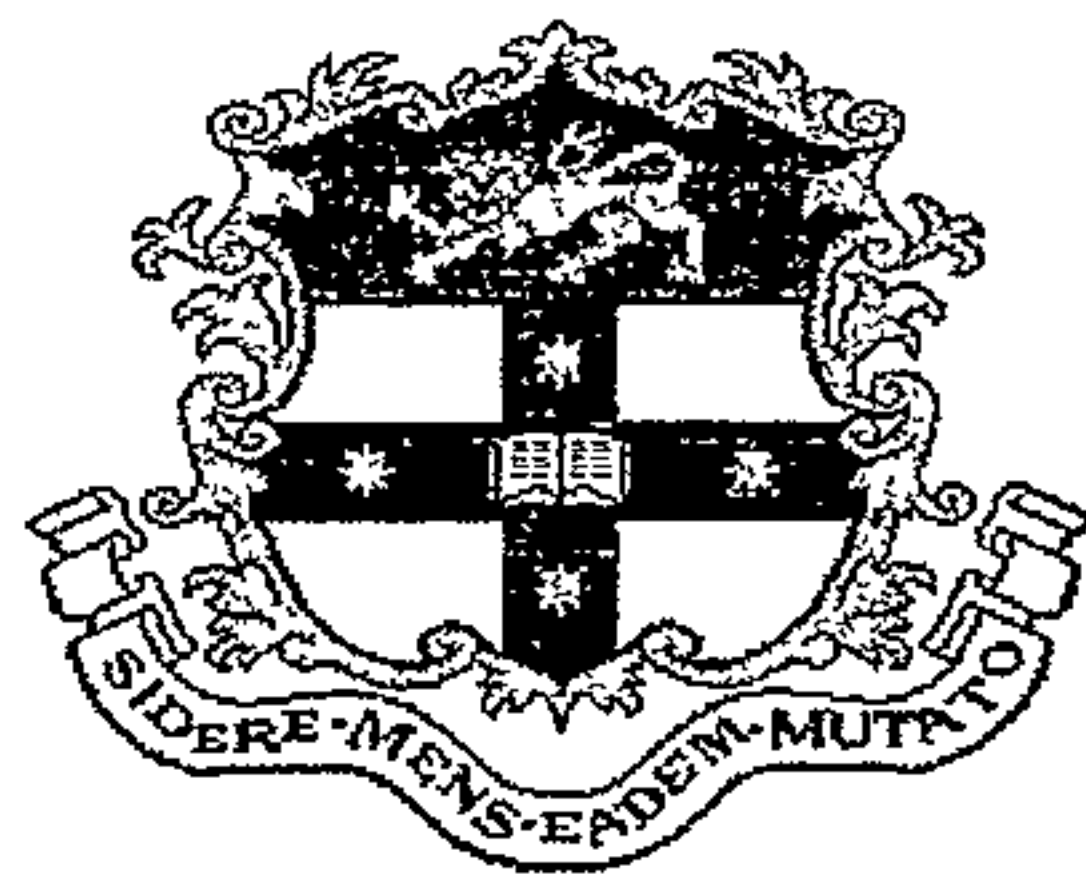


CHANGES IN REACTIVITY OF p53, Ki-67 AND PCNA ANTIGENS IN
ORAL EPITHELIUM FOLLOWING TISSUE FIXATION AND
DECALCIFICATION:
REVERSAL OF THESE EFFECTS BY MICROWAVE ANTIGEN RETRIEVAL



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February 1998

ABSTRACT

INTRODUCTION: Antigen preservation in soft and hard tissue specimens during tissue fixation and processing prior to immunohistochemical procedures is essential. Even though formalin is the most commonly used fixative for preservation of tissue morphology, it is clear that there is marked antigen loss during formalin fixation due to extensive cross linkage of proteins at the tertiary or quaternary level. Less attention has been given to the possible adverse effects on immunolocalisation of antigens during additional procedures following decalcification of tissue.

OBJECTIVES: Identification of any loss of reactivity (antigen masking) of three antigens, Ki-67, PCNA and p53 following formalin and EDTA-formalin fixation. Investigation of the possible reversibility of any antigen loss by the use of microwave retrieval technique.

METHOD: The investigation involved specimens of redundant human gingiva removed from six patients undergoing surgical treatment for periodontal diseases. The immunohistological detection of Ki-67, PCNA and p53 antigens in epithelium by appropriate antibodies has been compared in gingival tissue following:

- a) Snap freezing;
- b) Formalin fixation; and
- c) Exposure to a solution of EDTA (0.1 M EDTA) and fixative (10% formalin).

An attempt was made to enhance the immunolocalisation of the Ki-67, PCNA and p53 antigens using microwave antigen retrieval technique.

RESULTS: p53 antigen expression could only be found in snap frozen sections treated by microwaving. This antigen retrieval was consistent and showed typical localisation of the p53 antigen to nuclei of the basal and lower spinous cells of the epithelium. The present study indicated that the p53 antigen was still present in masked form and could be retrieved by microwave irradiation. Staining by PC10 antibody was apparent only in snap frozen, formalin and EDTA-formalin fixed specimens subjected to microwave irradiation, and not in non-microwaved specimens. In the microwaved sections, the percentage of PCNA positive basal epithelial cells (29%) in EDTA exposed specimens was significantly greater than that for the formalin fixed (9%) ($p < 0.01$) and snap frozen sections (4%) ($p < 0.001$). The Ki-67 protein was detected in both microwaved and non-microwaved sections and there was no significant difference between the proportions of positive basal epithelial cells stained in these two post fixation regimes.

CONCLUSIONS: It was concluded that fixation of tissue in formalin and EDTA-formalin solution causes antigen masking in gingival tissue and microwave antigen retrieval can effectively be used to unmask these antigens. The study findings confirm that the microwave antigen retrieval is an important step towards standardisation in immunohistochemistry and improved immunostaining of routinely fixed tissues.

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to my supervisor and local guardian, Associate Professor PD Barnard, Discipline of Public Health Dentistry, University of Sydney, for his support, patience, information, encouragement and advice in the preparation of this treatise.

I am deeply indebted to Professor DM Walker, Discipline of Oral Medicine & Oral Pathology, University of Sydney, whose guidance, care, and supervision throughout the preparation of this thesis have been more than I could ever ask for or expect.

Special thanks are in order to Miss Janice Matthews for her guidance and help throughout the investigation and literature review.

Finally, I would like to thank my parents and my friends, who helped me in less direct, but equally valuable ways.

STATEMENT OF ORIGINALITY

This treatise contains no material which has been accepted for the award of any degree or diploma at any University. To the best of my knowledge no data presented in this thesis has been previously published or reported other than the abstracts and posters that carry my name as co-author. Experiments presented in this thesis were performed by myself (unless otherwise acknowledged in the text) in the Oral Pathology Laboratory, Westmead Hospital Dental Clinical School, between July 1997 and October 1997.



Afzal Ahmed Sharafuddin
March 1998

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1. INTRODUCTION

Immunohistochemistry research has made important contributions to our understanding of cancer. Despite improvement in immunohistological techniques, major artefacts induced by fixation remain a significant problem. During fixation there is antigen masking due to extensive cross linkage of proteins at the tertiary or quaternary structural level (Cattorretti et al., 1993).

Even though new techniques for antigen unmasking have been developed, our understanding of the influence of fixation and decalcifying regimes (for hard tissue) remains incomplete (Arber et al., 1996). One of the biggest problems in immunohistochemistry has been how to obtain both good morphology and the immunoreactivity of antigens in tissue sections (Ezaki, 1996).

The present study has investigated the effects of formalin fixation and ethylene diamine tetra-acetic acid (EDTA) on three tumour markers PCNA, Ki-67 and p53. These tumour markers are helpful in understanding the biology of certain neoplasms and have been used in attempts to provide prognostic information. This study has been set up to compare the preservation of antigens with microwave antigen retrieval (MAR) with that in routinely processed tissue.

1.1 Immunohistochemistry and its importance

The development of immunohistochemistry has assisted in improving specificity and sensitivity in diagnosis of oral cancers, for example when used to recognise intermediate filament proteins characteristic of epithelium or mesodermal tissues

(Walker, 1997). Immunohistochemistry can make important contributions in the diagnosis of a wide range of tumour patholog when the peroxidase-antiperoxidase technique is used as an adjunct to the morphological assessment of tumours (Leong & Wright, 1987). One of the important uses of immunohistochemistry is the identification of tumour markers and cell markers and this is shown in Table 1.1 (Bancroft & Cook, 1984).

Table 1.1: Uses of immunohistochemistry.

1. Demonstration of immunoglobulins of lymphoreticular origin	Multiple myeloma Hodgkin's disease Lymphomas, etc.
2. Skin diseases (bullous disorders) a. Pemphigus b. Pemphigoid	IgG on prickle cell junctions IgG on basement membranes
3. Identification of hormone containing cells and tumours	Gastrin Calcitonin Testosterone, etc.
4. Tumour and cell markers	α -1-antitrypsin α -fetoprotein Carcino-embryonic antigen Factor viii, etc.
5. Identification of organisms	Hepatitis B, antigen Herpes simplex, etc.
6. Location of enzymes	Most common enzymes

Source: Bancroft & Cook (1984). *Manual of histological techniques*, p. 195.

These markers have been essentially used in diagnosis, but certain molecules associated with cellular proliferation such as PCNA & Ki-67 or tumour suppressor genes such as p53 may permit the assessment of the biological potential of neoplasms (Mehregan & Mehregan, 1996). The immunohistochemistry technique is used for localisation of substances within tissues by applying the principles of chemistry and biochemistry.

Some of the main advantages of immunohistochemistry include its ease of use, cost benefit and accuracy of staining which provides important and essential information of therapeutic and prognostic relevance (**Leong & Wright, 1987**).

1.1.1 Antigens

Antigens are substances which stimulate the formation of antibodies. Antigens comprises molecules of different chemical types (usually proteins) capable of stimulating the immune system of an animal to produce a response specifically directed at the inducing substance (**Weir, 1973**).

In most tumours, tumour specific antigens have not been defined. It is possible to produce monoclonal antibodies against various epitopes on tumour associated antigens (**Sivolapenko et al., 1989**). These antigens may be seen in some normal tissues, but their expression in tumours may be altered allowing their use as tumour marker (**Sivolapenko et al., 1989**).

1.1.2 Antibodies

Antibodies or immunoglobulins are complex protein molecules produced by plasma cells and certain lymphocytes, found in germinal centers of lymphnodes, follicles of spleen and other tissues (**Bancroft & Cook, 1984**).

1.1.3 Antigen Retrieval

In the late 1980s, because of the antigen masking due to formaldehyde fixation, only a limited range of antibodies was available which could be used successfully in

immunohistochemistry and it was thought that these epitopes were irreversibly denatured by formaldehyde (Werner et al., 1996).

This theory of irreversible alteration of epitopes was changed by the development of antigen retrieval methods. These antigen retrieval methods proved that the reactivity of the epitopes with their respective antibodies were only masked or concealed and not lost completely (Werner et al., 1996).

The advancement of new antigen retrieval protocols resulted in major advances in the use of paraffin-embedded tissues in immunohistochemistry. During antigen retrieval there is cleavage in the bonds induced by fixation and reconstruction of the original three-dimensional structure of the epitopes (Werner et al., 1996).

Various techniques to retrieve the immunoreactivity of antigens (unmasking) after routine tissue preparations, such as fixation, dehydration and decalcifying regimes, have been devised and are now finding their way into use for immunostaining in biology and pathology (Ezaki, 1996).

Enzyme pre-digestion of tissue sections with proteases such as trypsin, pronase, proteinase K or pepsin, was the common method of antigen retrieval in formalin fixed tissues (Werner et al., 1996). Antigen retrieval using new techniques based on the exposure of routine sections to high temperatures using Bunsen burner, microwave irradiation, pressure cooker or autoclaving for variable times was proposed during the early 1990s (Pileri, 1997).

The mechanisms by which these approaches facilitate the detection of most antigens are not yet completely defined. Antigen retrieval combining heat and a buffer promises to be superior to enzymatic pre-digestion (**Cattoretti et al., 1993**).

Antigen retrieval has been widely used in surgical pathology and has proved itself to be a simple and effective method for immunohistochemistry on archival tissue sections. The development of new antigen retrieval techniques like the microwave antigen retrieval has reduced the detection thresholds (increased sensitivity) of immunohistochemical staining for a wide range of antibodies (**Shi et al., 1997**).

The possible mechanisms whereby, or by which, antigen retrieval may occur have been summarised by **Suurmeijer and Boon (1993a)** as follows: (a) breaking of the formalin-induced crosslinkage between epitopes and unrelated proteins; (b) extraction of diffusible blocking proteins; (c) precipitation of proteins and; (d) rehydration of the tissue sections, allowing better penetration of antibodies and increasing accessibility of epitopes.

1.1.4 Tumour markers

Pressman and Keighley in 1948 introduced the use of radiolabelled antibodies in tumour localisation. Since then several workers have demonstrated the potential of both monoclonal and polyclonal antibodies in the diagnosis and treatment of cancer (**Sivolapenko et al., 1989**). In addition to the help in assessment of lineage of neoplasms, markers are now available that have the potential to identify molecules of prognostic significance (**Mehregan & Mehregan, 1996**).

The in vitro production of monoclonal antibodies (**Kohler & Milstein, 1975**) acted as a catalyst in the growth of immunohistochemistry. Monoclonal antibody derived by this technique from cultured fused cells in unlimited amounts may be directed against a specific antigenic determinant or a tumour marker.

1.1.4.1 Oncogenes

Oncogenes are specific DNA sequences associated with the malignant transformation by accelerating cell growth and division. The exact mechanisms of malignant transformation are still being investigated (**Watson & Sikora, 1989**). However, it appears that many oncoproteins expressed by oncogenes function by dysregulating cell cycle control. Tumour formation may be promoted when these genes are activated by the process of amplification, mutation, translocation and deletion (**Watson & Sikora, 1989**).

Monoclonal antibodies have been developed against oncogene products and these novel antibodies have been used to investigate oncogene function in normal and neoplastic tissue and have already demonstrated their potential for purifying and analysing oncoproteins (**Doolittle et al., 1983**). The first connection between oncoproteins and proliferation control was made in 1983, when c-sis was shown to encode a subunit of Platelet Derived Growth Factor (**Doolittle et al., 1983**).

p53

The p53 gene codes for a tumour suppressor protein which is a 53 kilodalton nuclear phosphoprotein. **Nigro et al., (1989)** documented the occurrence of p53 mutation in

different human tumours arising from various sites such as the breast, lung, colon, and mesenchyme. In their review Levine et al (1991) has stated that wild type p53 acts as a tumour suppressor gene in the normal form, but as an oncogene in its mutant form.

The wild type p53 protein is essential for normal cell growth and the eventual suppression of the malignant phenotypes (Levine et al., 1996). Inactivation of p53 induces the development of malignancy (Levine et al., 1991). Where DNA is defective or scarce, p53 acts as the guardian of the genome (Hall et al., 1991) to arrest the cell cycle at a G1 checkpoint "Knock-out". The p53 gene product also diverts cells with mutational errors into apoptosis, another means whereby formation of neoplastic clones may be inhibited (Donehower et al., 1992).

p53 expression is not usually detectable by immunohistochemistry in normal tissues as the wild type p53 protein has only a half-life of approximately 15 to 30 minutes before degradation (Hall et al., 1991). Mutant p53 may be more stable and more resistant to degradation, extending its half-life and thus becoming more easily detectable by immunostaining (Mehregan & Mehregan, 1996). Immunohistological evidence of p53 protein expression is not invariably due to mutant protein, as p53 may be stabilised by binding and inactivating viral or heat shock proteins or complexing between p53 molecules. Conversely p53 gene deletion will result in negative immunoreactivity (Mehregan & Mehregan, 1996).

There are several possible mechanisms through which p53 aberrations can influence cellular proliferation (Weinberg, 1991). Mutation of these cells results in diminished

control of cellular proliferation. P53 is also influenced by human papillomavirus, which can induce an increase in p53 degradation, thereby diminishing control of cellular proliferation (**Kao, 1991**).

p53 genetic alterations have been identified as the most common mutations in human cancer (**Frank et al., 1994**). Previous studies have shown direct correlation between abnormal p53 protein expression and gene mutation in head and neck squamous cell carcinoma (**Somers et al., 1992**). In some tumours, such as prostatic carcinoma, p53 expression has been correlated with prognosis, but in general p53 has not been a reliable guide to the prognosis (**Mehregan & Mehregan, 1996**).

1.1.4.2 Growth fraction

The proliferation rate of a tumour is an important facet of its biological behaviour (**Stenzel et al., 1996**). An assessment of the rate of cell proliferation of tumours can be determined by the ratio of the number of cycling cells to the total cell population (growth fraction). Other variables such as the cell cycle time could also affect the estimate of the growth fraction. As in the present study growth fraction have been estimated enumerating the proportion of cells expressing Ki-67 and PCNA. These are cell cycle associated antigens.

The immunoreactivity of Ki-67 has been well established in frozen sections and PCNA in formalin-fixed, paraffin-embedded tissue (**Cox & Walker, 1996**). Immunohistological detection of these proteins represents a useful marker of the proliferative status of the investigated lesion (**Kurki et al., 1988**).

Ki-67 Antigen

Ki-67 antibodies recognise a human nuclear antigen which is absent in G₀ (resting) phase and expressed during the G₁, S, G₂, and M phases of the cell cycle (see review by Mehregan & Mehregan, 1996). It denotes a nuclear protein forming part of a DNA replicase complex. The Ki-67 antibody reacts with a human nuclear cell proliferation-associated antigen that is expressed in all active parts of the cell cycle (see review by Mehregan & Mehregan, 1996).

Determination of the proportion of cells with Ki-67 positive nuclei offers a rapid, simple, and reliable means of evaluating the growth fractions of normal and malignant human cell populations. The Ki-67 index has been shown to correlate with prognosis in certain circumstances (see review by Mehregan & Mehregan, 1996).

In bone tumours, the level of Ki-67 expression correlates with the grade of malignancy and is diagnostically and prognostically useful (Scotlandi et al., 1995). Cell kinetic data on archival material collected in histopathology departments all over the world can be compared or aggregated through the detection of Ki-67 antigen. Use of Ki-67 is highly reproducible, easy to perform at low cost, and no additional technical skill is needed because after microwave treatment, routine immunohistochemical methods are used (Cattoretti et al., 1992).

The availability of Ki-67 monoclonal antibody opened new possibilities for an extensive analysis of cell kinetics in human neoplasms (Scotlandi et al., 1995).

Proliferative Cell Nuclear Antigen

PCNA is a protein which is required for DNA synthesis and repair. It is an accessory protein of DNA polymerase delta, a 36 kd nonhistone protein (McCormick & Hall, 1992). PCNA expression is increased in G1 phase, is negligible in G0 phase and reaches a maximum in the S phase of the cell cycle, then declines in G2 phase (Mehregan & Mehregan, 1996). Immunohistochemically, PCNA was also found in noncycling cells as it is also involved in DNA repair (Mehregan & Mehregan, 1996).

The percentage of PCNA positive cells can be quantitated by manual counting or by image analysis technique. The great advantage of using PCNA over other methods is the ease of application to routinely processed specimens or archival tissue. PCNA may be a useful marker of the proliferative state of neoplastic tissues (Sabine et al., 1994). For example the prevalence of PCNA-expressing cells also appears to correlate with grade of carcinoma, with high-grade carcinomas displaying a higher proportion of PCNA positive cells (Robbins et al., 1987).

1.2 Oral Cancer

Oral cancer is the sixth most common cancer in the general population in developed countries, and the third most common cancer in developing nations (Silverman, 1988). "Oral cancer has been defined as cancer of the oral cavity and pharynx, including cancer of lip, tongue, salivary glands, gum, floor and other areas of the mouth, oropharynx, nasopharynx, hypopharynx, pharynx and other buccal areas International Classification of Diseases ICD-9 140-149" (Wong et al., 1996).

Silverman & Gorsky (1990) state that 96% of all oral cancers are carcinomas out of which 91% are squamous cell carcinoma. The overall 5 year survival rate for patients with squamous cell carcinoma (SCC) in all stages and all grades is less than 50%, which is one of the lowest among the major cancers. Even one lymph node metastasis is associated with a 50% reduction in survival rate (**Diogene et al., 1996**).

In 1994, the NSW Cancer Council reported an incidence of 767 new cases of head and neck cancers, amounting to 3% of all new cases identified. Some 360 (3%) deaths due to head and neck cancer are reported annually in NSW (**Coates & Armstrong, 1997**). The prognosis for cancer in the oral cavity is amongst the worst for malignancies in general. It is clear that we need to advance our understanding of oral cancer aetiology and development before further improvement can occur (**Parkin et al., 1988**).

The work by **Grid et al (1993)** suggest that p53 mutation can serve as an indicator for potential malignancy development and possible recurrence in the oral mucosa. PCNA is considered as a useful tumour marker in delineating nonmalignant lesions from malignant lesions in the oral cavity (**Zain et al., 1995**). Ki-67 antigen could be considered as a valuable tool for cell kinetics reference and can be considered as a supplementary method to flow cytometry in diagnosis and therapy of oral cancer (**Zoeller et al., 1994**).

1.3 Formalin & antigen masking

For detecting specific biomarkers it is important to understand the limitations imposed by the fixation methods and processing of the tissues. **Ferdinand Blum** in 1893

discovered formaldehyde as a tissue fixative and it remains the commonest fixative used in pathology (Fox et al, 1985). Commercially available formalin is diluted to a 10% solution (3.7- 4% formaldehyde) for use as a fixative. One of the main advantages of formalin is its superior preservation of morphological detail (Shi et al., 1997).

The antigen preservation of soft and hard tissue specimens for immunohistology influences the accuracy of results. Even though formalin is accepted as the popular fixative for preservation of tissue structure, it is clear that there is marked antigen loss ("masking") during formalin fixation, due to extensive cross- linking among the amino-acid residues of proteins affecting the tertiary or quaternary structural level (Ezaki, 1996). Cross linkage is a special type of chemical modification of protein and can be defined as a process involving the joining of two molecular components by covalent bonds achieved by the use of crosslinkage reagents, such as tissue fixatives (Wong et al., 1991).

For a long time, it was thought that formalin-sensitive antigens might be irreversibly destroyed during the fixation process. The work of Malisius et al (1995) on retrieval and enhancement of hidden antigens proved that during fixation most cellular antigens were not irreversibly destroyed but only masked.

Arnold et al in 1996 compared different fixatives for demonstration of specific antigens. No single fixative proved to be best for all antigens. Buffered formalin was found to be the poorest fixative for maintaining antigenicity in immunohistochemistry.

1.4 Decalcification & antigen masking

For immunohistochemistry, specimens containing hard tissue components must first be decalcified, usually with dilute solutions of mineral or organic acids and usually after fixation and prior to processing and sectioning. The effects of decalcification on the immunoreactivity of formalin fixed tissue has received little attention and the information available in the literature is generally contradictory (**Matthew, 1984**).

Decalcifying regimes hinder the immunohistochemical analysis by masking or destruction of tissue antigen (**Erber, 1996**). The effect of decalcification regimes can be reduced by mild decalcification procedures but they are time consuming and in some instance a quick diagnosis is of vital interest to the patient (**Mullink et al., 1985**). Knowledge of the preservation of antigenic reactivity in decalcified tissue will be useful in the diagnosis of tumours of uncertain histogenesis and origin which affect calcified tissues (**Athanasov et al., 1987**).

1.5 Microwave antigen retrieval and unmasking of antigens

Shi et al (1991) described an antigen retrieval method using microwave irradiation, which could expose antigen binding sites concealed by the process of formalin fixation with its attendant alteration of protein tertiary structure. **Norton (1993)** has stated that heating may cleave the polypeptide backbone and disrupt cross-links produced by formalin. Unmasking of masked epitopes could significantly expand the range of antibodies useful in immunohistochemistry as well as reduce the incidence of false-negative results in over-fixed specimens (**Norton, 1993**).

The best method advocated for microwave antigen retrieval is irradiation of the slides in the microwave oven using the intermittent heating method of two 5min cycles with an interval of 1 min between the heat cycles (Shi et al., 1991).

Leong (1996) has mentioned microwave antigen retrieval as an important step towards standardisation and improved immunostaining of routinely fixed tissues. This procedure helps to increase the intensity and extent of immunostaining.

2. AIMS AND OBJECTIVES

With the growing interest in immunohistochemical staining procedures, there has been new development of a wide range of immunohistochemical methods which are of potential value for the surgical pathologist in diagnostic and investigative studies. Even though formalin remains the popular fixative used in pathology, it is often not the best choice, because of the intermolecular cross linkage during formalin fixation, for preserving antigenicity of the tissues (Shi et al., 1991).

The markers of cell proliferation and oncogene products have shown marked variations in staining in different neoplasms and between different laboratories. These differences reflect both true biological difference, between tumours but also artefactual differences imposed by fixation techniques (Mehregan & Mehregan, 1996). Choosing the correct fixative for minimal loss of antigenicity and maximal preservation of tissue morphology is therefore an important consideration in immunohistochemistry (Shi et al., 1991).

Microwave antigen retrieval has been introduced in immunohistochemistry for the uncovering of antigens which have been obscured due to formalin fixation. Increase in sensitivity in terms of intensity and extent of immunostaining without deleterious effects on the specificity and unmasking of masked epitopes could significantly expand the range of antibodies useful in immunohistochemistry as well as reducing the incidence of false-negative results (Shi et al., 1991).

As our current knowledge on the biology and aetiology of oral cancer is still limited, advances in the area still depend on the validity of the investigative technique used in immunohistochemistry (Mehregan & Mehregan, 1996). Investigation of the antigenicity of gingiva using the microwave antigen retrieval technique would be of value in expanding knowledge in this area.

The aims of the present investigation were:-

1. Identification of any loss of reactivity of three antigens, Ki-67, PCNA and p53 following formalin fixation.
2. Identification of antigen masking resulting from exposure to a solution of EDTA (0.1 M EDTA) and a fixative (10% formalin).
3. Identification of possible reversibility of any antigen loss by the use of microwave retrieval technique.

3. MATERIALS AND METHOD

The investigation involved (n= 6) specimens of redundant human gingiva removed from six patients undergoing surgical treatment for periodontal disease (n= 5) and cyclosporin associated hyperplasia (n= 1). Each of these specimen was divided into approximately three portions. These three portion of all the six specimens were processed in three different ways as follows (Figure 3.1).

Portion 1: This portion was fixed in neutral buffered 10% formalin (pH 7.2) at room temperature overnight.

Portion 2: This was incubated in a solution of neutral buffered (pH 4.5) 10% formalin- 0.1 M EDTA at room temperature overnight.

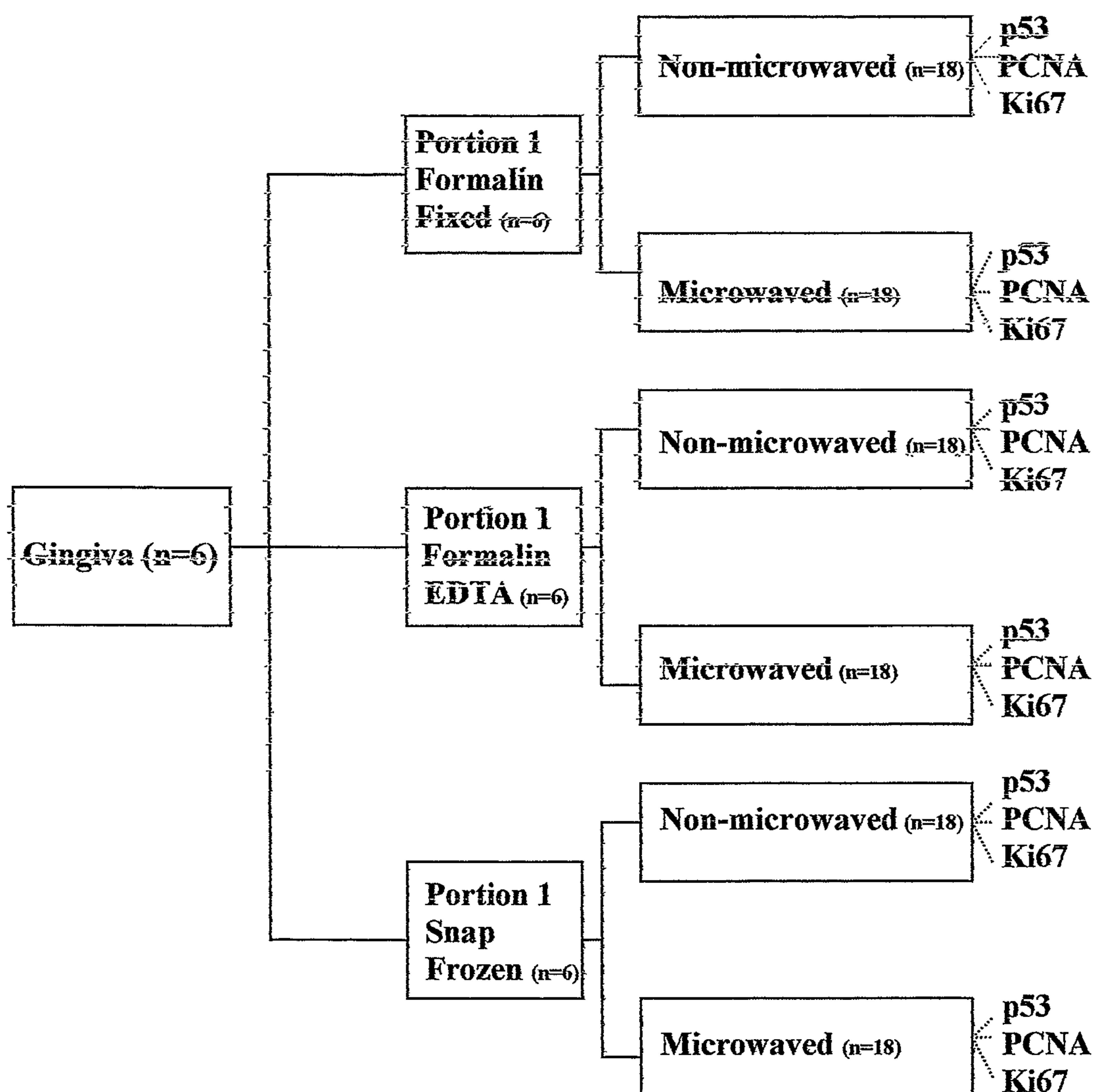
Portion 1 and portion 2 were routinely processed for wax embedding. The specimens were immersed in increasing strengths of ethyl alcohol (ethanol), as the water has to be removed from the tissue in order to embed the tissue in paraffin wax and this is know as dehydration. The tissues are then immersed for 2 hrs in xylene (wax solvent). Xylene is miscible with wax than alcohole and this step is also known as clearing as these wax solvents have the effect of raising the refractive index of the tissue, which makes them appear clear.

The tissues are then impregnated in a vacuum-embedding oven and given four changes of paraffin wax over a period of 2 hours. The tissues are finally transferred from the last wax bath to mould filled with molten paraffin wax. Once solidification of wax is complete, the block is removed from the mould (Culling, 1985).

Portion 3: The final portion was snap frozen in liquid nitrogen and stored at -70° C.

These three portions of specimens were examined for expression of p53, PCNA and Ki67 antigens using the immunohistological technique, with and without microwave antigen retrieval.

Figure 3.1: Summary of different regimes of tissue fixation, microwave irradiation and antigens investigated, in the 3 portions of each specimen of gingiva.



3.1 Paraffin embedded sections (Portions 1& 2)

Duplicate 4 micrometer thick sections were cut from each paraffin embedded tissue blocks at right angle to its epithelial surface. The sections are mounted on the slide using the water-bath method.

The processing of the sections are done in the following steps (**Key et al., 1993; Vojtesek et al., 1992; Hall et al., 1990**).

- The sides are then beacked in an oven at 60°c for 1 hour.
- Immerse in HistoClear (to remove paraffin) 5mts.
- Immerse in 2 changes of HistoClear for 1min each.
- Place in two changes of absolute alcohole for 1min each.
- Immerse in 75% alcohole for 1min.
- Wash in water for 1min and rinsed in posphate buffer saline (PBS).

3.2 Snap frozen sections (Portion 3)

For snap freezing small pieces of the fresh gingival tissue, not more than 1 mm in thickness, are placed in embedding matrix (Lipsaw) on an aluminium foil boat and immersed into a container with isopentane and lowered into liquid nitrogen at -160°C to -180°C . Once the blocks are frozen the specimens are removed from isopentane and stored in -70°C freezer.

Frozen tissues are tranferred into the block-holders using the embedding matrix and frozen in the cabinet of the cryostat at -20°C . Several 9 micrometer thick cryostat sections were cut of each specimen. The sections were mounted into Poly-L-lysine coated slides. The sections are air-dried for 5 min before being fixed in acetone at room temperature for 5 minutes.

3.3 Microwave antigen retrieval

Tissue sections to be exposed to microwave irradiation were incubated in sodium citrate buffer (2.94g/litre; pH 6.0) and irradiated for two 5 min. cycles each at full power. Between cycles the fluid was replenished with hot sodium citrate buffer. The sections were allowed to stand for 20 minutes undisturbed, following the microwave treatment.

- The microwaved and non-microwaved tissue sections were subsequently rehydrated in PBS (pH 7.2).
- The primary antibodies are applied (monoclonal or polyclonal primary antibodies to p53, PCNA, Ki67 as detailed in **Table 3.1**) and incubated in a humid chamber at room temperature for 1 hour. Mean time PBS is applied to negative control sides.

Table 3.1: Specificity, dilution and incubation period of primary antibodies used in the study.

PRIMARY ANTIBODY	ANTIGEN	DILUTION	INCUBATION PERIOD	TEMPERATURE
<u>Monoclonal</u>				
DO7 (1)	p53	1:100	1Hour	Room temperature
PC10 (2)	PCNA	1:200	1Hour	Room temperature
<u>Polyclonal</u>				
Ki-67 (3)	Ki-67	2.125	1Hour	Room temperature

(1, 2 and 3: Novocastra Laboratories Ltd., Newcastle upon Tyne, UK.)

- The sections were washed in a water bath for 2 min.
- Then the sections are immersed in 3% hydrogen peroxide (blocking agent) for 5 min

to block endogenous peroxidase activity.

- After washing again with tap water and twice with PBS for 1 min each the sections were incubated with secondary antibodies, biotin-conjugated goat anti-mouse monoclonal antibody or goat anti-rabbit polyclonal antibody for 1 hour (as detailed in **Table 3.2**).
- The sections were then rinsed in tap water for 2 min and and rinsed 1min in PBS.
- Then apply peroxidase conjugated streptavidin, diluted 1:4000, and incubate for 1 hour at room temperature.

Table 3.2: Specificity, dilution and incubation period of secondary antibodies used in the study.

SECONDARY ANTIBODY	ANTIGEN	DILUTION	INCUBATION PERIOD	TEMPERATURE
<u>Monoclonal</u> GOAT ANTI-MOUSE ANTIBODY(1)	p53 & PCNA	1:250	1Hour	Room temperature
<u>Polyclonal</u> GOAT ANTI-RABBIT ANTIBODY(2)	Ki-67	1:250	1Hour	Room temperature

(1 & 2: Biosource International, Camarillo, CA 93012, USA.)

- Wash with tap water for 2min.
- Stain slides with diaminobenzidine (DAB) for 5min.
- The sections were then lightly counter-stained with haematoxylin (1 dip).
- This step was followed by a thorough washing in running tap water for 2min.
- Then the slides were immersed in blueing solution for 1min.

- Wash in tap water for 1min.
- Immerse the slides for 2 changes in absolute alcohol for 1min each.
- Followed by three changes of histoclear for 1min each.
- Mount slides with DPX and coverslip.

Each set of experiments included positive and negative controls.

3.4 Positive controls

SW480 cell line is derived from a human adenocarcinoma of the colon. They have a defined p53 mutation. These cells were revived from liquid nitrogen following established protocols.

Cell line	Code mutation
SW480	273 CGT → CAT (Arg → His)

Reference: (Nigro, 1989)

3.5 Negative control

p53 (SOAS-2)

1. Null allele human osteosarcoma cell line (SOAS-2) served as one negative control.
2. Omission of the primary antibody to the sections.

3.6 Grading

The minimum size of the sample, 700 basal epithelial cells, to show any intergroup difference compared with the intra-observer variation had been determined by the method of accumulative means (Cox & Walker, 1996). Using numbered high power

field examination from random number tables selected or atleast 700 basal epithelial cells were counted.

The prevalence of cells expressing p53 protein detected by DO7 antibody assessed was using a grading system in which the specimens of redundant human gingiva, graded 0-3, according to the proportion of basal epithelial cells expressing p53 protein on tissue subjected to microwave antigen retrieval or without microwave antigen retrieval.

The proportion of immunoreactive basal epithelial cells was graded as 0= <1, 1= 1-10%, 2= 11-50%, 3= > 50% of basal epithelial cells demonstrating immuno-reactivity for DO7 antibodies. The growth fractions were expressed as percentage of basal epithelial cells reacting with Ki-67 and PC10 antibodies. The proliferative activity of redundant human gingival specimens was estimated in microwaved and non-microwaved specimens.

3.7 Statistical Analysis

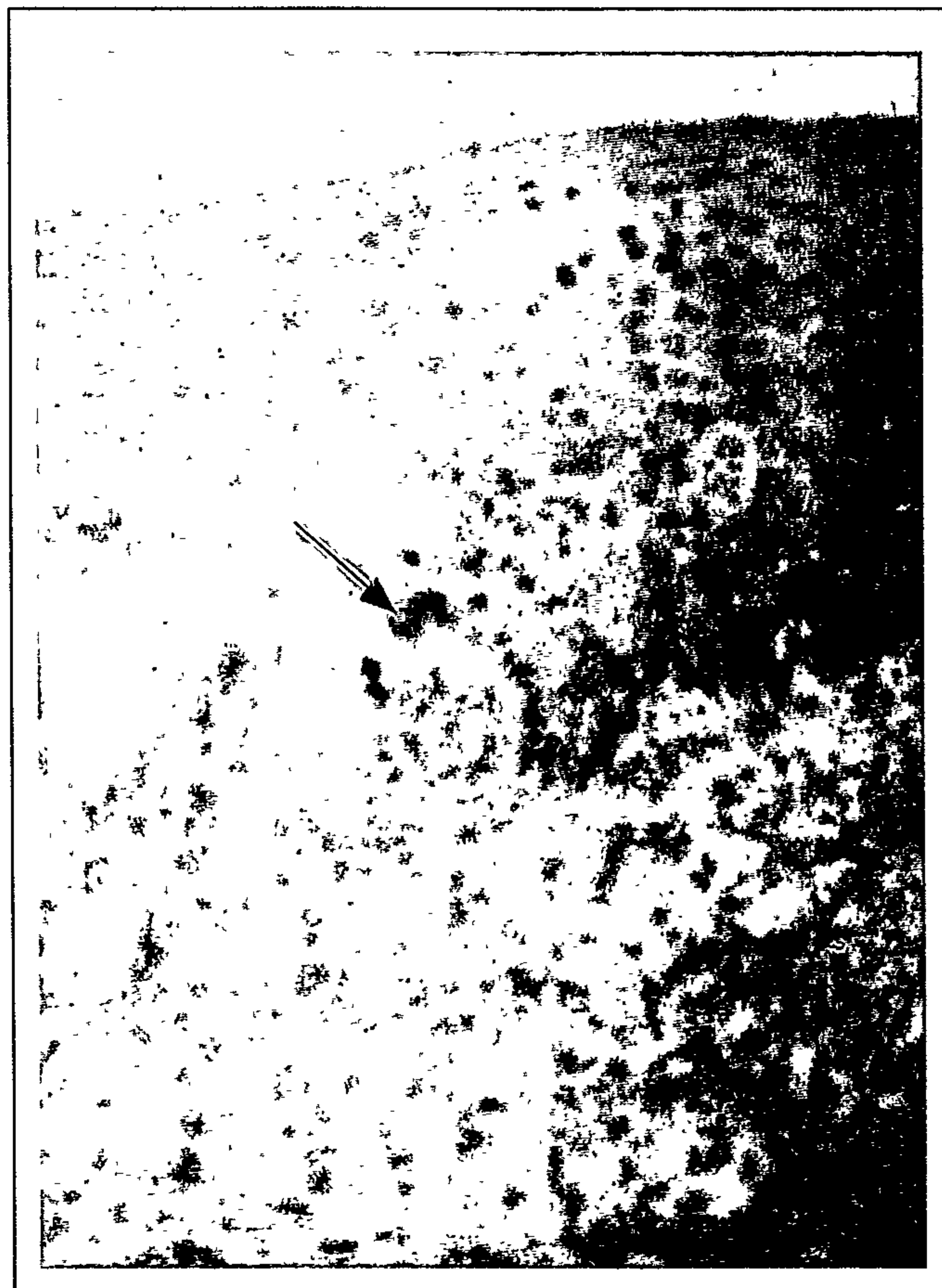
The statistical significance of any difference between the groups was assessed using the Mann-Whitney nonparametric test; p values of < .05 were accepted as significant.

4. RESULTS

4.1 p53 expression

The formalin-fixed and formalin-EDTA fixed specimens of gingiva excised during routine periodontal surgery for non-neoplastic disease had as expected no detectable p53 antigen by immunohistology using the DO7 antibody with or without microwave treatment. Non-microwaved snap frozen and acetone fixed sections showed only non-specific background staining without nuclear staining. When the acetone-fixed snap frozen specimens were subjected to microwave antigen retrieval however five out of six sections expressed p53 staining characteristically localised to the basal and parabasal cell layer with mild cytoplasmic staining (**Figure 4.1**).

Figure 4.1: Basal and parabasal epithelial cells with p53 detectable (arrow) by DO7 antibody in microwaved snap frozen sections of gingiva. Counterstained with heamatoxyline (Magnification = 50).



Grading of prevalence of p53 positive epithelial cells

3(50%) out of the five specimens were graded 2 (11-50%, positive basal epithelial cells), one specimen (16%) was as graded 3 (>51%), and one (16%) specimen was graded 1 (1-10%) and one (16%) specimen as 0 (<1%) (**Table 4.1**). p53 was detected only in the microwaved frozen specimen of gingival epithelium and was found in 5 of the 6 specimens tested. The difference in p53 expression mostly depended on the type of tissue processing and the microwave irradiation. The proportion of basal epithelial cells in each specimen showed statistically significant difference ($p < 0.01$).

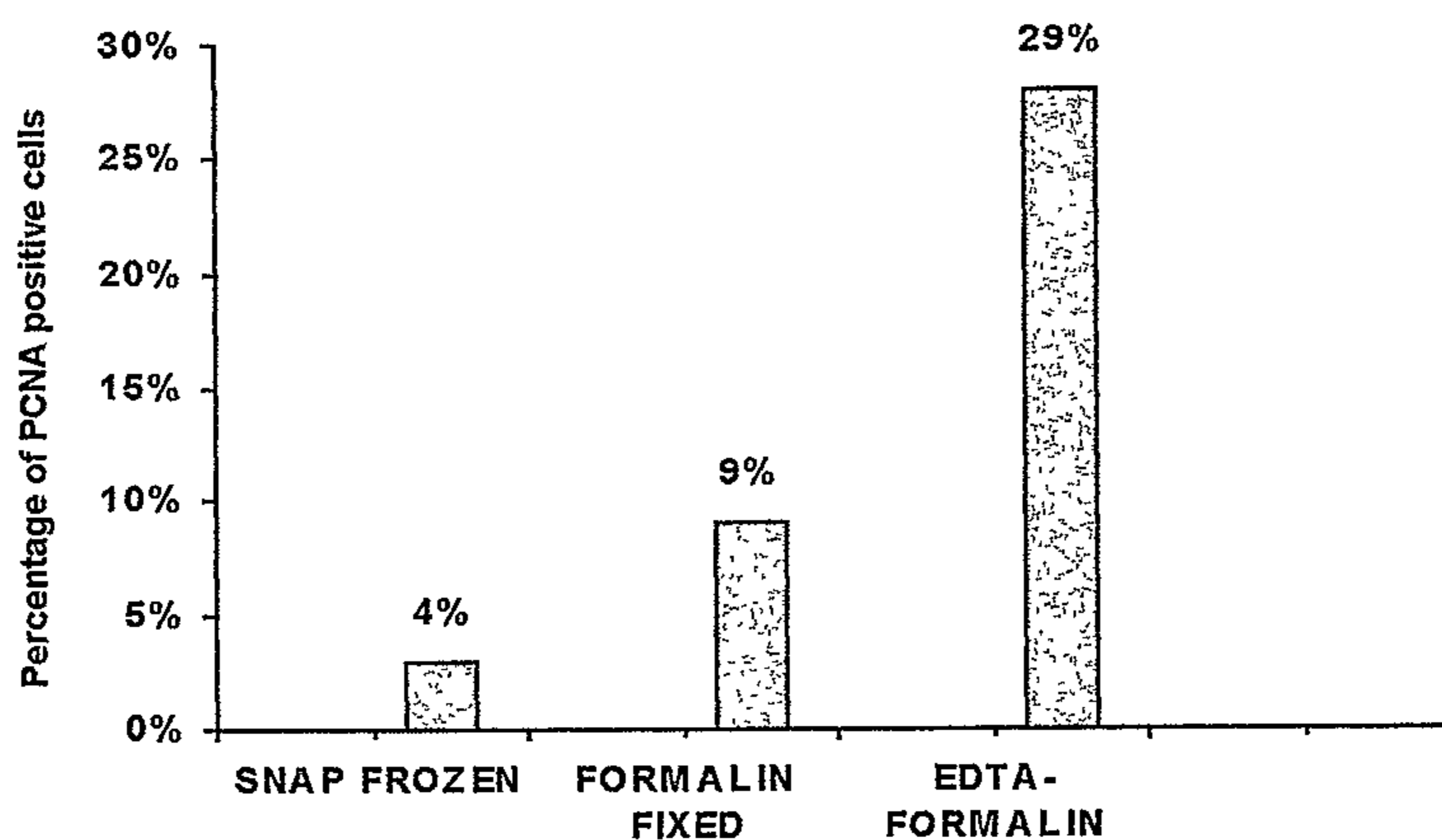
4.2 PCNA expression

For semiquantitative analysis of PCNA expression, cell counts of the positive basal cells were performed on at least 700 basal epithelial cells. In snap frozen sections, none of the 6 specimens of the non-microwaved tissue had any nuclear staining. There was non-specific background staining with stroma showing positive inflammatory cells.

None of the tissue preserved by snap freezing, formalin or formalin-EDTA but not microwaved, reacted with the PC10 antibody for PCNA. By comparison with non-microwaved tissue, the proportion of basal cells with PCNA positive nuclei in the microwaved specimens of gingiva was significantly increased and estimated as 3.6% for snap frozen tissue ($p < 0.01$), 8.6% for formalin fixed tissue ($p < 0.001$) and 28.7% for formalin-EDTA fixed tissue ($p < 0.01$) (**Fig 4.3, Table 4.2**) was difference. Cell staining in the microwaved tissues was not only more prevalent but more intense in positive cells.

Specimens fixed in EDTA-formalin solution and routinely processed for paraffin embedded sections failed to stain with the PC10 antibody for PCNA. When these sections were subsequently exposed to microwave irradiation, the tissue reacted with this antibody and this was a significant difference ($p < 0.01$) from the non-microwaved specimens. In the microwaved sections, the percentage of positive basal epithelial cells stained in EDTA exposed specimens for PC10 antibody (29%) significantly exceeded that for both formalin-fixed (8.6%) and snap frozen sections (3.6%) respectively. The comparison is shown on **Figure 4.2**.

Figure 4.2: Comparison of the percentage of positive basal epithelial cells stained in microwaved snap-frozen, formalin-fixed and EDTA-formalin fixed sections for PC10 antibody.



Positively staining cells were not frequent in the basal layer and had the most intensely staining nuclei but PCNA-positive cells were also, and consistently, found extending through the lower two-thirds of the spinous layer.

Table 4.3: Proliferative activity of gingiva, (a) non-microwaved, (b) microwaved, expressed as mean percentage (growth fractions), of basal epithelial cells reacting with PC10 antibodies.

		PCNA				
Tissue process	Number of specimens	(a) Non-microwaved		(b) Microwaved		p-value of microwaved vs non-microwaved
		Number of positive sections	Mean proportion of positive cells (growth fraction) % & Standard deviation (±)	Number of positive sections	Mean proportion of positive cells (growth fraction) % & Standard deviation (±)	
Snap frozen	6	0/6	0%	5 in 6	4%(1)	<0.01*
Formalin fixed	6	0/6	0%	6 in 6	9%(7.4)	<0.001*
Exposed to EDTA	6	0/6	0%	5 in 6	29%(24.8)	<0.01*

* Statistically significant difference
 NS: Not significant

Figure 4.3: PCNA positive basal epithelial cells (arrow) in formalin-EDTA fixed and microwaved gingiva, as detected by PC10 antibody. Counterstained with haematoxylin (Magnification= 50) .



4.3 Ki-67 expression

Ki-67 expression was evident only in the snap-frozen tissue (Figure 4.4). Both the microwaved and non-microwaved tissue had positively reacting basal layers. The mean proportion of positive cells in snap-frozen sections without microwaving was 21% and after microwaving was 34%. There was a significant statistically difference in the proportion of positive cells in microwaved and non-microwaved sections ($p < 0.05$) (Table 4.3). Positive cells were not detected in the (Table 4.3) formalin fixed and formalin-EDTA exposed gingiva with or without microwave antigen retrieval. The experiments demonstrated that the Ki-67 antibody used for the purpose was not suitable for conventionally fixed tissue.

Table 4.3: Proliferative activity of gingiva, (a) non-microwaved, (b) microwaved, expressed as mean percentage (growth fractions), of basal epithelial cells reacting with Ki-67 antibodies.

Tissue process	Ki-67						p-value of microwaved vs non-microwaved
	(a) Non-microwaved			(b) Microwaved			
	Number of specimens	Number of positive sections	Mean proportion of positive cells (growth fraction) % & Standard deviation (±)	Number of positive sections	Mean proportion of positive cells (growth fraction) % & Standard deviation (±)		
Snap frozen	6	4 in 6	21% (1)	4 in 6	34% (21.5)	<0.05*	
Formalin fixed	6	0/6	0	0/6	0	> 0.05 <1, NS	
Exposed to EDTA	6	0/6	0	0/6	0	> 0.05 <1, NS	

* Statistically significant difference
 NS: Not significant

Figure 4.4: Immunoreactive basal epithelial cells (arrow) in microwaved snap-frozen gingiva incubated with Ki-67 antibody and counterstained with haematoxylin (Magnification= 100).



4.4 Controls

(a) Positive control

The adenocarcinoma cell line SW480 reacted positively to DO7 antibodies for all methods of tissue preservation, with or without microwave antigen retrieval expression (Table 4.1).

(b) Negative control

The p53 null-allele cells reacted negatively with the DO7 antibodies as predicted. Absence of staining was experienced when the primary antibody was omitted.

5. DISCUSSION

The investigation involved specimens of redundant gingiva removed after periodontal surgery for non-neoplastic disease, in the Periodontics Department, Westmead Hospital Dental Clinical School, from 6 patients undergoing surgical treatment for chronic periodontal disease (n= 5) and cyclosporin-associated hyperplasia (n= 1). The following discussion is based on p53, PCNA and Ki-67 detection in the above specimens processed in three different ways (formalin fixed, formalin-EDTA fixed and snap frozen) with and without microwave irradiation

5.1 p53 detection

Mutation in the p53 protein is the most common genetic alteration in human cancer (**Lane & Bechimol, 1990**). **Hall et al (1991)**, state that p53 expression is not usually detectable by immunohistochemistry in normal tissues as the wild type p53 protein has a half-life of only approximately 15 to 30 minutes due to its rapid degradation. It would have been preferable to include some normal tissue in addition to the specimens of hyperplastic gingiva in case the p53 persistence was due to some inflammatory stimulus.

Increased levels of p53 protein are often found in malignant tumors, but rarely in benign tumors and normal or non-neoplastic tissue (**Langdon & Partridge, 1992**). The disclosing of p53 protein in the frozen sections of hyperplastic gingiva in the present study indicates that failure to detect p53 in non-neoplastic states may alternatively be due to antigen masking. In the present investigation, p53 protein could not be detected in acetone-fixed frozen sections of the non-neoplastic gingiva by

immunohistochemistry, but its presence was confirmed in this tissue after microwaving in 5 out of 6 specimens, a statistically significant difference. The protein was not identified in formalin-fixed paraffin- embedded tissues. The reason for this expression only in microwaved snap frozen sections remains to be determined but it is suggested that it is due to the unmasking of p53 antigenic determinants in the snap-frozen tissue by the microwave treatment.

The possibility that this could represent false positive staining has to be considered but this seems an unlikely explanation considering that the p53 expression was restricted to its usual distribution in nuclei of the basal and lower spinous cells of the epithelium. The positive results in control adenocarcinoma cell line and the negative findings in the control null allele SOAS-cell line or when the primary antibodies were omitted indicated that the staining was specific for p53.

Binding to viruses such as SV40, or formation of oligomers of p53 itself, will like, the effect of most p53 mutations result in immunohistochemical detection of the protein due to an extended half-life of the protein. Positive staining for p53 needs therefore to be supplemented by mutational analysis by molecular biology techniques such as the polymerase chain reaction-single stranded conformation polymorphism analysis (Walker, 1997).

5.2 PCNA detection

In the present study PCNA reactivity was enhanced with microwaving, in agreement with the findings of Shin et al (1991). The present study compared non-microwaved

and microwave based antigen-retrieval of the snap frozen, formalin fixed and EDTA-exposed specimens. The ability of microwaving to unmask antigenic determinants in snap frozen, formalin-fixed and paraffin-embedded tissues for immunostaining was also demonstrated in the case of the PCNA antigen. Exposure of the tissues to the decalcifying agent EDTA did not abolish this enhanced staining.

Staining by PC10 antibody was only detected in snap frozen and formalin fixed specimens following exposure to microwave irradiation. The differences in the percentage of positive basal epithelial cells (respectively $p < 0.01$, $p < 0.001$), were significantly different between microwaved and non- microwaved specimens.

As a result of microwave irradiation, the growth fraction estimated for the growth fraction of the oral epithelium was increased. However the improved sensitivity of detection of the PCNA antigen even in trace amounts, may conversely reduce its usefulness for assessment of growth fraction, as the half life of the protein may approach or exceed the duration of the cell cycle.

The study by Steinbeck (1993) has shown 17% of staining in normal, 49% in mild, 54% in moderate and 73% in severe dysplasia. It is concluded that the PCNA immunoreactivity in the basal cells increased with the increasing grade of dysplasia.

The present investigation also showed that fixation and tissue processing influenced the results of PCNA immunohistochemistry. Differences in tissue handling and staining must be kept in mind in evaluating determination of growth fraction reported by

different investigators. Compared with non-microwaving, microwave heating using citrate buffer solutions provides enhanced but specific staining with the clearest background in formalin fixed paraffin sections (**Mehregan & Mehregan, 1996**).

Although heat treatment may enhance the sensitivity of some reagents, it is important when employing microwave antigen retrieval to use appropriate controls to monitor the specificity of antibody binding in case the normally masked epitopes are exposed giving rise to false-positive results with previously trusted antibodies (**Norton, 1993**).

5.3 Ki-67 detection

The present study has confirmed that immunolocalisation of Ki-67 in paraffin embedded tissue requires the ancillary aid of microwave antigen retrieval, in accord with **Mokry and Nemeck (1995)** who stressed that the staining intensity may be influenced by the kind of fixation and the length of microwave pre-treatment. However despite the manufacturer's claim that the antibody did react with formalinised antigen, in the current investigation Ki-67 was only expressed in snap frozen sections and the values for the growth fraction calculated for microwaved and nonmicrowaved epithelium in snap frozen sections did not differ significantly. The percentage of Ki-67 positive cells expressions increased according to the degree of epithelial hyperplasia and the grade of malignancy in the oral cavity (**Zoeller et al., 1994**).

Accordingly some, but not all, authors have found that the number of PCNA positive cells significantly exceeded the number of Ki-67 positive cells, suggesting that PCNA may not yield an entirely accurate index of cellular proliferation (**Mehregan &**

Mehregan, 1996), whereas the shorter-lived Ki-67 antigen resulted in a more reliable assessment of the proportion of actively cycling cells (**Mokry & Nemeck, 1995**). In the present investigation, the growth fraction determined by PCNA counts of labelled cells in microwaved EDTA-formalin treated gingival epithelium were comparable with those for Ki-67 in frozen tissue, microwaved or non-microwaved.

Ki-67 is regarded as a reliable marker of proliferating cells, and growth fractions determined by Ki-67 detection have been shown to be significantly correlate with *in vivo* initiated thymidine labeling data, which are regarded as the gold standard in epithelial cell kinetics (**Gerdes et al., 1984**). However *in vivo* initiated thymidine labelling of clinically normal mucosa poses ethical problems.

5.4 Tissue processing & antigen retrieval

There is no universal fixative in immunohistochemistry capable of processing all antigens. Formalin remains the standard fixative for use in routine histopathology. False negative results in immunohistology on formalin fixed tissues are commonplace. The determination of optimal fixation regimes for different human tumours and normal tissue for each antibody is time consuming and not achievable in routine investigation. Therefore the ability of high temperature microwave heating to uncover many antigens in formalin-fixed tissue represents an important advance in immunohistochemistry.

The duration of fixation also remains a critical variable. Longer fixation times generally result in diminished immunoreactivity and may necessitate vigorous antigen retrieval procedures (**Mehregan & Mehregan, 1996**). **Ezaki (1996)**, advised therefore that the

period of fixation is to be kept to a minimum. In the present study the specimens were fixed over night with formalin.

Neither p53 nor Ki-67 were expressed in EDTA-treated specimens. Non-microwaved, EDTA exposed formalinised specimens probed for PCNA were negative. Microwave heating reversed this negative finding with a mean proportion of positive cells (growth fraction) of 29%. The preservation of antigenicity of tissues treated with decalcifying agents are problematic, because of masking (Erber, 1996) of antigenic sites resulting in false-negative staining.

In the present investigation it was found that antigen masking can be reversed by using microwave antigen retrieval technique in PCNA, p53 and Ki-67 antigens. The result of this study support Shi et al's study in 1991.

Although antibody staining of Ki-67 in frozen sections has remained the standard for the evaluation of cell-cycle associated antigens in tissues, poor cytological detail is one of the limitations of this technique (Beckstead, 1994; Taylor, 1986). Routine paraffin-embedded sections are widely used for morphological examination of tissues but are not optimal for antigen preservation (Beckstead, 1994).

This particular investigation has used a citrate buffer solution, pH 6. Although the assessment of the optimal conditions for the microwave treatment were not a part of this study it is known that, the pH of the buffering solution can influence the immunoreactivity of the antigens used in the study (Shi et al., 1995).

Even though the introduction of monoclonal antibodies had led to better reproducibility of immunohistochemical results between various laboratories, discrepancies still occur, even with the same antibody, due to differences in method. Standardisation of technique and reagents are essential for meaningful comparison of data from different workers (**Elias et al., 1989**).

Some practical difficulties faced in the present investigation were the lack of time available for the study. The labour-intensive nature of the procedure and the number of slides that could be reliably heated in the microwave ovens were relatively small. Dislodgment of sections from the slide by boiling of the buffer and rotatory action of the microwave plate was a frequent problem. Although microwave antigen retrieval constitutes an important technical advance, considerable problems remain to be overcome, for example the marked variations in staining that can be encountered between different antibodies systems to detect the same antigen and in the different immunohistochemical techniques used.

6. SUMMARY

6.1 Oncogene

6.1.1 p53 antigen

- p53 antigen expression could only be found in snap frozen sections treated by microwaving. This antigen retrieval was consistent and showed typical localisation of the p53 antigen to nuclei of the basal and lower spinous cells of the epithelium.
- It had previously been assumed that wild-type p53 is not detectable in normal or non-neoplastic tissue due to rapid degradation but the findings of the present study indicate that the p53 antigen is still present in masked form and can be retrieved by microwave irradiation.

6.2 Cell cycle proliferation associated antigens

6.2.1 PCNA antigen

- None of the specimens of non-neoplastic gingiva fixed in EDTA-formalin solution and conventionally processed for paraffin embedded histological sections showed any immunostaining by the PC10 antibody for PCNA. When these sections were subsequently exposed to microwave irradiation, 5 out of 6 specimens (**Table 4.2**) reacted with this antibody and this was a significant difference ($p < 0.01$).
- Staining by PC10 antibody was apparent only in snap frozen and formalin fixed specimens subjected to microwave irradiation, and not in non-microwaved

specimens. The differences in the proportion of positive specimens and the percentage of positive basal epithelial cells in frozen and formalinised material were statistically significantly ($p < 0.01$, $p < 0.001$ respectively).

- In the microwaved sections, the percentage of PCNA positive basal epithelial cells (29%) in EDTA exposed specimens, was significantly greater than that for the formalin fixed sections (9%) ($p < 0.01$) and snap frozen sections (4%) ($p < 0.001$).

6.2.2 Ki-67 antigen

- The Ki-67 antibody used for the investigation proved to be reactive with snap frozen sections. The Ki-67 protein was detected in both microwaved and non-microwaved sections and there was statistically significant difference ($p < 0.05$) between the proportions of positive basal epithelial cells stained in these two post fixation regimes.

7. CONCLUSIONS

- This immunohistological investigation has confirmed previous reports that the three antigens, p53, PCNA and Ki-67 become masked during fixation in formalin or EDTA-formalin and that these effects could be generally reversed by microwaving of the section.

However the finding that p53 antigens are not in fact quickly degraded in non-neoplastic frozen tissue but are masked, is novel and questions the validity of the use of this antibody in screening for p53 mutation.

It could be concluded that microwave antigen retrieval increases the sensitivity of p53 immunoreactivity, but p53 expression is not a specific marker of gene mutation and should be interpreted with caution and supplemented with molecular biology techniques where a genetic error is suspected.

- With the microwave antigen retrieval technique, defined antigens can be reproducibly detected in formalin-fixed, paraffin-embedded tissues and snap frozen tissues, with the effect of enhancing the sensitivity and specificity of the method. The study findings confirm the statement by **Leong (1996)** that microwave antigen retrieval is an important step towards standardisation in immunohistochemistry and improved immunostaining of routinely fixed tissues.

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