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Research Article

Morphometric Analysis of Nitrergic and Cholinergic Neurons of Myenteric Plexus of Enteric Nervous System of Rat Colon In Chronic Stress

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Key words : Atrophy, Colon, Dismotility, Morphometry, Passive Avoidance Stress

Abstract: Stress is a response of the organism which alters the homeostasis. Role of chronic stress in the modulation of common gastrointestinal disorders has already been established. Long lasting stress affects the synaptic plasticity, dendritic morphology and neurogenesis. The present study was conducted to evaluate the morphological changes in the enteric nervous system of rat colon to understand the basis of functional and physiological alterations in response to chronic stress. Seven male wistar rats were subjected to chronic passive avoidance stress for 6 hours daily for 42 days and they were compared with equal number of controls. Colonic tissue was collected for histochemistry of nitrergic and cholinergic neurons of myenteric plexus of enteric nervous system and evaluated both by light and electron microscope. The number and size of the neurons were evaluated. The area, perimeter and maximum diameter or ferret diameter were measured according to the principles of stereology for neuronal profile. There was atrophy of both nitrergic and cholinergic neurons as well as reduction in number in the myenteric plexus in rat colon. There were bouts of diarrhoea followed by constipation in the stressed animals. Psychological stress is known to cause oxidative stress to the cells, which damages the DNA structure leading to cell death. Thus apoptosis could be responsible for reduction of neuronal cell population and size. The dismotility may be due to imbalance between inhibitory (nitrergic) and excitatory (cholinergic) neurons due to their atrophy.

Stress is a defensive response of the organism to various stimuli (psychological, chemical or physical), that may alter the homeostasis of the organism (Chrousos GP and Gold PW 1992; McEwen BS 2000). Psychological stresses have always been acknowledged as potential harm to health like chronic disorders of the digestive system including functional gastrointestinal disorders (FGD), inflammatory bowel diseases (IBD), gastro-esophageal reflux (GERD) and peptic ulcer disease (PUD) (Brandspiegel HZ 1998; Wittstein IS 2005). Several literatures have indicated that long lasting stress affects the synaptic plasticity, dendritic morphology, and neurogenesis in animals and humans (Kim JJ and Yoon KS 1998; Sapolsky RM 1996). The stress system is activated when homeostasis is challenged by extrinsic or intrinsic forces. The digestive tract is unique among internal organs because it is exposed to a large

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number of physio - chemical stimuli and certain stressful life events have been associated with the onset or exacerbation of some of the most common chronic disorders of the digestive system as mentioned above. There is sufficient literature describing the physiological and functional changes of gut due to different types of stressors. The present study has attempted to evaluate the morphological changes, if any, that occur in the gut wall and the enteric nervous system of the colon, with a view to understand the basis of functional and physiological alterations.

Materials and Methods

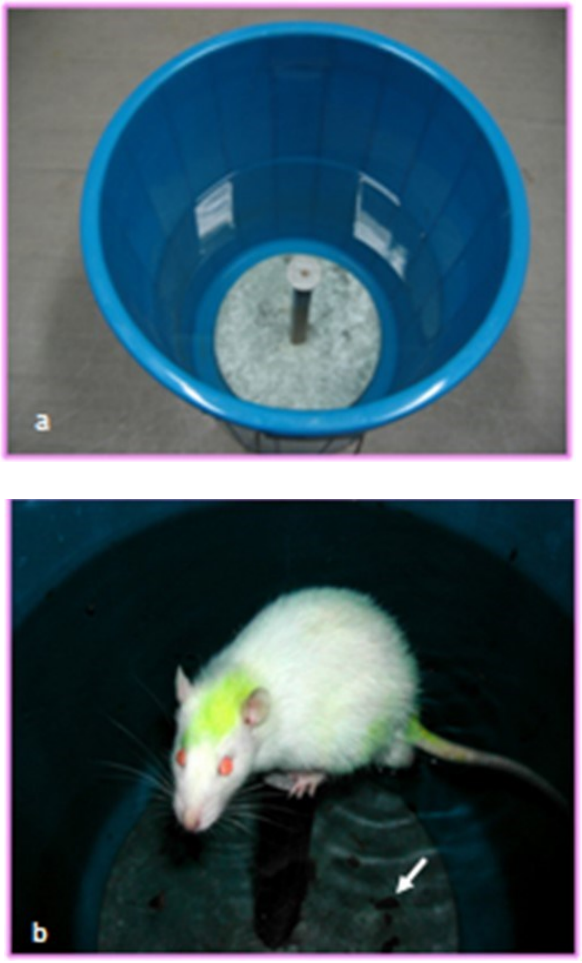
All procedures were explained and approved by the Animal Ethics Committee, All India Institute of Medical Sciences, New Delhi.

Total fourteen male Wistar rats weighing 150 to 200 gm and aged 6 to 10 weeks were included and devided as control and experimental group (n=7). All the rats were kept in animal house providing separate cage for each rat maintaining 12:12 hour dark/light cycle. Appropriate humidity and temperature were maintained with food and water ad libitum to both experimental and control except the period from 10.00 am to 4.00 pm.

Stress Protocol

The experimental group of animals was subjected to passive avoidance stress as per the protocol described by Enck, P et al in 1989 for 6 hours daily for a period of 42 days (Enck P et al. 1989). Each experimental rat was made to sit on a small platform (diameter 4 cm) elevated one inch above the water level in a barrel partially filled with water (Figs. 1a, b). After experiment they were returned to their home cages and provided with food and water. The controls were also kept in the same room and not given any food (except water) while the experimental animals were undergoing stress. Body weight was recorded before and 42 days after the experiment of both groups. Number of faecal pellets was also counted (Fig.1b).

Fig. 1 Photographs represent sress model



(a) and Rat is in a stress model (b). White arrow indicates the number of fecal pellets (b).

Tissue collection and Processing

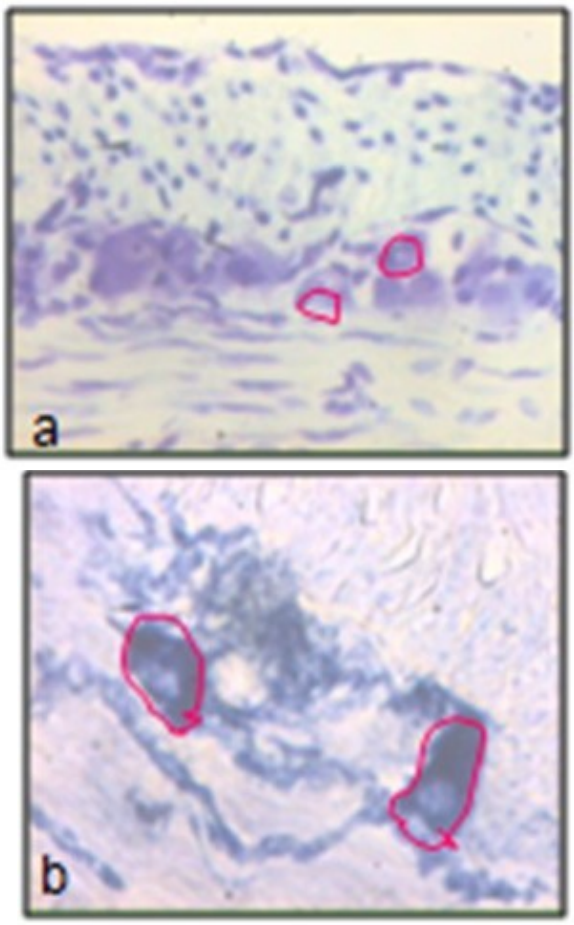
At the end of the experiment, animals were anaesthetized by ether inhalation and perfused with 4% buffered paraformaldehide. Whole colon was identified and taken 2-3 cm away from the caecum. The lumen was cleaned gently with 0.1 M phosphate buffer. Sections were stained by Cresyl violet and Masson's

trichrome. Cryosections were stained by NADPH Diaphorase and Acetyle choline esterase (AChE). All sections were examined under light and electron microscopes.

Morphometry and application of stereology

The images were analysed using ImageJ (available at http://rsb.info.nih.gov/ij/). The myenteric neurons, plexuses and nitrergic & cholinergic neurons were identified and accurately outlined and measured for the cell profiles like area, perimeter and ferret diameter and mean point count for neurons (Figs. 2a, 2b).

Fig. 2 Photographs represent outlines of neuron used for stereological analysis



Nissle stain (a) and NADPH stain (b)

STATISTICAL ANALYSIS

STATA software was used for statistical analysis and data was expressed as mean ± standard deviation. Independent sample t test (Student's t-test) was used to determine the statistical significance between the means. Standard error of mean (SEM) and the 95% confidence interval (CI) of the difference between the means were noted. Probability levels of less than or equal to 5 % (two-tailed p value < 0.05) were considered to be significant.

Observation

It was noticed that the respiratory rate and the movements of the whiskers were increased when the animals were going through stress period. Initially the fecal output was increased in experimental animal during both stress and non stressed period. Later it was noticed that there was alternate diarrhoea and reduced excretion (constipation). Towards the end of the phase of stress period the fecal excretion was decreased. There was no alternate diarrhoea and constipation in the control animals. It was noticed that the food intake reduced by the experimental than control animals.

Observation of Body weight & adrenal gland weight

The net body weight gain in experimental group was lesser than the control as food intake was less in former group.

Relative weight of adrenal gland

The relative weight of adrenal gland was expressed as: adrenal weight in mg × 100 per unit body weight in gm (Table 1). In experimental animal relative weight of adrenal gland was increased ($p = 0.035$) than the control (Fig. 3). In chronic stress there was hypertrophy of adrenal glands.

Table 1. Relative adrenal gland weight to the body weight in experimental (E) and control (C) Rat

Animal no	Adrenal weight (mg)	Body weight (gm)	Relative adrenal wt to body wt
C1	0.020940	170	0.0123221
C2	0.032019	225	0.0142310
C3	0.037830	250	0.0151321
C4	0.026293	230	0.0114321
C5	0.032697	225	0.0145321
C6	0.039471	245	0.0161110
C7	0.039372	234	0.0145179
E1	0.028600	195	0.0146670
E2	0.034400	180	0.0191111
E3	0.035400	195	0.0181538
E4	0.028000	150	0.0186667
E5	0.025900	195	0.0132821
E6	0.027000	150	0.0180000
E7	0.029800	165	0.0180606

Table shows increased relative adrenal gland weight to the body weight in experimental (E) than control (C) rat. Mean relative adrenal gland weight to body weight in control is 0.0139 and in experimental group is 0.0169. (t=-2.442 & p=0.035*).

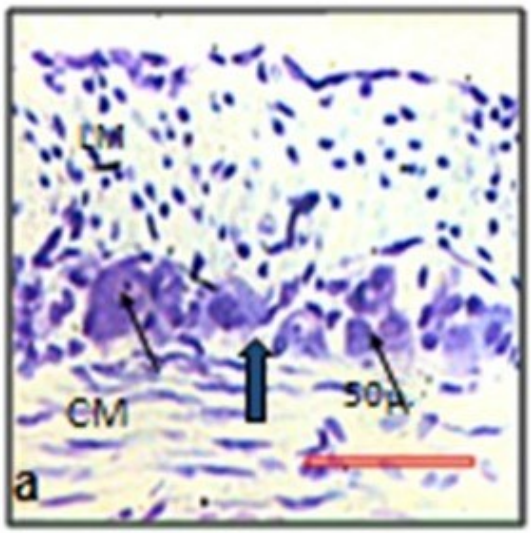
Fig. 3 Kidney with adrenal gland indicated with arrow and shows hypertrophy of adrenal gland in chronic stress. (K = kidney)

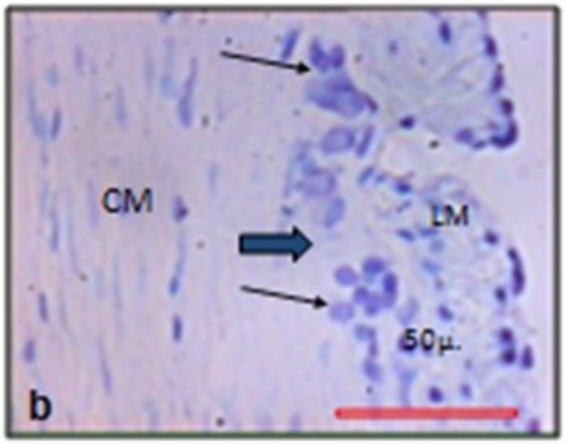
Morphometry in (Cresyl violet) Nissl stained sections

The size of the myenteric ganglia was reduced in experimental animal (Fig. 4). In control the mean point count of myenteric ganglia was 15.19 (SD 12.61, min 0, max 66) and in experimental it was 13.96 (SD 12.37, min 0, max 57). The mean point count of neuronal cells in the myenteric plexus of control rat was 6.56 (SD 6.10, min 0, max 32). In experimental animals it was diminished to 4.85 (SD 4.97, min 0, max 29) (Figure 4). The myenteric ganglia (t=0.17 and p= 0.85) and the myenteric neurons (t=0.53 and p= 0.60) were reduced in chronically stressed rat.

The mean area and perimeter of the neuronal profiles of myenteric neuronal cell in cresyl violet stained sections were compared. The mean ± SD area in non-stressed or control animal was 26.4 ± 21.91 μ^2 (median 21.42 μ^2, min 7.56 μ^2, max 268.3 μ^2). In stressed rat it was 21.4 ± 11.93 μ^2 (median 18.38 μ^2, min 3.66 μ^2, max 130.01 μ^2). Perimeter of the myenteric neuron in control was 22.01 ± 6.94 µ (median 20.53 µ, min 11.58 µ, max 71.44 µ). In the experimental it was 18.02 ± 4.83 µ (median17.11 µ, min 7.84 µ, max41.73 µ). The overall size of neuronal cell profiles i.e.area (t=0.49 & p= 0.63) and perimeter (t=1.16 & p=0.27) in myenteric ganglion is reduced after chronic stress (Table 2).

Fig.4 Myenteric ganglia in Cresyl violet stain.





Black arrows indicate the neuron. Thick black arrow indicates the myenteric ganglion. Control (a) and experimental (b). (LM = Outer longitudinal muscle; CM = Inner circular muscle)

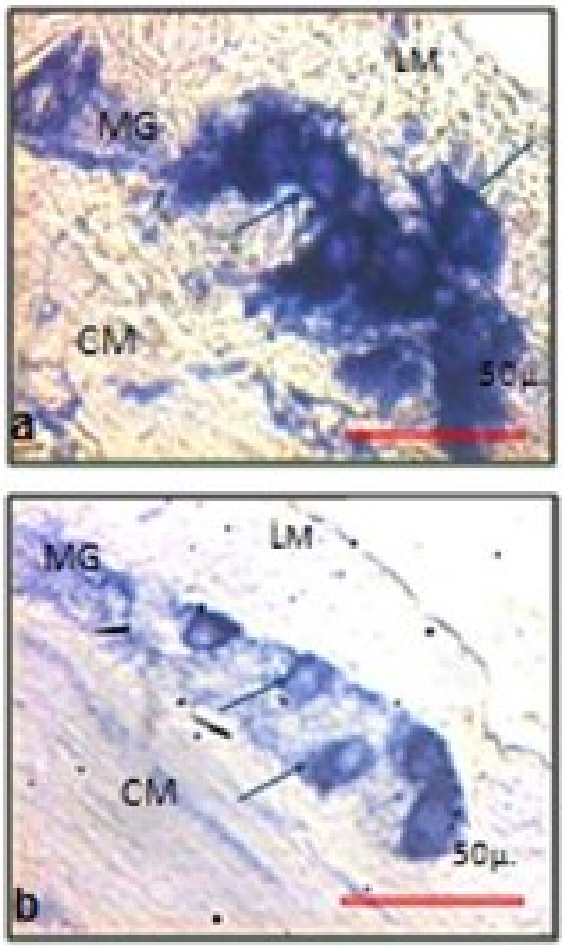
Morphometry of diaphorase positive neurons

The mean area of neuronal somata of nitrergic myenteric neurons was 274.51± 116.41 μ^2 (median 262.65 μ^2, min 71.79 μ^2, max 948.05 μ^2) in control, where as that of the experimental animals was 191.43±94.67 μ^2 (median 168.32 μ^2; min 29.48 μ^2; max 739.31 μ^2) . The perimeter of the neuronal soma was 65.26± 14.7 µ (median 64.27 µ; min 34.11 µ; max 127.36 µ) in the control group and in experimental animal it was 54.95 µ ± 13.79 µ (median 53.44 µ; min 22.61 µ; max 108.45 µ) (Figure 5). The ferret diameter of neuronal soma in control was 24.25± 5.76 µ (median 23.79 µ; min 11.58 µ; max 48.26 µ). In chronic stressed rat it was 20.87± 5.72 µ (median 20.37 µ, min 7.64 µ, max 41.02 µ). There is a decrease in the mean area (t=1.357 & p=0.204), perimeter (t=1.258 & p=0.236) and ferret diameter (t=1.063 & p=0.312) of the myenteric nitrergic neurons in stressed rats compared to the control animals.

The mean point counts of nitrergic neurons in myenteric ganglion in colon was reduced in experimental animals (mean ± SD) 4436.67 ± 2665.16, (median 3850, min 1200, max 10400) compared to the control (mean ± SD) 11369.23 ± 6438.62 (median10800, min 3200, max 30500). The number of nitrergic (inhibitory) neurons of

myenteric ganglion was significantly (t=-2.436 & p=0.03*) reduced in chronic stress (Fig. 5) (Table 2).

Fig.5 Myenteric ganglion in NADPH - diaphorase stain.

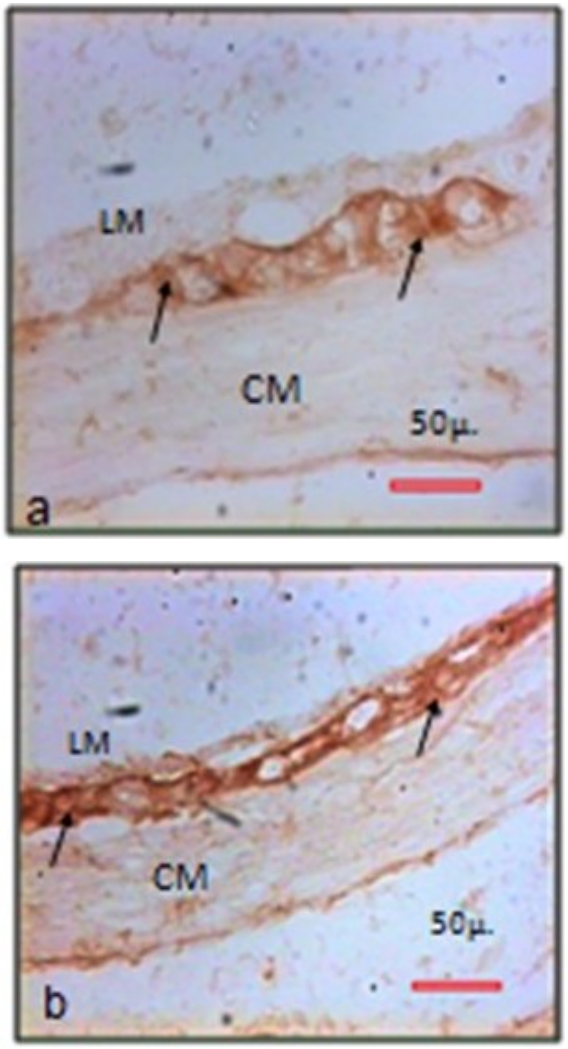


Note the decreased size of the NO (nitric oxide) positive neurons in chronic stressed rat (b) than control (a). Black arrows indicate the neurons and unstained area is nucleus (LM = Outer longitudinal muscle; CM = Inner circular muscle; MG = Myenteric ganglia)

Morphometry of the cholinergic myenteric neurons

Morphometric evaluation of the Acetyle choline esterase (AChE) positive neuronal cells in the myenteric ganglion was also performed. The mean area of cholinergic neurons in the myenteric ganglion was 272:27 ± 122:18 μ^2 (median 250:85 μ^2; min 72:71 μ^2; max 791:59 μ^2) in the control and in the experimental animals it was 224:63 ± 114:02 μ^2 (median 199:15 μ^2; min 47:98 μ^2; max 730:04 μ^2).

Fig. 6 Myenteric ganglion in AchE stain.



Black arrows indicate the cholinergic neurons and unstained area is nucleus. Control (a) and experiment (b).

The mean area (t=0.698 & p=0.50) of cholinergic neurons was reduced in chronically stressed rats. The mean perimeter of the cell profiles in control animals was 62:91 ± 14:01 µ (median 61:42 µ; min 33:85 µ; max 113:22 µ) and in experimental it was 58:21± 15:11 µ (median 56:79 µ; min 28:59 µ; max 126:99 µ). The mean ferret diameter of neuronal profiles in the control group was 24:04 ± 5:91 µ (median 23:18 µ; min 12:42 µ; max 45:86 µ) and in the experimental it was 22:59 ± 6:47µ (median 22:09 µ; min 10:75 µ; max 53:25 µ). The perimeter (t=0.55 & p=0.58) and ferret diameter

(t=0.40 & p=0.69) of the myenteric neuronal profiles in the myenteric ganglion was also reduced in experimental animal colon. It has been noted that excitatory cholinergic neurons also atrophied in chronic stress (Fig. 6) (Table 2).

Table 2: Comparisons of different neuronal cell profiles of myenteric plexus in different types of neurons

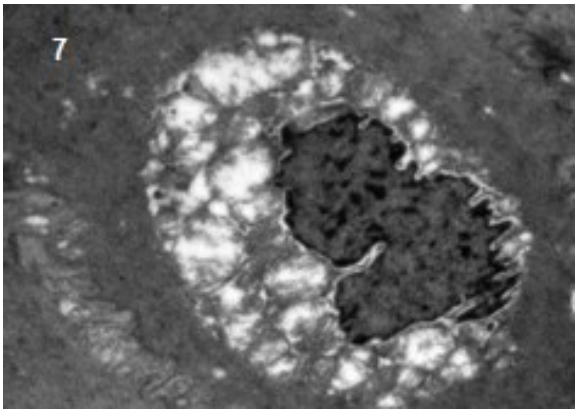
Staining procedure	Section in	(Mean) Cell profiles	Experimental	controls
Cresyl violet	Paraffin sections	point count of myenteric ganglia	13.96	15.19
		point count of neuronal cells in the myenteric plexus	4.85	6.56
		area	21.4 ± 11.93 μ^2	26.4 ± 21.91 μ^2
		Perimeter	18.02 ± 4.83 μ	22.01 ± 6.94 μ
Nitrergic myenteric neurons (NADPH) positive	Cryosections	point counts of nitrergic neurons in myenteric ganglion	4436.67 ± 2665.16,	11369.23 ± 6438.62
		area	191.43±94.67 μ^2	274.51± 116.41 μ^2
		perimeter	54.95 μ ± 13.79 μ	65.26± 14.7 μ
		ferret diameter	20.87± 5.72 μ	24.25± 5.76 μ
Cholinergic myenteric neurons (AChE) positive	Cryosections	area	224:63 ± 114:02 μ^2	272:27 ± 122:18 μ^2
		perimeter	58:21± 15:11 μ	62:91 ± 14:01 μ
		ferret diameter	22:59 ± 6:47μ	24:04 ± 5:91 μ

Table shows there was reduction of all neuronal parameters as mean area, perimeter, ferrate diameter and point count in both inhibitory nitrergic and excitatory cholinergic neurone of myenteric plexus rat colon in chronic stress

Electron microscopy

In electron microscopy it was noted that the number of mast cells was increased in experimental animal. The microvillus architecture of epithelium was distorted in stressed colon. The numbers of apoptotic cells in the colon of experimental animal were more than those in the control animals (Fig. 7).

Fig. 7 Electron microscopic picture of an apoptotic cell.



Discussion

There are numerous reports on the functional disturbances of the gastrointestinal system in stress, but the literature is scant on the structural changes in the enteric nervous system of rat colon after chronic stress. An appreciation of these changes is required for

understanding of the disturbances of the GI system. Hence this study was undertaken to evaluate the effects of chronic stress on the morphology of the enteric nervous system in the rat colon. Chronic passive water avoidance stress has already been used to study the stress-induced changes of gastrointestinal function (Enck P et al 1989). We have adopted same model but studied morphometric changes as well of chronic stress on the enteric nervous system.

The enteric nervous system is connected bi-directionally to the brain via autonomic pathways forming the brain-gut axis (Bhatia V and Tandon RK 2005). The myenteric plexus is mainly concerned with motor activity of muscularis externa (Berezina TP and Ovsiannikov VI 2011; Ovsiannikov VI and Berezina TP 2007), and the submucous plexus with various activities of mucosa of the gut wall. It is evident that the different kinds of stress with different amplitudes have profound role in motor activity, secretion, permeability and immunological functions of the gut wall (Caso JR et al. 2008). As these functions are coordinated by the ENS, it is likely that stress directly or indirectly affects the Enteric Nervous System via HPA axis and its neurotransmitters (Monnikes H et al. 1994). Corticotropin releasing factor (CRF) is reported to be the key mediator of the central stress responses (Suto G et al.1994; Takeda E et al. 2004). Two CRF receptor subtypes, R1 and R2, have been described which mediate increased colonic motor activity and delayed gastric emptying, respectively, in response to stress. The weight of the adrenal gland relative to the body weight ($p<0.035$) was increased (Table 2) in the animals subjected to chronic stress. There is an increase in CRH which results in hypertrophy of the adrenal gland. The relative weight of adrenal gland to body weight is a better marker than the blood levels of corticosterone in rats as it tends to return to basal levels in chronic stress (Yvonne M and Ulrich-Lai 2006). During the initial phase of the experiment the animals subjected to passive avoidance stress exhibited anxiety and tried to escape from the situation. After a few days their food intake was reduced. Takeda E et al reported that stress induces loss of appetite due to anorectic effects of CRF and delayed gastric emptying (Takeda E et al. 2004). There was diarrhea in stressed animals due to increased colonic motor activity during the initial phase of the experiment. Delayed gastric emptying and increased colonic motility were reported in both animals and humans under stressful conditions (Da Silva MS et al. 2002; Gue M et al. 1991; Tache Y et al. 1999). Multiple neurotransmitters play active role in the propulsion of the bolus from oral to aboral direction in the intestines. The progressive aborally directed peristaltic movement of the bolus is effected by a net balance between excitatory cholinergic and inhibitory nitrergic neurons. The muscle wall of the intestine contracts behind the bolus under the influence of the excitatory cholinergic neurons and a receptive relaxation of the wall occurs in front of the bolus by diffusing NO (nitric oxide) produced by the nitrergic neurons. The ring of contraction behind the bolus migrates aborally propelling the bolus behind the distal wave of relaxation.

The morphology of both the cholinergic and nitrergic neuronal categories was evaluated following exposure of the animals to chronic stress in our study. In the current study chronic stress resulted in the atrophy of the myenteric neurons of the enteric nervous system as shown by a reduction of neuronal cell size in myenteric plexus (Figs. 2, 3). In these animals there was a reduction of nitrergic neuronal size in the experimental group (Fig. 2). The atrophy of nitrergic neurons was responsible for constipation after a bout of diarrhoea due to the delay in the relaxation of the bowel after contraction. Chronic stress had profound effect on the myenteric cholinergic neurons. The size of the excitatory cholinergic neurons in the myenteric plexus was reduced (decreased area, perimeter and diameter of the neuronal profiles) in stressed animals

(Fig. 3). Magarinos, AM et al (1996), McEwen, B.S. et al (1999) and Czeh, B et al (2001) have shown that stress had profound effects in neuronal morphology and function in several forebrain systems including limbic structures and the prefrontal cortex. Studies in animal models of chronic stress and stress hormones have demonstrated stress induced atrophy in CA3 pyramidal neurons, decrease in adult neurogenesis in the dentate gyrus and reduction of total hippocampal volume. It has been proposed by Jacobson, L. et al (1991) that these morphological changes might interfere with the negative regulation of the stress response that is induced by the hippocampus via the hypothalamic-pituitary-adrenal axis. More recently it has been shown that in rat the amygdala and prefrontal cortex are also morphologically affected by stress (Wellman CL 2001 and Wittert GA et al. 1996). Mechanisms similar to those implied in these reports may well be responsible for the atrophy and apoptosis of the neurons of the ENS as they closely resemble the neurons of the CNS, rather than the neurons of the peripheral nervous system, in their structural characteristics and neurotransmitter.

Conclusion

It was known that chronic stress leads to physiological disturbances like adrenal hyperplasia, anorexia, gastric stasis, increased colonic motility and inflammation, diarrhoea and/or constipation and reduction of body weight consequently. In the present study it is demonstrated that chronic passive avoidance stress causes atrophy and reduction in the numbers of cholinergic as well as nitrergic myenteric neurons in the rat colon. The apoptosis could be responsible for reduced neuronal cell population and atrophy. The mechanisms responsible for these changes are likely to be similar to those operating in the CNS in stress. However, they need to be investigated and confirmed by ultra structural and molecular biological techniques with large number of sample.

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Declaration

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Research Article

Demonstration of Hippocampal Neurons Using Golgi-Cox and Rapid-Golgi Staining Methods – A Report on Their Practical Implications

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Key words : Neuro histology, Golgi Cox, Rapid Golgi, Neuron Staining

Abstract: Golgi staining is one of the unique staining procedures to study the morphology of a complete neuron. Random selectivity of only a few neurons to get stained of all the neurons is one of the intriguing facts of this staining method. This empirical randomness questions the veracity of this procedure. To add to this, there are many recipes available to do this procedure. Hence confusions prevail over which method to follow to get a reliable and reproducible Golgi technique of neuronal staining. Present study took the two most popular methods to analyze as follows: A. Rapid-Golgi method (unfixed tissue-immersion fixation) which uses silver nitrate as the base chemical to form the black stain over the neuronal cells. B. Rapid-Golgi method with perfusion fixed tissue and C. Golgi-Cox method using mercuric chloride as the base chemical to form the black staining over neurons. These methods were tried on one of the complex neuronal region of brain i.e. hippocampus. It is observed that Golgi-Cox method seem to give more reliable and appreciable results when compared to that of Rapid-Golgi method.

Hippocampal region of the brain is considered to be one of the hot-seat of today's neuroscience research if not in the life science research itself. Facts that, comparative simpler organization, limited connections and strongly identifiable individual function make it a widely researched structure for understanding the complex brain in its whole (Andersen *et al*, 2006). Even though many newer and sophisticated techniques were being developed to visualize this part of the brain, a long practiced, simple but yet powerful technique is Golgi staining (Heinz, 2005). Invented more than a century ago, it lost its importance only to regain in the later part of 20th century after the correlation with memory and the dendritic arborizations in hippocampus were found (Ferrer and Gullotta, 1990). Discovered by Camillo Golgi (Golgi, 1873), widely applied and perfected by Ramon Y Cajal (Cajal, 1909), Golgi staining of neurons brought them their combined Nobel Prize in Physiology or Medicine for the year 1906. This staining helps to visualize the neuronal soma and the processes of the neurons to its whole extent and opened avenues towards understanding the dendritic arborization (Cajal et al., 1999).

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The mechanism of this staining procedure falls under silver impregnation technique i.e. by redox reactions (Chan and Lowe, 2002). But the exquisite nature of this procedure lies in the randomness of selection of neurons to get itself stained (Lorente de, 1938). This enables a stained neuron to stand out with all of its process in a clear background. Due to the various factors that could influence this procedure such as pH (Angulo et al., 1994), temperature, duration (Orlowski and Bjarkam, 2009), concentration of the reactants, nature of the tissue, other interferences and contaminants (Spacek, 1992). These variants give great scope for customizing this procedure with application principle, chemicals used, timing and physical factors. Thus many modifications and enhancements were still being tried to complicate and confuse which one to follow (Heinz, 2005). In this study we have tried to compare the **A.** standard Rapid-Golgi method (unfixed tissue-immersion fixation) which uses silver nitrate as the base chemical to form the black stain over the neuronal cells. **B.** Rapid-Golgi method with perfusion fixed tissue and **C.** Golgi-Cox method (Cox, 1891) using mercuric chloride as the base chemical to form the black staining over neurons. Wistar albino rat hippocampus was used for the study.

Materials and Methods

The tissues used in the study were obtained from young male Wistar Albino rats used as control rats for other studies.

Rapid-Golgi method: Under profound sedation rats were decapitated. The skin over the skull was retracted by a midline incision and the brain exposed by cutting the skull open with scissors and forceps. The brain was sliced across the optic chiasma and dorsal to it two slices of 5 mm each was sectioned out with a sharp razor blade.

This method was done as described earlier (Rao and Raju, 2004). The slices were dropped in Rapid-Golgi fixative (Potassium Dichromate-5g, Chloral Hydrate-5g, Gluteraldehyde-8ml, Formaldehyde-6ml, Dimethyl Sulfoxide-10 drops, All mixed in 100ml of Distilled water) kept in an Amber coloured bottle. From second day the tissue was changed to freshly prepared fixative (every time) for the next three days. On the 5th day, the tissue was rinsed with 0.75% aqueous solution of silver nitrate and using a soft brush all deposits over the tissue were wiped.

For the next two days, tissue was kept under 0.75% of silver nitrate solution in a dark place. Tissue was again brushed and then subjected for dehydration with absolute alcohol for 10 minutes and embedded with paraffin wax. Thick sections (>50µm) were taken using rotary microtome (Leica, Germany), sections were collected in a gelatin coated glass slide and cleared with EZ-DeWax ™ (BioGenex,USA) or xylene (optional). Sections were mounted with DPX and cover slipped, and examined using light microscope (Nikon Corporation, Japan).

Perfusion fixed Rapid-Golgi method: The animals were given overdose (euthanasia dose) of anesthesia, after cessation of respiration transcardial fixation was performed using 10% formalin (otherwise known as 4% formaldehyde) (Morest and Morest, 1966). All the above procedure (*vide supra*) for Rapid-Golgi was followed thereafter.

Golgi-Cox method: This method was followed as described by McDonald *et al* (2005) with modifications in developing the stain. Solutions of 5% potassium dichromate, 5%mercuric chloride and 5% of potassium chromate were prepared and mixed in the ratio of 5:5:4 to 10 parts of distilled water to prepare Golgi-Cox solution (GC solution). Under euthanasia dose, the animals were transcardially perfused with 0.9% saline and the brain was removed. It was cut roughly into three equal parts and the middle part containing hippocampus was immersed in a vial containing GC solution. It was kept in complete darkness and the GC solution was changed during every alternate day for a fortnight. Then the tissue was

transferred to a vial containing 30% sucrose and stored at 4°C for a week or so. Then the tissue was sectioned using a vibratome (Leica, Germany) at 200µ thickness and sections were collected in a trough filled with 6% sucrose solution and washed with distilled water. Without exposing much to light, the sections were treated with 22.5% Ammonium Hydroxide solution for 30 minutes and then with 5% Thiosulphate solution for 30 minutes. Then it was rinsed with distilled water for 2 minutes and exposed to light. The sections were collected in glass slides and processed for mounting with DPX and coverslipped for observations.

All the above histological preparations were photographed under 4x, 10x, 40x and 100x. The hippocampal regions were analyzed using following parameters *viz*., background staining of chromating fluid, uniformity of neuronal staining, complete staining of an individual neuron, clarity in staining with dendritic spines and for any artifact.

Observation

In both the Rapid-Golgi methods there were excess chromating fluid staining seen in the hippocampal region. Isolated spots of an orange hue staining were seen in the Rapid-Golgi methods. However, in perfusion fixed Rapid-Golgi method these deposits were less than the standard Rapid-Golgi method. In both these methods, the periphery of the hippocampus that is close to the lateral ventricles showed a thick deposit of the chromating fluid almost masking the visibility of this region. But in the Golgi-Cox method there was a uniform distribution of the background staining and providing a clear visibility throughout the hippocampal region. There were no focal islands of chromating fluid depositions nor specific contrast in the peripheries (Fig. 1).

In Rapid-Golgi methods, focal black deposits were seen but no such over staining were seen in the Golgi-cox method. In the Rapid-Golgi method the soma of the neurons showed intense dark staining (densely aggregated around it). Under high power magnification (100x), the Rapid-Golgi methods showed poor resolution of the dendritic spines but these were clearly appreciable in Golgi-Cox method.

Discussion

In this study we have tried to find the reliable and seamless Golgi neuron staining method by comparing two different methods i.e. Rapid-Golgi and Golgi-cox. It was obvious from our observations that mercuric chloride based Golgi-Cox method was superior than silver nitrate based Rapid-Golgi method. Golgi neuron staining is cherished as one of benchmark methods in staining the neurons and their connections and forms an important tool when comes to correlating with morphological and behavioral neuroscience; however, there are some glitches yet to be resolved. In spite of using these methods for 100 years the exact mechanism of the staining is still not deciphered (Pasternak and Woolsey, 1975). This is considered as an empirical method by some.

A major advantage, as well as the downside in this method are that the randomness of the selectivity for staining particular set of neurons in a given sample, through an unknown mechanism and a factor that cannot be controlled by the investigator; however, completely stains the selected neuron which gives a complete idea on its topography (Angulo *et al,* 1996). This property to expose the whole neuron is the highlight of this staining method.

There is general consensus that this property or mechanism of staining in the tissue happens from within the neuron and not from outside the neuron (Chan and Lowe, 2002). Overall the neuron which takes the stain is completely stained without any degree of discrepancy or breach, whereas the other neurons which do not take the stain is absolutely unstained and gives a clear background without any interference in viewing the stained neurons.

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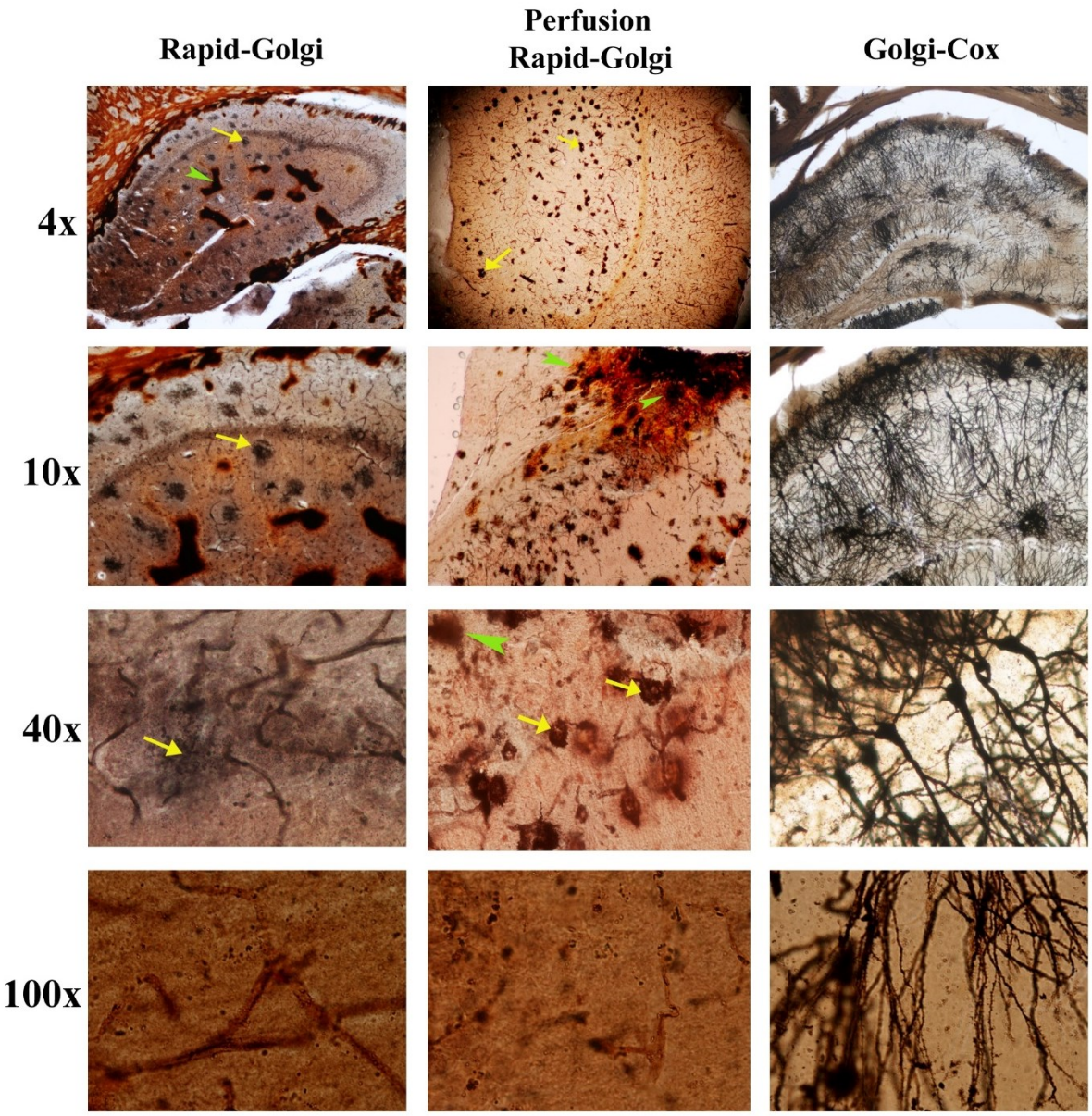
Fig. 1 Photomicrographs showing of different types of Golgi staining of Hippocampal neurons in various magnifications.



Green arrow heads shows the excessive remains of chromating fluid giving an uneven background and the yellow arrows shows patchy silver aggregations

The available explanation for the principle of Rapid-Golgi staining is that when the tissue is incubated in an aldehyde solution with chromium salts, initially and then exposed to silver nitrate. By a redox reaction, the metallic silver is impregnated in the neuron and gives a black colour (Chan and Lowe, 2002). Whereas in the case of Golgi-cox method the mercuric chloride incubation with chromium salts develops mercurous chloride, which is also known as calomel. On exposure to ammonia, the white coloured calomel changes into black coloured metallic mercury in the neuron (Rosoklija et al., 2014).

The following are the commonly encountered problems in Rapid-Golgi staining

1. Rarely the tissue shows no uptake of color in any of the neuron - reason not known.
2. Relatively dark staining in first few sections and in the peripheries of all the sections.
3. Wherever there is a discontinuity (e.g. para-ventricular regions of the brain) in the tissue, the homogenous distribution of the stain is lost.
4. Mostly there will be patchy aggregation of the black color around the neuronal soma.
5. Even with consistent standardized procedure or time schedule reproducibility of the staining intensity is not guaranteed, which makes it difficult to compare across the experimental groups.
6. The complexity lies in the double incubation of chromating solution followed by silver nitrate solution. With larger tissue slabs which could give room for inconsistent infiltration, resulting in uneven chromation coloring of the tissue. This may be due to factors like texture, make-up and natural partitions of the tissue etc.

Thus the lack of reproducibility in Golgi staining put a question on its reliability (Rosoklija et al., 2003).

While comparing standard Rapid-Golgi method and Perfusion fixed Rapid-Golgi method, the latter one shows relatively better results with an even background, as well as in discrete staining of the neurons and its arborizations. This may be because of the fact that pre infiltrated aldehyde in the fixative increases the penetration of the chromating fluid which also has the aldehydes gluteraldehyde, formaldehyde and chloral hydrate (Angulo *et al,* 1996). As this accomplishes the initial phase of the process successfully, the chances of hindering the outcome is considerably reduced and so the results are better compared to standard Rapid-Golgi method.

In case of Golgi-Cox method, the 'calomel' filled tissues are sectioned by a vibratome, after the first incubation. This increases the penetration of ammonia to the maximum, thus converting all the calomel filled in the neurons to black metallic mercury (Stean, 1974). Subsequent chemical washes stops further reaction and washes the residues leaving behind the metallic mercury in the neuron alone. Thus it gives a uniform staining all around the section and allows us to compare different sections across the experimental groups.

While comparing the Rapid-Golgi and Golgi-Cox methods on the grounds of practical difficulties and applicability, it is obvious that Rapid-Golgi can be done with a simple rotary microtome itself commonly found in any histology lab, whereas Golgi-Cox needs a sophisticated microtome i.e. vibratome for sectioning. However, the chemicals used in Golgi-Cox are readily available when compared to Rapid-Golgi method. The use of chloral hydrate in Rapid-Golgi pose a problem, as it falls under the category of narcotic. Apart from this considerable amount of skill is required in handling the thin sections obtained from vibratome in Golgi-cox method.

Conclusion

In conclusion analyzing the pros and cons of these staining gives insight into practical understanding associated in its usage. One should give due consideration for all the factors to get a successful histological picture using Golgi staining.

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Research Article

Delayed Transplantation of Human Amniotic Epithelial Cells in Transient Focal Cerebral Ischemia Induced Rat Brain Can Survive and Ameliorate Functional Recovery

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Key words : human amniotic epithelial cells, cerebral ischemia, transplantation, motor function

Abstract: Cerebral ischemia sets off a series of interrelated events resulting in neuronal injury and functional deficits. Human amniotic epithelial cells found to be immunologically naïve and proved to be an effective substitute to adult, embryonic and fetal stem cells for neurological diseases, when transplanted in different animal species. In this study cerebral ischemic reperfusion injury was created in the wistar albino rats and after 7 day of reperfusion HAE cells was transplanted in the ischemic area and the HAE cell survival and motor behavioral outcome was assessed through neurobehavioral tests. HAE cell was found to survive and migrate in the ischemic brain till 30 days after transplantation and also they showed improvement in rotorod test performance and reduction in the foot fault error in the narrow runway task. The outcome of this study confirms that HAE cell is a potent substitute for neural cells and can survive in the adverse environment of ischemic brain ameliorating functional recovery.

Brain tissues are more susceptible to any small alteration in their normal environment and produce drastic changes in the general body systems. Amongst the neuronal diseases or disorders, stroke or the cerebrovascular disorder stands the world's third leading dreadful disease and a leading cause for long-term morbidity among the world population (Lo *et al.,* 2003). Increase tobacco use, diet leading to overweight and obesity, high blood pressure (BP), high blood cholesterol and less physical activity are the major risk factor for stroke. Stroke is 5 to 10 percent higher in the countries such as Russia, India, China, Pakistan and Brazil when compared to UK and USA (Gorelic, 2009).

During cerebral ischemia as the supply of oxygen and glucose to the brain tissue is lost the production of high energy phosphate compounds such as adenosine triphosphate (ATP) falls, leading to failure of energy dependent processes necessary for neural cell survival. This sets off a series of interrelated events that result in neuronal injury. These include the failure of mitochondrial function, electrolyte imbalances in brain cells, increase in the intracellular calcium level, release of

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excitatory neurotransmitters, production of oxygen free radicals and other reactive oxygen species.

In the fully matured brain, the striking disadvantage is inability of neurons to regenerate, so when there is a permanent loss of neuron, it is not substituted by a new generation of similar cells. In this instance the transplantation of embryonic stem cells had made a tremendous benefit in substituting the neuronal loss and regaining the function loss. But they have their own limitation on ethical issues and species specificity. The transplantation of adult, embryonic and fetal stem cells has shown promising development in improving the behavioral deficits in experimental models of neurological diseases. Although ethical and political issues have limited the use of fetal stem cells; cell resources and rejection have restrained the use of adult stem cells.

The human placental derived amniotic epithelial cells isolated from the placenta after delivery has been proved as a potential graft source which express markers for both neural and glial cells and when transplanted show no immune rejection (Sakuragawa, *et al.,* 1996,1997). Further studies on Human amniotic epithelial (HAE) cells have shown that they has the potentiality to synthesize and secrete various neurotransmitters such as epinephrine, nor-epinephrine, dopamine (Elwan, 1998) acetyl-choline (Elwan and Sakuragawa., 1997) and erythropoietin (Terada *et al.,* 2000). HAE cells was found to lack histocompatitbilty complex antigen (Terada et al.,2000), secrete neurotrophic factors such as brain-derived neurotrophic factor, neurotrophin-3, and nerve growth factor (Uchida *et al*., 2000). They do not express telomerase and found to be non-tumerogenic upon transplantation (Miki *et al.,* 2005). Transplantation of HAE cells into rat midbrain prevented the degeneration of nigrostriatal dopamine neurons after injection of 6-hydroxydopamine (Kakishita *et al.*, 2003). When HAE cells transplanted in to the monkey spinal cord after injury, it reduced the formation of glial scar and attracted the growth of new collateral sprouting and improved the hind limb motor function (Sankar and Muthusamy. 2003). Moreover the HAE cells are useful and noncontroversial source of stem cells for all transplantation and regenerative medicine (Miki.T *et al.,* 2005) and do not invoke any religious, ethical or legal issues like human fetal cortical tissue.

Considering the above facts, This study was designed to evaluate the efficacy of HAE cells by delayed transplantation in the ischemic brain of wistar albino rat after transient focal cerebral ischemia induced by middle cerebral artery occlusion.

Materials and Methods

Animals and Experimental design.

Healthy adult male wistar albino rats weighing about 250 to 300 grams of body weight were used for this study. Rats were housed in polypropylene cages with paddy husk, maintained under standard atmospheric condition of 12 hour light and dark cycle at a constant temperature of $25^0\pm 3^0$C and 30% to 60 % humidity. Rats were fed with standard rat pellet diet and water ad libitum. This entire study was approved by the Institutional Animal Ethical committee. All efforts were made to minimize both the number of rats used for this study and unwanted stress or discomfort to the rats during experimental procedures.

Rats were grouped as (i) Sham (Rats underwent the surgical procedure except Middle cerebral artery occlusion(MCAo)), (ii) Lesion (Rats exposed to 2 hours of MCA occlusion and reperfusion) and (iii) HAE Transplantation group (Transplantation of HAE cells on day 7 following 2hrs of MCAo and reperfusion).All the rats were assessed

for motor behavioral function and were sacrificed for histological assessment on day7 and 30 after transplantation.

Induction of transient focal cerebral ischemia.

To create transient focal cerebral ischemia in wistar albino rats, the surgical procedure suggested by Longa, *et al* (1989), by intraluminal filament occlusion of Middle Cerebral Artery was adopted. Rats were deprived of food but not water for 8 hours prior to surgery and anesthetized with intraperitoneal administration of Thiopental sodium (40mg/kg body weight) with a pre anesthetic intramuscular administration of Atropine (0.5 mg /kg body weight,). The ventral aspect of the neck was shaved thoroughly and cleaned with antiseptic solution. Then a ventral midline incision was made in the neck and the underlying connective tissue layers were incised and separated in layers. The right common carotid arteries (CCA), internal carotid artery (ICA), external carotid artery (ECA) were isolated from the surrounding nerves plexus and connective tissues. The branches of ECA were isolated and cauterized using micro diathermy bipolar electrodes (Martin - Germany). Distal segment of the ECA was ligated using 4 0 silk suture and a loose not was made around the proximal segment of ECA. The CCA and ICA were temporarily occluded using micro vascular clips (FST ,USA) and a small nick was made in the ECA and the pre prepared 4 0 nylon monofilament (Belayev, *et al.,* 1996) was introduced in to the ECA and the lose knot near the proximal segment was tightened. The distal segment of ECA was detached from the proximal segment and the micro vascular clips from CCA and ICA were removed. Then the Monofilament was redirected in to the ICA till a resistance is felt (Approximately 18mm - 19mm length). The surgical area was closed and sutured in layers, retaining the monofilament in position. After 2hrs of occlusion the monofilament was withdrawn and reperfusion was established.

Human Amniotic Epithelial (HAE) cells isolation and transplantation

HAE cells isolation was done as described by Sakuragawa *et al.,* (1996). The connective tissue from the amniotic membrane was scrubbed and removed. The membrane was than cleaned with phosphate buffered saline (PBS) thoroughly and trypsinised in 0.125% trypsin (Hi-media) in PBS for 3 changes of 20 minutes each. The pellets so obtained after each treatment were re-suspended in PBS and pooled together and washed in fresh PBS for 3 times. The HAE cells so obtained were suspended in Dulbaco's Modified Eagle Medium (DMEM) with HEPES (Hydroxy ethyl piperazine sulphonic acid) buffer (Himedia, India) and supplemented with 10% fetal bovine serum. The HAE cells were then maintained in a CO_2 incubator in a humidified atmosphere of 5% CO_2 in air at 37º C. The culture was maintained till the host animal was ready for transplantation. HAE cells viability was assessed before transplantation through Trypan blue exclusion method and cells was used for transplantation only when the viability was more than 85%.

After 7 days of reperfusion the group iii rats were anesthetized with Ketamine, Xylazine at a dose of 100 mg/kg body weight and 10mg/kg body weight respectively. HAE cells was injected in to the ischemic area of rat brain through small burr holes with the aid of stereotaxic apparatus (Inco, India) based on the following coordinates (i) Antero-posterior (AP) = 1.08 , Right lateral (RL) = 3 mm, and Dorsoventral (DV) = 6 mm from the dural surface; (ii) AP = -1.08 mm, RL = 3 mm, and DV = 4mm from the dural surface. Using a 10µl Hamilton syringe fitted to the manupulator of the stereotaxic apparatus 2µl of cell suspension (1 X 10^4 cells/µl) was slowly injected into each site of the brain as

per the above mentioned coordinates. After transplantion, the needle was left in place for 10 minutes and then withdrawn slowly. The surgical incision was closed in layers. The rats were left undisturbed for two hours and then they were taken for post-operative management.

Neurobehavioral assessments.

All the rats were assessed for their motor behavior through the following tests by experimentally blind investigators.

Rotarod Test (Chen, et al., 2001)

In this test the rats were placed on the rotarod cylinder and the time the animal remained in the rotarod was measured at the constant speed of 20 rpm for maximum of 5 minutes. The trial will be ended if the animal falls off the cylinder before 5 minutes or spuns around the beam for more than 2 consecutive rotations without attempting to walk. The rats trained priorly for this test alone were included in the experimental groups. After lesion as per the scheduled groups and period of study, three trials were conducted and the mean value was recorded. A score of 0,1,2,3,4,5,6 was assigned to the performance of the rats as tabulated below.

Animal status	**Score**
Balances with steady posture	0
Balances the beam ,but grasps the side of the beam	1
Hugs beam and 1 limb falls off the beam	2
Hugs beam and 2 limb falls off the beam or spins on beam (>60s)	3
Attempts to balance the beam ,but falls off(>40s)	4
Attempts to balance the beam ,but falls off(>20s)	5
Attempts to balance the beam ,but falls off(<20s)	6

Narrow Runway Test (Kunkel, et al., 1993)

The narrow runway is a wooden beam of 2.5 cm wide, 100 cm length and 10 cm height. Rats were made to run on the beam and the duration to cross the beam, number of steps and forelimb errors were measured during the evaluation. An error was counted when the animal foot misses the beam and slips over the side. Before lesion rats were trained for 7days and the mean scores were recorded for three trials. After lesion as per scheduled groups and study period rats were consecutively assessed for three trials and the mean value was recorded.

Histological and immunohistochemical analysis.

The rats were euthanized using thiopental sodium at a dose of 80mg/Kg body weight intrperitoneally on scheduled study periods, rats were transcardially perfused with 0.1 M Phosphate buffered saline (PBS) and then with 4% paraformaldehyde in PBS and the rats were decapitated and the brain was removed and stored in 4% paraformaldhyde in PBS at 4^{o}C. Histological staining was performed through paraffin processing as described previously. Briefly, sections were cut in the coronal plane at 10 micron thickness and mounted on slides. Sections were deparaffinated and rehydrated. Sections were stained with hematoxylin–eosin (H & E) for morphological analysis.

To confirm the survival of HAE cells in the group iii rats the brain block selected from the lesion site was processed for immunohistochemical staining. The brain tissue was impregnated in 25% sucrose in PBS till the section sinks completely in to the solution. Then the brain tissue was embedded with Bright Cryo-M-Bed (Bright instrument company Ltd, England) and 20 µm thick section were taken in a cryostat (Leica CM 1510S, Germany) for immunohistochemical staining. The tissue sections obtained were stained for Human

cell marker with Mouse anti Human nuclei monoclonal antibody(Anti HuNu, Chemicon International Inc) and Rabbit anti mouse IgG conjugated with TRITC was used as the secondary antibody. The brain section was observed under Nikon labophot episcopic fluorescence attached microscope. For TRITC Nikon G2A; Excitation filter 510 ~ 560; Barrier filter 590.

Statistical Analysis

All the data were expressed as Mean ± SEM and was analyzed by two way analysis of variance (ANOVA) followed by Bonferroni test and p values ≤ 0.05 were considered as statistically significant.

Observation

Human Amniotic Epithelial (HAE) cell transplantation and animal behavior:

In the rotarod test (FIG. 1) the score of HAE cell transplanted rats on day1 after transplantation was not significant when compared to lesion rats. But performance of the HAE cell transplanted rats on day 7 and 30 there was a significant improvement in the rotarod performance score when compared with Lesion.

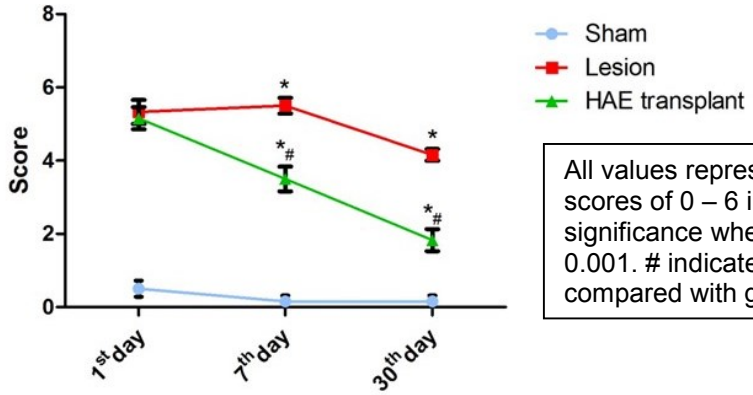
In the narrow runway test (FIG.2), the HAE cells transplanted rats showed a marginal reduction in the foot fault errors on all the study periods when compared to the lesion rats; but the values were not statistically significant.

Fate of Human Amniotic epithelial cells transplanted in the ischemic cerebral hemisphere:

The HAE cells transplanted on day 7 after MCAo in the ischemic cerebral hemisphere of wistar albino rats was found to survive, migrate and integrate with the host cells. On day 7 after transplantation HAE cells were observed in cluster at the site of injection in the ischemic rat brain. In the H&E stained section of the transplanted Ischemic cerebral hemisphere, HAE cells appear in clusters with shrunken and basophilic cytoplasm (FIG 3A). A few cell groups were found to be surrounded by the macrophages already existing in the ischemic area. Further the positivity of Anti Human nuclei (HNu) antibody (FIG.3C) confirms the survival of HAE cells at the transplanted site on day 7 after transplantation.

On day 30 after transplantation, the overall transplanted HAE cell population was found to be reduced (FIG.3B). The HAE cell morphology was altered from round to oval shape. The cells were found to be enlarged and migrated towards the ischemic area (FIG.3B). A few cells showed a synaptic network with the existing viable neurons in the ischemic hemisphere. The Anti HNu antibody positive cells were found to be dispersed in the ischemic zone confirms the survival and migration of HAE cells on day 30 after transplantation (FIG.3D).

Fig. 1 Rota-rod test performance of experimental groups



All values represent Mean ± SEM of graded scores of 0 – 6 in rota rod test. * indicates its significance when compared with group I $p \leq 0.001$. # indicates its significance when compared with group II, $p \leq 0.001$.

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Fig 2 Narrow run-way test performance of experimental groups

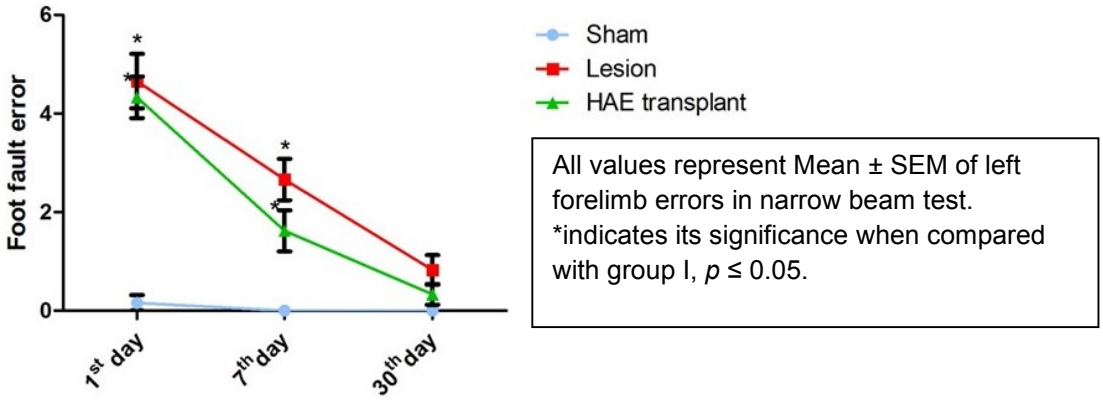
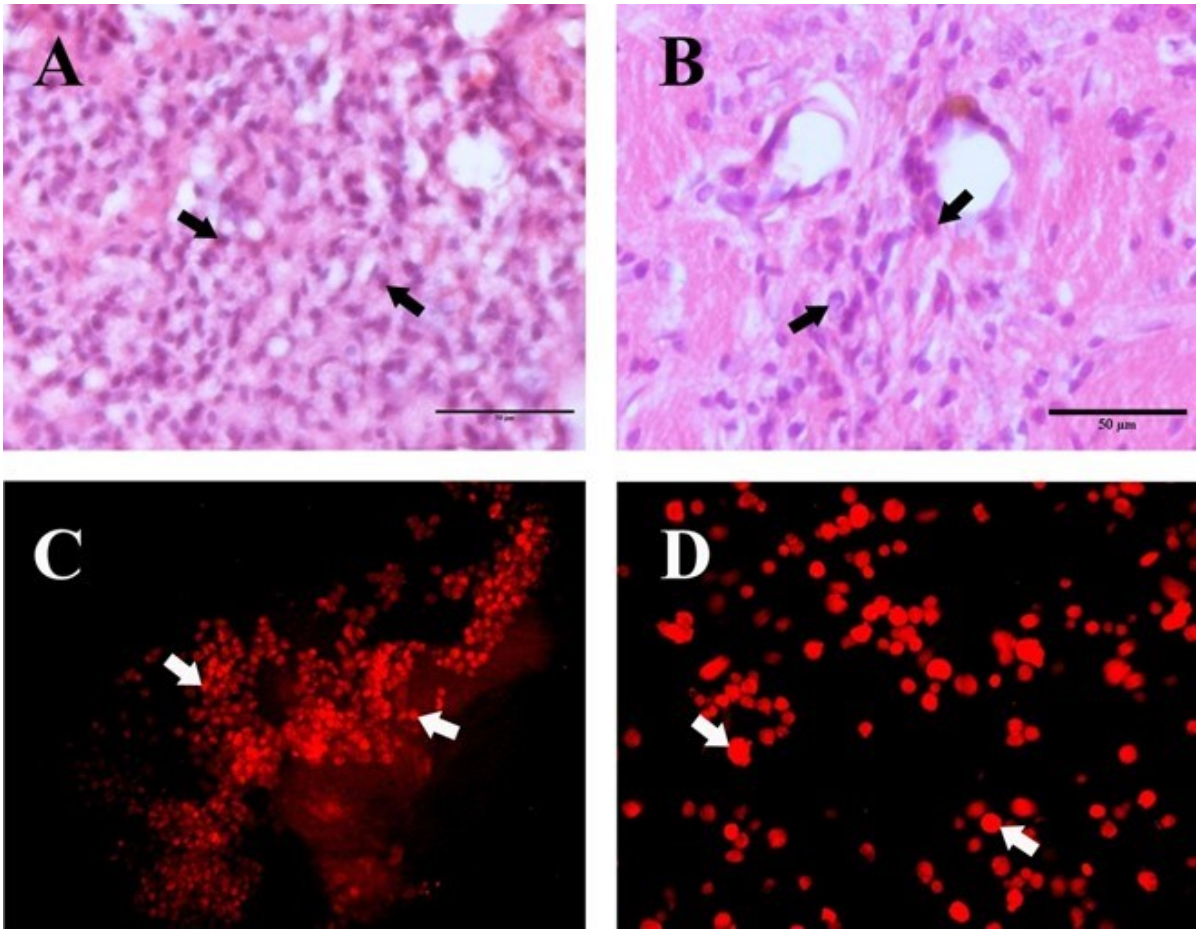


Fig 3. HAE cells transplanted in the Ischemic cerebrum of wistar albino rats



(A) H& E stained section shows HAE cells in ischemic cerebrum on day 7 after transplantation, arrow represents the HAE cells in clusters. (B) H& E stained section represents HAE cells on day 30 in the Transplanted site, Arrow indicates the dispersed HAE cells from the transplanted site . (C & D) Immunohistochemically stained with Mouse Anti Human Nuclei monoclonal primary antibody and Anti Mouse IgG conjugated with TRICT secondary antibody to confirm the survival of HAE cells in the transplanted site on day 7 and 30 respectively.

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Discussion

There is no effective means for replacing brain cells and restoring its function after permanent loss due to neural disorders. Fetal tissue transplantation made a remarkable development in substituting damaged brain tissues and improving the functional deficits. Various experimental studies have been conducted to evaluate the status of embryonic and foetal neural tissue transplantation in the ischemia induced neuronal damage has shown promising development in improving the behavioral deficits in experimental models of neurological diseases (Hadani *et al*., 1992; Koide *et al*., 1993; Athara *et al*., 1994). But ethical and political issues have limited the use of fetal stem cells, cell resources and rejection have restrained the use of adult stem cells. Human amniotic epithelial (HAE) cells , derived from the placenta after parturition was considered to be the alternative source for fetal and embryonic stem cells in substituting the neural loss due to various neurological disorders. Human amniotic epithelial cells are immunologically naïve (Adinolfi *et al.,* 1982). Invitro and invivo studies on HAE cells has shown remarkable outcome by expressing neural and glial markers and also found to express certain neurotrophic factors and neurotransimitters, when transplanted in various neurological disease animal models (Sakuragawa. *et al.,*1996; Terada *et al*., 2000; Kakishita *et al.*, 2003)

In this study, rats that were intracerebrally injected with HAE cell on 7th day after MCAo, showed improved motor performance assessed through rotarod stability test and narrow runway for foot fault error test till 30 days after transplantation. A similar study on HAE cell transplantation in MCAo rats and assessment for 2 weeks was shown to enhance motor function through different neurobehavioral tests (Liu et al., 2008)

In the present study on day 1, the HAE cell transplanted animals did not show any difference in performance of the behavior task, this may be due to non-integration or delayed adaptation of HAE cells with the host brain. This study also document the role of HAE cells in improving the motor function of rats after focal cerebral ischemia through morphological modification and integration with the surviving host neuronal cell population. We were able to detect some HAE cells dispersed from the site of injection to the peripheral infarct zone on day 7 and 30 after transplantation. This demonstrates that intracerebral delivery of HAE cells promotes survival and potential migration into the rat brain. In earlier studies the presence of Human umbilical cord blood cell in the ischemic rat brain was located by the mouse anti human nuclei antibody (Chen, *et al.*, 2001). Similarly in this study the mouse anti human nuclei antibody, a human cell marker made a significant contribution to locate the dispersed HAE cells in the ischemic brain.

Furthermore, HAE cells increased the ability to improve the behavioral recovery of ischemic rats and reduced the infarct size rapidly and stably. These results support the assertion that HAE cells may be a cell resource for cell therapy and useful vehicles for gene therapy in neurodegenerative disease (Liu.,et al 2008).

After ischemia a vast population of neural glial cells undergoes permanent damage and further evokes inflammatory reactions and apoptotic cell death, worsening the functional loss and increasing the infarct area progressively. The known neurotrophic factors BDNF, NT- 3 expressed by HAE cells (Uchida, *et al.*, 2000) and other growth factors secreted by HAE cells may partially explain their therapeutic mechanism, but the mechanism of functional recovery after transplantation of HAE cells is still questioned and need to be answered through

further investigations. This study has given a primordial document to show the survival of HAE cells till 30 days after transplantation in the ischemic brain after delayed transplantation.

Conclusion

In conclusion the HAE cells as a substitute to embryonic stem cells can be an effective stem cell for neurodegenerative disorders, but require further validation on its long term benefits and status in animal models for successful application in clinical trials.

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Research Article

Effect of Ethanol Exposure on Heart Development in Zebra Fish *(Danio rerio)* Embryos

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Key words : Ethanol, zebrafish, Heart, Development, Fetal Alcohol Syndrome

Abstract: The objective of the present study is to evaluate the effect of ethanol on developing zebra fish heart. Male and female fishes are allowed to breed normally and the fertilized eggs were collected and it was exposed to 3 % of ethanol (EtOH) for one hour once in 24 hours for 96 hours. The embryos were subjected to various analyses related to heart development like external morphology, heart morphology, heart rate, heart looping formation, heart length and ventricular stand still. The data showed significant alteration in the length of whole embryo or larva, heart length and heart rate of EtOH exposed embryo when compared to control. The looping also altered like string-like or straight tube appearance, with the ventricle located distinctly anterior to the atrium. Further, the ventricle appeared smaller than normal, the atrium is elongated, and both chambers having a narrow width. The incidence of ventricular standstill and valvular regurgitation is occurring in EtOH treated embryo when compared to control. Taken together, the steady decrease in heart size coupled with the severe defects in ventricular function, failure of cardiac looping formation, heart rate, ventricular standstill and retrograde blood flow would be expected to have a substantial impact on the ability of zebra fish to circulate blood. From the present study it is concluded that EtOH exposure during development results in structural and functional impairment in heart that mimic malformations that occur in patients with fetal alcohol syndrome.

Prenatal alcohol exposure has been associated with inauspicious effects like developmental delays and infant mortality, leading known cause of mental retardation in human fetuses (Abel and Sokol 1991). Gestational alcohol exposure has been shown to contribute symptoms of fetal alcohol syndrome (FAS) (Jones et al., 1973). The FAS produce lots of alteration like growth retardation, facial abnormalities, sensory deficits, impaired fine motor skills, and learning deficits, including mental retardation (Stratton et al., 1996). Previously it was believed that FAS was the result of alcohol abuse, but it is now believed that smaller doses of alcohol also can have alcohol-related birth defects (ARBD) or alcohol-related neurodevelopmental disorder

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(ARND) (Stratton et al., 1996). Recently, the zebra fish (*Danio rerio*) has become an important animal model in developmental biology and neuroscience (Bilotta et al., 2001). Also, zebra fish have transparent eggs and the embryos are semi-transparent. Thus, the stage of embryonic development of an individual embryo can be determined without interfering with development. Several studies have shown that EtOH exposure can affect zebra fish embryonic development. Bilotta et al., (2004) had reported that the EtOH exposure produces cyclopia (fusion of the two eyes). Then also reported that EtOH exposure impair the development of nervous system and behavioral deficit in zebra fish embryo (Cole et al., 2012). Carvan et al., (2004) also reported that EtOH exposure alters the neurobehavioral and skeletal morphogenesis. Another study also documented that it produces severe body malformations (Baumann and Sander, 1984). Daft et al., (1986) had reported that gestational acute EtOH exposure produced abnormal heart and great vessel development in mice. Fetal alcohol spectrum disorder (FASD) describes a range of birth defects, including various congenital heart defects (CHDs). However the exact mechanisms of FASD-associated CHDs are not fully understood. The objective of the present study to analyze the effect of EtOH on developing heart using zebrafish embryos as *in-vivo* experimental model

Materials and Methods

Zebrafish husbandry and embryo collection

Adult zebrafish (Daniorerio) were purchased from a local authenticated supplier and housed. Zebra fish were reared in 2.0 l polycarbonate tanks on a recirculating system in which the water was maintained at 28±1 °C and at pH of 7.0±0.2. The fish were fed twice daily. Eggs were obtained by random pairwise mating of zebra fish. Three adult males and four females were placed together in small breeding tanks the evening before eggs were required. The breeding tanks (L 26 cm; H 12.5 cm; W 20 cm) had mesh egg traps to prevent the eggs from being eaten.

The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (50 eggs per dish) containing 40 ml fresh embryo buffer. Eggs were washed four times to remove debris, unfertilized, unhealthy and dead embryos under a dissecting microscope. At 3.5 hours, post fertilization (hpf), embryos were again screened and any further dead and unhealthy embryos were removed. Throughout all procedures, the embryos and the solutions were kept at 28.5°C, in the incubator under a light cycle of 14 h light: 10 h dark (lights on at 8 h). Embryo buffer was refreshed every 24 h. Normal dividing and spherical embryos at the 256 cell stage (2.5 hpf) through the oblong stage (3.7 hpf) were selected and utilized for all of the studies described. Embryos were staged using the pectoral fin, yolk sac, anal pore, and swim bladder as indicators of developmental stage.

Ethanol exposure

The washed forty embryos in triplicates at 3.7 hpf stage were used for the present study. The embryo was exposed to 3 % of EtOH for 1 hour at 28.5°C. After the incubation the embryos were washed 3 – 4 time with a fresh embryo buffer. Embryos were kept in an incubator at 28.5°C, with a refreshment of the buffer once in a day for 96hpf. Every 24 hpf the embryo were observed and analyzed for morphological ad, developmental changes of heart.

Morphological analysis

The EtOH exposed embryos were subjected to morphological analysis like viability of the embryo, length of the embryo, status of pericardial sac and heart shape. The embryo was placed in a lateral position and was analyzed qualitatively and quantitatively.

Heart rate

Zebrafish embryos were positioned in 3% methylcellulose and kept on the lateral side to get a better view of functioning heart (Duan et al., 2013). Ventricles beats are counted in 20s periods. At least thirty measurements were taken and their average was used in statistical analysis. In these experiments the heat rates for EtOH exposed embryo were measured in the same observation session under identical conditions like control.

Estimation of SV-BA distance

Changes in heart morphology caused by EtOH exposure were measured (Mehta et al., 2008) by positioning embryos in 3% methyl cellulose to allow capturing of lateral view images and measurement of the distance between the sinus venosus (SV) and bulbus arteriosus (BA) regions of the heart. The image was captured and was subjected to distant measurement using stage and occulo meter scale.

Incidence of ventricular standstill.

Zebra fish larvae were assessed for the incidence of ventricular standstill at 96 hpf as described previously (Antkiewicz et al., 2005). The embryo or larvae were observed for larvae persistent lack of visible ventricular contraction and scored as exhibiting the ventricular standstill. At least three separate experiments were performed (n =40 larvae per each EtOH exposed embryo per experiment), and the average percentage of larvae exhibiting ventricular standstill for each group was calculated.

Heart looping formation

To assess the angle of looping between the atrium and ventricle, the developing heart will be studied through recordings of the beating heart and image will be analyzed with manual method by analyzing the orientation of each chamber.

Peripheral blood flow

Video images of blood flow in the posterior inter segmental vein in the trunk of embryos will be captured and the number of red blood cells passing a chosen landmark in the posterior inter-segmental vein during 10 s will be counted.

Statistical analysis

The significant difference between the mean value of control and experimental groups were analyzed according to the method of Zaire (1974). All the data were subjected to Student‘t’ test and data showing p value < 0.05 was considered as statistically significant.

Observation

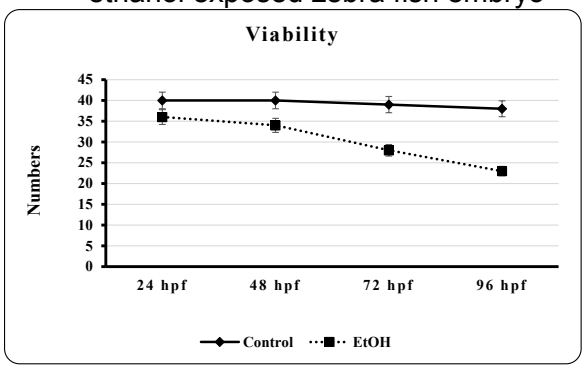
Morphological analysis

The effect of EtOH on morphology of the developing zebra fish embryo was studied. The viability and total length of the embryo was found to be significantly ($p<0.001$ and $p< 0.001$) reduced in EtOH exposed embryo than the control embryo (fig. 1 & 2). Additionally, edematus pericardial sac was observed in EtOH exposed embryo than the control (Plate. 1 & 2). Ethanol exposure significantly ($p< 0.001$) prolonged the hatching and also lead to embryo malformation when compared to control (fig. 3 & Plate 1).

Effects of EtOH on Heart Morphology

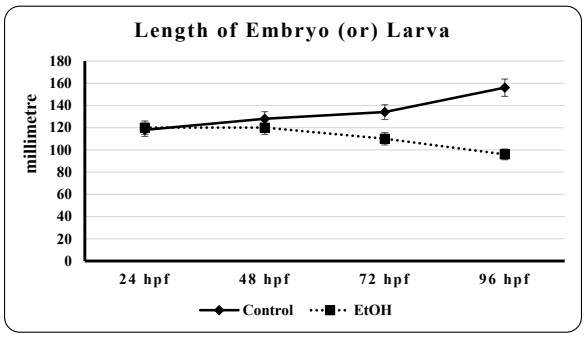
Zebrafish embryos exposed to 3 % EtOH showed pericardial edema and a significant decrease in blood flow by 72 hpf. This change became progressively more pronounced and was quite obvious by 96 hpf. Instead of the looped and S-shaped hearts that was seen in the control fish, hearts from EtOH exposed embryos were found to be elongated and string-like straight tubes (Plate. 2). In control embryos, the normal looping process places the ventricle and atrium side by side, so that the two chambers largely overlap each other in lateral view. In contrast, the EtOH hearts were significantly lengthened (fig. 4).

Fig 1. Showed the viability of the control and ethanol exposed zebra fish embryo



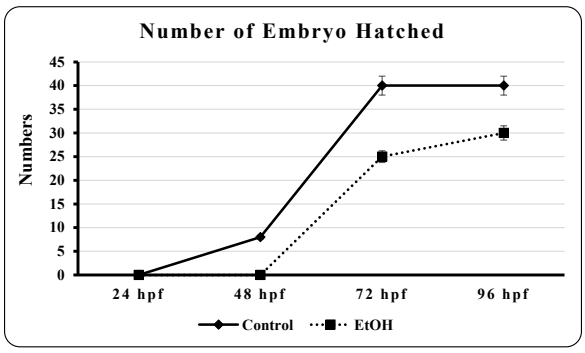
Each point is indicates the mean ±SEM (n=40), EtOH – ethanol.

Fig 2. Showed the length of embryo or larvae of the control and ethanol exposed zebra fish.



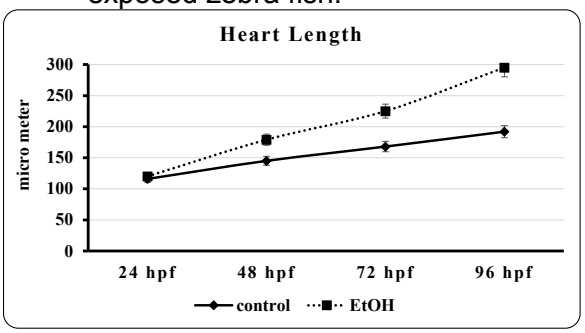
Each point is indicates the mean ±SEM (n=40), EtOH – ethanol.

Fig 3. Showed the number of embryo hatched in control and ethanol exposed zebra fish.



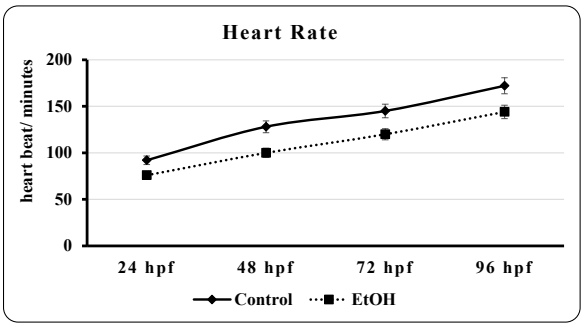
Each point is indicates the mean ±SEM (n=40), EtOH – ethanol.

Fig 4. Showed the length of heart in embryo or larvae of the control and ethanol exposed zebra fish.



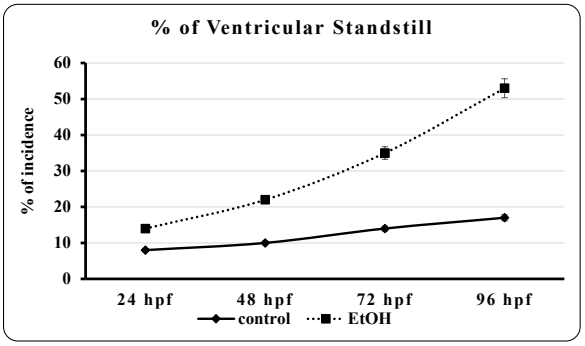
Each point is indicates the mean ±SEM (n=40), EtOH – ethanol.

Fig 5. Showed the heart rate of embryo or larvae of the control and ethanol exposed zebra fish



Each point is indicates the mean ±SEM (n=40), EtOH – ethanol.

Fig 6. Showed the percentage of incidence of ventricular standstill in embryo or larvae of the control and ethanol exposed zebra fish.



Each point is indicates the mean ±SEM (n=40), EtOH – ethanol.

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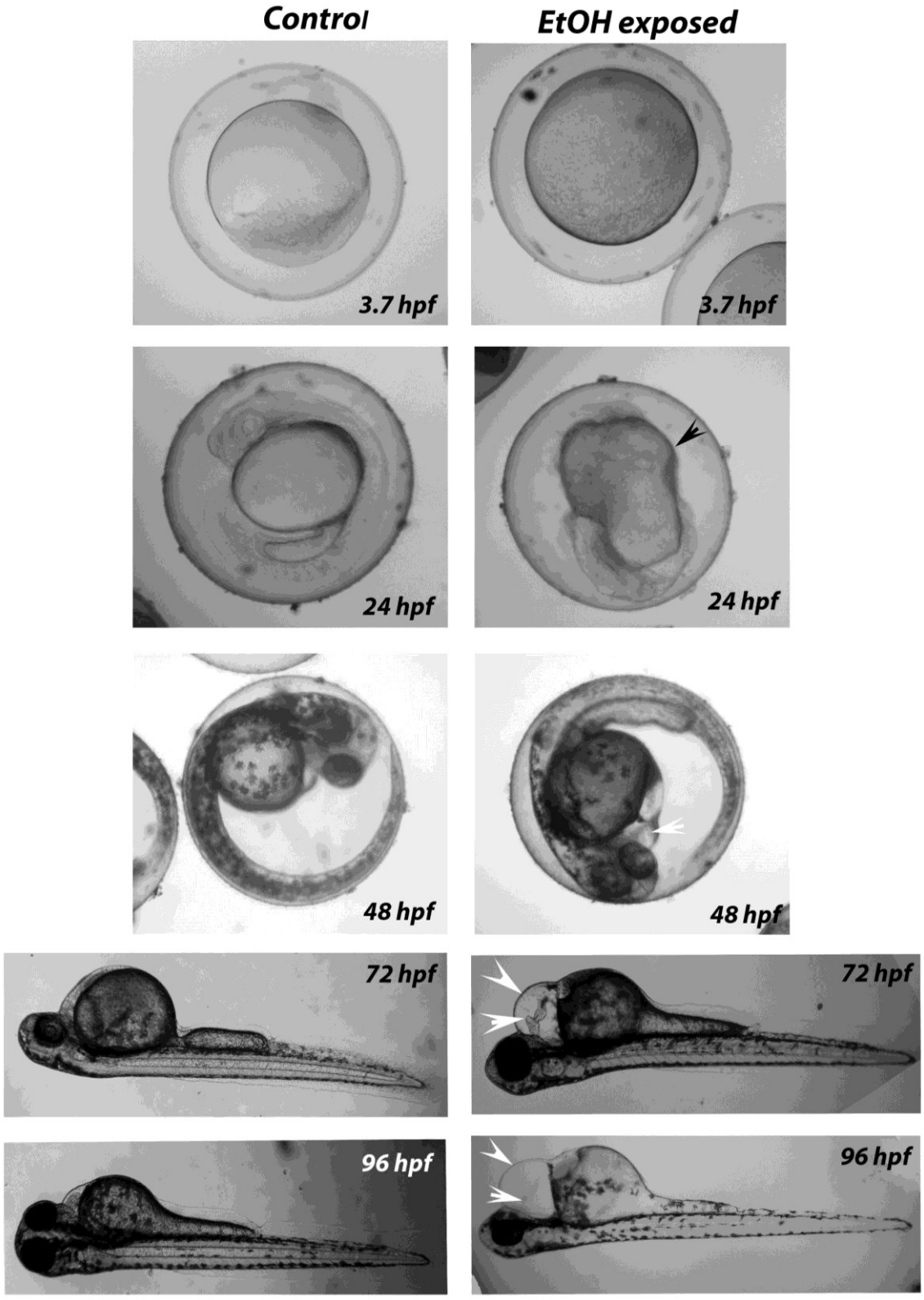

Plate 1. Showed various morphological analysis of control and ethanol exposed zebrafish embryo or larvae. The black arrow indicates the malformation of embryo, white arrow indicates the pericardial edema and cardiac malformation, EtOH – ethanol and hpf – hours post fertilization.

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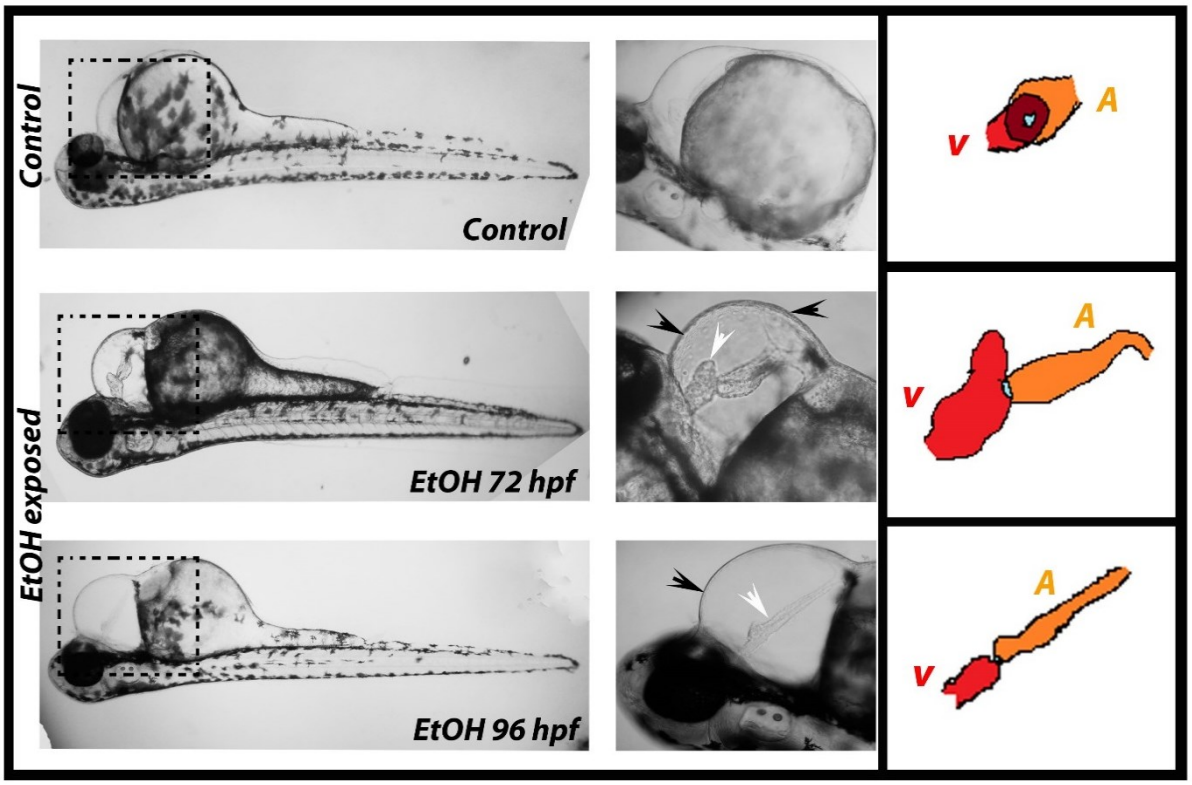

Plate 2. Showed cardiac looping and heart length of control and ethanol exposed zebrafish embryo or larvae. The black arrow indicates the pericardial edema, white arrow indicates the and cardiac malformation, EtOH – ethanol, hpf – hours post fertilization.

In the EtOH treated embryos the looping disturbance creates alteration such that the ventricle was positioned anterior to the atrium. Thus, in the EtOH exposed embryos the chambers can be easily distinguished without overlap (Plate. 2). Moreover, in the EtOH exposed animals the atria were thin and elongated and the ventricles appeared smaller and more compact than normal (fig. 4 and Plate. 2).

In order to quantify the effect of EtOH on zebra fish hearts, the distance between the junction of the heart with the inflow tract at the sinus venosus (SV) and the junction with the outflow tract in the region of the bulbus arteriosus (BA) was determined. The resulting numbers provides an index of the change in heart morphology due to the EtOH treatment, and reflect the change in cardiac looping. The EtOH exposure produced significant increases in the BA-SV distance at all the time point when compared to controls (Fig.4 & Plate 2). More specifically, while the control hearts underwent the process of looping and compaction, this process was not observed in the EtOH treated hearts.

Effect of EtOHon Heart Rate

The effects of EtOH exposure on the developing zebra fish heart is not limited morphological alteration. We extend to analyze the heart function by measuring the heart rate and ventricular stand still. The data showed that the EtOH exposure profoundly reduced the heart rate and produce ventricular regurgitation in all the time point when compared to control (fig. 5). The control heart exhibited normal contraction, with the ventricles contracting immediately after the atrium. But the EtOH treated heart showed delays in the contraction of ventricle followed by atrium contraction (fig. 6). This effect is more prominent in 120 hpf were It also found that the 120 hpf were the ventricles had ceased beating almost completely (data not shown).

Peripheral blood flow

The effect of EtOH on peripheral blood flow was analyzed using zebra fish embryo. There is a significant ($p<0.001$) reduction of blood flow in EtOH exposed embryo (5.5 ± 1.2) when compared to control (23.5 ± 2.10).

Discussion

The effects of EtOH on the developing zebra fish heart are evident by various morphological and functional and developmental defects. These effects include altered looping of heart tube into a string-like or straight tube like appearance, with the ventricle located distinctly anterior to the atrium. Furthermore, the ventricle appears smaller than normal, the atrium is elongated, and both chambers have a narrow width. Interestingly, the EtOH treated heart showed valvular regurgitation of blood and a striking ventricular standstill at all the time point, which clearly demonstrate that teratogenic effect of EtOH on developing heart.

The elongation of the heart by EtOH might be due to EtOH induced failure in morphogenetic movement of cells, which leading to deficiency in attachment point between the heart and the common cardinal vein (CCV) to migrate dorsally, thus mechanically stretching the heart. From approximately 72 to 96 hpf, the heart's attachment point to the inflow tract migrates dorsally, in a process that appears to contribute to the heart's looping and compaction within the pericardium (Antkiewicz et al., 2005).

Furthermore, the cardiac morphological defect in heart morphology is more obvious even at 48 hours and it was altered more dramatically in all the experiment time point. Thus, the alteration of heart morphology is present from the beginning of the process of dorsal migration, and therefore cannot be secondary to the disruption of CCV regression. However, the CCV dorsal migration might contribute to the elongated heart morphology at later time points. Similarly, the formation of pericardial edema could move the inflow and outflow attachment points apart, causing the elongated heart morphology. Additionally, EtOH exposure produces the looping impairment which further facilitates the lengthening and alters the inflow and outflow tract (Samoa and Marrs, 2013). Defects in the heart might well be expected to produce a concurrent reduction in circulation. In the present study, we also find that impairment of peripheral blood flow in EtOH exposed embryo when compared to control. This clearly indicated and further emphasized that circulation disturbance plays an additional factor for impairment in structure and function in developing heart (Hove et al., 2003).

The present data clearly shows that EtOH exposure has a striking effect on the ventricles beat (ventricular standstill). Consistent with the present study an earlier report by Bilotta et al., (2004) reveled EtOH exposure to the developing embryo reduces the heart rate. These effects might be due to the increasing pressure (Hove et al., 2003) of the pericardium by pericardial edema, as it could be inhibiting the heartbeat by compressing the heart and preventing filling in a process analogous to cardiac tamponade. We consider this mechanism unlikely because such a mechanism should affect the atrium at least as much as the ventricle, yet the atrial beat is unaffected. However, EtOH exposure might block the impulse propagation at the AV node only if we conclude that the observed phenomenon as an AV conduction block (Antkiewicz et al., 2005). But, the study does not show any progressive arrhythmias, which are commonly associated with AV block. Thus, possible mechanism to explain the ventricular standstill is a failure of the action potential to travel beyond the AV junction. Further, elaborate study is needed to explore how the EtOH alters the AV block and ventricular dysfunction.

Additionally, the valvular regurgitation of blood flow also observed as early as 48hpf in EtOH exposed heart compared to control. It further enhanced in

72 and 96 hpf EtOH exposed heart, in particular, decrease in cardiac output and blood flow becomes more dramatic, the movement of blood cells between the atrium and the ventricle, as well as between the ventricle and the outflow tract can be observed in most embryos. This could be due to an EtOH mediated defect in valve function (Karunamuni et al., 2014) or adaptive response to overcome elevated afterload due to a block in circulation (Hove et al., 2003). Since we could not analyze the vascular flow in 120 hpf due to lack or nil circulation and heartbeat. However, the present study showed retrograde blood flow at 48, 72 and 96 hpf, when blood flow is still substantial and heart contractility seems unaffected. This suggests that the developing valves are not functioning properly, perhaps due to altered valve cushion development, or a loss of contractility at the sites of the nascent valves.

Conclusion

Taken together, the steady decrease in heart size coupled with the severe defects in ventricular function, failure of cardiac looping formation, heart rate, ventricular standstill and retrograde blood flow would be expected to have a substantial impact on the ability of zebra fish to circulate blood. A disruption of heart development in zebrafish embryos exposed to EtOH is particular importance in light of the growing body of evidence demonstrating that the cardiovascular system is also a key target of EtOH toxicity in humans. From the present study it is concluded that EtOH exposure during development results in structural and functional impairment in heart that mimic malformations that occur in patients with fetal alcohol syndrome. Further studies are necessary to identify the molecular mechanism behind how the EtOH affect developing heart.

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Research Article

Tori in dry skulls of the Southern Indian population-anthropologic and clinical emphasis

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Abstract: Tori are benign bony exostoses with limited bone marrow. The prevalence of Torus palatinus (TP) and Torus mandibularis (TM) has been extensively studied in various ethnic populations to demonstrate racial differences. The aim of our study was to demonstrate the occurrence of TP and TM in South Indian dry skulls. For this purpose, hard palate and mandible of seventy eight articulated and disarticulated dry skulls were examined macroscopically. Further, the location, morphology and morphometry of tori were evaluated. A bilateral, symmetrical, elongated, multi-lobular form of TM and a large, bilateral, asymmetrical, right ridge and left lobular forms of TP were observed with prevalence of 1.28 % and 1.28 % respectively. TM was located at the lingual surface of mandible along the incisor, canine and premolar teeth. TP was located on either side of inter-palatine and inter-maxillary sutures of hard palate. The respective length, breadth and height of the right and left TP were found to be 20 mm, 10 mm & 10 mm; 22 mm, 21 mm & 15 mm. The present study signifies that the examination of tori in dry skulls might be advantageous over that in living subjects for the better evaluation of occurrence, morphology and morphometry of torus. The present study emphasizes the surgical removal of torus since it might cause serious unpredicted challenges during endo-tracheal intubation and fabrication of prosthesis. To the best of author's knowledge, this is the first study demonstrating the occurrence of tori in South Indian dry skulls

Tori are benign bony exostoses that are developed as dense cortical protruberances with limited bone marrow. The torus is often considered as an anatomical variant (Woo, 1950) or as a physiological phenomenon (Suzuki and Sakai, 1960) than as any pathology. Torus palatinus (TP) is usually located along the inter-maxillary and the inter-palatine sutures of hard palate and Torus Mandibularis (TM) is usually located at the lingual surface of mandible along the canine or premolar teeth (Garcia et al., 2010).Torus is covered by a thin and less vascularised mucous membrane that might

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be lacerated easily by the denture (Suzuki and Sakai, 1960)and might also suffer ulcerations due to traumatized tori (Al-Quran and Al-Dwairi, 2006). Further, tori might pose challenge in the construction of prosthesis in edentulous subjects (Al-Quran and Al-Dwairi, 2006). The presence of large torus might interfere with a clear view of glottis (Best et al., 2014) and pose fatal challenge to anaesthesiologists for a successful intubation. Tori are considered to affect speech, deglutition and mastication and also might obscure radiographic details of premolars and maxillary air sinus (Seah, 1995). The aetiology of torus is not clearly understood but currently interplay between genetic and environmental factors are considered to play a major role (Gorsky et al., 1996; Ismail and Hamad 2009) though reports also suggest functional stress to be a cause (Hasset, 2006).A relationship between increased bone density and the presence of tori in post-menopausal women has also been reported (Belsky et al, 2003).

The torus mandibularis (TM) and torus palatinus (TP) were of anthropological interest since 1814 (Seah, 1995). TM is a non-metric trait found to vary in occurrence among different ethnic populations and frequently recorded during the archaeological assessment of skeletal remains (Hasset, 2006). Both tori are extensively studied to demonstrate racial variations (Table 1). The present study was aimed to demonstrate the occurrence of TP and TM in dry skulls of the Southern Indian population. This study provides a baseline data that of anthropological significance.

Materials and Methods

Sample Collection

Seventy eight articulated and disarticulated dry skulls with well-preserved hard palate were procured from the department of Anatomy of Karpaga Vinayaga Institute of Medical Sciences and Research center. The hard palate and the mandible of dry skulls were examined macroscopically for the presence of bony exostoses.

The morphological study

a. Torus mandibularis

All the seventy eight dry mandibles were macroscopically inspected for the presence of cortical bony exostoses. Mandible with torus was visually examined for its form, location in relation to the teeth, and its unilateral or bilateral presence. The form of unilateral torus was evaluated on the basis of number of nodules viz., uni-lobular, bi-lobular or multi-lobular forms and the length was categorized as solitary when the location is related to one or two teeth and as elongated when extends over two teeth (Ihunwo and Phukubye, 2006). Further, the sex of the mandible with torus was determined by analyzing standard anatomical features.

b. Torus palatinus

The hard palate of all dry skulls was inspected macroscopically for the presence of torus. Further, the palate with torus was visually inspected for its form, location and its unilateral or bilateral presence. The morphology of TP was categorized as ridge or lobular forms. A torus which was relatively wide and uniform in width was considered to be lobular. A torus that was relatively narrow in width throughout its length was considered to be ridge. The size of the palatine torus was determined to be small, medium or large according to Woo's (1950) method of classification. Further, the sex of the skull with TP was determined by analyzing standard anatomical characteristics.

The bilateral tori were considered to be symmetrical when the form of torus on

both sides was more or less similar and as asymmetrical if tori exhibit dissimilar forms.

The morphometric study

a. Torus Mandibularis

No measurements were made for mandibular torus with regard to the prevalent idea that these characteristics are non-metrical and should be assessed by standard procedure of inspection (Ihunwo and Phukubye, 2006).

b. Torus Palatinus

The exact location of the torus in the hard palate was determined by measuring its distance from the incisive foramen (I), from the alveolar margins of hard palate (A) and from the posterior free margin of hard palate (P). The length, breadth and height of the torus were measured. All the measurements were done with vernier calipers

Observation

Occurrence of torus

The examination of the hard palate and mandible in dry skulls showed 1.28 % and 1.28 % occurrence of TP and TM respectively (Table 2).

The morphological study results

a. Torus Mandibularis

The examination of mandibles revealed one mandible with bilateral symmetrical tori at its lingual aspect. The torus was multi-lobular in form and elongated in length (Table 2). The bilateral tori were present along the incisor, canine and two premolar teeth (Fig 1). The sex of the mandible with torus was evaluated to be male.

b. Torus Palatinus

The examination of hard palate of dry skulls showed one skull with a bilateral asymmetrical palatine torus on either side of the inter-maxillary and inter-palatine sutures of hard palate. The posterior ends of the torus was fused but separated anteriorly by a deep groove. The right and left tori exhibited ridge and lobular forms respectively (Table 2 & Fig. 1). The size of the torus was evaluated to be large. The sex of the skull with palatine torus was evaluated to be male.

The morphometric study results

Torus palatinus

The distance of right and left TP from I, A and P was found to be 7 mm and 4 mm, 10 mm and 4 mm; 6 mm and 7 mm respectively. The respective length, breadth and height of the right and left palatine tori were found to be 20 mm, 10 mm &10 mm; 22 mm, 21 mm & 15 mm (Table 2).

Discussion

The etiology of tori was not well understood in the past due to lack of proper evidence. However, currently the reports suggest genetic factors to play a possible role (Sierra and Jackson, 1992). The development of torus is reported to be an autosomal dominant trait (Suzuki and Sakai, 1960; Gorsky et al., 1998; Yoshinaka et al., 2010). However, studies also suggest that the interplay between environmental and genetic factors and functional stress might be the cause and have reported an association between oral tori and Temporo-mandibular disorders (Hasset, 2006; Ismail and Hamad, 2009). Cagirankaya (2004) in his study has indicated a relationship between the presence of torus palatinus and development of maxilla in female subjects.

The prevalence of tori has been extensively studied in various ethnic populations to demonstrate racial differences [Table 1]. The reports have shown high prevalence in Mongoloid race than Caucasians (Woo, 1950). Nevertheless, high prevalence has been reported in the Caucasian race when compared to Negroid (Kolas et al., 1953; Dosumu et al., 1998).

Table 1 Data of prevalence of torus mandibularis and torus palatinus in various ethnic populations.

Study	Year	Population	Sample size	Torus Palatinus %			Torus Mandibularis %		
				%	M	F	%	M	F
Kolas *et al.,*	1953	American	2478	20.9	14.7	26.7	-	-	-
Skrzat	1956	Polish	98 skulls	67 (In No.)	27 (In No.)	28 (In No.)			
Shah *et al.,*	1992	Western Indian	1000	9.5	-	-	1.4	-	-
Gorsky *et al.,*	1996	Israelis	1002	21	16.4	24.9	-	-	-
Gorsky *et al.,*	1998	Israelis	168	38.7	38	39.3	-	-	-
Dosumu *et al.,*	1998	Nigerian	2506	2	1.5	2.4	3.2	2.4	3.9
Yildiz *et al.,*	2005	Turkish	1943	30.9	28.1	34.3	-	-	-
Ihunwo and Phukubye	2006	South African	284	-	-	-	28.7	-	-
Al-quran	2006	Jordanian	338	29.8	No difference		42.6	No difference	
Ismail and Hamad	2009	Iraq	932	5.3	2	6.8	7.2	4	8.4
Sawair *et al.,*	2009	Jordanian	618	25.7	No difference		15.4	No difference	
Mirza *et al.,*	2013	Pakistani	909	3.7	35.3	64.7	-	-	-
Patil *et al.,*	2014	Northern Indian	3087	1.3	-	-	6.9	-	-
Present study	2014	South Indian Dry Skull	78	1.28	-	-	1.28	-	-

M – Male; F - Female

Table 2: Data of occurrence, morphology and morphometry of Palatine and Mandibular tori in South Indian dry skulls

TORUS	FREQUENCY	RIGHT TORUS (mm)				LEFT TORUS (mm)			
		Form	Length	Width	Height	Form	Length	Width	Height
TM	1.28%	Multi-lobular	Elongated	-	-	Multi-lobular	Elongated	-	-
TP	1.28%	Ridge	20	10	10	Lobular	22	21	15

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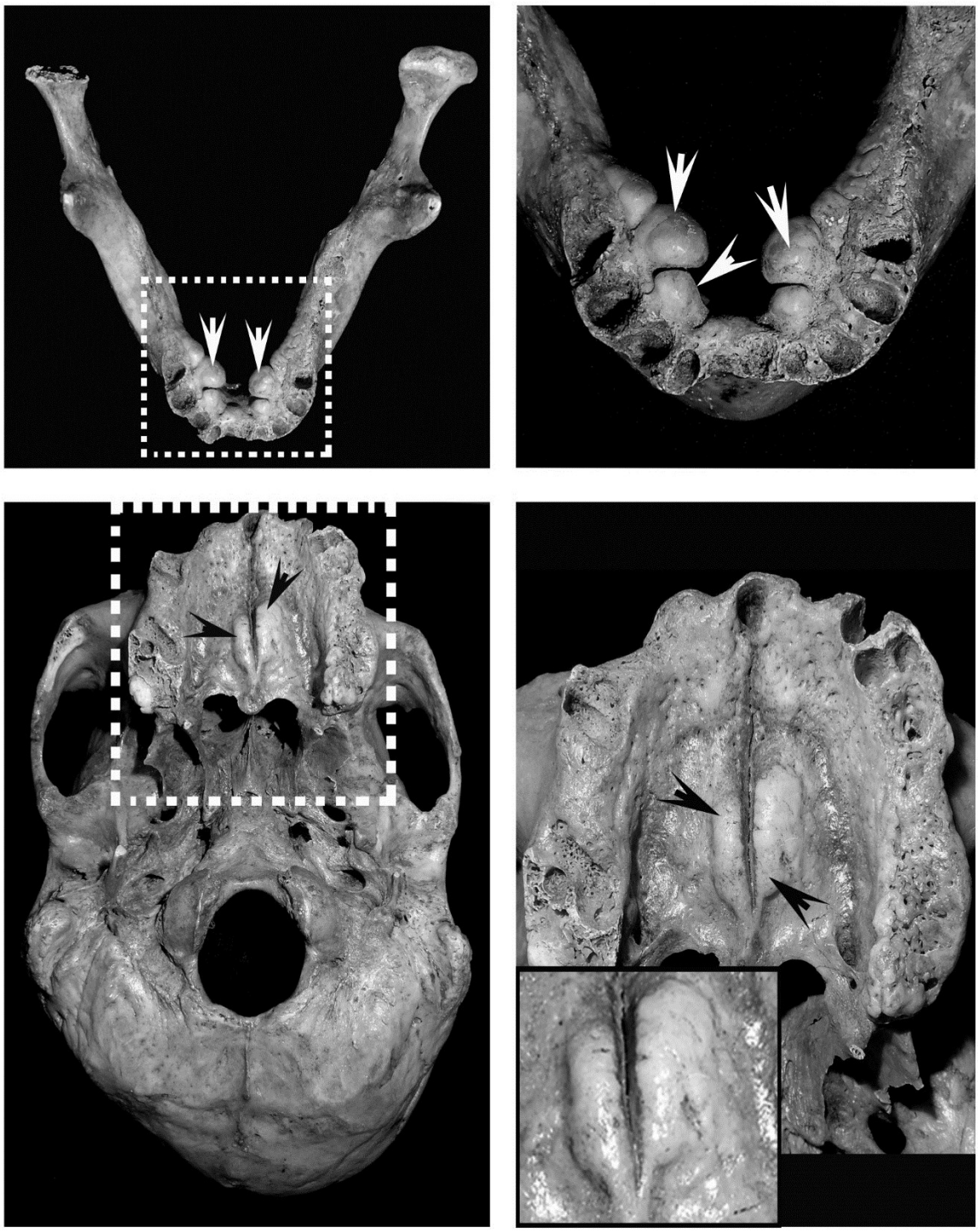
Fig 1 Morphological image of mandible and hard palate with torus.



The mandible showed a bilateral, elongated multi-lobular torus (white arrow) at the lingual surface on either side of symphysis menti. The hard palate showed right ridge and left lobular forms of bilateral torus (black arrow) on either side of inter maxillary and inter palatine sutures.

Further, the frequency of the torus has been reported to vary significantly in both sexes with high prevalence of tori in females than males (Kolas et al., 1953, Shah et al., 1992; Gorsky et al., 1996; Sebaie and Al-Wrikat, 2011) whereas few reports have shown high prevalence in males than females (Ruprecht et al., 2000; Patil et al., 2014). Nevertheless,

some literatures indicate no significant sex differences in their prevalence (Skrzat et al., 2003; Al-Quran, 2006; Igarashi et al., 2008). The study by Shah et al. (1992) have reported 9.5 % and 1.4 % prevalence of TP and TM respectively in the living subjects of the Eastern Indian population whereas the similar study carried out in the Northern Indian population have shown low frequency of TP and high frequency of TM viz., 1.3 % and 6.9 %respectively (Patil et al., 2014). The present study has demonstrated the occurrence of tori in the dry skulls of the Southern Indian population unlike the earlier studies in living subjects of India. The prevalence of tori in dry skulls was found to be higher than that in the living subjects (Seah, 1995). This might be due to the fact that the mucous membrane and glands obscure the small tori and reduce the possibility of their detection by visual oral inspection or palpation. This might result in confusions among different observers in the detection of torus and hence might lead to wide variations in occurrences among various populations. Further, TM less than 3 mm was reported to be undetectable in radiographic study (Ruprecht et al., 2000). The examination of dry skulls might be advantageous over that in living subjects for a direct and better evaluation of the morphology and morphometry of tori. The present study indicates comparatively low frequency of 1.28 % and 1.28 % of TP and TM respectively. From this data, it may seem that the frequency of tori in the Southern Indian population is comparatively less than that of the Northern and the Western populations of India. Nevertheless, this view needs further authentication by studies on larger samples from the Southern parts of India.

Literatures have reported the flat palatine tori to be the most frequent followed by the spindle or ridge form whereas the lobular or the nodular forms are considered to be larger and infrequent (Woo, 1950; Kolas et al., 1953; Seah, 1995; Gorsky, 1996; Patil et al., 2014).The present study has shown bilateral right ridge and left lobular forms of TP that are less frequent. The torus is believed to start at puberty with progressive growth in size with increase in age (Maclinns et al., 1998) but also suggested to develop at birth (Garcia et al., 2010). Though the tori are considered to be clinically insignificant but its growth in size might pose a challenge in Prosthodontics during the fabrication of Prosthesis (Yildiz, et al., 2005; Yoshinaka et al., 2010). The thin poorly vascularized mucosa covered torus when interferes with denture might cause tissue inflammation (Al-Quran and Al-Dwairi, 2006). Hence, it is advisable for the dentists or prosthodontists to recommend the patients for surgical resection of tori to avoid untoward inconveniences and clinical complications. Further, the presence of mandibular tori might cause difficult laryngoscopy with subsequent complication in successful intubation that might pose serious or fatal challenge to anaesthesio-logists. The mucosa covering these exostoses might be subjected to lacerations and hemorrhage during laryngoscopy, intubation and extubation or any other intra-oral treatment or management procedures. Also, large palatal tori may interfere with laryngeal mask airway placement (Sarma, 2014).Cases of difficult intubation due to the presence of TM are reported in literatures (Woods, 1995; Durrani et al., 2000; Takasugi, 2009; Best, 2014; Sarma, 2014). Anaesthesiologists should be aware of these cortical exostoses to prevent unpredicted challenges that they pose.

To the author's best of knowledge, this is the first study demonstrating the occurrence of tori in dry skulls of the Southern Indian population. The present data indicates low frequency of tori but to authenticate the geographical variations in the occurrence of tori, studies on large samples of South Indian population need to be carried out.

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