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RESEARCH ARTICLE

A STUDY OF EVALUATION OF AGNOR IN SALIVARY GLAND TUMORS.

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Abstract

Objective: The aim of this study was to evaluate the application of AgNOR in various benign and malignant salivary gland tumors by quantitative analysis of AgNOR and to determine its relation to histopathologic findings. As well as compare with normal salivary gland tissue as a control.

Study design: Forty- cases of benign and malignant salivary gland tumors were retrieved and the mean AgNOR count was calculated for all cases.

Results: Mean AgNOR counts were 1.72+_0.29, 2.76+_0.75, and 1.3+_ 0.86 in benign, malignant and normal salivary gland tissue respectively .Unpaired 't' test and ANOVA test was applied to test significance. A significant difference was observed in the mean AgNOR counts among the3 groups ($P < 0.001$). But among benign and malignant group there was no significant difference.

Conclusion: It can be suggested that the AgNOR technique can be applied on routine histological samples to assess the growth potential of salivary gland tumors. However, its prognostic value should be validated with clinical studies and survival analyses.

Introduction:-

Salivary gland tumors often pose considerable difficulty in differential diagnostic and prognostic assessment based on histomorphologic grounds alone. Histomorphohogy may poorly correlate with clinical outcome and the tumors in the same type of classification schedule exhibit different clinical courses. Prognostic relevance of various cell proliferation markers has been investigated in many types of human cancers, recently including salivary gland tumors. Evaluation of DNA content by flow cytometry, cytophotometry, AgNOR technique and immunohistochemical detection of antigens in cycling cells such as the Ki-67 antigen proliferating cell nuclear antigen (PCNA) have been applied to a variety of benign and malignant salivary gland tumors in only few studies so far.

In view of the above recent diagnostic and prognostic strategies, argyrophil staining for nucleolar organizer regions called as AgNOR technique has a promising future.(1,2,3,4) Nucleolar organizer regions are loops of ribosomal DNA occurring in the nucleoli possessing the genes for synthesizing rRNA. They are an essential part of the

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machinery of the nucleolus.(5) The number of NORs per nucleus has been related to the degree of cell activity and metabolism and subsequently to the malignancy.

The study was undertaken to correlate AgNOR count with the histologic findings of different salivary gland neoplasms and to evaluate the application of AgNOR for the assessment of benign and malignant salivary gland tumors.

Thus, this study aims to determine the importance of AgNOR staining technique in assessing various benign and malignant salivary gland tumors.

Materials and Methods:-

In the present study staining for nucleolar organizer regions was done in 40 cases for which formalin fixed paraffin embedded blocks were retrieved from the archives of department of oral pathology. Out of which 30 cases were of benign and malignant tumors of salivary gland and 10 blocks of normal salivary parenchymal tissue which were stained as controls.

Of 30 cases, 11 cases were benign salivary gland tumors, which consisted of 9 pleomorphic adenoma and 2 basal cell adenoma. In 19 malignant salivary gland tumors 4 cases were of mucoepidermoid carcinoma, 2 cases of adenoid cystic carcinoma, 3 cases of acinic cell carcinoma, 2 cases of polymorphous low grade adenocarcinomas 4 cases of adenocarcinoma and one each of myoepithelial carcinoma, salivary duct carcinoma, basal cell adenocarcinoma and epi-myoepithelial carcinoma.

Four micron sections were stained with silver nitrate solution method for visualization of NORs. No counter staining was performed. Control sections were also stained with H & E and AgNORs method. In the tumors, 100 cells from the lesional areas were observed under 100X oil immersion objective and counting of nucleoli was done. Subsequently data was analyzed statistically with unpaired 't' test and ANOVA test.

The silver reaction product was seen as discrete black dots at the light microscope level and was enumerated using a X100 oil immersion lens.

The counting protocol suggested by Crocker, et al. (6) was followed.

Observation and Result:-

In all specimens, clearly defined black dots of varying size recognized as AgNORs were observed in all of the yellow stained nuclei. The slide background was clean, with little or no extraneous silver precipitation. The nuclear AgNORs dots were easily countable.

There was little variation in terms of AgNORs numbers among the different cell types of pleomorphic adenoma within the same tumor, such as ductal cells, myoepithelial cells and squamous metaplasia cells except in chondroid area where AgNOR count was less as compared to cellular areas. The AgNORs in pleomorphic adenoma including 3 different cell types was 1.66 per nucleus (range 1-5 dots/nucleus). Moreover, pleomorphic adenoma rarely contained more than 4 dots/nucleus. The dots in pleomorphic adenoma and basal cell adenoma were round with smooth margins and their sizes were nearly the same. (FIG NO.1b)

2 cases of basal cell adenoma were studied in which mean AgNOR count was 2.025 dots/nucleus (range 1-5 dots/nucleus). (FIG. NO.1c, TABLE NO. 1.)

4 slides of mucoepidermoid carcinoma were studied which included 2 cases of low grade mucoepidermoid carcinoma and one case of intermediate and high grade mucoepidermoid carcinoma each. Mean count of low-grade mucoepidermoid carcinoma was 1.96 dots/ nucleus and intermediate grade mucoepidermoid carcinoma 3.60 dots/nucleus and the highest number of AgNORs were seen in high-grade mucoepidermoid carcinoma, which was 4.25 dots/nucleus (range 2 - 8 dots/nucleus). In the mucoepidermoid2 distinct cells types could be determined on the basis of AgNOR staining. The first was epidermoid cells, which contained mean 3.60 dots/nucleus and the second was mucous secreting cells, which contained a mean number of 1.96 dots/nucleus. (FIG. NO.2a, 2b,2c)

2 cases of adenoid cystic carcinoma were studied, mean count of cribriform area was 2.7 dots /nucleus and solid area numbers of dots were 3.20/nucleus (range 1-6 dots / nucleus). AgNORs per nucleus was seen as irregularly distributed silver stained material throughout the nuclei. (FIG. NO. 2f)

2 cases of polymorphous low grade adenocarcinomas were studied. Mean AgNOR count was 2.375 dots / nucleus (range 1-5 dots / nucleus). (FIG NO. 2 d, 2e)

Each case of acinic cell carcinoma showed different histologic pattern i.e. solid, tubular/microcystic, follicular and papillary. There was no significant difference between these different patterns and mean AgNOR count was 2.166 dots /nucleus (range 1-4 dots / nucleus). It was difficult to count dots in case of papillary type of acinic cell carcinoma. (FIG.NO.3a).

3 cases of adenocarcinoma were stained which showed mean AgNOR count of 2.54 dots /nucleus. Dots /nucleus were highly irregular and randomly distributed throughout the nucleus. As compared to other malignant tumors the sizes of AgNORs were less distinct and were smallest than those observed in other malignant tumors. (FIG. NO.3c)
One case each of salivary duct carcinoma, basal cell adenocarcinoma, malignant myoepithelioma and epi-myoepithelial carcinoma were studied. The mean AgNOR count in these tumor were 2.90 dots /nucleus, 4.28 dots /nucleus 1.85 dots /nucleus and 2.75 dots /nucleus (range 1-5 dots/nucleus). In case of basal cell adenocarcinoma AgNORs dots were large, distinct and range of dots was 1-4 dots /nucleus. The dots in the malignant neoplasms had a greater variability in size and shape compared to those in the benign tumors. (FIG.NO. 3f, 3b, 3e, 3d GRAPH NO.1)

The mean AgNOR count in benign salivary gland tumors was 1.84 dots / nucleus whereas in malignant salivary gland tumors mean AgNOR count was 2.46 dots /nucleus. The mean AgNOR count in the control specimens was 1.3 dots/nucleus (range 1-3 dots/nucleus). (FIG. NO. 1a). It has been observed that less number of AgNOR count was found in control specimens as compared to benign salivary gland tumors, which indicates the proliferative activity seen in benign tumors. Also there is definite increase in mean AgNOR count in malignant tumors as compared to benign tumors, which gives clarification of the difference in behavior of these tumors. Statistically analysis of AgNOR count: (Table no. 1 & 2). The differences between the groups were subjected to analysis by the unpaired ‘t’ test and ANOVA test. The difference in the mean numbers AgNOR between normal, benign and malignant tumors was statistically highly significant ($P < 0.01$). But difference between the mean numbers AgNOR among the benign tumors was non-significant. In case of malignant tumors difference between the mean numbers AgNOR was statistically significant only in case of adenocarcinoma and adenoid cystic carcinoma, adenocarcinoma and acinic cell carcinoma. The result between adenocarcinoma and polymorphous low grade adenocarcinomas was borderline i.e. (P value 0.06).

Discussion:-

Tumors of the salivary glands constitute an important area in the field of oral and maxillofacial pathology. A number of investigators have published their findings on salivary gland neoplasms, but a comparison of these studies is often difficult. Some studies have been limited to only the major glands or have not included all the minor salivary gland sites. In addition, the ever-evolving classification system makes an evaluation of some older studies difficult, especially when we try to compare them with more recent analyses. Notwithstanding these difficulties, it is still helpful to compare these studies because they provide a good overview of salivary neoplasia in general.

The NORs are so named, as the nucleolus is formed after mitotic division, in the two daughter cells at these specific sites on the five acrocentric chromosomes resulting in 10 NOR bearing chromosomes during metaphase. (2, 3 7, 8, 9) Though the exact nature of the NORs is not known as yet, they are thought to be associated with certain acidic or non-histone proteins like C23, B23 and RNA polymerase I. These proteins are thought to play a role in RNA transcription. They have an affinity for silver and bind to the argyrophilic stain, which is used to demonstrate them in the AgNOR technique. (10) Cytological studies have shown that NORs have a very high protein concentration and thereby a very high concentration of all kinds of silver reactive protein groups. (11)

Reduction of the silver ions at localized places occurs initially, which function as starting points for further deposition of reducible silver by growing faster and faster in an eventually auto catalytic process. Microscopically visible silver deposits may be formed. The carboxyl groups and sulphydryl group associated with NORs are thought to be responsible for this reaction.

In a normal diploid cell the individual NORs usually are not discernable because they are tightly aggregated in one or two nucleoli normally present in a cell. Active proliferation may be accompanied by nucleolar dissociation resulting in dispersed AgNORs throughout the nucleus. This as well as increase in transcriptional activity will result in an increase in the mean AgNOR count of a cell population. In malignancy, the AgNOR, tend to become more dispersed throughout the nucleus and thus more readily discernible. Thus the cancer cells are characterized by a higher numbers of NORs with a smaller size and a more irregular distribution than hyperplastic and normal cells proving it to be an indicator of the degree of malignancy.(11)

In the present study AgNOR counts for benign, malignant tumors and normal salivary gland parenchyma ranged from 1.1 to 2.15, 1.85 to 4.28, and 1.3 + 0.86 respectively. (Table no. 1 and 2)

According to Morgan DW, et al., (10) Epivatianos A, et al. (12), Wang SZ, et al. (13), Irani S et al. (14) and Adeyemi et al. (15) differences between the numbers of AgNORs in the benign and malignant groups, were highly significant.

The findings of present study also correlate well with the results of the above authors and difference between AgNOR count of benign and malignant salivary gland tumors was statistically significant ($P < 0.011$).Morgan DW, et al. (11) had suggested that difficulty is sometimes encountered in distinguishing between pleomorphic adenoma, Adenoid cystic carcinoma and adenocarcinoma, especially in small biopsies from salivary glands. But AgNOR staining technique is of diagnostic help in distinguishing between these salivary gland tumors.

In present study, we also found statistically significant difference between adenocarcinoma and acinic cell carcinoma ($p < 0.007$) and adenoid cystic carcinoma and acinic cell carcinoma ($p < 0.05$).

Epivatianos A, et al. (12) had concluded that the AgNOR, technique can be used as a diagnostic aid in differentiating between benign and malignant salivary gland tumors, and possibly the salivary duct carcinoma from adenoid cystic carcinoma and poorly differentiated mucoepidermoid carcinoma.

The mean AgNOR count for pleomorphic adenoma and adenoid cystic carcinoma was 1.6 + 0.28 and2.8 + 0.35, which is consistent with the findings of Fujita, et al. (16), Matsumura, et al. (17) and van Heerden, et al. (18)

The mean AgNOR count for epi-myoepithelial carcinoma in the present study was 2.75, which is consistent with findings of van Heerden, et al. (18)

In present study we found a low AgNOR count in polymorphous low grade adenocarcinoma as compared to adenoid cystic carcinoma but the difference was statistically non significant. This is in contrast to van Heerden, et al. (18) and Freitas RA, et al. (19) who detected statistical significant difference between the mean AgNOR count in polymorphous low grade adenocarcinoma and adenoid cystic carcinoma. (Table no. 3)

According to this author the higher count in adenoid cystic carcinoma could probably be related to the more aggressive behavior of this neoplasm when compared to polymorphous low grade adenocarcinoma.

The disparity in the result of our study could be due to scarcity of adequate number of cases.

Thus the mean AgNOR count in various benign and malignant salivary gland tumors correlate well with various other studies except in mucoepidermoid carcinoma. In the case of mucoepidermoid carcinoma a significant difference was found in similar studies carried out by Morgan DW, et al. (10), Matsumura, et. al. (17), and Alaeddini et al. (20). This can be attributed to the fact that histological grading done with present study was not consistent with that of above mentioned authors.

Fonseca I, et al. (21) and Landini G, et al. (22) evaluated the mean AgNOR count in normal salivary gland parenchyma and compared it with adenoid cystic carcinoma and pleomorphic adenoma respectively. The mean AgNOR count for adenoid cystic carcinoma was 4.2 ±99 and in normal salivary tissue was 1.21± 1.4 and for pleomorphic adenoma 2.06 for solid / ductal areas and 1.30 for chondroid areas, normal salivary gland it was 1.5 respectively. The findings of mean AgNOR count in the present study for normal salivary gland tissue correlate well with this study.

In the present study we found a gradual increase in mean AgNOR count from normal salivary gland parenchyma to benign and further to malignant salivary gland tumors also found statistically significant difference in the above three groups. Therefore, the above findings suggest the significance of this technique as an adjuvant diagnostic tool.

According to Landini G. (22) in the benign lesions, particularly pleomorphic adenoma in which the mitotic activity is very low, the AgNOR techniques provides a good method for a quantitative measurement of the cell activity.

Warnakulasuriya (12) has opined that "dispersion of AgNORs over the nucleoplasm appears to be a hallmark of malignancy".

Pich A et al. (23) in his studies on pharyngeal carcinoma suggested that AgNOR plays an important role in proliferative activity by putting forward the equation (PA = Ki-67 or MIB-1 scores X AgNORs), thus explaining the importance of AgNOR technique in order to estimate the actual proliferating activity of the cell.
The distinction between adenoid cystic carcinoma (ACC) and polymorphous low grade adenocarcinoma (PLGA) is important because ACC has a much more aggressive clinical course. In general ACCs are slow-growing tumors with prolonged survival, even in the face of metastatic disease.

However, ACCs are frequently relentless tumors with multiple recurrences and these tumors decrease patient's long-term survival rates, despite all attempts at therapeutic intervention.35 Almost all patients succumb sooner or later to this insidious tumor, which has been characterized by the term "wolf in a sheep's skin".

In general, the distinction between ACC and PLGA can be based on histologic examination. Cribriform, tubular and solid tumor cell arrangements can be found in both tumors. Mitotic activity can also be seen in both tumors.

Polymorphism is seldom seen in ACC. The nuclei of ACC are usually more hyperchromatic and more angular than those of. PLGA. ACC shows basophilic pools of glycosaminoglycans, which are not typical of PLGA.

Cytoplasmic staining of PLGA is eosinophilic to amphophilic, whereas ACC exhibits very pale to a clear staining. Immunohistochemistry may assist in their differentiation. Despite these differences, it can be very difficult to distinguish between ACC and PLGA, especially when only a small tissue fragment is submitted for histologic examination. Therefore it is essential to use a definite diagnostic technique, which will differentiate not only between benign and malignant tumors but also between the more aggressive and less aggressive tumors like ACC and PLGA respectively.

van Heerden, et al. (18) and Freitas RA, et al. (19) have found that solid and cribriform patterns of ACC have a higher AgNOR count indicating increased cellular activity as compared to tubular ACC and PLGA.

Thus suggests that AgNOR count in these neoplasms can be used a one of the criterion to establish a final diagnosis.

Conclusion:-

Thus the AgNOR staining can be useful in detecting increase proliferative activity of the tumors and can also be used in differentiating certain malignant salivary gland tumors especially ACC and PLGA as they have different prognostic implications. This AgNOR staining technique is one of the important research tools, which is simple, accurate and remarkably specific. In line with the observations it can be concluded that there is a definite difference in the AgNOR count of benign and malignant salivary gland tumors and between certain malignant salivary gland tumors like adenocarcinoma, acinic cell carcinoma and adenoid cystic carcinoma which show a distinct difference in AgNOR count. Although this technique is beneficial in differentiating between normal salivary gland parenchyma and benign and malignant salivary gland tumors, the overlapping of the AgNOR count between various tumors prohibited the use of this technique as absolute criterion in establishing a final diagnosis. However it is proposed that the AgNOR technique, which is rapid, simple and inexpensive, may be an useful adjuvant diagnostic tool in the differential diagnosis of benign and malignant salivary gland tumors and to differentiate between certain malignant salivary gland neoplasms.

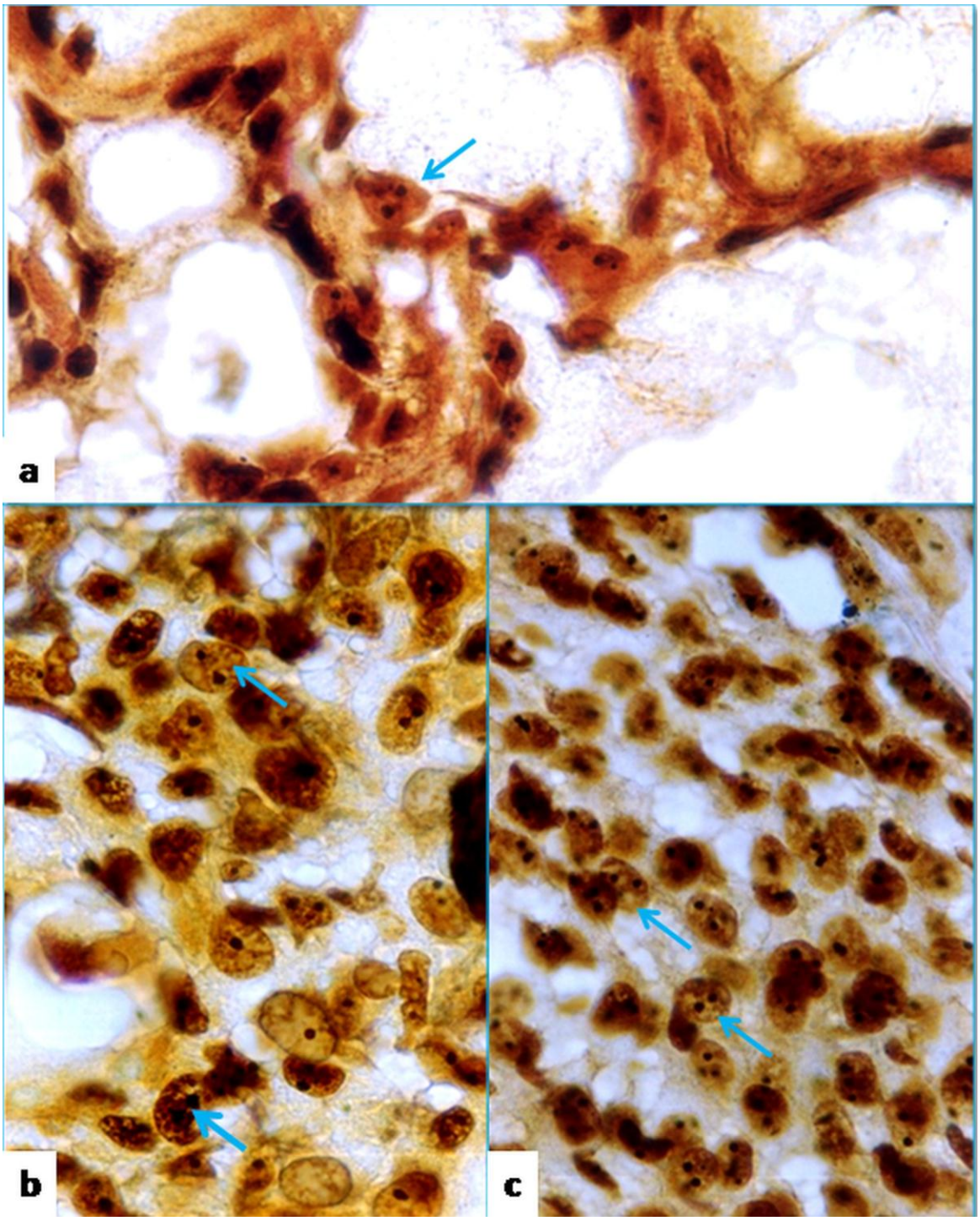


Fig. No. 1a:- AgNOR dots in normal salivary gland acini – range : 1-3 dots/nucleus (x100oil immersion)
Fig. No. 1b:- AgNOR dots in pleomorphic adenoma – range : 1-5 dots/nucleus (x 100oil immersion)
Fig. No. 1c:- AgNOR dots in basal cell adenoma – range : 1-5 dots/nucleus (x 100oil immersion)

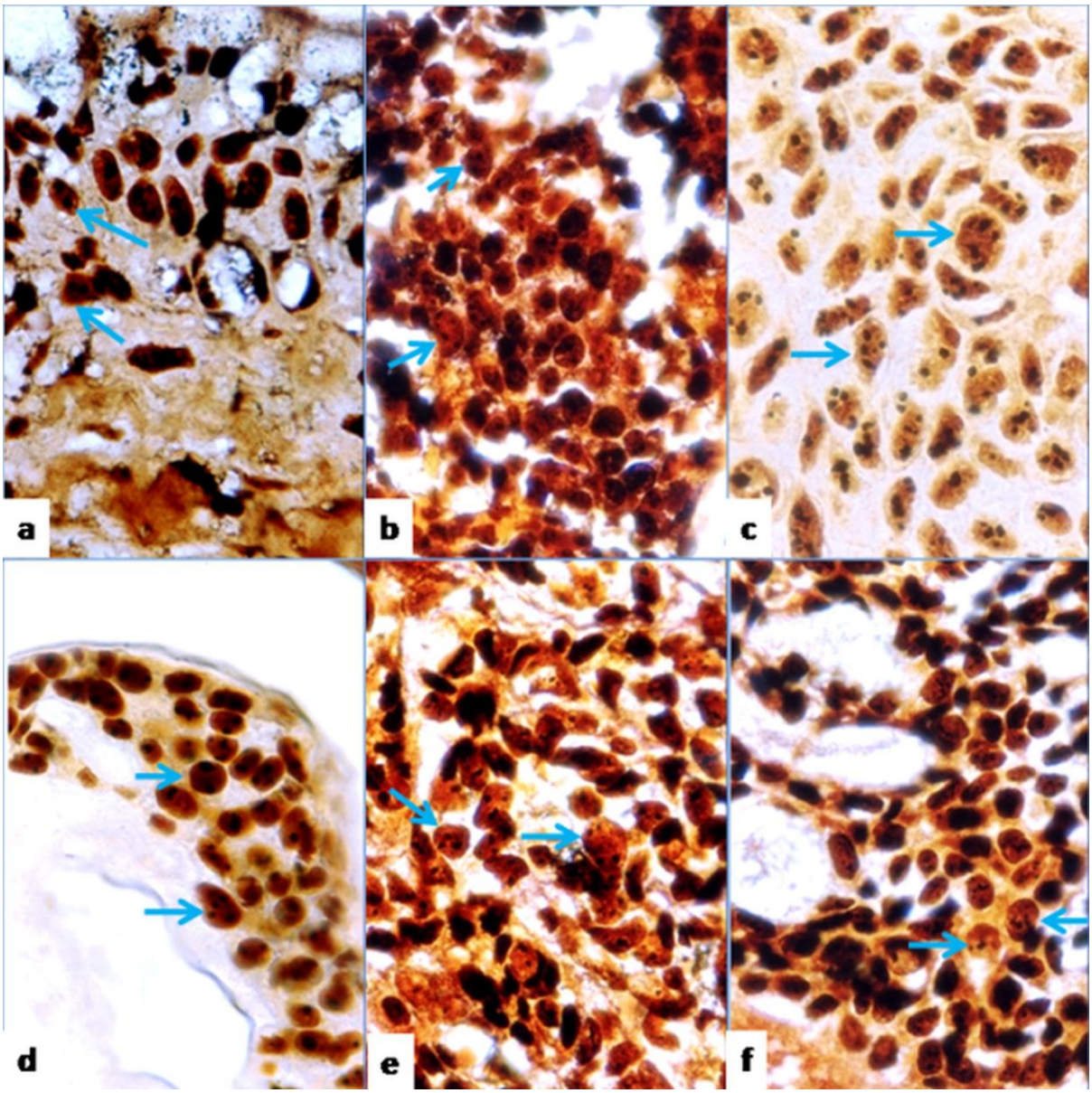


Fig. No. 2 AgNOR **a:-** dots in low (l) mucoepidermoid carcinoma – range : 1-4 dots/nucleus (x100oil immersion)
Fig. No. 2b:- AgNOR dots in intermediate (i) mucoepidermoid carcinoma – range : 1-5 dots/nucleus (x100oil immersion)
Fig. No. 2c:- AgNOR dots in high (h) mucoepidermoid carcinoma – range : 2-8 dots/nucleus (x100oil immersion)
Fig. No. 2d:- AgNOR dots in plga cribriform pattern – range : 1-5 dots/nucleus (100oil immersion)
fig. No. 2e:- AgNOR dots in plga lobular pattern – range : 1-5 dots/nucleus (x100oil immersion)

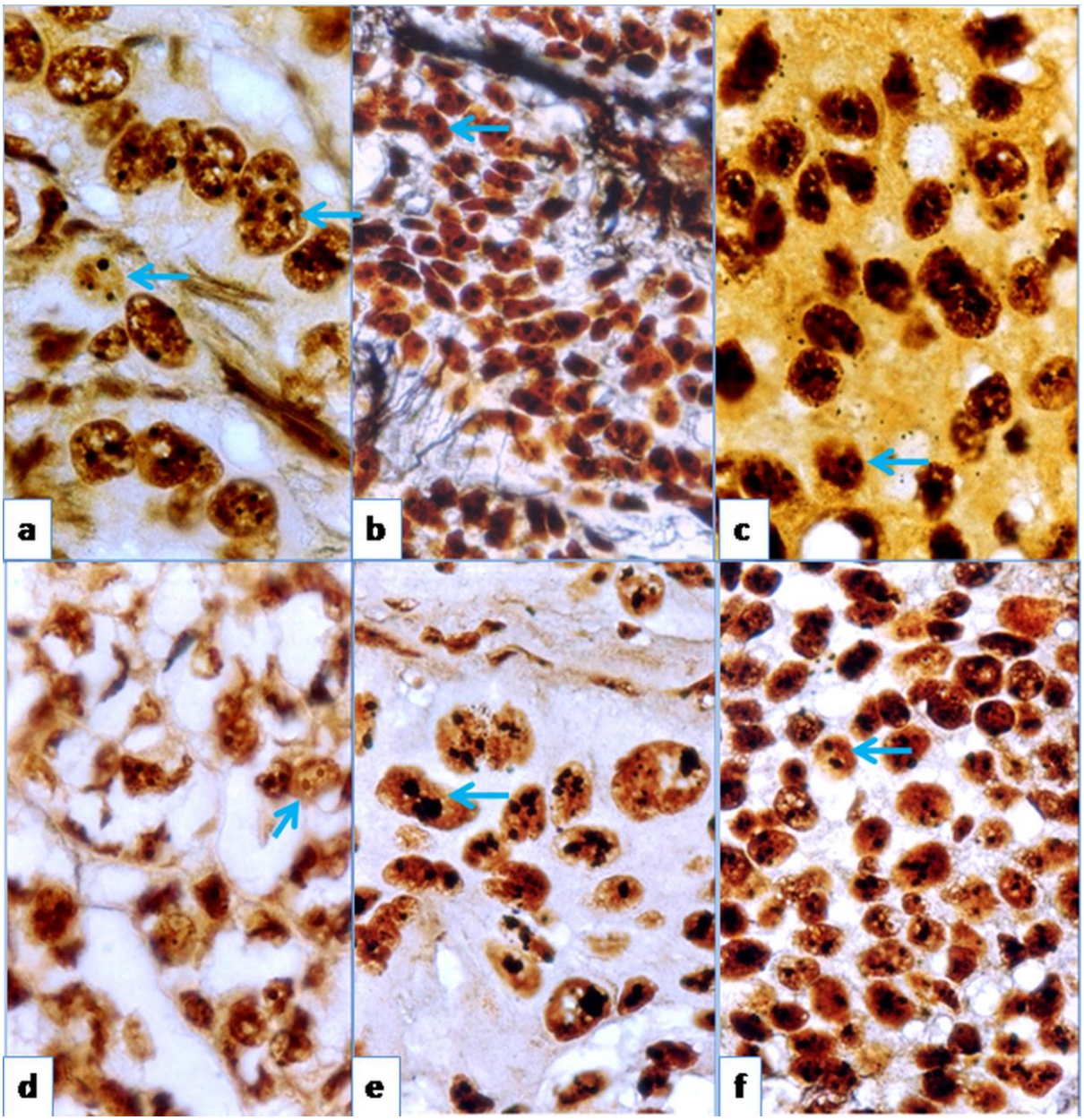
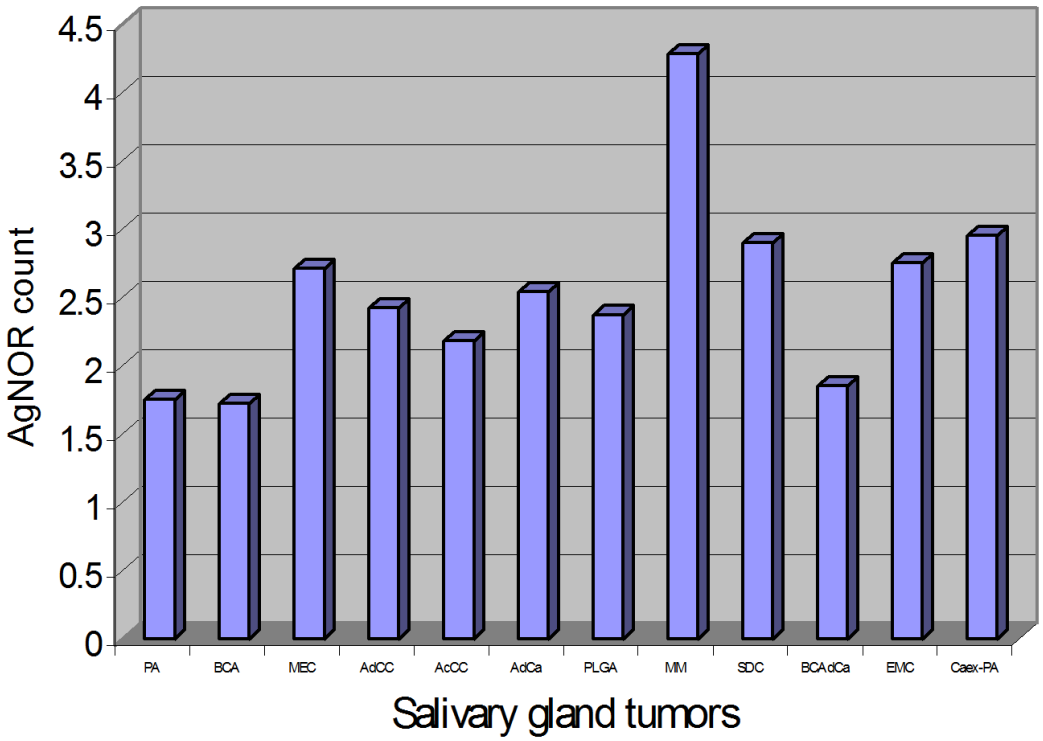


Fig. No. 3a:- AgNOR dots in acinic cell carcinoma – range : 1-4 dots/nucleus (x100oil immersion)
Fig. No.3b:- AgNOR dots in basal cell carcinoma – range : 1-4 dots/nucleus (x100oil immersion)
Fig. No. 3c:- AgNOR dots in adenocarcinoma – range : 2 - 6 dots/nucleus (x100oil immersion)
Fig. No. 3d:- AgNOR dots in epi-myoepithelial carcinoma – range : 1- 5 dots/nucleus (x100oil immersion)
Fig. No. 3e:- AgNOR dots in malignant myoepithelial carcinoma – range : 2-7 dots/nucleus (x100oil immersion)
Fig. No. 3f AgNOR**f:-** dots in salivary duct carcinoma – range : 2-5 dots/nucleus (x100oil immersion)



Graph no. 1:- AgNOR count in benign and malignant salivary gland tumors

Table no. 1:- AgNOR Count in Normal Salivary Gland Parenchyma

Sr. No.	AgNOR count	Mean ± SD
1	1.25	1.3 ± 0.86
2	1.25	
3	1.30	
4	1.70	
5	1.20	
6	1.8	
7	1.1	
8	1.1	
9	1.2	
10	1.2	

Table no. 2:- AgNOR Count in Benign and Malignant Salivary Gland Tumors

Sr. No.		AgNOR count	Mean ± SD	Mean of AgNOR count of benign and malignant tumors ± SD
I.	**Benign**			
Pleomorphic adenoma				
1		1.54	1.6 ± 0.28	1.72 ± 0.29
2		1.65		
3		1.70		
4		2.15		
5		1.80		
6		1.75		
7		1.75		
8		1.1		

9		1.5		
Basal cell adenoma				
10		2.10	2.02± 1.16	
11		1.95		
II.	Malignant			
Mucoepidermoid carcinoma				
1		3.60	2.94 ± 1.16	2.76 ± 0.75
2		4.25		
3		1.95		
4		1.97		
Adenoid cystic carcinoma				
5		2.55	2.8 ± 0.35	
6		3.05		
Acinic cell carcinoma				
7		2.3	2.16 ± 0.15	
8		2.2		
9		2.0		
Adenocarcinoma				
10		3.09	3.34 ± 0.37	
11		3.27		
12		3.75		
13		3.02		
Polymorphous low grade adenocarcinoma				
14		1.95	2.375 ± 0.60	
15		2.8		
Malignant myoepithelioma				
16		4.28	4.28	
Salivary duct carcinoma				
17		2.90	2.90	
Basal cell adenocarcinoma				
18		1.85	1.85	
Epi-myoepithelial carcinoma				
19		2.75	2.75	

Table No. 3:- Mean AgNOR count of certain individual benign and malignant salivary gland tumors of the present study and of some other authors.

	Present Study	van Heerden et al(18)	Morgan et al (10)	Matsumura et al (17)	Fonseca et al (212	Landini .(23)	G	Alaeddini et al (20)
						Solid / ductal area: 2.06 ± 0.25		
Pleomorphic adenoma	1.66□0.28	1.52□0.32	1.47	1.62				
						Chondroid area: 1.31 ± 0.12		
Warthin tumor	--			1.72				
Basal cell adenoma	2.22□0.10			--				
								L 0.64+-0,15
Mucoepidermoid carcinoma	2.94□1.66	1.93□0.55	4.25	2.05				I 1.04+-0.21
								H1.42+-0.23
Adenoid cystic carcinoma	2.82□0.35	2.83□0.89	3.92	2.78	4.2□0.90			
Acinic cell carcinoma	2.16□0.15							
Adenocarcinoma	3.34□0.37							
Polymorphous low grade adenocarcinoma	2.37□0.60	1.90□0.31						
Malignant myoepithelima	4.28							
Salivary duct carcinoma	2.90							
Epi-myoepithelial carcinoma	2.75	2.23						
Basal cell Adenocarcinoma	1.85							
Carcino ex-pleomorphic adenoma		2.05 □ 0.55						

Undifferentiated carcinoma				3.13							
Normal			1.37□0.86				1.21□1.4	1.51 ± 0.12			

Conflicts of Interst:-

1. Author Manisha Aba Sardar declares that she has no conflicts of interest.
2. Author Dr. V. K. Hazarey declares that he has no conflicts of interest.
3. Author Dr. S. M. Ganvir declares that she has no conflicts of interest.

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Ethical Approval:-

The above study has been approved by the Institutional Ethics Committee of Government Dental College & Hospital, Nagpur and is in compliance with the seventh revision of Declarations of Helsinki.

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