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Silver Stained Nucleolar Organising Region (AgNOR) Count and its Correlation with Colposcopy in Suspicious Cervical Pathology

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Abstract
Introduction: *Colposcopy is considered a sensitive tool for screening of cervical malignancy. Colposcopy directed biopsy helps in confirming diagnosis. Silver stained nucleolar organising region (AgNOR count) of dysplastic cells further enhances the diagnosis.*
Aim: *To study the correlation of AgNOR count and colposcopy in suspicious cervical lesions.*
Materials and methods: *200 women of reproductive age group were screened for cervical cancer and colposcopy directed biopsy taken. AgNOR count studied and correlated with HPE and Modified Reid'scolposcopic index.*
Results: *There was a good correlation between AgNOR count and the degree of dysplasia. Also there was a good correlation of AgNOR count and Modified Reid'scolposcopic index.*
Conclusions: *AgNOR count is simple, effective method and it adds strength to histopathology and colposcopy and also assess different grades of cervical intraepithelial neoplasia.*
Keywords: *colposcopy, cervical intraepithelial neoplasia, AgNOR count, Reid's colposcopic index.*

Introduction

Early Diagnosis of precancerous lesion of cervix and treatment leads to 100% cure rate. The cervical malignancy is ideal disease for screening as it has long preinvasive period hence the screening of the cancer cervix is emerged as the important part of non-communicable disease screening. Colposcopy is one of the most important and highly sensitive methods to visualize the ectocervix under magnification and to find out cervical suspicious lesion. Histopathological prediction of colposcopy is obtained by Modified Reid's Colposcopic Index [1]. But the final definitive diagnosis is by colposcopy guided cervical biopsy and histopathology. Sometimes during histopathological examination there may be few confusions to arrive at exact diagnosis, at that occasions there may be few adjunct methods like immunohistochemistry, tumour marker, silver staining are used.

AgNOR count indicates proliferating activity of cell. Nucleolar Organising Regions [2] are the loops of DNA coding for ribosomal RNA and they are closely bound within nucleoli in normal cells. But in highly multiplying cells like malignant cells, Nucleolar Organising Regions are loosely dispersed and they have great affinity for silver that is they are

argyrophilic[3],[4], hence AgNOR count increases from benign to malignant condition hence it has an important role in diagnostic oncopathology. In this study correlation between AgNOR count and clinical prediction of doubtful lesions of cervix using colposcope is analysed.

Aim of the Study

- Aim of this study is to correlate AgNOR count and colposcopy in suspicious cervical lesion.

Objectives

- To determine the diagnostic accuracy of colposcopy.
- To study the variation in number of AgNOR count as the disease progresses from benign to malignant lesion.
- To find the correlation between colposcopy and AgNOR count in suspicious lesions of Uterine Cervix.
- To find out the usefulness of AgNOR dot count in classifying different grades of cervical intraepithelial neoplasm.

Materials and Methods

This study was conducted on 200 women of reproductive age group from 30 years to 45 years of age. Inclusion Criteria include women with complaints of abnormal vaginal discharge, low backache, intermenstrual spotting and post coital bleeding. Exclusion Criteria is frank cancer cervix on Speculum examination

Study method

1. The patients are included in the study after explaining them about the procedure and benefits and after getting consent. Privacy maintained.
2. A detailed history including age, parity, menstrual history, marital history, medical history, age at first intercourse, frequency of coitus and prior history of cervical screening, STD's, personal habits, socioeconomic status and literacy are elicited.
3. They are subjected to detailed clinical examination including abdominal examination, per speculum, vaginal examination. On speculum examination, original squamous epithelium appears pinkish in colour and metaplastic epithelium appears pinkish white in colour.
4. They are subjected to COLOPOSCOPIC examination.

Colposcope is a low power field binocular microscope which is stereoscopic and having a light source of adjustable intensity used to examine lower genital tract. The working distance that is the distance between the patient and objective should be accurate and it is usually 250 to 300 mm. Patient should be in Modified lithotomy position. Requirements for colposcopic examination: All the instruments are placed in a tray. They include

- Cusco's speculum - Bivalve self-retaining
- Cotton swab
- Vaginal side wall retractor or condom
- Sponge holding forceps
- Endocervical speculum
- Cervical polyp forceps
- Punch biopsy forceps
- 3 per cent acetic acid solution
- Lugol's Iodine
- Normal Saline
- Formalin solution
- Cervical cytology brush.
- Bottle containing alcohol for fixing cytological smears
- Monsel's paste

Basic principles: Examination of cervix was done using normal saline, 3 percent acetic acid and lugol's Iodine solution. Normal Saline is applied to study the vasculature in the stroma beneath the epithelial layers. The green or blue filter is used to see the vessels clearly by removing the red light. Three percent acetic acid can be prepared by adding 3 ml of glacial acetic acid to 97 ml of distilled water and the resulting solution is mixed well. At the end of a day, remaining solution should be discarded. Application of 3% acetic acid is by using a cotton swab in sponge holder.

Procedure of Colposcopy:

i. Insert the bivalve cusco's speculum to visualise the cervix and look for abnormal discharge, polyp, ectopy, leukoplakia, nabothian follicle, hyperkeratosis, any vaginal ulcer and condyloma etc. speculum is lubricated with warm water.

ii. Using a cotton swab soaked in normal saline, excess secretions are removed. Dry swabs should not be used as it may traumatise the cervix.To define the transformation zone,the proximal border is taken as new squamocolumnar junction and the distal border that is the original squamocolumnar junction is found by line joining nabothian cyst or crypt openings over the ectocervix. If new squamocolumnar junction is not visualised completely then the colposcopic examination is unsatisfactory and it is visualised by using endocervical speculum to retract the external cervical os to visualise new SCJ in endocervical canal.

iii. Acetic acid application using cotton swab or sprayer. It helps to visualise transformation zone, to find abnormal acetowhite areas, to coagulate and clear the mucus for better visualisation of cervix. The Ace to white areas will appear slowly over one minute and may disappear afterwards, so reapplication using a cotton swab may be required every 2 to 3 minutes from the acetic acid pooled in posterior fornix.

iv. Lugol's Iodine application – Abnormal areas have little or lacks glycogen. So abnormal areas may appear partially brown to mustard yellow depending on severity and degree of differentiation.

v. Immature squamous metaplastic epithelium appear on acetic acid application as pinkish white, translucent areas intervening with areas of columnar epithelium with ill-defined margins and smooth surface with openings of crypts and effect lasts for less than 1 minute, confined to transformation zone and Iodine uptake depends on glycogenation. Mature squamous metaplastic epithelium appear on acetic acid application light pinkish areas with ill-defined margins smooth surface containing crypt openings and nabothian follicles, confined to transformation zone and take up lugol's iodine to form mahogany brown or black areas. Inflammatory lesions appear on acetic acid application pale patchy areas with islands of necrosis with ill-defined margins, variegated surface, not confined to transformation zone and the effect last for less than two minutes, finepunctations may be seen and show partial iodine uptake on lugol's iodine application.

Abnormalities after applying normal saline, 3% acetic acid and lugol's iodine were documented and colposcopic prediction of histopathological diagnosis was obtained on the basis of Modified Reid's Colposcopic Index [4]. Modified.RCI includes 4 categories of abnormalities, each were given the score of 0 to 2 and the grade of cervical intraepithelial lesions were predicted by adding scores of all four categories.

REID'S COLPOSCOPIC INDEX

Point	Colour	Vessels	Border	Iodine staining
0	Faint,shiny, snow white	Fine	Feathery, indistinct	Mahoga n-y brown
1	Grey white	No surface vessels	Smooth, straight	Tortoise shell
2	Oyster grey	Punctuatio ns Mosaic atypical	Internal borders rolled, edges peeling	Mustard yellow

4. Cervical biopsy is taken from abnormal areas using punch biopsy forceps under colposcopic guidance. If multiple abnormal areas are present then more than one biopsy was taken from the region showing worst abnormalities and those that are near the squamocolumnar junction.

5. The specimen is kept in 10% formalin. The specimen should be deep enough to have adequate stroma. While taking biopsy repeated crushing and cutting of tissues was avoided. This procedure is painless.

6. Ferric subsulphate (Monsel's) paste is applied firmly over biopsy site using a cotton swab to obtain haemostasis after cervical biopsy
7. Biopsy specimens are preserved in formalin solution and sent to pathology laboratory.
8. Usual Paraffin sectioning is done. Routine haematoxylin and eosin staining is done and histopathological diagnosis is made.
9. Silver staining is done on the sections made from same paraffin block using one step silver staining method.
10. In a slide, AgNOR dots are counted as black dots per 100 cells using 100× oil immersion lens.
11. AgNOR count is obtained as mean of AgNOR dots in 100 cells.
12. AgNOR count along with histopathological diagnosis are correlated with colposcopic prediction of histopathology to show significant increase in AgNOR count as the lesion progresses from benign to malignant.

After collecting information, all the data are tabulated in master chart and analysis done using statistical package for social science version17.0. Statistical analyses are carried out by using chi-square test, ANOVA, tucky HSD.

AgNOR– Technical Aspects: Method used in AgNOR staining is one step silver staining method. The advantages are it is a simpler method. The disadvantages are time consuming and inter observer variation in counting small dots. A mixture of 50% silver nitrate solution (Solution A) and 1% formic acid in 2gms% of gelatin solution (Solution B) (colloid stabilizer) is taken. These solutions are separately prepared and mixed just before staining. Histological slides are incubated for 45 min to one hour in the above mentioned solution and then washed, dehydrated. Then it is cleared and mounted under light microscopy for examination. This method is more specific method in detecting inter phase or metaphase NORs. On light microscopy, NORs are visualised as discrete black / brown dots in a background of pale yellow. AgNOR dots can be counted by using 100x oil immersion lens. AgNOR dots counted on 100 cells and mean is found and the results are expressed as mean number of AgNORs counted per nucleus. Lymphocytes are routinely used as internal controls.

Results

1. In this study, women of reproductive age between 30 and 45 were included and the mean age was 36.97 years. Among 101 HPE positive women, 35 women were between age group of 30 and 35 years, 45 women were between 36 and 40 years and 19 women were between 41 and 45 years.
2. Majority of women, about 53.5% were para 3 or more.
3. Most of the women, 78.5% women belonged to class IV socioeconomic status according to Modified Kuppusamy socioeconomic scale [5].
4. 62% of women were illiterate. Among 124 illiterate women, 72 were positive on biopsy.
5. Only 11.5% of women had history of previous cervical screening.
6. Minimum age of first intercourse was 15 years and maximum age was 25 years. The mean age of first intercourse was 19.84
7. Out of 200 cases, 99 women had chronic cervicitis, 36 had CIN I, 31 had CIN II, 18 had CIN III and 16 had squamous cell carcinoma.
8. Most common symptom was abnormal vaginal discharge (75.5%) followed by intermenstrual bleeding (12 %), low back ache (8%) and post coital bleeding (4.5%).
9. 9.Accuracy of colposcopy (Table-1): Sensitivity of colposcopy- 94.1%, Specificity of colposcopy-63.6%, Positive predictive value of colposcopy-72.5% and Negative predictive value of colposcopy-91.3%
10. Association between colposcopy and histopathology: As shown in table 2, Out of 69 cases with Reid's index 0 to 2 (colposcopy negative), only 6 cases were CINI. Out of 71 cases with Reid's index 3 to 4, 36 cases were chronic cervicitis, 30 cases were CIN I and 5 cases were CIN II. Out of 60 cases with Reid's index 5 to 8, about 26

cases were CIN II, 18 cases were CIN III and 16 cases were squamous cell carcinoma.

11. Association between AgNOR count and histopathology. All 99 cases with AgNOR count of 0 to 2 were chronic cervicitis. All 36 cases with AgNOR count of 2 to 4 were CIN I. Cases with AgNOR count of 4.1 to5, about 31 were CIN II and 8 cases were CIN III. Cases with AgNOR count greater than 5, about 10 were CIN III and 16 cases were invasive carcinoma.
12. Association of AgNOR count and colposcopy: The mean AgNOR count rose from 1.55 in cases with Mod. Reid's index of 0 to2 to5.05 in cases with Mod.Reid's index of 5 to 8 as shown in figure [i].
13. Table 3 Shows the correlation between modified RCI and AgNOR count with pearson correlation coefficient of 0.910 and P value of <0.001 which is statistically highly significant.
14. The mean AgNOR count increased with the severity of cervical pathology from 1.4 in chronic cervicitis to 6.2 in squamous cell carcinoma as seen in figure [ii].
15. Table 4 shows there is significant difference in mean AgNOR count as the disease progresses from chronic cervicitis to CIN-I to CIN-II to CIN-III to Squamous cell carcinoma which is statistically highly significant with pvalue<0.001.

Discussion

Accuracy of colposcopy

The following table showed sensitivity and specificity of colposcopy in various other studies.

Author	Sensitivity %	Specificity %
Stewart[6]et al	89	52
Lozowski[7]et al	96	29
Present study	94.1	63.6

Mean AgNOR count: In this study, the mean AgNOR count increases as disease progresses from chronic cervicitis to CIN I to CIN II to CIN III to squamous cell carcinoma. Samarth [8] et al (2012) conducted a study on 50high risk women. They took 2 cervical smears. One was used to obtain histopathological diagnosis and another one was used for AgNOR count. The mean AgNOR count showed progressive increase with the severity of the lesions .Normal cervix -1.75, chronic cervicitis-2.09, low squamous intraepithelial lesion (LSIL)-2.81, High Squamous Intraepithelial Lesion (HSIL)-4.17 and carcinoma cervix-5.98. This study also showed significant difference in AgNOR count between various lesions of cervix. MisraJS [9] et al., in 2005, 50 cervical smears were studied. The AgNOR count of normal smear and inflammatory smears was from 1 to 2. The AgNOR count of LSIL ranged from 2 to 4 and the HSIL ranged from 6 to 8. The squamous cell carcinoma had 10 AgNOR dots. This study also correlated well with present study. Singh Uma [10] et al., in 2006, conducted a study by using cervical biopsy specimen. The AgNOR count of CIN I was 1.64, that of CIN II was 2.68 and that of CIN III was 4.3.In a study conducted by Egan and Crocker [11] et al mean AgNOR count of CIN I is 2.3, CIN II is3.5 and CIN III is 4.7 (1990). Egan et al found mean AgNOR count increased from CIN I to CIN III, whereas the mean size of the AgNOR dots decreased from CIN-I to CIN-III. This also correlated well with present study. In a study conducted by Pratibha and Kuruvilla [12] (1995) showed mean AgNOR count increases as the pathology progresses to more severe lesion. The mean AgNOR count of normal cervix is 1.2 that of CIN I, II is 1.8, that of CIN III is 3.0 and mean AgNOR count of carcinoma is 4.3.Kaushik [13] et al found mean AgNOR of 1.36 in case of normal cervix, 2.56 in case of CIN-I, 4.28 in case of CIN-II, 5.16 in case of CIN-III, and 6.40 in case of invasive lesion.

Reference Studies	CIN I	CIN II	CIN III
Singh U et al	1.64	2.68	4.3
Crocker J et al	2.3	3.5	4.7
Kaushik et al	2.56	4.28	5.16
Present Study	2.52	4.32	5.05

Table showing mean AgNOR count in different studies

Significant difference in AgNOR count according to CIN: The present study shows there is statistically significant difference between mean

AgNOR count of different grades of cervical intraepithelial neoplasms and the p value is <0.001.In a study conducted by K C Shivraj et al showed there was significant difference between mean AgNOR count of different grades of lesions with the p value of <0.01 except there was not much difference between chronic cervicitis and low grade squamous intraepithelial lesions. In another study by Prathiba and Kuruvila also showed there was significant difference in mean AgNOR count between benign, premalignant and malignant lesion.

Correlation between colposcopy and AgNOR count: There exist a significant correlation between colposcopy and AgNOR count. Pearson correlation coefficient is .910 with the p value of <0.001.In a study conducted by Ritu Goyal [14] et al also showed there is significant correlation between colposcopy and AgNOR count with pearson correlation coefficient of 0.892. In this study, the mean AgNOR count increases as disease progresses from chronic cervicitis to CIN I to CIN II to CIN III to squamous cell carcinoma. The mean AgNOR count for chronic cervicitis was1.48, the mean AgNOR count for CIN I was 2.52, mean AgNOR count for CIN II was 4.32, mean AgNOR count for CIN III was 5.05 and mean AgNOR count for squamous cell carcinoma was 6.20.The present study shows there is statistically significant difference between mean AgNOR count of different grades of cervical intraepithelial neoplasms and the p value is <0.001.

Conclusion

One effective way to prevent cervical cancer is to diagnose at preinvasive stages of disease. Colposcopy is a simple and inexpensive method of visualising the doubtful cervix under magnification and it is an effective tool in screening of suspicious lesions of cervix and to take biopsy for histopathological diagnosis. When histopathological diagnosis is difficult and doubtful, it can be combined with AgNOR count. As AgNOR count is a marker of proliferative activity of a cell. Our study showed there is statistically significant difference in mean AgNOR count between different grades of cervical intraepithelial neoplasia. Our study also showed there is statistically highly significant correlation between colposcopy and AgNOR count. All these observations support AgNOR count is simple, most effective and reproducible method and it adds strength to histopathology and colposcopy in suspicious cervical lesions and to assess different grades of cervical intraepithelial neoplasia.

Table 1 Accuracy of colposcopy

Colposcopy	HPE		Total	P Value
	Positive	Negative		
Positive	95	36	131	<0.001**
Negative	6	63	69	
Total	101	99	200	

** denotes significant at 1%

Table 2 Association between colposcopy and histopathology

HPE	Modified RCI			Total
	0-2	3-4	5-8	
CC	63	36	0	99
CIN I	6	30	0	36
CIN II	0	5	26	31
CIN III	0	0	18	18
SCC	0	0	16	16
Total	69	71	60	200

Table 3 Correlation between colposcopy and AgNOR count

Variables	Correlation Coefficient	P Value
Mod. RCI and AgNOR	0.91	<0.001**

Table-4 Significant difference in AgNOR count with regards to HPE

(I)HPE	(J) HPE	AgNOR count		P Value	95% Confidence Interval	
		Mean difference (I-J)	Std. Error		Lower Bound	Upper Bound
CC	CIN I	-1.0443	0.04698	<0.001	-1.1736	-0.9149
CIN I	CIN II	-1.8012	0.05914	<0.001	-1.9641	-1.6384
CIN II	CIN III	-0.7268	0.07153	<0.001	-0.9238	-0.5299
CIN III	SCC	-1.1488	0.08293	<0.001	-1.3772	-0.9205

Fig [i] AgNOR count and colposcopy

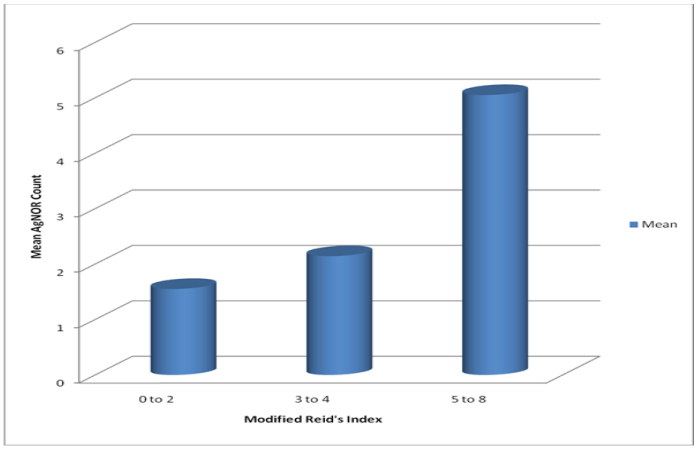
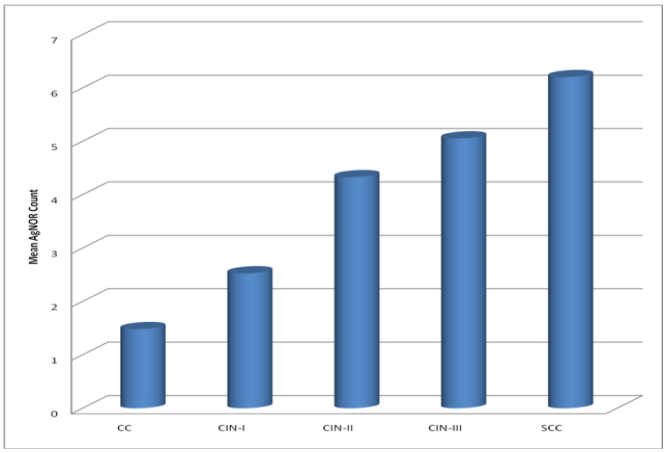


Chart 2 Mean AgNOR Count for each Cervical Pathology



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