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Evaluation of Silver-Nucleolar Organizing Region in Trachea-esophageal Region of Chick Embryo (*Gallus domesticus*)

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Abstract : The study aimed to evaluate the proliferative activity of the pharyngeal walls by using the silver-staining technique (Ag-MOR). It revealed that the mean of Ag-NORs count was higher in the 3rd day of embryonic development as compared to that of 2nd day of development. The Ag-NORs counts in 2 day embryo ranged between (2.123-2.924 dot/ cell), (2.535-3.369 dot/ cell), (4.5-5.078 dot/ cell) and (2.833-3.585 dot/ cell) in region I, II, III and IV respectively. While the count was ranged between (2.262-2.393 dot/ cell), (2.903-3.546 dot/ cell), (3.867-4.484 dot/ cell) and (4.56-5.108 dot/ cell) in region I, II, III, and IV respectively in 3edday of incubation. The differences in the total Ag-NORs count between two and three day of development was statistically significant at $P \leq 0.05$. The count was higher in pharyngeal epithelial wall of the three day embryos when compared with the two day embryos. Also the statistical analysis show significant differences in means of Ag-NORs counts between the part of pharyngeal wall and regions.
Keywords: Ag-NOR, histology, histochemical study ***trachea-esophageal region***, chick embryo, Iraq.

Introduction

The digestive and respiratory systems have different physiological functions and are generally considered to be and studied as two independent systems. Although at birth they are separated, they both derive from a common and transiently developed structure, the foregut, which is the anterior part of the gastrointestinal (GI)tract[1].

The primitive gut tube is initially patterned into three broad domains along its anterior– posterior (AP) axis: the fore- from which the respiratory system is derived, mid-, and hindgut. As they develop, each region of the gut is characterized by unique mesodermal and endodermal morphologies, which can be easily discerned by gross and microscopic examination. Specifically, these tissues show regional differentiation along the AP axis that specifies pharynx, esophagus and stomach (the foregut), small intestine (the midgut), and large intestine (hindgut)[2].

The respiratory system originates from the formation of an endodermal diverticulum in the ventral wall of the foregut, whereas the esophagus forms from the foregut dorsal wall. The foregut endoderm evaginates and pushes the surrounding mesenchyme to form the 2 presumptive lung buds. In avian embryos, these processes manage to form 2 independent and separate endodermal structures, dorsally the esophagus and ventrally the lung buds. Later, the lung buds grow caudally, leading to the formation of a temporary trachea-esophageal (TE)

septum. The appearance of the TE septum is followed first by the expansion of the trachea and second by the final separation of the two endodermally derived systems[3].

When the newborn baby takes its first breath, air travels down the trachea into the lungs. When it suckles, milk passes along the esophagus into the stomach. The critical functions of breathing and eating thus depend on two distinct epithelial tubes, the dorsal esophagus and the ventral trachea, that develop from a common progenitor- the anterior foregut tube[4]. Defects in foregut development underlie the relatively common spectrum of human malformations referred to as esophageal atresia and tracheal esophageal fistula. While the pathological nature of these abnormalities is obvious, the molecular and cellular basis of these anomalies and even of normal tracheal and esophageal development, are poorly understood[5]. This Study aimed to detect the proliferative activity that could be related to trachea-esophageal formation.

Nucleolar Organizer Regions (NORs)

Nucleolar Organizer Regions are loops of DNA that encode ribosomal RNA and are considered important in the synthesis of protein[6,7]. They are located on the short arms of acrocentric chromosomes 13, 14, 15, 21 and 22[8].

The number of NOR-bearing chromosomes varies depending on the species, ranging from 1 in haploid yeast cells to 10 in human somatic cells (acrocentric chromosomes) .The number of NORs at any given stage of cell cycle appears to be inversely proportional to cell cycle time[9], thus the higher the number of NOR, the shorter the cycle time, and the faster the rapidity of cell proliferation as recognized, but the situation is not the same in anuloploidy cells (abnormal DNA contents), because an increase in Ag-NOR count may be due to real increase in NOR bearing chromosomes.

The active NORs are detected by aspecific silver staining procedure, designated Ag-NOR staining[10]. The silver staining technique identifies neither rRNA nor rDNA but the acidic proteins associated with these sites of rRNA transcription, these proteins are designated as B23, C23,"Ag-NOR protein" and RNA polymerase I[11].

The Ag-NOR are argyrophilic non histone proteins whose precise biochemical nature is not well understood. However they are easily demonstrated by the simple, specific, one-step staining technique used in this study[12].

Material and Methods

Chick embryos: Fertilized chick eggs were obtained from local hatchery and incubated at 38 C°. Chick embryo were removed and staged according to the criteria of Hamburger and Hamilton[14]. Embryonic tissue were fixed in Bouin's solution for 8 hours at room temperature and were processed for paraffin sectioning[15]. Nucleolar Organiser Region Staining Procedure was done according to[16]. The stained sections were seen under microscope in different magnification power and photographed by using digital camera (Sony DSC-W350). Ag-NOR counting was done as per the method proposed by[8] under 100X objective, under oil immersion.

Statistical analysis

The data of Ag-NORs counting were expressed in mean. The statistical analysis of the obtained data was performed by using the Analysis of Variance (ANOVA) test at least significant differences (L.S.D) according to Snedcor and Cochran[17]. The level of significance was accepted at $P \leq 0.05$.

Results:

Silver staining proved to be a simple technique for the quantitative evaluation of Ag-NORs. The Ag-NORs appeared as dark brownish to black dots with varying size within nuclei which stained pale yellow (Figure 1). They are widely distributed in the nuclei. Non-specific silver staining was, in most sections, minimal or absent. Overall, this method permitted accurate counting of Ag-NORs both within nucleoli and lying free within nuclei.

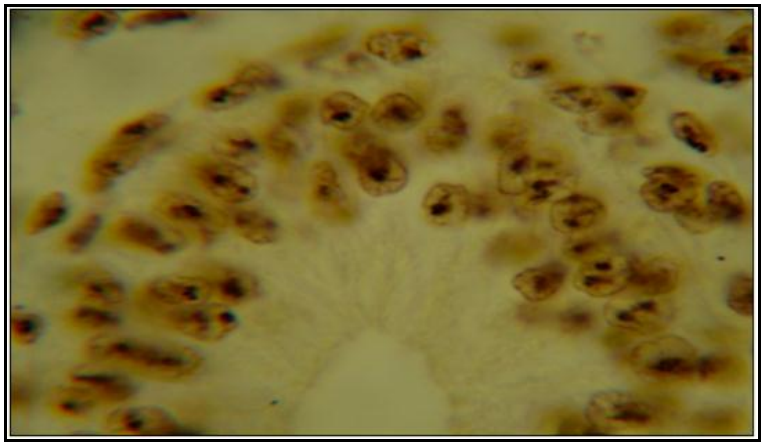
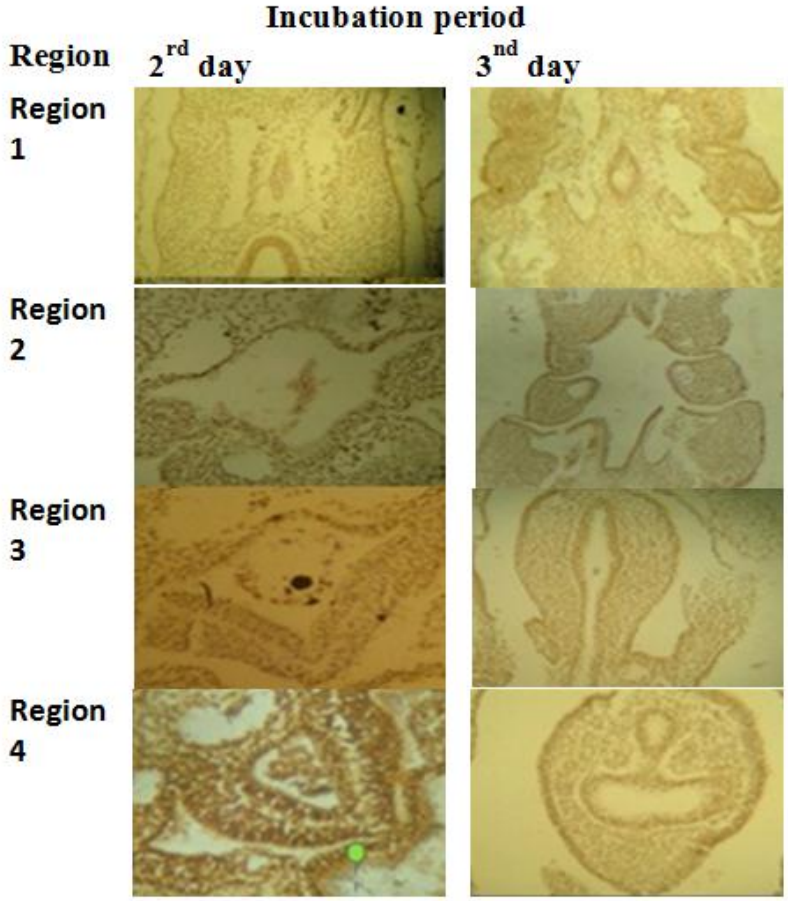


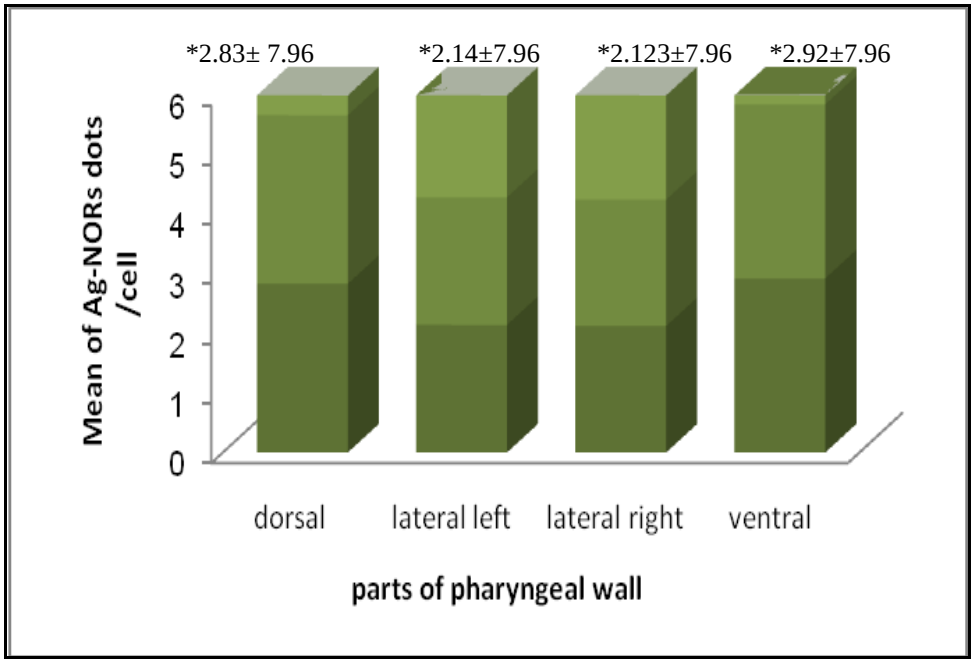
Figure (1): A Section of Pharyngeal epithelial Tissue showing the type of Ag-Nors Stained by Silver Staining. 100X.

Ag-NOR Count of Two Day Chick Embryos

The sections at the cranial part of the pharynx at region I (Figure 2&3) show significant variability in the pattern of proliferative activity in the epithelial cells of the pharyngeal wall indicated by the Ag-NORs count, the mean number of Ag-NORs count ranged between 2.123-2.924 dots/cell. The Ag-NORs count in the pharyngeal pouches wall at region II (Figure 2&4 revealed significant differences, it demonstrated that the epithelial of the wall of bilateral pharyngeal pouches have the least proliferative activity proved by the smaller of the number of Ag-NOR dots (2.633 and 2.535 dots/ cell). The walls of the ventral pharyngeal pouch revealed high proliferative activity (3.29 dots/ cell). This count was increased more in the dorsal pouch (3.369 dots/ cell) in the early embryonic stages. The next caudal sections at region III (Figure 2&5) at the pharyngeal walls with a shallow bilateral pouches showed significant differences at $p \leq 0.05$, The dorsal aspect of the pharyngeal wall have the low proliferative profile, it was of (5.05 dots/ cells), the bilateral pharyngeal pouch demonstrate equilateral proliferative activities (5.16 and 5.078 dots/ cell) that approximated the activity at the ventral aspect of this pharyngeal wall (4.5 dots/cell).In the region IV (Figure 2&6), the proliferative activity revealed significant variability at $p \leq 0.05$, The proliferative activity was the least in the ventral aspect of pharyngeal wall (2.833 dots/ cell), while it was higher at the bilateral pouches of pharyngeal walls (3.585 and 3.544 dots/ cell), also it was of a comparable value in the dorsal pouch of pharyngeal wall (3.454 dots/ cell).

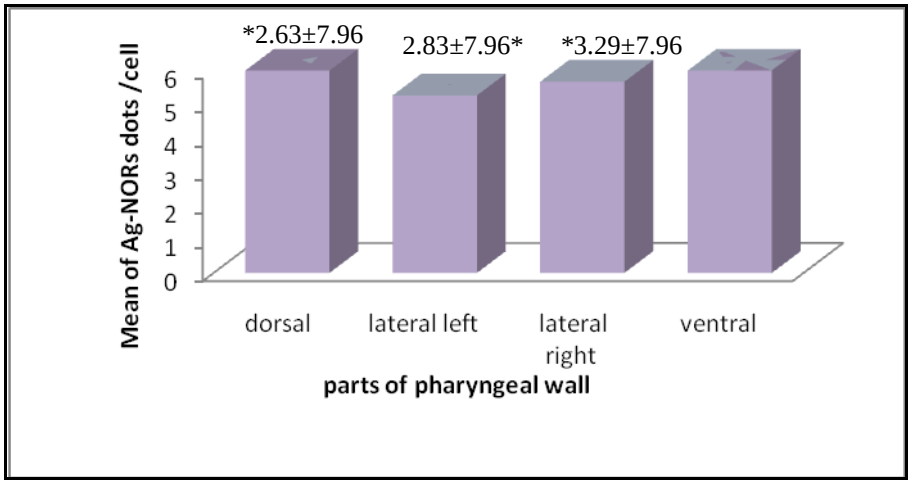


Figuer (2): Regional configuration of the pharyngeal primordia during the 2nd and 3rd days of incubation. 40X.

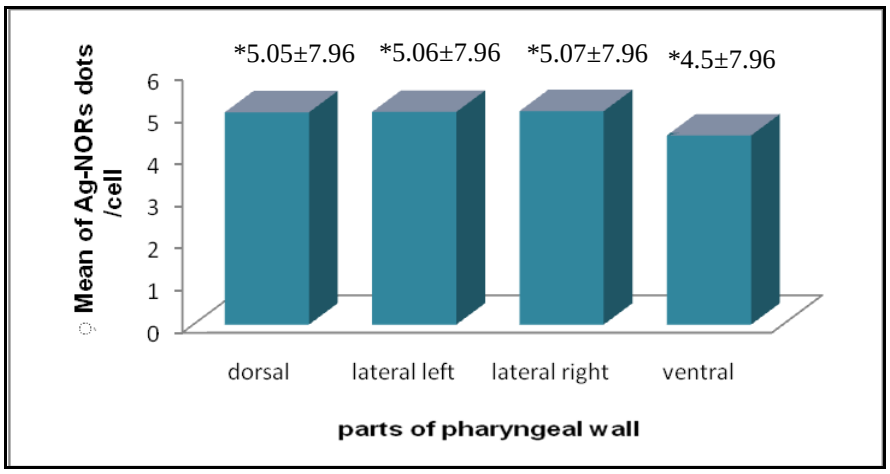


L.S.D. under P≤ 0.05 for the number of section and region= 0.176. , *=Mean± Standard Error (SE)

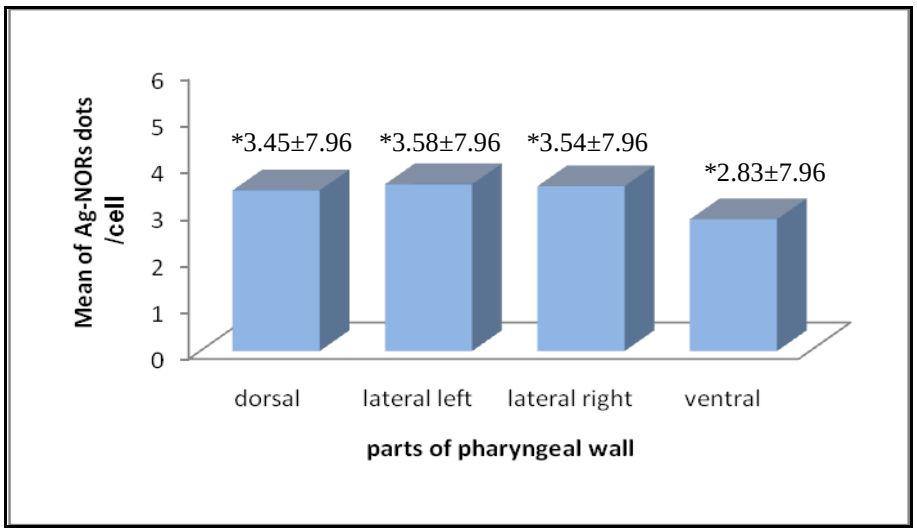
Figure (3): Ag-NORs count in region I of two day chick embryo.



L.S.D. under P≤ 0.05 for the number of section and region= 0.176., *=Mean± Standard Error (SE).
Figure (4): Ag-NORs count in region II of two day chick embryo.



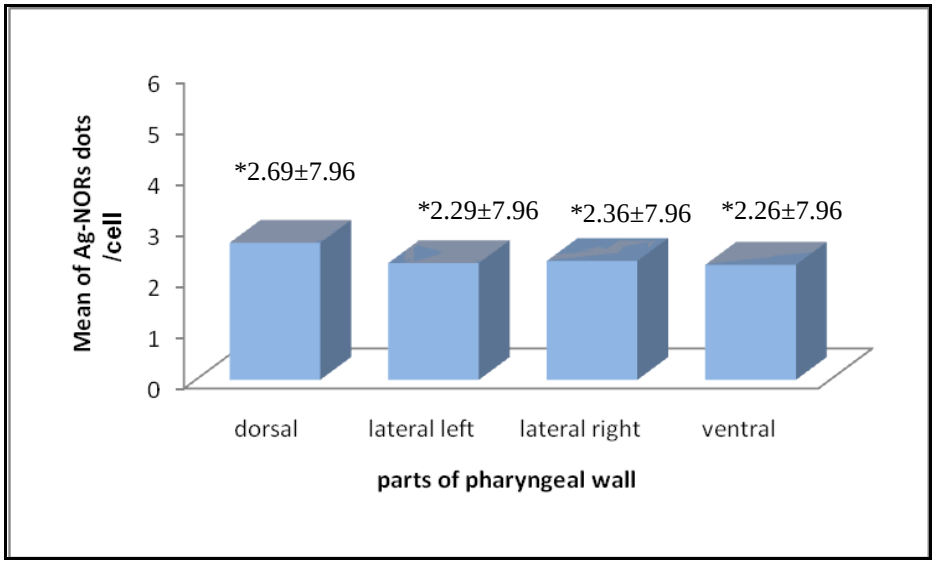
L.S.D. under P≤ 0.05 for the number of section and region= 0.176., *= Mean± Standard Error (SE).
Figure (5): Ag-NORs count in region III of two day chick embryo.



L.S.D. under P≤ 0.05 for the number of section and region= 0.176., *=Mean± Standard Error (SE).
Figure (6): Ag-NORs count in region IV of two day chick embryo.

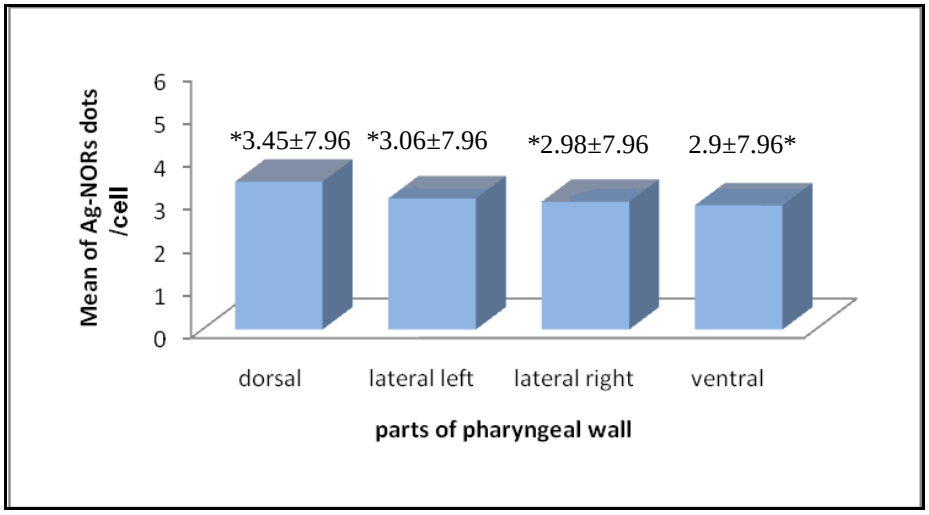
Ag-NOR Count of Three Day Chick Embryo:

In 3rd day embryo, the region I and IV have no significant differences at p≤ 0.05 , while the region II and III showing significant differences at the same statistical level. In the embryonic sections of 3rd day incubation at region I (Figure 2&7), the proliferative activity was (2.393 dots/ cell) in the dorsal pharyngeal pouch, (2.293 and 2.336 dots/ cell) in the bilateral pouches, and (2.262 dots/ cell) in the ventral pouch of pharyngeal wall. In region II(Figure 2&8), The mean of Ag-NORs count was increased significantly at P≤ o.o5, it was of (3.546dots/ cell) in the dorsal pharyngeal pouch, while it was less in lateral pouches of the pharyngeal wall (3.063 and 2.988 dots/ cell) and (2.903 dots/ cell) in ventral pouch of the pharyngeal wall. In more caudal section at region III (Figure 2&9), the proliferative activity was significant (4.484 dots/ cell) in the dorsal pouch, (4.081 and 4.224 dots/ cell) in the bilateral pouches of pharyngeal wall, and (3.867 dots/ cell) in the ventral pouch of pharyngeal wall. In the region IV (Figure 2&10) the proliferative activity of pharyngeal wall was (5.108 dots/ cell) in the dorsal pouch, (5.027 and 4.974 dots/ cell) in the bilateral pouches, and (4.56 dots/ cell) in the ventral pouch.



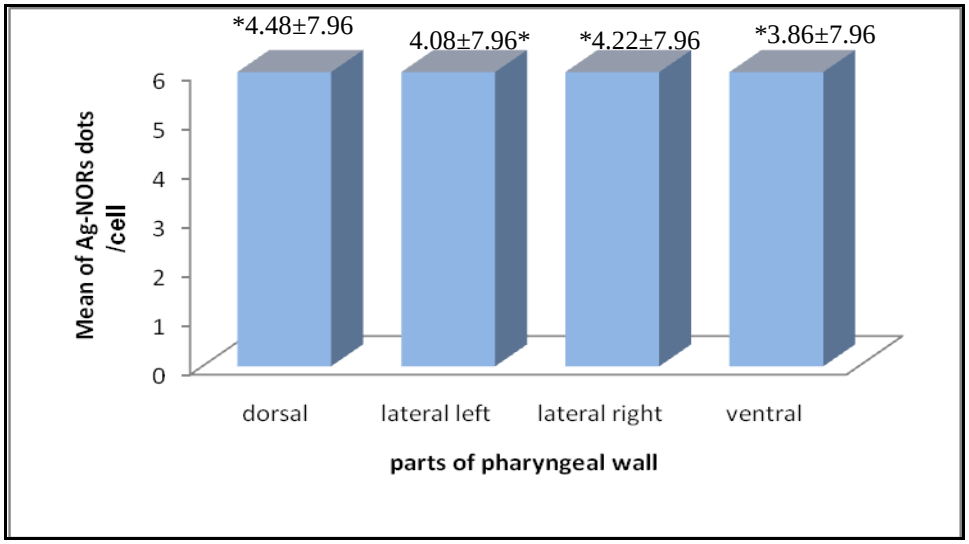
*= Mean± Standard Error (SE).

Figure (7): Ag-NORs count in region I of three day chick embryo.



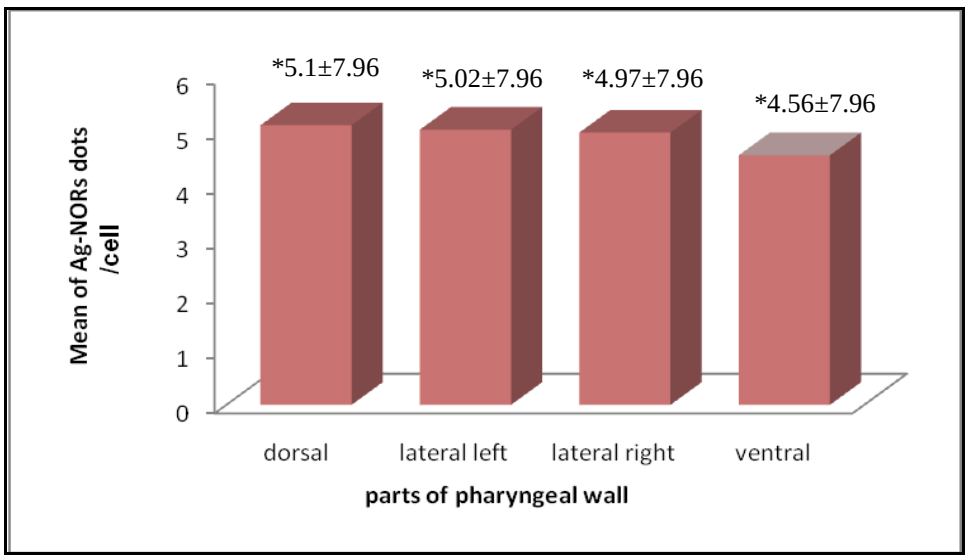
L.S.D. under P≤ 0.05 for the number of section and region= 0.176., *= Mean± Standard Error (SE).

Figure (8): Ag-NORs count in region II of three day chick embryo.



L.S.D. under P≤ 0.05 for the number of section and region= 0.176., *= Mean± Standard Error (SE).

Figure (9): Ag-NORs count in region III of three day chick embryo.



*=Mean± Standard Error (SE).

Figure (10): Ag-NORs count in region IV of three day chick embryo.

Discussion

The proliferative activity of the embryonic tissues was evaluated by counting the number of Ag-NORs dots[16], this method represent the best to be used in analyzing the proliferative growth in the embryonic structures at the trachea-esophageal region. The Ag-NORs during 2nd and 3rd days of embryonic development were counted in the cells of the pharyngeal walls at four regions as a number of Ag-NOR dots per cell. The Ag-NOR counts indicated that the cranial part of the pharynx (region I) have higher significant proliferative activity in the dorsal pharyngeal wall during the second day when compared with the dorsal wall in the third day of development. The proliferative activity indicated by the Ag-NORs dots showed increment in the epithelial wall of the dorsal pharyngeal pouch in pharyngeal walls at region II in comparison to that of the lateral pharyngeal pouches during both the second and third day of development. The ventral pharyngeal pouch showed the least proliferative activity. This conclusion may suggest an explanation for the caudal extension of this higher dorsal proliferative part from the configuration of region II during the second day to the configuration of region II

during the third day of development. The region III, showed low proliferative activity at the dorsal walls compared to the ventral and lateral wall during the second day of development, while it was higher in the dorsal pharyngeal pouch during the third day of incubation. This proliferative activity could be the basic developmental factors that cause the dorsal prolongation of the dorsal pharyngeal wall to become slit-like during the third day. The proliferative activity was higher in dorsal and bilateral wall of the slit-like pharyngeal walls as compared with ventral wall during the third day of development. At region IV of 2 day embryo, the proliferative activity show no significant differences in dorsal and lateral wall of the pharynx , but significant when compared with ventral wall.

At region IV, the wall of the respiratory diverticulum maintained comparable rate of proliferative activity that equivalent to that of the pharyngeal wall. This may suggest that the respiratory diverticulum (namely the tracheal bud) developed as a bud from the ventral aspect of the caudal part of the slit-like pharynx with a rate of proliferative activity similar to that of the pharyngeal wall. However; the orientation of the growth of the respiratory diverticulum differs from that of the pharyngeal wall, the respiratory diverticulum showed ventral growth. The result of Ag-NORs showed the evidences that support the result of the histological study which suggested that the respiratory diverticulum is a bud. Also the Ag-NORs result was making clear that the mesenchymal lectin binding goes with the idea that there is no septum formation.

The use of silver staining to identify nucleolar organizer regions (NORs) has increased dramatically in recent years. Ag-NOR method is cheap, simple and applicable to paraffin sections without the need of image analysis systems and can be performed in any laboratory. NORs are cellular structures present during the mitotic and G1 phases of cell division. Nucleolar organizer regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods. Each silver-stained dots corresponds, at the ultra structural level, to a fibrillar centre with a closely associated dense fibrillar components (17).The number and size of silver stained NORs (Ag-NORs) has been associated with increased rates of proliferation and decreased nuclear stability.

The Ag-NOR Technique provides an assessment of cell activity by indicating the degree of rDNA transcription. This provides a quantitative index of cell protein biosynthesis since ribosomes are essential in this process[17-21]. This method is highly specific and it identifies NOR-associated proteins rather than NORs themselves[22].

Conclusions:

Ag-NORs counting is a satisfactory method to evaluate the variation in the proliferative activity of pharyngeal epithelium according to both site and stage of development.

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