats whose mothers were given orally the aqueous extract of the *Boswellia serrata* (0.1 g/kg/day) during gestation (3 weeks) were anesthetized and transcardially perfused with phosphate-buffered solution of 4% formaldehyde and 1% glutaraldehyde (n=8). Each brain was removed and divided into two hemispheres. One hemisphere selected at random for estimating the volumes of CA3 layers, and the other for morphometric analysis of CA3 neuronal dendrites. The Cavalieri principle employed to estimate the volumes and a quantitative Golgi study used to analyse the dendritic arborizations.

Results

Comparisons revealed that the control rats had lower volumes than the experimental animals in all layers of CA3 ($p < 0.05$). It was also indicated that the neurons of CA3 in experimental rats had more dendritic segments (31.25 ± 3.33) than the controls (27.5 ± 2.67), $p < 0.05$. The dendritic branching density was higher in experimental rats relative to that found in the control rats.

Conclusion

Results of this study provide a neuroanatomical basis that may be relevant to the early reported enhancement of learning and memory abilities in offspring.

Keywords: *Boswellia serrata*, Hippocampus, Morphometry, Pregnancy

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Introduction

The *Boswellia* species (Burseraceae), which are trees native to Ethiopia, Somalia, India, and the Arabic peninsula, produce a gum resin that is known as olibanum (frankincense). The resin of *Boswellia serrata* has been traditionally applied in folk medicine for centuries to treat various chronic inflammatory diseases. Experimental data from animal models and studies with human subjects confirmed the potential of *Boswellia serrata* extracts for the treatment of not only inflammation but also of cancer (1, 2). An investigation of *Boswellia*'s analgesic and psychopharmacologic effects noted that it "was found to exhibit marked sedative and analgesic effects" in animals (3).

However, Iranian traditional literature referred to frankincense as a herbal drug for increasing memory power. It is also suggested that pregnant women may improve the learning and memory abilities in their child by consuming frankincense (4). It has previously been shown that the offsprings whose mothers were administered frankincense, during gestational period, had better learning and memory performance (5) associated with an increase in the somal volume of hippocampal neurons in CA3 (Cornu Ammonis) (6). It has been well known that the hippocampus is a sensitive region of the brain with high plasticity involving in certain aspects of learning and memory (7). The hippocampal circuit integrity is crucial for learning and memory (8). The dendritic systems are the functional core of neuronal ensembles as they represent almost 90% of the receptive surface of neurons. There is also substantial evidence that organization of neuronal receptive surfaces is crucial for integration and transfer of information at the synaptic level (9).

On the basis of this background, in continuing the previous work, the volumes of the CA3 hippocampal field, i.e. Stratum

oriens, Stratum pyramidale and Stratum radiatum lacunosum molecular (Figure 1) and their dendritic arborizations examined, in the young male rats whose mothers were administered with frankincense during gestation for 3 weeks. The Cavalieri principle (10), an unbiased stereological method, employed to estimate the volumes and a quantitative Golgi study was used to analyse the dendritic arborizations.

Materials and Methods

Animal and Treatment

Two months old female Wistar rats (from animal house of Isfahan Medical Faculty - Iran) weighing 200 to 220g were used. They were kept in a controlled animal room at a temperature of 22-24 °C, with a 12 - hours day / night cycle (light on at 07.00–19.00 hours) and provided food and water ad libitum.

One week after adaptation to environment, one male Wistar rat was put into each cage containing two females. All pregnant dams assigned randomly to control and experimental groups. Animals in the experimental group were given the aqueous extract of *Boswellia* gum resin in a daily dose of 0.1 g/kg body weight during gestation (3 weeks). The Control animals received a similar volume of saline. The olibanum resin with certified botanical origin, *Boswellia serrata*, purchased from Gol darou (Isfahan, Iran). The aqueous extract of *Boswellia* gum resin was prepared in pharmacognosy laboratory of pharmacy faculty of , Isfahan University of Medical Sciences (for drug preparation see ref. 11). Drug and saline were administered orally (by feeding needle).

On the day 21 after birth, all offspring were weaned, female and male offsprings were separated and housed, four in each cage. A maximum of two males were taken from each litter to remove any "litter effects" (12).

The animals exposed to normal animal room conditions until testing at 2 months of aging. Animal cares and handling were in accordance with the rules approved by Local Research Council on Isfahan Medical University of Iran.

Histological procedures

Rats were deeply anesthetized with urethan (Merk -Germany) and transcardially perfused with a phosphate-buffered solution of 4% formaldehyde and 1% glutaraldehyde. Each brain was numbered and cerebellum and olfactory bulb were removed. The brains divided into hemispheres. One hemisphere was selected at random for estimating the volumes of CA3 layers, and the other for morphometric analysis of CA3 neuronal dendrites. They were postfixed in the perfusate, overnight. Posterior portion of each hemisphere, which contained hippocampus, was taken.

Nissl staining

Each brain block was embedded in 5% agar. Coronal sections of 100 μm thickness cut serially with a calibrated Bio-Rad Polaron H1200 vibratome and collected along the entire extent of the hippocampus. Using systematic uniformly random sampling (10), every 5th section (with an interval of 500 μm) was taken for Nissl staining. The sections were mounted on gelatin-coated object glasses immediately after the sectioning and fixed on these by air-drying at room temperature. The dry sections stained using hematoxylin: dip in distilled water, 4 minutes in hematoxylin, washed in running tap water for 10 minutes, rinsed with distilled water, dehydrated in 70% (10 minutes), 96% (2×5 minutes) and 99% ethanol (2×8 minutes), cleared 15 minutes in xylene; and coverglasses were mounted.

Golgi impregnation

The hippocampal formation postfixed in fresh fixative for 1 week and Golgi impregnated according to the Eckenhoff and Rakic (13), which relies on the use of high concentrations of aldehydes in Golgi solution. Briefly, the hippocampus was immersed for 72 h in 100 ml of a solution: 10 ml glutaraldehyde (25%), 10 ml formaldehyde (39%), 5 g potassium dichromate, 5-9 drops dimethylsulfoxide, and made to 100 ml with distilled water. During this period the solution was changed twice. The specimens were then transferred into 1.5% silver nitrate and stored in the dark for 3-5 days. The silver nitrate solution was changed every two days. The tissue was wrapped in paraffin shell and sliced in the horizontal plane at nominal thickness of 100 μm . The tissue slices were dehydrated, cleared and mounted on slides and coverslipped. The slides were blind coded for quantitative analyses.

Volume estimation

Discrimination between the different subdivisions of the hippocampal formation (Figure 1) was made according to cell morphology (14). The Cavalieri principle (10) used to estimate the reference volume of the constituent layers of the CA3 of the hippocampal formation. A grid with a tessellation of points, randomly positioned on each section, and the points hitting each layer of CA3 were counted. The number of points, ΣP , multiplied with the area associated with each point, $a(P)$, to obtain an unbiased estimate of sectional area of each profile. The sum of sectional areas of the layers was used to estimate reference volume, $V(ref)$, from the following relationship, where t represents the distance between sections.

$$V(ref) = t. \Sigma P. a(P) = t. \Sigma A$$

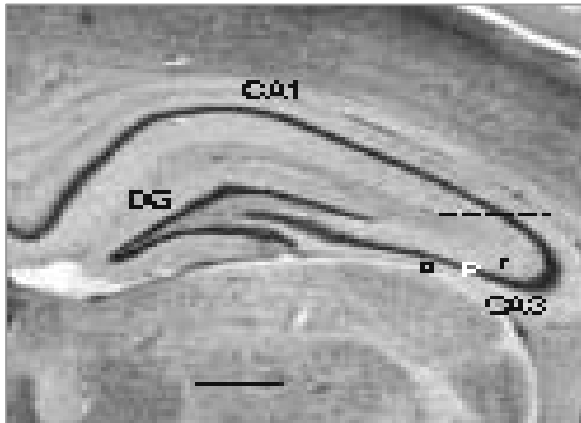


Figure 1. Low power micrograph of a 100 µm thick vibratome section through the hippocampus of a wistar rat (hematoxylin stain, broken line delineates CA3 from CA1, Scale bar = 0.5 mm). DG, Dentate gyrus, CA1 and CA3; Superior and inferior regions of Cornu Ammonis, respectively. o; Stratum oriens, p; Stratum pyramidale, r; Stratum radiatum+lacunosum + molecular.

Morphometric analysis of CA3 dendrites

From the CA3 pyramidal cell layer, ten pyramids were selected and pooled per animal in a single group. The criteria employed for selecting the neurons to be measured were as follows (15, 16):

(i) dark and consistent impregnation throughout the extent of dendrites, (ii) cell bodies located in the middle part of the section thickness in order to minimize branch segments cut off at the plan of the section, and (iii) relative isolation from neighboring impregnated cells in order not to have overlapping dendrites of adjacent cells. Because these criteria fulfilled solely by apical dendrites, the basal dendritic trees of pyramidal cells were not included in the estimations.

The presence of cut terminal segments on a neuron was not considered as a criterion for its exclusion from the estimations because the elimination of these neurons would have biased the sample towards smaller neurons (17). As it appeared that in the Golgi sections of experimental and control rats there was a similar percentage of cut branches (18%), the likelihood that these cut branches could have interfered with the final results is negligible (17). The dendritic trees of the CA3 pyramidal

cells were drawn with the aid of a camera lucida, at a final magnification of $\times 640$.

Number of dendritic segments per cell

The centrifugal ordering of dendritic trees was used to estimate the number of dendritic segments per cell (18). Accordingly, order 1 assigned to the dendrites arising from the soma and the successive orders were sequentially attributed to each branching point up to the terminals. In pyramidal neurons, the same criteria used and therefore there was no distinction between oblique dendrites, apical shafts and terminal tufts. The total number of segments per cell calculated by summing the number of dendritic segments of all orders.

Dendritic branching density

The branching density of dendritic trees was evaluated by applying the method of concentric rings (17). The number of dendritic intersections crossing each concentric ring centered in the cell body was counted. The concentric rings were calculated at interval of 25 µm. whenever the dendrites extended beyond 375 µm (circle 15) they were included in circle 15.

Statistical Analysis

The Coefficient of Error (CE) of the individual estimates calculated according to Gundersen and Jensen (19). The Coefficient of Variation (CV) determined as described by West and Gundersen (20). The Students t-test was performed on data from the experimental and control rats. Differences were considered to be significant if $p < 0.05$.

Results

The volumes of the different layers of the CA3 are shown in Table 1. Statistical analysis revealed a significant effect of prenatal administration of Boswellia gum resin on the volume of the layers of the CA3 hippocampal field of young offsprings. Comparisons between two groups revealed that the control rats had lower volumes than the experimental animals in all layers of CA3, i.e. Stratum Oriens, Stratum Pyramidale, and Stratum Radiatum Lacunosum Molecular ($p < 0.05$).

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Table 1. Volumes of the layers of the CA3 pyramidal cell (mm³) in experimental (prenatally *Boswellia* administrated) and control rats.

Hippocampal CA3 field	Control (n=8)		Experimental (n=8)		p
	Mean (CV)	CE	Mean (CV)	CE	
Stratum Oriens	2.23 (0.08)	0.03	2.56 (0.10)	0.05	<0.05
Stratum Pyramidale	1.45 (0.11)	0.03	1.66 (0.10)	0.03	<0.05
Stratum Radiatum + Lacunosum + Molecular	5.53 (0.07)	0.05	6.14 (0.10)	0.05	<0.05

Coefficients of Error (CE) is the estimated intra- animal coefficient of error
Coefficients of Variation (CV) is the observed inter-animal coefficient of variation

No areal shrinkage correction was used in the study because of the insignificant magnitude of the shrinkage and because no difference in shrinkage was found between groups (Mean areal shrinkage of 3.5% detected). The results of this study also showed that a significant impoverishment of the dendritic trees of CA3 pyramidal cells (Figure 2) was present in sections from the control group as compared to the experimental group. Comparisons between two groups revealed that the neurons of CA3 in experimental rats had more dendritic segments (31.25 ± 3.33) than the controls (27.5 ± 2.67), $p < 0.05$. Study of the dendritic intersections showed that the effect of maternal *Boswellia* treatment was significant for circles 10, 11, 12, 13, 14 and 15 (Figure 3). The number of intersections in these circles in the study group was higher than the control group ($p < 0.05$).

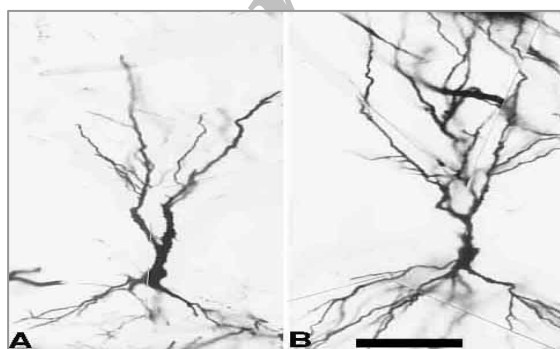


Figure 2. Photomontages of Golgi-impregnated CA3 neuron from control (A) and prenatally *Boswellia* administrated young rats (B). CA3 pyramidal cells from control rats display an obvious impoverishment of their terminal dendritic arborizations, when compared to similar neurons from the prenatally *Boswellia* administrated rats. Scale bar = 75 μ m and applies to both frames.

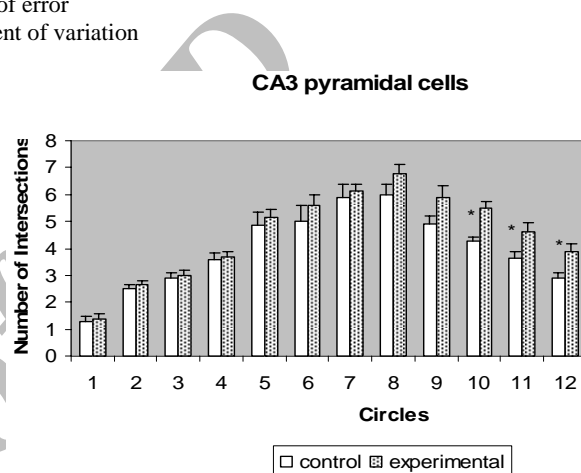


Figure 3. Graphic representation of the dendritic branching density of CA3 hippocampal neurons of experimental (prenatally *Boswellia* administrated young rats) and control. Vertical bars represent SEM. Circles 10, 11, 12, 13, 14 and 15, $p < 0.05$.

Discussion

The present study demonstrated that chronic maternal administration of *Boswellia* gum resin in gestational period induced significant volumetric alteration in the layers of the CA3 hippocampal field in young male rats.

Quantitative morphological analysis of dendritic architecture of Golgi-impregnated CA3 hippocampal neurons also indicated that young rats whose mothers were *Boswellia* treated during gestation showed more dendritic branches in the CA3 pyramidal neurons.

Although the precise biological mechanism behind the *Boswellia*-induced neuritic enhancement is unclear, the psychopharmacological and sedative action of *Boswellia* gum resin (3) may help to

explain enhancement of CA3 dendritic arborization.

These explanations can be given for the findings of present research: obviously, both the experimental and control mother rats had been exposed to undesired various physical and emotional stresses (oral gavages, habitat, environmental noise exposure, fearing and so on) during pregnancy. It is well known that prenatal stress affects adversely the function and structure of hippocampus in developing and adult mammals (21, 22). For example, it is reported that prenatal noise exposure (23) or prenatal saline injection (24) produced structural alterations in the hippocampus of developing rats. Recent studies have shown that stress during pregnancy induces the morphological changes in processes of CA3 hippocampal neurons in the adult offspring (25, 26).

It is relatively easy to speculate that anxiety, stress are lessened (reversed) when the pregnant rats supplemented with *Boswellia* gum resin, due to *Boswellia*'s sedative and psychopharmacologic effects (3). Consequently, a neuroprotective effect from prenatal stress by *Boswellia* could also be presumed. In the other word, *Boswellia serrata* could decrease the resulting hippocampal damage due to prenatal stress.

To date, there is no study on the effect of prenatal administration of herbal drugs on offspring's brain structure, to compare with the results of this research.

It is noteworthy that the quantitative techniques used here were sufficiently sensitive to detect even little changes in the volumes. Coefficients of error (CE) provide a standardized statistic for evaluating the precision of volume estimates derived by

modern stereological techniques (27). Upon completion of the analysis, it was found that the means of CEs are for the volume estimates of the three layers of CA3 ranged from 0.03-0.05 with an overall average of 0.04. The inter-animal Coefficient of Variances (CVs) for the three regions varied from 0.07-0.11 with a mean of 0.09. The ratio CE^2/CV^2 is about 0.20, indicating that the precision of the estimates may be even better than needed for optimal sampling (19).

In the earlier research, it was observed that maternal administration of *Boswellia serrata* during gestation induces significant increase in the individual somal volume of hippocampal neurons only in the CA3 (6). This finding likely reflect profound alteration in the neurites of CA3 hippocampal cell, a finding that is strongly supported by the data reported in the present study.

Conclusion

Results of this study provide a neuroanatomical basis that may be relevant to the early reported enhancement of learning and memory abilities in offspring. The constituents and precise mechanism responsible for the efficacy of *Boswellia serrata* are still a matter of debate and remain to be clarified. Further studies are being conducted to investigate the mechanism of action, and side effect of this natural product.

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References

- 1- Duwiejua M, Zeitlin IJ, Waterman PG, Chapman J. Anti-inflammatory activity of resins from some species of the plant family Burseraceae. *Planta Medica* 1993; 59: 6-12.
- 2- Huan Mt, Badmaev V, Ding Y, Liu Y, Xie JG, Ho CT. Anti-tumor and anti carcinogenic activities of triterpenoid, beta boswellic acid. *Biofactors* 2000; 13: 225-30.

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- 3- Menon MK, Karr A. Analgesic and psychopharmacological effects of the gum resin of *Boswellia serrata*. *Planta Medica* 1971; 4: 332-341.
- 4- Zargari A. Herbal Drugs. Vol. 1, Fifth edition, Tehran: University publication; 1989. 550-553 (In Persian).
- 5- Hosseini-sharifabad M, Esfandiary E, Alaei H. Effect of frankincense aqueous extract during gestational period on increasing power of learning and memory in adult offspring. *J Isfahan Med Sch* 2004; 71: 16-20 (In Persian).
- 6- Hosseini-sharifabad M, Esfandiary E. Quantitative analysis of hippocampal neuron number and size following prenatal administration of *Boswellia serrata* gum resin in adult rats. *J Isfahan Med Sch* 2005; 77:58-63.
- 7- Eichenbaum H. The hippocampal system and declarative memory in animals. *J Cogn Neurosci* 1992; 4: 217-231.
- 8- Rusakov DA, Davies HA, Harrison E. Ultrastructural synaptic correlates of spatial learning in rat hippocampus. *Neuroscience* 1997; 80: 69-77.
- 9- Horner CH. Plasticity of the dendritic spine. *Prog Neurobiol* 1993; 41: 281-321.
- 10- Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS* 1988; 96: 379-394.
- 11- Hosseini-sharifabad M, Esfandiary E, Alaei H, Moatar F. Effect of maternal consumption of aqueous extract of the gum resin of *Boswellia serrata* during lactation on increasing power of learning and memory in adult offspring. *IJBMS* 2003; 6(3): 207-211 (In Persian).
- 12- Chapman RH, Stern J. Maternal stress and pituitary-adrenal manipulations during pregnancy in rats: effects on morphology and sexual behavior of male offspring. *J Comp Physiol Psychol* 1978; 92:1074-1083.
- 13- Eckenhoff M F, Rakic R. Radial organization of the hippocampal dentate gyrus: a Golgi, ultrastructural and immunocytochemical analysis in the developing Rhesus monkey. *J Comp. Neurol* 1984; 223: 1-21.
- 14- West MJ, Slomianka L, Gundersen HJG. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec* 1991; 231: 482-497.
- 15- Green EJ, Jurska JM. The dendritic morphology of hippocampal dentate gyrus cells varies with their position in the granular cell layer: a quantitative Golgi study. *Exp Brain Res* 1985; 59: 582-586.
- 16- De Ruiter JP, Uylings HBM. Morphometric and dendritic analysis of fascia dentata granule cells in human aging and senile dementia. *Brain Res* 1987; 402: 217-229.
- 17- Uylings HBM, Ruiz-Marcos A, Van Pelt J. The metric analysis of three-dimensional dendritic patterns: a methodological review. *J Neurosci Meth* 1986; 18: 127-151.
- 18- Uemura E, Carriquiry A, Kliemann W, Goodwin J. Mathematical modeling of dendritic growth in vitro. *Brain Res* 1995; 671: 187-194.
- 19- Gundersen HJG, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J Microsc* 1987; 147: 229-263
- 20- West MJ, Gundersen HJG. Unbiased stereological estimations of the number of neurons in the human hippocampus. *J Comp Neurol* 1990; 296: 1-22.
- 21- Weinstock M. Alterations induced by gestational stress in brain morphology and behaviour of the offspring. *Prog Neurobiol* 2001; 65: 427-451.
- 22- Van den Hove DL, Steinbusch HW, Scheepens A, Van de Berg WD, Kooiman LA, Boosten BJ, et al. Prenatal stress and neonatal rat brain development. *Neuroscience* 2006; 137(1):145-155.
- 23- Kim H, Lee MH, Chang HK, Lee TH, Lee HH, Shin MC, et al. Influence of prenatal noise and music on the spatial memory and neurogenesis in the hippocampus of developing rats. *Brain Dev* 2006; 28:109-14
- 24- Schmitz C, Bultmann E, Gube M, Korr H. Neuron loss in the mouse hippocampus following prenatal injection of triated thymidine or saline. *Int J Dev Neurosci* 1999; 17: 185-190.
- 25- Fujioka A, Fujioka T, Ishida Y, Maekawa T, Nakamura S. Differential effects of prenatal stress on the morphological maturation of hippocampal neurons. *Neuroscience* 2006; 141, 907-915.
- 26- Hosseini-sharifabad M., Hadinedoushan H. Prenatal stress induces learning deficits and is associated with a decrease in granules and CA3 cell dendritic tree size in rat hippocampus. *Anatom Sci Intl* 2007; 82:211-217
- 27- Hosseini-sharifabad M, Nyengaard JR. Design-based estimation of neuronal number and individual neuronal volume in the rat hippocampus. *J Neurosci Meth* 2007; 162: 206-214.