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ANALYSIS OF MITOTIC ACTIVITY IN EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA : A HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY

Oral Pathology

Dr. Sonal Lahoti M.D.S Oral Pathology and Microbiology

Dr Kavita Rao* M.D.S Oral Pathology and Microbiology *Corresponding Author

Dr. Rajiv Ranjan Sinha M.D Anatomy

Dr Kumar Ashish MBBS

ABSTRACT

Introduction- This retrospective laboratory based study was performed to evaluate and correlate the mitotic activity count and Ki67 labeling index in epithelial dysplasia and oral squamous cell carcinoma cases.

Methodology- 75 cases were retrieved from the archives of Department of Oral Pathology and Microbiology. All the slides were stained with Crystal Violet special stain and Ki67 antibody. The Mitotic Activity Count was performed using an occulometer grid and Ki67 count was done using an image pro-express software in 10 consecutive high power fields.

Results - Statistically significant difference in the values of mitotic activity index and Ki-67 labeling index between epithelial dysplasia and oral squamous cell carcinoma cases were observed.

Discussion- Mitotic activity using Crystal Violet stain and Ki67 labeling index were significantly increased from epithelial dysplasia to poorly differentiated oral squamous cell carcinoma cases. A positive correlation between mitosis and Ki67 count indicates the usefulness of Crystal violet as a selective stain for mitotic figures.

KEYWORDS

epithelial dysplasia , squamous cell carcinoma , mitotic figures , Ki-67 , Crystal violet

INTRODUCTION

Mitosis of cells gives rise to tissue integrity. The various chromosome arrangements in mitotic cells are referred to as mitotic figures.[1] Defects of mitosis results in various nuclear abnormalities, namely, micronuclei, binucleation, broken egg appearance, pyknotic nuclei and increased number of and / or abnormal mitotic figures.[2] These abnormal mitotic figures are commonly seen in oral epithelial dysplasia and squamous cell carcinoma.[2] The distinction between a pyknotic nucleus, an apoptotic cell and a mitotic cell in a routinely stained tissue section may pose a problem. Errors in identifying a mitotic cell can thus weaken the reliability of histological grading due to the loose use of morphologic criteria. Combination of stains and modification of the existing histochemical techniques can overcome these problems.[2] Crystal violet stain is a step ahead of the standard H&E stain, which facilitates the identification of mitotic figures even at lower magnification as compared to H&E stain.[3] Crystal violet is a selective stain for analyzing mitotic activity (mitotic figures) as it has a high affinity for the highly acidic chromatin of mitotic cells. Mitotic cells are stained magenta and stand out distinctly against a light blue background of resting cells.[2]

Hyperproliferation is thought to be an early marker of disorderly growth. [5] Ki-67 is a human nuclear antigen and is believed to be a reliable marker of cell proliferation as this nonhistone protein can be expressed at all the stages of the cell cycle except G0.[5] Presence of brown precipitate at the site of target antigen (nucleus) is indicative of positive immunoreactivity.[6] Therefore this present study analyzes mitotic activity in epithelial dysplasia and oral squamous cell carcinoma using 1% Crystal Violet stain and Ki67 immunomarker and correlates mitotic counts obtained by using 1% Crystal violet and Ki-67 immunopositive mitotic cells.

1. MATERIALS & METHODS

This retrospective study was carried out on tissue sections obtained from diagnosed cases of leukoplakia with epithelial dysplasia and Oral squamous cell carcinoma (OSCC), retrieved from the archives of Department of Oral Pathology and Microbiology , V.S Dental College and Hospital, Bangalore. The study group comprised a total of 75 cases, 15 cases of normal gingival tissue , 15 cases of leukoplakia with dysplasia , 15 cases of well differentiated SCC , 15 cases of moderately differentiated SCC and 15 cases of poorly differentiated SCC. Two sections of the positively diagnosed cases were made. One section was stained with 1% crystal violet stain and the other section was stained with antibody against Ki67 nuclear antigen , an immunohistochemical marker.

1.1 VIEWING AND ASSESSMENT : COUNTING OF MITOTIC FIGURES

Mitotic figures were assessed in 10 consecutive high power fields of Crystal violet stained section using an occulometer grid , according to the Vandiest et al criteria in 4 different groups (Group I leukoplakia , Group II Well differentiated SCC , Group III Moderately differentiated SCC , Group IV Poorly differentiated SCC) .

Mitotic Activity Index was determined as given by Jannink et al[7] : Number of mitotic figures / 10 hpf

2.1.1 Factors considered while counting mitotic counts

1) Tumor sections that contain the most abundant mitosis should be used since cell proliferation is subjected to significant regional differences.
2) The interval between death and fixation of tumor tissue should not be prolonged , late or insufficient fixation of tumor especially in the center of node that have not been sectioned results in lower mitosis rate.
3) Observer should not be restricted to single phase mitosis such as metaphase and anaphase ,falsely low values will be obtained.
4) Do not involve the karryorhectic and pyknotic nuclei.[8]

2.1.2 Protocol for proper mitotic counting

1) Microscopic requirements
2) Slide quality
3) Selection of the areas for counting
4) Criteria for selection of mitotic figures
5) Counting procedure [9]

MICROSCOPIC REQUIREMENTS

For the counting of mitotic figures , an ordinary light microscope should be used with a 10x ocular and a 40x objective , a numerical aperture of 0.75 and the field diameter of 450um.[9]

SLIDE QUALITY

The paraffin sections on the slide should be an adequately stretched section with a standard thickness of 5um. In a section of 5um , the objective has a depth of field at around 1 um. Even though mitotis is better appreciated when the depth of focus is varied, mitosis should be counted within one depth of field range.[9]

SELECTION OF THE AREAS FOR COUNTING

1) The most cellular region of the lesion must be selected , preferably at the periphery of the tumor avoiding those regions showing

necrosis , inflammation or calcification as much as possible. As the periphery of the tumor is the most proliferating part of the carcinoma, as the peripheral region is more closely related to blood vessel supply.

2) All the fields which contain an area of less than 50% of the tumor cells must be omitted out from inclusion into the counting procedure
3) If several areas of tumor have met these criteria, the area subjectively found to have the highest density of mitotic figure is chosen
4) In the most cellular region , a counting area of approximately 0.5*0.5 diameter is marked. If this area does not contain 10 HPF's , 2 or more areas that meet the above criteria are selected. If 10 HPF's cannot be selected , the counting is performed in the available fields and the number of mitosis then found is extrapolated to 10 HPF's to obtain the mitotic index.[9]

2.1.3 Counting Procedure

The high power field in which counting is to be done is bisected by a linear eyepiece micrometer. The mitotic figures intercepted by this line are counted first. Then the mitotic figures adjacent to the mitotic figures in central lines are counted carefully to avoid the inadvertent recounting of mitotic figures .This procedure is followed for all the 10HPF's.[9]

2.2 VIEWING AND ASSESSMENT : Ki67

IHC stained sections were evaluated for Ki67 positive cells in basal , parabasal and suprabasal layers of the epithelium using an Image proexpress software in 4 different groups(Group I leukoplakia , Group II Well differentiated SCC , Group III Moderately differentiated SCC , Group IV Poorly differentiated SCC). Cells were considered positive for Ki67 antigen , if there was intranuclear DAB staining . Brown to dark brown nuclear staining cells were scored in 10 consecutive high power fields and Ki67 labeling index was determined as given by Karring and Loe[10]

Number of positive nuclear profiles / mm^2

2.3 Inclusion Criteria

1) Normal oral mucosa obtained from patients undergoing extraction for orthodontic purpose or Impacted teeth
2) Epithelial dysplasia
3) Incisional and excisional biopsies of primary squamous cell carcinoma

2.4 Exclusion Criteria

1) For normal oral mucosa, biopsy of the subjects with signs of inflammatory gingival and periodontal disease.
2) Secondary and Metastatic tumors of Oral Squamous Carcinoma
3) Cases with Hypertension, Diabetes Mellitus and Bleeding disorders

2. RESULTS

In our study , we had a sample size of 75. The sections were stained with Crystal Violet special stain as seen in Fig 1 ,Fig 3 , Fig 5 , Fig 7 and Ki67 immunohistochemical marker as seen in Fig 2 , Fig 4 , Fig 6 , Fig 8 Mitotic Activity Index and Ki67 Labeling Index were quantified in 10 hpf for each of the study group.

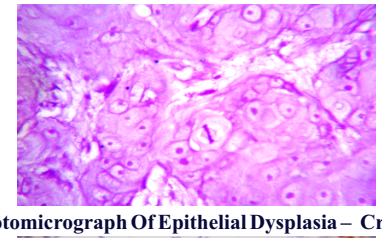

Fig 1 : Photomicrograph Of Epithelial Dysplasia – Crystal Violet

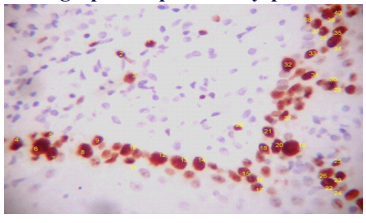

Fig 2: photomicrograph of epithelial dysplasia – ki 67

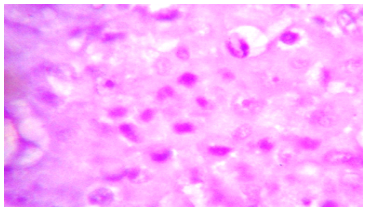

Fig 3: photomicrograph of wdscc – crystal violet

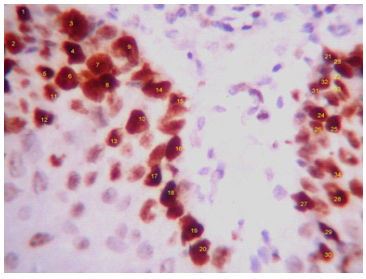

Fig 4: PHOTOMICROGRAPH OF WDSCC – Ki67

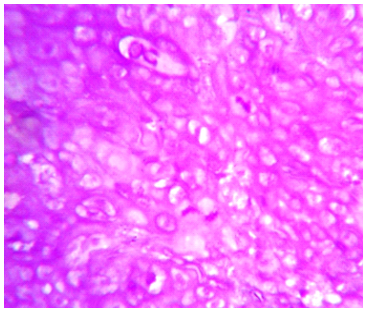

Fig 5: PHOTOMICROGRAPH OF MDSCC- CRYSTAL VIOLET

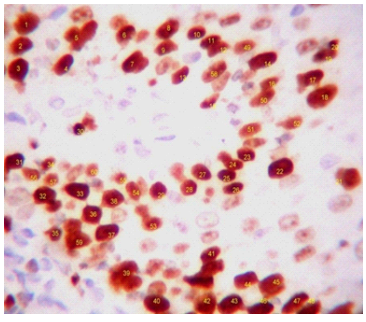

Fig 6 : PHOTOMICROGRAPH OF MDSCC – Ki67

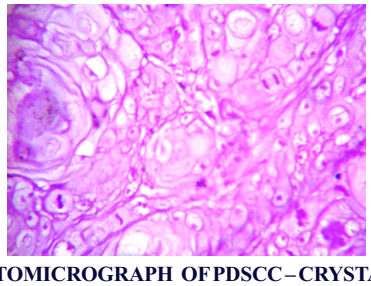

Fig 7 : PHOTOMICROGRAPH OF PDSCC – CRYSTAL VIOLET

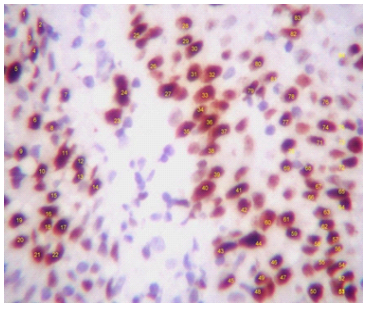

Fig 8: PHOTOMICROGRAPH OF PDSCC- Ki67

Cases were divided into 4 groups (Table 1)

Table 1 : Division of 4 study groups

	GROUP
Epithelial dysplasia	1
Well differentiated Squamous Cell Carcinoma	2
Moderately differentiated Squamous Cell Carcinoma	3
Poorly differentiated Squamous Cell Carcinoma	4

For statistical analysis, Anova test and Pearson's Correlation Coefficient test were applied. Mitotic Activity Index(Table 2) and Ki67 Labeling Index (Table 3) are compared in various groups. Mitotic Activity Index and Ki 67 Labeling Index are then correlated in various groups (Table 4)

Table 2 compares Mitotic Activity Index in epithelial dysplasia and different grades of OSCC. It shows that there is statistical significance of MAI within various groups.

Groups	N	Mean	SD	Median	Min.	Max.	F value	'p' value
Epithelial dysplasia	15	1.00	1.558	.00	0	4	3.593	0.019
Well differentiated OSCC (WDSCC)	15	1.00	1.000	1.00	0	2		
Moderately differentiated OSCC (MDSCC)	15	1.53	1.598	1.00	0	5		
Poorly differentiated OSCC (PDSCC)	15	2.87	2.642	3.00	0	8		

Table 3 compares Ki67 Labeling Index in epithelial dysplasia and different grades of OSCC. It shows that there is statistical significance of Ki67 LI within various groups.

Groups	N	Mean	SD	Median	Min.	Max.	F value	'p' value
Epithelial dysplasia	15	15.80	16.411	13.00	0	47	6.147	***0.001***
Well differentiated OSCC (WDSCC)	15	74.20	105.744	37.00	0	403		
Moderately differentiated OSCC (MDSCC)	15	103.53	93.092	112.00	0	300		
Poorly differentiated OSCC (PDSCC)	15	163.40	129.322	140.00	0	503		

Table 4 : Correlation of Ki67 LI with MAI in epithelial dysplasia and different grades of OSCC.

GROUPS	Mitotic Activity index	Ki67 Labeling index	Correlation Coefficient	'p' value
Epithelial dysplasia	0.1	15.8	-0.453	0.090
WDSCC	0.1	74.2	-0.609	0.016
MDSCC	0.153	103.5	-0.107	0.706
PDSCC	0.286	163.4	-0.211	0.451

DISCUSSION

Oral potentially malignant disorders are altered epithelial lesions with an increased propensity towards squamous cell carcinoma. Oral SCC is recognized as a disease resulting from genetic damage , leading to uncontrolled proliferation of damaged cells.[11] Tumor cell proliferation activity is believed to indicate the degree of aggressiveness of the tumor.[12] Therefore measures of proliferation are often incorporated into histological grading systems.[13] The simplest and most widely used method is the mitosis counting which despite many potential technical limitations correlates with prognosis in many neoplasms.[13]

The most obvious histological marker of stage in the cell cycle is the mitotic figure[14]. Increased mitotic figures and nucleoli counts are recognized cellular changes in oral epithelial dysplasias. Cells in the mitotic phase can be identified because of the typical appearance of the chromosome sets during the different subphases of the M-phase. This has been the basis for mitotic counting.[15] Counting of mitotic figures is the oldest way of assessing proliferation and has been applied as a diagnostic tool , especially in tumor pathology.[16] Even though many other ways of assessing proliferation have become available , the ease with which mitosis can be recognized without special equipment apart from a decent microscope and a well stained section has led to the increasing popularity of counting of mitotic figures.[15]

Baak et al recommended the use of the Mitotic Activity Index , measured in ten fields in the most cellular parts of a tumor.[17] The Mitotic Activity Index was determined in 10 consecutive high power fields (* 40 objective , n.a.0.75 , field diameter 450um) starting at the spot within the measurement area.[18] In the ocular of the microscope , a 10* 10 squared grid was placed to simplify counting the number of nuclei. In order to avoid double counts , only clear nuclei within the grid squares and/or hit by the right or bottom line of the squares were counted.[18] Our study followed the same protocol as mentioned above. A literature search revealed numerous selective stains like crystal violet , toluidine blue and giemsa , which highlight chromatin patterns. These stains have been used in brain tissue , uterus and breast carcinoma.[19,20,21] Several studies have been performed to evaluate the accuracy of Crystal violet stain though none of these studies correlated it with an established proliferative marker like ki67. In addition to mitotic activity , other methods to assess proliferative activity include flow cytometric analysis of the fraction of cells in the S-phase , synthesis of DNA content using Thymidine labeling or Bromodeoxyuridine incorporation , immunohistochemical analysis of proliferation antigens like PCNA and ki67.[17,22] If an antigen specific to a phase of the cell cycle can be detected, it may be possible to raise antibodies to it which can be used in immunohistochemical identification of proliferating cells.[14] A particular advantage is that the immunohistological demonstration of cell cycle related antigens allows spatial orientation to be shown, and the phenotype of proliferating cells can be determined by double staining methods.[22] . Ki67 is a more accurate proliferative index because it stains all the phases of cell cycle except G0.[23] Ki-67 levels are low in the G1 and S phases and rise to their peak level in mitosis. Later in the mitotic phase , a sharp decrease in Ki-67 levels occur.[24] The monoclonal antibody Ki-67 was first described in 1983 by Johannes Gerdes and colleagues. The Ki-67 antigen was named after its place of characterization at Kiel in Germany and because the clone-producing antibody was grown in the 67th well of the tissue – culture plate.[6] The Ki-67 protein, detected by immunolocalization of the Ki- 67 antigen, is located in the nucleus and its gene is located on chromosome 10q25-ter.[6] The prototypical antibody, Ki67, is only effective in fresh or frozen tissue, whilst newer antibodies such as MIB1 react with different epitopes and can be used in fixed tissue.

Many studies have been carried out using Ki-67 as a diagnostic and prognostic marker in various neoplasms. In our study , quantification of the ki67 positive cells was performed in randomly selected fields , rather than the invasive tumor front or the center of the tumor sections. This approach was decided on for the following reasons: 1)To study the proliferative cells in incision biopsy specimens where obtaining the invasive tumor front is generally not possible.2) Inability in locating the invasive tumor fronts in poorly differentiated squamous cell carcinomas.3) Difficulty in obtaining invasive tumor fronts in extensive tumor. Either mitotic counts or Ki-67 labeling indices have been reported to be of diagnostic or prognostic significance however pathologists who favour one criterion apparently tend to object to the other so few studies have been performed to correlate Mitotic count and Ki67 labeling index.[25] Of these, some described a close concordance of the mitotic activity and Ki67 positivity whereas others found only a weak correlation and in one investigation , mitotic index and Ki67 score were found to be inversely correlated to the clinical outcome.[25] Therefore the aim of present study was to use a simple, cost effective technique for studying mitosis in epithelial dysplasia and various grades of OSCC using 1% Crystal Violet stain. Also one of our objective was to correlate mitotic counts obtained by using 1% crystal violet with the cells labeled with ki67 which is an established

proliferative marker. This was done to validate the usefulness of crystal violet as a stain for evaluating proliferation and mitosis. In our study the mean MAI was found to be same for epithelial dysplasia and well differentiated OSCC and then significantly increased from well differentiated to moderately differentiated to poorly differentiated OSCC. A statistically significant mean difference was seen between epithelial dysplasia and poorly differentiated SCC. This was in accordance with the previous studies carried out by Madhuri Ankle et al[2] and Kiran B Jadhav et al[4] The significantly increased mitotic counts with 1% Crystal Violet suggests that this stain provides a crisp staining facilitating the identification of mitotic figures even at lower magnification as compared to an H&E stained section. Also the mean Ki67 LI was found to be significantly increased from epithelial dysplasia to well differentiated to moderately differentiated to poorly differentiated OSCC. A statistically significant mean difference was seen between epithelial dysplasia and poorly differentiated SCC. This was in agreement with the previous studies conducted by Premlata B et al[6]. Further we correlated MAI with Ki67 LI in various groups , a statistically significant positive correlation was observed in epithelial dysplasia , well differentiated , moderately differentiated and poorly differentiated OSCC. These results were in accordance with Noel Weidner et al[26] in breast cancer , Akyildiz EU et al[27] in meningiomas , A.P. Dutral et al[28] in canine mammary gland tumors. However these results were in contrast with those by Pierre Rudolph et al[25] who showed that in squamous cell carcinomas , the two parameters were inversely correlated.

CONCLUSION

Thus as our study has shown a significant correlation between mitosis and Ki67 LI , it can be said that mitotic cell counting is the easiest ,cheapest and fastest way of assessing proliferation. The use of 1% Crystal Violet as a selective stain for mitotic figures helps in the distinction between a pyknotic nuclei , an apoptotic cell and a mitotic cell compared to routinely used H&E stained tissue section. It can be reproducible when precisely standardized staining techniques and identification criteria are strictly followed. The present study recommends the use of 1% Crystal Violet as a selective stain for mitotic figures.

REFERENCES

1) Arthur Worth Ham, David H Cormack, Ham's Histology,1987;198. Lippincot publishers.
2) Ankle M , Kale A , Charantin S. Comparison of staining of mitotic figures by Hematoxylin and eoisn and crystal violet stains , in oral epithelial dysplasia and squamous cell carcinoma. Indian Journal Of Dental Research.2007; 18(3): 101-105.
3) Sangeeta J Palaskar, Swati Patil, Bindiya Narang, Prashant Prabhu, Pargatsingh Kathuriya, Rasika Pawar. Efficacy of Various Stains to Study Mitotic Figures in Oral Epithelial dysplasia - A Pilot Study. Journal Of Dental And Allied Sciences. 2013;3(2): 46-48.
2. Kiran B. Jadhav, Ahmed Mujib B. R.1, Nidhi Gupta. Crystal violet stain as a selective stain for the assessment of mitotic figures in oral epithelial dysplasia and oral squamous cell carcinoma. Indian Journal Of Pathology & Microbiology. 2012 :55(3).
3. Veena Maheshwari, SC Sharma , Varsha Narula , Sachin Verma , Anshu Jain , Kiran Alam. Prognostic and Predictive impact of Ki67 in premalignant and malignant squamous cell lesions of oral cavity. International Journal Of Head & Neck Surgery.2013;4(2):61-65.
4. Premalatha B.R. K. Uma. Analysis of KI-67 antigen in human Oral Squamous Cell Carcinoma – An immunohistochemical Study. .Journal Of International Oral Health.2012;2(1).
5. Jannink , Diest P J van , Baak J P A. Comparison of the prognostic value of Mitotic Frequency and Mitotic Activity Index in breast cancer. The breast.1996;5: 31-36.
6. DB Nandini. RV Subramanyam. Nuclear features in oral squamous cell carcinoma. JOMFP.2011;15(2):177-181.
7. K Shailja Yadav , Sudhir Gonuguntla. Assessment of interobserver variability in mitotic figure counting in different histological grades of oral squamous cell carcinoma.The Journal Of Contemporary Dental Practice.2012;13(3):339-344.
8. Navya Narayanan , Ciji Jose , Sathi PP , Joji I Maliekkal. Effect of delay of fixation on mitotic counts in histopathological sections of tumors. International Journal Of Pharma Research and Health Sciences. 2014;2(2):131-137.
9. V.Tumuluri , G.A Thomas , I.S Fraser. Analysis of KI67 Antigen at the invasive tumor front of human oral squamous cell carcinoma. J Oral Pathol Med 31:598-604.
10. Vijay Tumuluri , Graham A. Thomas , Ian S. Fraser. The relationship of proliferating cell density at the invasive tumor front with prognostic and risk factors in human oral squamous cell carcinoma. J Oral Pathol Med.2004;33:204-208.
11. Hideo Kurokawa et al. The relationship of the histologic grade at the deep invasive front and the expression of Ki-67 antigen and p53 protein in oral squamous cell carcinoma.J Oral Pathol Med. 2005; 34:602-607.
12. Daniela Daiconescu. The value of mitotic counting in Prostate Carcinomas. International Journal of Mathematical models and methods in applied sciences. 2011;5(2).
13. J Bury , S Cross. Molecular biology in diagnostic histopathology. Current Diagnostic Pathology.2003;9:266-275.
14. Paul J van Diest , Proliferation markers in tumours: interpretation and clinical value. J Clin Pathol,1998;51:716-724.
15. Laura Medri et al. Prognostic relevance of mitotic activity in patients with node - negative breast cancer. Mod Pathol 2003;16(11):1067-1075.
16. Chandrakant Patel et al, Role of mitotic counts in the grading and prognosis of the breast cancer. Indian J Pathol Microbiol.2002;45(3): 247-254.
17. I Jannick et al. Comparison of the prognostic value of mitotic frequency and mitotic activity index in breast cancer. The Breast 1996;5:31-36.
18. Fraser FJ. Selective stain for mitotic figures , particularly in developing brain. Stain Technol.1982; 57(4):219 -24.
19. Dutt MK. Basic dyes for the staining of DNA in mammalian tissues and absorption spectra of stained nuclei in the visible light. Microsc Acta.1982; 86(1):59-68.
20. Donhuijsen K , Scmidt U , Hirche H , Van Beuningen D ,Budach V. Changes in mitotic rate and cell cycle fractions caused by delayed fixation. Hum Pathol. 1990; 21: 709-7
21. P A Hall, D A Levison. Review: Assessment of cell proliferation inhistological material. J Clin Pathol.1990;43:184-192.
22. Surbhi Goyal , Usha Rani Singh , Correlation of mitotic indices , AgNor count , Ki67 count and Bcl-2 with grade and stage in papillary urothelial bladder cancer. Urology Journal.2014;11(1).
23. W Jonat et al. Is the Ki67 labeling index ready for clinical use. Annuals of oncology 2011;22.
24. Pierre Rudolph et al. Correlation between mitotic and Ki67 labeling indices in paraffin embedded carcinoma specimens. Human Pathology,1998;29(11).
25. Weidner N , Moore D , Vartanian R. Correlation of ki67 antigen expression with mitotic figure index and tumor grade in breast carcinomas using the novel paraffin-reactive MIB 1 antibody. Human pathology.1994;25(4): 337-42.
26. Akyildiz EU , Oz B , Comunglu N , Aki H. Clinical study: the relationship between histomorphologic characteristics and ki67 proliferation index in meningiomas. Bratskil Lek Listy.2010;111(9): 505-509.
27. Dutral A.P ,Junior G.M , Schmitt F.C , Cassali 1 G.D. Assessment of cell proliferation and prognostic factors in canine mammary gland tumors. Arq. Bras.Med.Vet.Zootec.2008;60(6);1403-1412.
28. Dutral A.P ,Junior G.M , Schmitt F.C , Cassali 1 G.D. Assessment of cell proliferation and prognostic factors in canine mammary gland tumors. Arq. Bras.Med.Vet.Zootec.2008;60(6);1403-1412