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Research Article

EVALUATION OF MICRONUCLEI IN ORAL EPITHELIUM AND IN PERIPHERAL BLOOD OF TOBACCO USERS:- A CROSS SECTIONAL OBSERVATIONAL STUDY

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ABSTRACT

Aim: To assess and compare the number of micronuclei in exfoliated buccal oral epithelial cells and in peripheral blood lymphocytes in habitual tobacco users.
Methodology: A total of 90 participants (sample size, N=90) were drawn from the patients/ accompanying person, based on their habit history of tobacco use, who reported to the Department of Oral Medicine and Radiology. The participants were divided into 3 groups- Group 1-smokeless tobacco users (n=30), Group 2-smokers (n=30) and Group 3- individuals with no tobacco habit (n=30). Micronucleus assay was carried out in exfoliated buccal cells and in peripheral blood lymphocyte of Giemsa stained slides. A minimum of 500 cells each, from buccal mucosa and from peripheral blood lymphocytes were studied after observer blinding**.**
Results: The results obtained indicated that smokeless tobacco users and smokers showed significant increase in micronuclei count in oral epithelium and in peripheral blood lymphocytes when compared to the controls**.**
Conclusion: The results of this study offer convincing evidence of the genotoxic potential of tobacco (smokeless and smoked). The genotoxic chromosomal damage caused by tobacco in the epithelial cells of the buccal mucosa and in peripheral blood lymphocytes is reflected in the increased micronuclei count in smokeless tobacco users and in smokers. This may serve as a useful 'risk assessment tool' in individuals with tobacco habit.

INTRODUCTION

Cancer is arguably the most challenging disease to have afflicted the human race and how a single aberrant cell proliferation in an uncontrolled manner evades all the defence mechanisms to overcome the human body remains an enigma. Head and neck cancers account for 30 % of all cancers. Worldwide, approximately 5,60,000 new cases of head and neck cancer are diagnosed and 300,000 deaths occur each year. More than 90% of all head and neck cancer are oral cancer.[1]

Etiology of oral cancer is multifactorial and complex. Tobacco use is an epidemic and global public health problem and the use of tobacco products is the major cause of oral cancer.[2] The human health risk caused by tobacco smoking is not limited to smokers but also to non-smokers who are exposed to environmental tobacco smoke. Exposure to any type of tobacco is associated with, and/or increases the risk of various cancers. The nicotine and carcinogen alliance is detrimental to human health and causes loss of productivity and is also responsible for millions of preventable and premature death each year.[3] Screening for Oral cancer and potentially malignant disorders should include a thorough history, clinical examination and relevant investigations. Investigations include vital staining methods, visualization adjuncts, histopathology, imaging and cytopathology that includes micronucleus assay.[4] Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from lagging chromosomes at anaphase or from accentric chromosome fragments. The fragments of DNA left behind are incorporated into secondary nuclei. These are much smaller than the main nucleus of cells, are known as micronuclei. Micronuclei may be formed as the result of agents that cause chromosome break (clastogens) or as the result of agents that cause damage to the spindle apparatus (aneugens). Micronuclei in exfoliated cells are a promising tool for the study of epithelial carcinogens and can be used to detect chromosomal damage .[5]

Genotoxic agents are capable of causing structural damage to DNA and are also potentially carcinogenic. The presence of micronuclei indicates chromosome damage or spindle dysfunction and may thus provide a marker of early-stage of

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carcinogenesis. Thus the frequency of micronclei in the cells of a tissue serves as marker of exposure and of biological response to genotoxic agents. These are good indicators for genomic instability and have been used by researchers to assess genotoxic cell damage. Direct visibility and ease of access allows a unique opportunity for the collection of epithelial cells from the oral cavity for the assessment of micronuclei.[6,7] Micronuclei assay is a sensitive, non-invasive, ease of scoring and low cost technique that offers a very simple method for obtaining information on the status of epithelial cells.[5]

The assay of micronuclei, as a measure of genotoxic damage, in different target cell populations has been the subject of scientific research for many years. Researchers studies have found increased number of micronuclei in peripheral blood lymphocytes of smokers.[6,8,9]. However very few studies have assessed the effect of smokeless tobacco in peripheral blood lymphocytes in tobacco chewer.[10,11]

There is paucity of scientific literature on the comparative effects of smoking and smokeless tobacco on micronuclei in peripheral blood lymphocytes. Data correlating the mutagenic effects of tobacco use on cells of 2 different lineages (Oral epithelium and reticuloendothelial system) is also limited.[10,12,13]

The present study was conducted to assess and compare the number of micronuclei in exfoliated buccal oral epithelial cells and in peripheral blood lymphocytes in habitual tobacco users.

MATERIALS AND METHODOLOGY

A cross-sectional observational study was conducted in the Department of Oral Medicine and Radiology and the Department of Oral Pathology and Microbiology, Manav Rachna Dental College, Faridabad. The clinical material for the present study comprised of total of 90 participants (N=90). The participants for this study were drawn from the patients/ accompanying person, based on their habit history of tobacco use. Participants were divided into group 1 Smokeless tobacco users (N=30), group 2 Smokers (N=30), group 3 Non-habiters (N=30)

The study was approved by Institutional Ethical Committee and informed consent was taken from each participant of the study.
The inclusion criteria included Smokeless tobacco users (any form) using smokeless tobacco 3 or more times a day for last 2 years or more (Group-1) and Smokers of 20 or more beedis or cigarettes /day for last 2 years or more (Group-2). Individuals consuming both forms of tobacco (smokeless and smoking), history of past or present potentially malignant disorders/malignancy of the oral cavity or orofacial region, medication for systemic disease (cardiovascular, diabetes mellitus, asthma etc.), history of consuming alcohol, history of radiotherapy in Head & Neck area and pregnant and lactating women were excluded from the study.

After taking the informed consent of participants, each subject rinsed their mouth thoroughly with tap water. Exfoliated buccal cells were obtained by gently scraping the inside of the cheek with moistened wooden spatula, smeared on to a clear glass slide and immediately fixed with 95% ethanol for a minimum of 1 minute and stained using Giemsa for 20-30 minutes. Peripheral blood was collected from patient by venepuncture and transferred into an EDTA (ethylene di-amine tetra- acetic acid) vial. A buffy coat preparation was made by centrifuging EDTA blood sample and was quickly smeared on-to a glass slide. The slides were stained using Giemsa solution for 20-30 minutes air dried, and mounted in Canada balsam. Both slides from each participant were coded. Scoring was done for micronuclei according to criteria developed by Tolbert *et al*.[14]

a.	Intact cytoplasm and relatively flat cell position on the slide.
b.	Little or no overlap with adjacent cells
c.	Little or no debris.
d.	Nucleus normal and intact, nuclear perimeter smooth and distinct.

Scoring of Micronuclei

The criteria developed by Tolbert *et al*. was used for micronucleus scoring[13] (table 1)

Table 1 Comparison of Mean Micronuclei Count in Oral Epithelium of Smokeless Tobacco Users, Smokers and Controls

Sample	Mean Micronuclei Count in Oral Epithelium(Buccal) per 500 cells counted	p-value (ANOVA)
Group 1 Smokeless tobacco users (N=30)	4.03	
Group 2 Smokers (N=30)	2.77	
Group3 Control group (N=30)	0.20	<0.001

Parameters for cell inclusion in cells to be scored For each participant, a minimum of 500 cells each from the buccal mucosa and from peripheral blood were examined for detecting the presence of micronuclei. Slides preparedfrom the buccal mucosa were examined by light microscope for presence of micronuclei with a magnification of 400x (objective 40x and eyepiece 10x).The blood smears were examined with 1000x magnification under oil emulsion. Statistical analysis was performed using ANOVA test for correlation coefficient test. $P < 0.05$ was considered as significant.(Fig 1 and Fig 2).

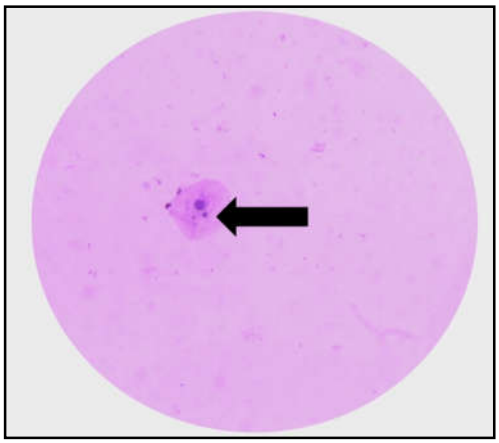


Fig 1 Photomicrograph Showing Micronucei In Buccal Oral Epithelium

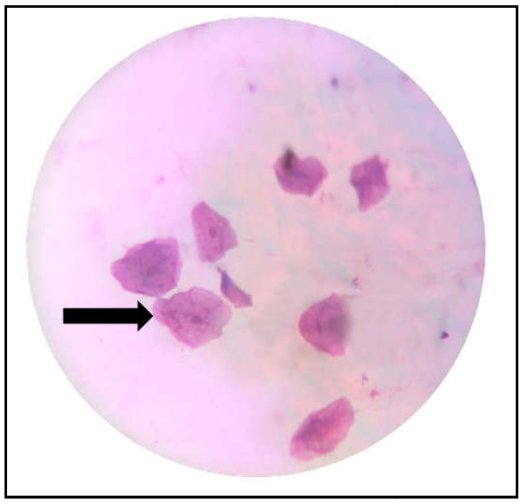


Fig 2 Photomicrograph Showing Micronucei In Buccal Oral Epithelium

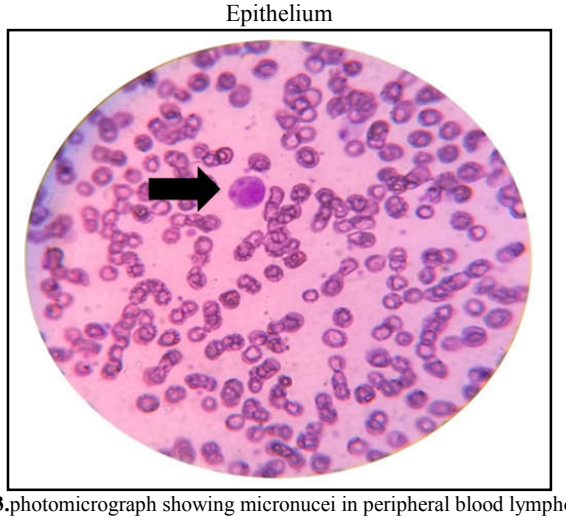


Fig3.photomicrograph showing micronucei in peripheral blood lymphocytes

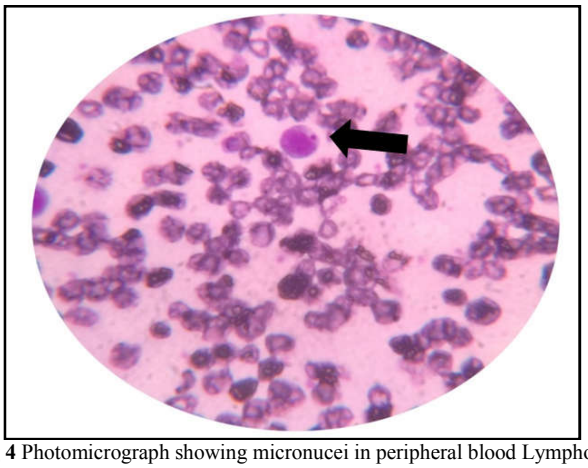


Fig 4 Photomicrograph showing micronucei in peripheral blood Lymphocytes

Statistical Analysis

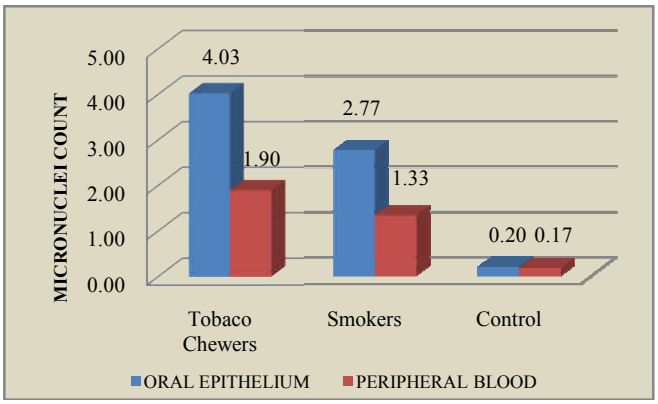
The statistical analysis was carried out using Statistical Package for social Sciences (SPSS, version 18.0 for Windows) with suitable statistical formula's. A p-value of less than 0.05 was considered to be statistically significant. Paired t-test and ANOVA (Analysis Of Variance) was performed for comparision of groups.

RESULTS

Table 1 and Bar Graph 1 shows mean micronuclei count in oral epithelium of Group 1 was 4.03, in Group 2 was 2.77 and in Group 3 was 0.20. The difference in micronuclei count in buccal oral epithelium of tobacco users (Group 1 & 2) and non-habiters (Group 3-control) was statistically significant ($p<0.001$). The micronuclei count in buccal oral epithelium of Group 1 was higher (mean count=4.03/500 cells counted) than that in Group 2(mean count=2.77/500 cells counted) and Group 3(mean count=0.20/500 cells counted). This was found to be statistically significant ($p<0.001$).

Table 1 Comparison of Mean Micronuclei Count in Oral Epithelium of Smokeless Tobacco Users, Smokers and Controls

Sample	Mean Micronuclei Count in Oral Epithelium (Buccal) per 500 cells counted	p-value (ANOVA)
Group 1 Smokeless tobacco users (N=30)	4.03	
Group 2 Smokers (N=30)	2.77	<0.001
Group3 Control group (N=30)	0.20	



Bar Graph 1 Comparison of Mean Micronuclei Count In Oral Epithelium and In Peripheral Blood Lymphocytes of Smokeless Tobacco Users, Smokers and Controls

Table 2 and Bar Graph Bar graph 1 shows mean micronuclei count in peripheral blood lymphocytes of Group 1 was 1.90, Group 2 was 1.33 and in Group 3 was 0.17.

Table 2 Comparison of Mean Micronuclei Count In Peripheral Blood Lymphocytes of Smokeless Tobacco Users, Smokers and Controls

Sample	Mean Micronuclei Count in Peripheral Blood Lymphocytes per 500 cells counted	p-value (ANOVA)
Group 1 Smokeless tobacco users (N=30)	1.90	
Group 2 Smokers (N=30)	1.33	<0.001
Group 3 Control group (N=30)	0.17	

The difference in micronuclei count in peripheral blood lymphocytes of tobacco users (Group 1& 2) and non-habiters (Group 3- control) was statistically significant ($p<0.001$).The micronuclei count in peripheral blood lymphocytes of Group 1 was higher (mean count=1.09/500 cells counted) than that in Group 2 (1.33/500 cells counted) and Group 3(0.17/500 cells counted). This was found to be statistically significant ($p<0.001$).

DISCUSSION

Oral cancer in India has a distinct demographic profile with some variation. It is emerging as a major public health problem in India. Tobacco use (smoking and smokeless) is perhaps the single most closely linked etiologic factor to the development of oral cancer. Other etiologic factors include alcohol consumption, nutritional deficiencies, viral infection etc. acting on a predisposed genetic framework confounded by socioeconomic, cultural, religious, racial and geographic variation, all contributes to a multifactorial pathogenesis of this deadly disease.[8]

Head and neck cancers account for 30 % of all cancers. Worldwide, approximately 5,60,000 new cases of head and neck cancer are diagnosed and 300,000 deaths occur each year.[2] Globally, oral cancer in the sixth most common cause for mortality.[4] Overall 57.5% of global head and neck cancers (excluding oesophageal cancers) occur in Asia especially in India, for both the sexes.[5] More than 90% of all head and neck cancer are oral cancer. In India oral cancer constitutes 12% of all cancers in men and 8% of all cancers among women.[6] Early detection of cancer is the most effective means of reducing mortality because its five- year survival rate is directly related to stage of diagnosis, prevention and early. When detected early, the probability of surviving from oral cancer is remarkably better than for most other cancers.

Screening for oral cancer and potentially malignant disorders should include a thorough history, clinical examination and relevant investigations. Investigation techniques include vital staining methods, visualization adjuncts, histopathology, imaging and cytopathology that includes micronucleus assay.[11] Chromosomal damage is an early indicator of carcinogenesis and this can be used as a tool for identifying high risk patients. Many biomarkers have been identified and among them, micronuclei are one of the most reliable biomarker for genotoxicity.[12]

Micronuclei are indicators of chromosomal damage due to genotoxic agents that cause chromosome breaks or spindle dysfunction. Micronuclei consist of small amount of DNA that arises in the cytoplasm when chromatid/ chromosomal 42 fragments or chromosomes are not incorporated into daughter nuclei during mitosis. These are much smaller than the main nucleus of cells. It is known that any agent capable of causing structural damage to DNA is also potentially carcinogenic, as most carcinogens are also mutagenic. Chromosomal aberrations are a frequent and significant response on exposure to mutagenic agents. They are of significance from the stand point of inherited human disease and have been implicated in carcinogenesis.

Tobacco and its constituent derivatives have been reported as potent mutagenic agents which are thought to be responsible for the induction of chromosomal aberrations resulting in production of micronuclei.[12] Tobacco carcinogens have the potential to act directly on the epithelium at the site of contact and they may additionally have a genotoxic effect on the circulating blood cells. Micronuclei (MN) in exfoliated cells and peripheral blood lymphocytes (PBL) have been used as biomarkers of chromosomal damage and genome stability in human population. [13,14]

On comparison the micronuclei count in buccal oral epithelium of smokeless tobacco users (Group 1) was higher (mean count=4.03) than that in smokers (Group 2) (mean count=2.77) and in Group 3(0.20). This was found to be statistically significant ($p<0.001$).

In the present study on comparison the mean micronuclei count in buccal oral epithelium of smokeless tobacco users (group 1) was higher (mean count=4.03) than that in smokers (Group 2) (mean count=2.77). This was found to be statistically significant ($p<0.001$).

The intergroup comparison of mean MN count in buccal oral epithelium between Group 1 (4.03) v/s Group 3(0.20), and Group 2(2.77) v/s Group 3(0.20) also revealed statistically significant increase in MN count in the study groups when compared to the controls ($p<0.001$).

The mean micronuclei count in buccal oral epithelium of Study Group (Group 1+Group 2) (3.40) was higher than that in Group 3(0.20). This was also found to be statistically significant ($p<0.001$).

The results of this study were in concurrence with previous studies reported by Motgi *et al* in 2014 [1]5Bansal *et al* in 2012[1]6, Balachandar V *et al* in 2008 17, Patel BP *et al* in 2009 18,Sudha S *et al* S *et al* in 2009 19.

An attempt was also made to assess the micronuclei count in peripheral blood lymphocytes in the study participants.
In the study on comparison the micronuclei count in peripheral blood lymphocytes of smokeless tobacco users (Group 1) was higher (mean count=1.90) than that in smokers (Group 2) (mean count=1.33) and in Control (Group 3)(0.17). This was found to be statistically significant ($p<0.001$)

The intergroup comparison of mean MN count in peripheral blood lymphocytes between Group 1 (1.90) v/s Group 3(0.17), and Group 2(1.33)v/s Group 3(0.17) also revealed statistically significant increase in MN count in the study groups when compared to the controls ($p<0.001$).

The micronuclei count in peripheral blood lymphocytes of Study Group (Group 1+Group 2) (1.62) was higher than that in Group 3(0.17). This was found to be statistically significant ($p<0.001$).

In the present study, the mean micronuclei count in peripheral blood lymphocytes of smokeless tobacco users (Group 1) was higher (mean count=1.90) than that in 46 smokers (Group 2) (mean count=1.33). This was found to be statistically significant ($p<0.001$).

Tobacco use is epidemic and contributes to preventable morbidity and mortality worldwide. The result of this study

offer convincing evidence of the genotoxic potential of tobacco (smokeless and smoked). Previous studies have clearly suggested a causal link between MN and cancer. It has been hypothesised about direct association between the frequency of MN in target tissues and cancer development, this is supported by increase in MN counts in target tissues and lymphocytes in cancer patients.

Tobacco smoke contains mixture of nicotine, carcinogens and toxicants. Nicotine is not a direct chemical carcinogen, however, it causes addiction leading the chronic exposure to tobacco smoke that increases cancer risk for tobacco users. Most constituents of tobacco smoke, detoxified or neutralize by metabolizing enzyme, are converted to more water-soluble products, which can be excreted from human body. However, during this process, certain reactive compounds may be formed as intermediates which may covalently bind to nucleophilic sites in DNA, causing DNA adducts. The DNA adducts can evade the repair system, and can cause miscoding during DNA replication resulting in a permanent mutation in the DNA sequence. Different forms of tobacco are associated with increased number of MN which is indicative of its genotoxic ability to cause cancer.[9]

Smokeless tobacco also contains carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines which are believed to be responsible for the induction of MN. These compounds are produced from nicotine by bacterial or enzymatic activity. The same formation occurs in the mouth under the influence of saliva.[16]

The risk of cancer in SLT users has been attributed to the presence of tobacco specific nitrosamines (TSNAs). In India, SLT processing is performed by individual farmers and small companies with little control over fermentation and curing, which increases the production of TSNAs.19 Furthermore, SLT use is not homogeneous in India, since the tobacco is often combined with betel leaf (Piper betle) and sliced areca nut (Areca catechu) and/or powdered slaked lime, which are additives that enhance the toxicity as well as the psychotropic effect of tobacco. Furthermore, studies have proven that the nicotine content of the Indian brands of smoking tobacco is slightly high as compared to that of the international brands.20 The nicotine content in commercially available chewing tobacco products was found to be much lower than that in the smoking form of tobacco, but the average daily consumption has made it comparable to the smoking form. According to the center for disease control, chewing tobacco which was used 7-8 times a day could be equivalent to smoking 30-40 cigarettes/day. Other factors such as use of slaked lime and continuous contact with the oral mucosa, led to more absorption of nicotine through SLT use. Additionally, in contrast to the smokers who absorbed nicotine primarily through the pulmonary vasculature, chewing tobacco users were found to absorb nicotine through the buccal mucosa and the gastrointestinal tract mucosa, which get further released into the blood circulation leading to genotoxic damage to peripheral blood lymphocytes.[16]

A reliable and minimally invasive biomarker is a necessity to improve the implementation of bio monitoring, diagnosis and treatment of diseases like oral cancer which are associated with genetic damage. MN assay has been rated as an appropriate tool to monitor cancer risks in humans caused by exposure to environmental factors or inherited genomic instability.

Evaluation of MN count in oral epithelium and in peripheral blood has an advantage that it is a relatively simple and short term procedure. Oral mucosal cells are the first barrier for inhalation or ingestion route and capable of metabolizing carcinogens. Blood cells are also subjected to genotoxic damage as tobacco products leach out into the oral cavity and are then absorbed through the epithelium into circulation. In addition, these epithelial cells are highly proliferative. Tobacco has known carcinogenic potential, buccal cells are the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products. Hence, oral epithelial cells are a preferred target site for early genotoxic events induced by carcinogenic agents entering the body through inhalation or ingestion. MN in the buccal oral epithelium and peripheral blood lymphocytes are also said to predict cancer risk for the upper aero digestive tract, including potentially malignant disorders such as oral leukoplakia. The result from the present study also confirm the genotoxic potential of tobacco(both smokeless and smoked) and indicate that MN assay can be used as screening, prognostic and educational tool in community center of oral pre cancer and cancer.

Buccal cells and peripheral blood lymphocytes are useful not only for characterizing the molecular mechanisms underlying tobacco-associated oral cancer, but also express diverse changes that appear promising as candidates for us as biomarkers for the early detection of oral cancer.

CONCLUSION

The results of this study indicate that the micronucleus test is a useful tool to assess the risk of genotoxic damage to oral epithelium in tobacco users. Further, peripheral blood lymphocytes may also be examined for the presence of micronuclei. Since blood investigation are routinely performed for various medical conditions examination of peripheral blood lymphocytes for the presence of micronuclei is recommended in tobacco users.

Limitations of the present study are that no attempt was made to assess the relationship of micronuclei count taking age and gender into consideration. Parameters for duration of exposure of tobacco use were also not considered for recognition of micronuclei count. Further research is necessary addressing the sources of variability on large population with comprehensive assessment of life style factors & health status.

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