Toxicity of Khat on Normal Human Oral Cells

In Vitro Studies Using Primary Human Oral Keratinocytes and Fibroblasts in Monolayer and Organotypic Cultures

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This work is dedicated to my wife Damaris Moraa and our daughters Maryam and Jameela.

Contents

ACKNOWLEDGEMENT **4**

SUMMARY **6**

LIST OF PUBLICATIONS **8**

LIST OF ABBREVIATIONS **9**

1. INTRODUCTION **11**

1.1 KHAT 11

1.2 TOXICITY 18

1.3 CELL CYCLE AND DIFFERENTIATION 20

1.4 APOPTOSIS 25

2. PROBLEM STATEMENT AND AIMS OF THE STUDY **38**

3. METHODOLOGICAL CONSIDERATIONS **40**

3.1 KHAT EXTRACTION AND HANDLING 40

3.2 CELLS AND ORGANOTYPIC TISSUES 41

3.3 ASSESSMENT OF CELL PROLIFERATION AND DIFFERENTIATION 45

3.4 MORPHOLOGICAL AND BIOCHEMICAL DETERMINATION OF CELL DEATH 47

3.5 ISSUES OF CLINICAL RELEVANCE IN THIS STUDY 52

4. RESULTS AND DISCUSSION **53**

5. CONCLUDING REMARKS **64**

6. FUTURE STUDY AREAS **65**

7. REFERENCES **66**

8. ORIGINAL PAPERS **76**

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Summary

Khat is an evergreen shrub of the *Celastraceae* family grown in parts of the Middle East and Eastern Africa where its use is important for the social and economic wellbeing of the communities. Fresh leaves and shoots of the khat plant contain the chemical cathinone which has a psychoactive effect comparable to amphetamine. Habitual chewing of khat is widespread in Yemen and the horn of Africa, and its use as a stimulant is gradually spreading to other parts of the world especially in immigrant communities. Prolonged khat use has been reported to have adverse effects on the central nervous, cardiovascular and reproductive systems. In the oral cavity, khat chewing has been associated with histopathological changes like hyperkeratosis, epithelial hyperplasia and mild dysplasia. A higher incidence of head and neck cancer has been reported among khat chewers compared to non-chewers. However, studies on the toxicological potential and mechanisms of actions of khat remain scarce. The aim of this study was to investigate the toxic effects induced by an extract of khat on primary normal human oral keratinocytes and fibroblasts in monolayer and in vitro reconstructed human oral mucosa.

Khat induced a concentration dependent inhibition of cell growth, with cells accumulating in the G1 phase of the cell cycle and showing an increased expression of cell cycle inhibitor proteins like p53, p21 and p16. The growth inhibition occurred earlier in fibroblasts when compared to keratinocytes. Unlike keratinocytes, fibroblasts also showed recovery of their proliferative potential on prolonged exposure. In reconstituted oral mucosa, khat induced a concentration dependent reduction in cell proliferation and a reduction in total epithelial thickness. An early and increased expression of p21, keratinocyte transglutaminase, involucrin and fillagrin, as well as decreased expression of cytokeratin 13 in tissues exposed to khat suggested premature differentiation and a switch from nonkeratinizing to keratinizing epithelium. These changes were accompanied by increase in p38 expression, and were reversed by inhibitors of p38. The results demonstrate the toxic potential of khat

to oral tissues and identify p38 MAP kinase signalling as the mechanism involved in stress induced by khat. At higher concentrations, khat induced cell death that showed morphological and biochemical features consistent with apoptosis. Khat induced an increase in cytosolic reactive oxygen species (ROS) and a depletion of intracellular glutathione (GSH). Antioxidants reduced ROS generation, GSH depletion and delayed the onset of cytotoxicity in both cell types. Generally, fibroblasts were more sensitive to khat-induced cytotoxicity than keratinocytes. Cell death induced by khat was caspase-independent and showed a swift and sustained decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) and release of mitochondrial apoptogenic proteins to the cytoplasm. The findings described in this study were observed at concentrations of khat comparable to those found in saliva among people chewing khat, and demonstrate the potential for khat to modulate key cellular functions such as proliferation, differentiation and cell death through specific signaling pathways. The results show that khat has toxic effects on human oral tissues and raises concerns about khat use and the development of various oral lesions.

Key words: Khat, oral, keratinocyte, fibroblast, organotypic, cell cycle, differentiation, senescence, apoptosis

List of publications

This thesis is based on the following original papers. The papers are referred to in the text by their roman numbers.

Paper I

Lukandu, O. M., Costea, D. E., Dimba, E. A., Neppelberg, E., Bredholt, T., Gjertsen, B. T., Vintermyr, O. K. & Johannessen, A. C. (2008) Khat induces G1-phase arrest and increased expression of stress-sensitive p53 and p16 proteins in normal human oral keratinocytes and fibroblasts. *Eur J Oral Sci,* **116,** 23-30.

Paper II

Lukandu, O. M., Costea, D. E., Neppelberg, E., Vintermyr, O. K. & Johannessen, A. C. Normal oral keratinocytes grown in organotypic co-cultures undergo premature differentiation and abnormal keratinization in response to khat-induced stress. *(Manuscript)*

Paper III

Lukandu, O. M., Costea, D. E., Neppelberg, E., Johannessen, A. C. & Vintermyr, O. K. (2008) Khat (*Catha edulis*) induces reactive oxygen species and apoptosis in normal human oral keratinocytes and fibroblasts. *Tox Sci,* **103,** 311-324.

Paper IV

Lukandu, O. M., Costea, E. A., Neppelberg, E., Bredholt, T., Gjertsen, B. T., Johannessen, A. C. and Vintermyr, O. K. Early loss of mitochondrial membrane potential in cell death induced by khat in primary normal oral cells. *(Manuscript).*

List of Abbreviations

AIF	apoptosis inducing factor
BA	bongkrekic acid
CHX	cycloheximide
CK	cytokeratin
CMF-DA	5-chromomethylfluorescein diacetate
COX IV	cytochrome c oxidase subunit IV
CsA	cyclosporine A
Cyt C	cytochrome c
DAPI	4',6-diamidino-2-phenylindole
DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
DHE	dihydroethidium
$DiOC_6(3)$	3,3'-dihexyloxacarbocyanine iodide
DMEM	Dulbecco's Modified Eagle's medium
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
GSH	reduced glutathione
GSSG	oxidized glutathione
H&E	hematoxylin and eosin
KSFM	keratinocyte serum free medium
MMP	mitochondrial membrane permeabilisation
mPTP	mitochondrial permeability transition pore
NAC	N-acetyl-L-cysteine
PBS	phosphate buffered saline
RNA	ribonucleic acid

ROS	reactive oxygen species
TBS	tris-buffered saline
TGase-K	keratinocyte transglutaminase
Tiron	4,5-dihydroxyl-1,3-benzededisulfonic acid
TUNEL	terminal deoxynucleotidyl transferase d-UTP nick end labeling
$\Delta\Psi_m$	mitochondrial membrane potential

1. Introduction

1.1 Khat

Khat (*Catha edulis* Forsskal) is an evergreen flowering tree or shrub of the *Celastraceae* family that grows in the equatorial climates mainly in the Arabian Peninsula and the regions around the horn of Africa (Al-Motarreb et al., 2002b) (Fig.1). The khat plant grows to a height of between 1 and 20 metres. Khat can grow both in arid and semi-arid areas making it an alternative to rainfall-dependent commercial crops like coffee and tea. Khat is known by various names in the different regions where it is grown such as Arabian Tea, Abyssinian tea, Tchat, Chak, Chaad, Khat, Miraa and Qat. Ethiopia, Yemen and Kenya are the three main khat-growing countries. Khat is also grown, or at times grows wild in Uganda, Tanzania, Rwanda, Zimbabwe and South Africa (Al-Hebshi and Skaug, 2005b).

1.1.1 History and socio-economic uses of khat

There is controversy as to the exact region where khat use and cultivation originated, with some authors suggesting Ethiopia and others suggesting Yemen (Al-Motarreb et al., 2002b, Al-Hebshi and Skaug, 2005b). It is however widely believed to have been cultivated long before coffee and to have been in use as early as the 14th century. Khat was first known to the western world through the work of a Swedish botanist Pehr Forsskal. His work was published posthumously by his friend Karsten Niebuhr in the botanical papers (*flora aegytico Arabia*) in 1775. The use and trade in khat as a drug was brought to international attention in 1935 when it was discussed at the League of Nations. Since then, several biochemical analyses of the plant have been carried out and published in bulletins by United Nations Office on Drugs and Crime (UNODC).

A

B

Figure 1: *Leaves of the khat plant (A) and khat leaves wrapped in banana leaves to prevent withering and enable easy transport (B)*

Mastication is the main method of khat use at present, but historical narratives suggest that dry khat leaves may have been used in a manner similar to tea leaves in the past periods (Al-Hebshi and Skaug, 2005b). The habit of chewing khat has an immense social importance within the communities where it is practiced. Fresh shoots and leaves of the khat plant are chewed mainly for their psychostimulant properties. In some places, khat chewers believe that khat has medicinal properties. The social gatherings where khat is chewed, otherwise known as *majlis al qat* (Yemen) or *bar'cha* (Ethiopia), help in strengthening social bonds, solving social problems, building relationships and reinforcing a sense of nationalism (Al-Motarreb et al., 2002b). Khat is also used by truck drivers and other workers who desire to keep awake and alert during the night. At present, khat is still largely used by communities in the regions where it is grown. However, advances in transport and mass movement of people have enabled spread of khat chewing to almost all parts of the world. Khat has huge economic benefits for the farmers, traders and even governments in form of taxes upon the khat business. Income from khat contributes substantial amounts of GDP in countries like Ethiopia, Yemen and Kenya (Al-Hebshi and Skaug, 2005b).

1.1.2 Contents and pharmacological aspects of khat

Khat contains three main phenylpropylamine alkaloids; (-)-S-cathinone, (+)-1S,2S-norpseudoephedrine (cathine) and (-)-1R,2S-norephedrine (Al-Motarreb et al., 2002b) (Fig. 2). Cathinone is the main psychoactive constituent in khat, but it is very unstable and rapidly decomposes into norpseudoephedrine and norephedrine as the leaves and shoots dry up. Khat grown in the Meru region of Kenya was found to contain phenylpentenylamine alkaloids like (+)-S-merucathinone, (-)-3S,4S-pseudomerucathine and (+)-3R,4S-merucathine.

Khat also has another group of alkaloids, the cathedulines, identified as K1, K2, K6 and K15 from the Kenyan khat, E2, E4, E5 and E8 from Ethiopian khat and Y1 from Yemeni khat (Al-Motarreb et al., 2002b). The phenypentenylamines and the cathedulines are thought to be of less significance compared to the

phenylpropylamines with regard to stimulant effects, but could probably play a role in inducing other effects in humans. As with other green plants, khat also has tannins, oils, vitamins, amino acids and minerals. The composition of these constituents can vary depending on the soil in which the khat is grown and the climatic conditions.

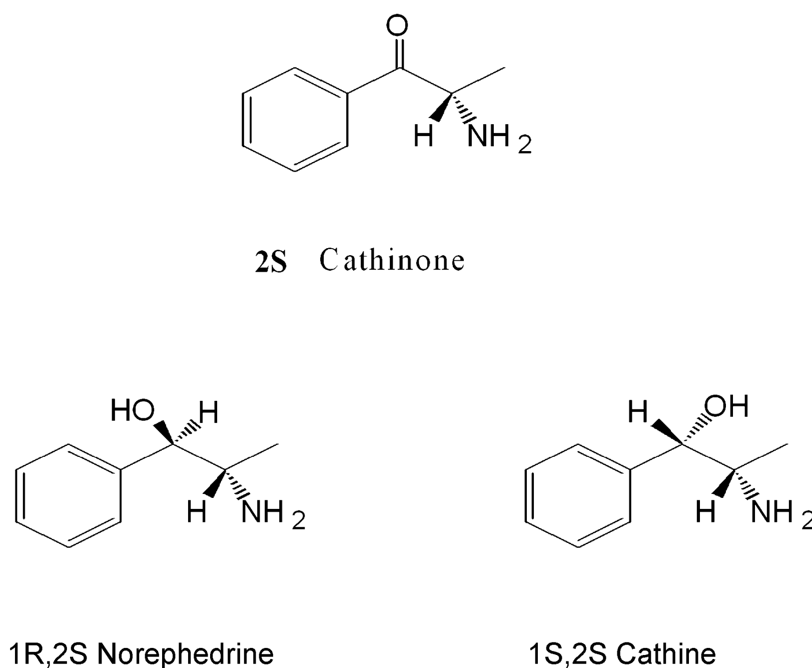

Figure 2: *Khat-specific phenylpropylamine alkaloids*

Mastication enables efficient extraction of the active constituents of khat into saliva, and the oral mucosa serves as the first and key absorption surface of these constituents. This allows rapid entry of cathinone and cathine into the bloodstream to achieve peak plasma levels within 1 – 4 h of chewing khat (Toennes et al., 2003). Once cathinone is absorbed, its sympathomimetic effects are achieved in a manner similar to other amphetamines i.e. by acting as an adrenergic receptor agonist and also by blocking norepinephrine transporter (to enhance norepinephrine effects). The alkaloids have short half-life (1.5 h for cathinone) due to rapid metabolism once in the blood (Widler et al., 1994, Toennes et al., 2003). This is partly the reason why khat has to be chewed continuously in order to elicit prolonged stimulant effects.

1.1.3 Potential adverse effects of khat

General medical and toxic effects of khat

The most widely studied health problems associated with khat chewing are the effects of cathinone on the central nervous system. In the short term, cathinone causes a feeling of euphoria, alertness, insomnia and anorexia. But unlike other drugs of abuse, it is controversial whether long term use can result in serious mental disease or dependence (Al-Habori, 2005). It has been suggested that the sympathomimetic action of cathinone on the central nervous system can result in functional mood disorders (Hassan et al., 2002). Some reports also suggest that khat may exacerbate symptoms in patients with pre-existing psychiatric disease, and that long term use could be a risk factor in the development of psychosis (Alem and Shibre, 1997, Awas et al., 1999, Odenwald et al., 2005). However, a recent critical look at the methodologies used in these earlier studies casts some doubt on the potential of khat to induce serious mental illnesses (Warfa et al., 2007). Neurologic effects of khat are thought to be responsible for peripheral effects like papillary dilatation (mydriasis) and dry mouth seen among khat chewers (Toennes and Kauert, 2004). In the cardiovascular system khat chewing induces vasoconstriction, tachycardia and hypertension (Al-Motarreb and Broadley, 2003). The increased coronary vasoconstriction associated with khat use (Al-Motarreb et al., 2002b, Al-Motarreb and Broadley, 2003, Baker et al., 2007) may partly explain the reported high incidence of myocardial infarction among khat chewers (Al-Motarreb et al., 2002a, Al-Motarreb et al., 2005). Cathinone has been reported to cause stomach irritation and delayed gastric emptying (Heymann et al., 1995). Antispasmodic effects and constipation induced by khat have also been reported in animal studies (Makonnen, 2000). Constipation is one of the major complains among khat chewers and it is thought to be caused partly by the tannins in khat and also by the effects of the alkaloids. Probably linked to this is the finding in some studies of a significant association between khat chewing and haemorrhoidal disease (Al-Hadrani, 2000). Other gastrointestinal effects include a potential risk in the development of duodenal ulcer disease (Raja'a et al., 2001) and the effects of khat on the bioavailability of

some antibiotics (Attef et al., 1997) among people using khat and taking medication at the same time.

Effects of khat on reproductive health are controversial. Khat chewing by pregnant women has a potential to affect the fetus (Abdul Ghani et al., 1987). So far, animal studies have highlighted the potential of khat to cause congenital abnormalities (Islam et al., 1994). Studies in albino mice show that khat reduces the possibility of pregnancy and increases the possibility of post-implantation abortion (Tariq et al., 1986). In males, khat is thought to cause impotence (Islam et al., 1990, Hakim, 2002) and several studies have shown degenerative changes in male sexual organs, low testosterone levels (Islam et al., 1990, Nyongesa et al., 2007, Nyongesa et al., 2008), and poor semen and sperm health (el-Shoura et al., 1995). However, other studies have reported opposite effects, including a report that some khat constituents could actually enhance sperm production, maturity and function in a way that can enhance fertilization (Al-Mamary et al., 2002, Adeoya-Osiguwa and Fraser, 2005, Mwenda et al., 2006, Abdulwaheb et al., 2007).

Khat affects blood formation in mice by inducing various chromosomal aberrations and suppression of bone marrow and also reduces the mitotic index of somatic cells (Qureshi et al., 1988). In the same study, khat was shown to induce chromosomal aberrations in gametes of mice. Khat has a potential to affect the systemic capacity to handle free radicals (Al-Qirim et al., 2002) but may also have some antioxidant effects owing to some of its antioxidant constituents such as flavonoids (Al-Zubairi et al., 2003). In an *in vitro* study, an organic extract of khat was shown to inhibit *de novo* RNA, DNA and protein synthesis in mammalian cells (Al-Ahdal et al., 1988). An extract of khat has been shown to induce caspase-dependent apoptotic cell death in human leukemia cell lines (Dimba et al., 2004a). Tests using khat extracts in mice have shown that the effects of khat are generally dose depended and tend to affect certain systems of the body more that others (Qureshi et al., 1988). Therefore, it is likely that the potential adverse effects of khat are limited by the inability of khat users to consume large quantities of khat.

Oral effects of khat use and potential risk for oral cancer

A number of studies have been done concerning the oral and dental health among khat chewers. Over 90 percent of the alkaloid content of khat is extracted into saliva during chewing and most of it is absorbed through the oral mucosa (Toennes et al., 2003). Therefore, oral tissues such as the oral mucosa are exposed to high doses of khat constituents during khat chewing rendering them susceptible to its potentially toxic effects. Some early studies describing effects of khat on oral tissues showed a lower dental caries rate among khat chewers (Hill and Gibson, 1987). Fluoride content in khat shoots and leaves is relatively low and this effect can therefore not be attributed to fluoride. Recently, a series of *in vitro* studies by Al-Hebshi *et al* revealed selective antibiotic effects of khat that could be of significance for periodontal and dental health (Al-Hebshi et al., 2005, Al-Hebshi and Skaug, 2005a, Al-Hebshi et al., 2006), but the active compound(s) in khat responsible for the antibiotic activity was not clear. Unfortunately, clinical studies carried out so far on periodontal health among khat chewers are generally controversial and inconclusive (Mengel et al., 1996).

Khat has been associated with oral keratotic white lesions which occur in the same region within the vestibule or buccal mucosa where the khat bolus is placed while chewing (Ali et al., 2004). Some of these lesions show histopathological changes like acanthosis, hyperkeratosis and mild dysplasia (Ali et al., 2004). The risk for developing these lesions was especially high among khat chewers who also used tobacco products (Ali, 2007). To the best of our knowledge, none of these lesions induced by khat has been considered to be premalignant (Ali et al., 2006). However, previous studies have found a higher incidence of head and neck cancer in khat users compared to non-users (Soufi et al., 1991, Nasr and Khatri, 2000). In some cases, the carcinoma developed in the same site where the khat bolus was placed while chewing. Isolated cases of oral cancer (Fasanmade et al., 2007), oral verrucous carcinoma (Awange and Onyango, 1993) and also plasma-cell gingivitis (Marker and Krogdahl, 2002) have also been reported in association with khat chewing. The genotoxic potential of khat constituents has been shown by a time dependent

induction of micronuclei in the buccal mucosal cells among khat users (Kassie et al., 2001a). However, due to a relatively small number of studies on khat (Carvalho, 2003) and the weaknesses of those studies already carried out, there is currently no consensus as to whether khat chewing is a potential risk factor for development of oral cancer. Large scale epidemiological studies and laboratory studies on cellular and molecular aspects of khat toxicity are therefore needed to examine the potential association between khat use and oral cancer (Al-Hebshi and Skaug, 2005b).

1.2 Toxicity

Toxicity refers to the potential of chemicals to cause injury or death to cells or tissues. This could result in abnormal functioning of these tissues and ill-health or death to the organism. Substances often need to be assessed for toxicity before they are allowed for use by humans and animals as foods, drugs and even cosmetics. However, *in vivo* toxicity testing can be considered unethical and a practical challenge because of the risk of injury and death of the test subjects, be it humans or animals (Welss, Basketter et al. 2004). For this reason, *in vitro* toxicity testing methods have been developed.

1.2.1 In vitro toxicity testing

Cytotoxicity

The effects of a toxic agent on cells can occur in two ways; 1) altering the metabolism and behavior of the cells and, 2) killing the cells. Parameters that may be assessed to determine alteration in cells include synthesis of DNA and proteins, cell cycling and differentiation, irritancy (e.g. release of certain cytokines) and also transformation (i.e. genetic mutations or malignant transformation). Cell viability and cell survival are used to assess the potential of toxic agents to kill cells in the short term and long term respectively. Parameters such as morphological changes, membrane integrity and perturbation in certain metabolic pathways are commonly

used in cell viability testing. Cell survival is normally assessed by clonal growth (plating efficiency).

Tissue toxicity

Tissue responses to toxic agents such as fibrosis, inflammation and structural changes can not be assessed using single cells in culture. Attempts have been made to culture organotypic (tissue) models that assemble various cells in order to mimic the *in vivo* situation. Some of the best advances in this area have been done on skin leading to the many types of reconstituted skin equivalents which are widely used in toxicity testing (Welss, Basketter et al. 2004). Models of oral mucosa have also been developed, and few studies have already used them for toxicity testing (Merne, Heikinheimo et al. 2004, Neppelberg, Costea et al. 2007). The parameters assessed in tissue toxicity testing include structural changes, cell death, expression of various proteins and release of cytokines.

1.2.2 Khat and cell proliferation

In view of the various effects of khat mentioned earlier, khat appears to have some potential to exert a general stress on cells in a manner that could affect cell cycle regulation and differentiation. In the literature, only a few studies have tried to investigate this potential. In one study, a reduction of cell proliferation was reported in studies where animals were exposed to khat (de Hondt et al., 1984). In another study carried out in plant roots, reduced proliferation was observed following treatment with cathinone (Al-Meshal, 1987). However, these previous studies did not focus on effects of khat on proliferation as a measure of toxicity, and did not attempt to address the specific mechanisms involved in the signaling leading to these effects. Therefore, little is known concerning the potential of khat constituents to modulate cell proliferation and differentiation both *in vitro* and *in vivo*.

1.2.3 Apoptosis as a mechanism of khat-induced toxicity

Apoptosis has been reported as a potential mechanism of cytotoxicity in mammalian cells following exposure to khat and khat-specific constituents (Dimba et al., 2003). Induction of apoptosis in leukemia cell lines by an organic extract of khat was reported to be swift, synchronous and concentration-dependent. Khat-induced effects were partly dependent on *de novo* protein synthesis, and were initially reversible upon washing away the khat (Dimba et al., 2004b). Prior to this study, there had been no evidence of apoptosis induced by khat constituents. However, other studies had reported induction of apoptosis by amphetamines (Stumm et al., 1999, Oliveira et al., 2002, Cadet et al., 2007), which were also accompanied by increased generation of reactive oxygen species. With regard to oral tissues, khat has been shown to induce genotoxic changes in cells of the oral mucosa (Kassie et al., 2001b). Genotoxic stress has the potential to induce differentiation and apoptosis. However, the mechanism of khat-induced cell death in oral cells has not been characterized in any previous study.

1.3 Cell cycle and differentiation

1.3.1 Cell cycle

The cell cycle is a sequence of irreversible events involving growth and replication that occur between one cell division and the next cell division in eukaryotic cells. It is controlled by a series of signals that eventually lead to inactivation of the retinoblastoma family of proteins, thus unblocking progression of the cycle (Weinberg, 1995). These signals are mediated by two types of regulators 1) promoters of the cell cycle known as cyclins and cyclin-dependent kinases (CDK) and, 2) CDK inhibitors like cip/kip family (p21, p27 and p57) and the INK4a/ARF family (p16INK4a, p14arf) (Fig. 3). p53 could also modulate the cell cycle by its regulatory effect on p21.

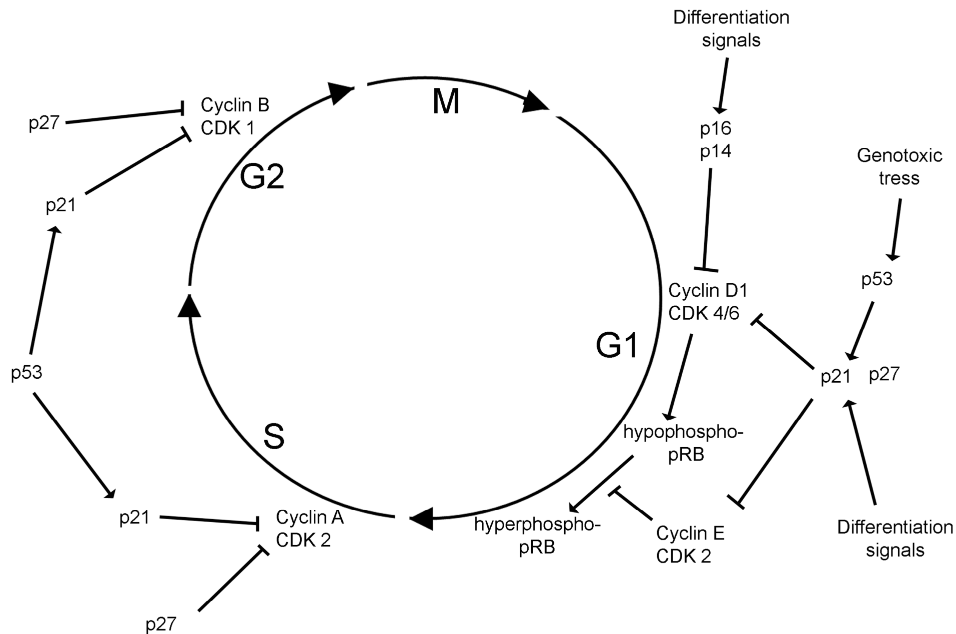

Figure 3: *Cell cycle and its regulation*

There are two checkpoints (G1/S checkpoint and the G2/M checkpoint) that occur at key sites within the cycle where the progress is assessed to ensure that no mistakes are made. Progress is not possible beyond these checkpoints unless all necessary conditions are met. An important event that takes place during cell division is DNA replication. Cells replicate their DNA and duplicate themselves in response to extracellular signals such as growth factors. Once DNA replication is initiated, these extracellular signals do not influence its progression (Blagosklonny and Pardee, 2002). However, damage to DNA resulting from toxic drugs and radiation could block entry of cells into the synthesis (S) phase. Determination of the amount of DNA in cells can thus provide information about the stage of the cycle where the cell are, and assess whether cells are arrested within a certain section of the cell cycle.

1.3.2 Differentiation and senescence

Normal eukaryotic cells have a limited potential to divide, and will eventually undergo irreversible cell cycle arrest determined by loss of telomere activity or telomere shortening (Herbig and Sedivy, 2006). The process that limits this potential

is termed replicative senescence, and it is accompanied by specific biochemical and phenotypic changes in the cell (Campisi, 1997). Cells can also undergo a permanent growth arrest in response to repeated or chronic non-lethal stresses. This is often called premature senescence or stress-induced senescence. Some of the known stresses include oxidative stress, DNA damage and sustained mitogen stimulation (Satyanarayana and Rudolph, 2004). The cell cycle inhibitor proteins mentioned above are known to be up-regulated at some stage in both replicative and stress-induced senescence (Loughran et al., 1996, Lee et al., 2000, Paramio et al., 2000, Campisi, 2001, Weinberg and Denning, 2002), and also in terminal differentiation (Parker et al., 1995, Martinez et al., 1999). However, the specific roles of these proteins in differentiation and senescence are still controversial (Harvat et al., 1998). It is possible that the role of each molecule may vary under different conditions or in different cell types, and also that they all cooperate during differentiation and senescence (Franklin et al., 1998, Paramio et al., 2001, Patil et al., 2005) (Fig. 4). For instance, it has been reported that p21 is responsible for the induction of terminal differentiation while p16 and p27 are responsible for maintaining the differentiated cells (Weinberg and Denning, 2002).

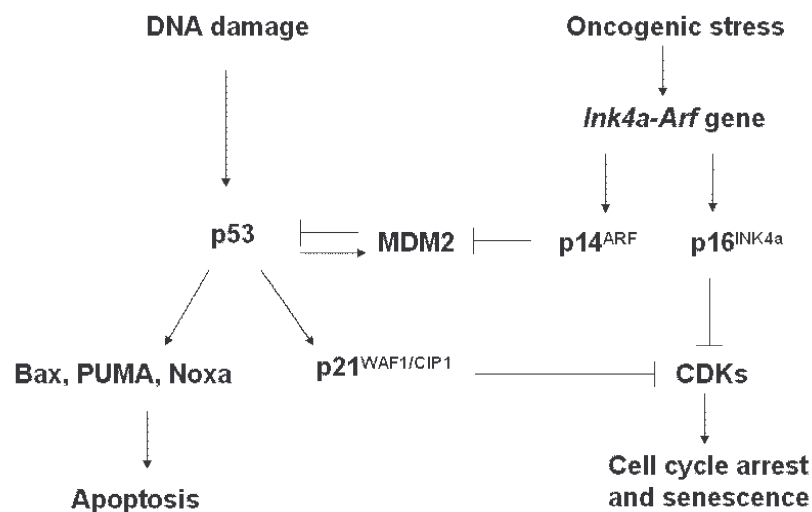

Figure 4: *Interconnectivity between pathways leading to cell cycle inhibition and cell death in response to cellular stress*

1.3.3 Epithelial differentiation and apoptosis

Differentiation refers to the process where a cell changes from a less specialized (stem-cell like) to a more specialized form in order to perform a specific function. In all epithelia, cell proliferation occurs in the basal area by epithelial stem cells and transit amplifying cells. The cells normally start to differentiate when they detach from the basement membrane and this progresses as they are pushed outward. Apart from influence from underlying connective tissue, epithelial differentiation is also controlled by local and systemic growth factors and cytokines. The differentiation pattern and phenotype in different epithelia can vary due to extrinsic factors such as influence from specific neighboring cells (e.g. fibroblasts), and also due to intrinsic factors specific to keratinocytes in the particular region (Gibbs and Ponec, 2000). These explain the variations in the types and degrees of proteins expressed in different epithelia.

Epithelial cell differentiation has been considered a special type of apoptosis (Haake and Polakowska, 1993, Teraki and Shiohara, 1999). The two mechanisms do share several signaling molecules and pathways. For instance, in response to cellular stress cells can initiate mitogen-activated (MAP) kinase signaling pathway which leads to the phosphorylation of either of the two downstream kinases, Jun N-terminal Kinase (JNK) and p38. Both of these proteins can induce apoptosis. In addition, p38 plays a role in both replicative senescence and ROS induced differentiation and senescence (Iwasa et al., 2003). The type and intensity of the stress will determine whether the cell will undergo proliferation, premature senescence, apoptosis or even necrosis (Toussaint et al., 2002a, Toussaint et al., 2002b). In keratinocytes, whereas stress-induced apoptosis occurs quickly, stress-induced differentiation is a much slower process that is associated with a strong induction of differentiation markers like involucrin and keratinocyte transglutaminase (TGase-K) (Sayama et al., 2001).

1.3.4 Differentiation in oral mucosa

As with all stratified squamous epithelia, the functional integrity of the oral mucosa is dependent upon the gradual differentiation of keratinocytes. The types of proteins expressed by keratinocytes vary at different stages during differentiation and also depending on whether the mucosa is keratinizing or non-keratinizing (Presland and Dale, 2000, Squier and Kremer, 2001). Cytokeratin (CK) 5 and 14 are normally expressed within the basal layers whereas involucrin, loricrin and TGase-K are expressed as differentiation progresses in the spinous and superficial layers in all regions. In most epithelia, the protein p21 is over-expressed just above the proliferating cells, and mildly expressed in the other regions of the epithelium (el-Deiry et al., 1995 Dotto, 2000, Weinberg and Denning, 2002). This is thought to be related to its role in induction of irreversible cell cycle arrest and differentiation. On the other hand, over-expression of p21 can inhibit further differentiation (Dotto, 2000). Thus, the increased expression of p21 is normally transient as it is subject to proteosome-mediated degradation (Blagosklonny et al., 1996).

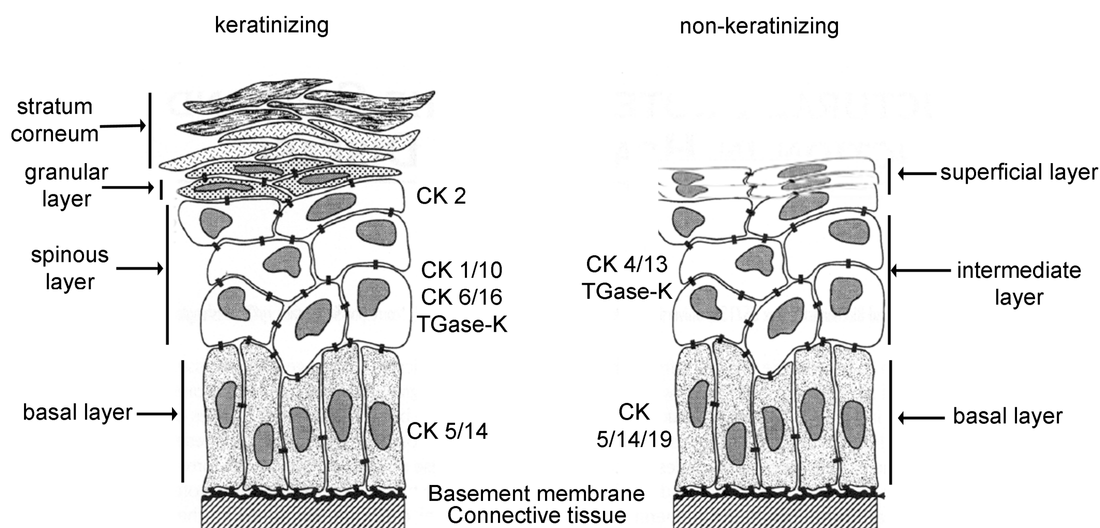

Figure 5: *Keratinizing and nonkeratinizing oral epithelia (modified from Presland and Dale 2000)*

The expression of p16 during differentiation in oral keratinocytes has been shown to vary depending on the factors inducing the differentiation (Lee et al., 2000). The spinous and superficial layers in keratinizing oral epithelia also express CK 1, 2, and 10 whereas non-keratinizing epithelia express CK 4 and 13 (Fig. 5). A protein such as filaggrin is expressed in the superficial layers of keratinizing oral epithelia and is normally absent in non-keratinizing regions.

1.4 Apoptosis

The term apoptosis was first used by Kerr (Kerr et al., 1972) to describe a particular type of cell death accompanied by rounding up of the cell, retraction of pseudopodia, reduction in cellular volume (shrinkage), condensation of chromatin (pyknosis), fragmentation of the nucleus (karyorhexis) and plasma membrane blebbing (Fig. 6). The term apoptosis is therefore a morphological description of a type of cell death, and the Nomenclature Committee on Cell Death recommends keeping it morphological, irrespective of the biochemical picture that it may present (Kroemer et al., 2005). Apoptosis is programmed genetically in every cell (Kroemer et al., 2007) and it is a key mechanism in embryonic development, post embryonic reorganization and immune function in multicellular organisms. It also plays a role in many pathologic conditions (Cooper et al., 1975) and therapeutic function of many drugs (Zamzami et al., 1995a, Zamzami et al., 1995b). The key morphological feature of apoptosis as described by Kerr et al is cell shrinkage. Cells also form plasma membrane blebs, and the chromatin condenses (pyknosis) and breaks up into small pieces (karyorhexis), but cells still maintain functionally intact plasma membranes especially in the early phases of cell death. Finally, the entire cell breaks up into membrane-bound fragments called apoptotic bodies (Kerr et al., 1994). In an *in vivo* environment, these apoptotic bodies are rapidly phagocytosed by neighboring cells preventing an inflammatory response. The process of apoptosis could take as little as 1-3 h in lymphocytes, or last as long as 3 days in keratinocytes (Haake and Polakowska, 1993).

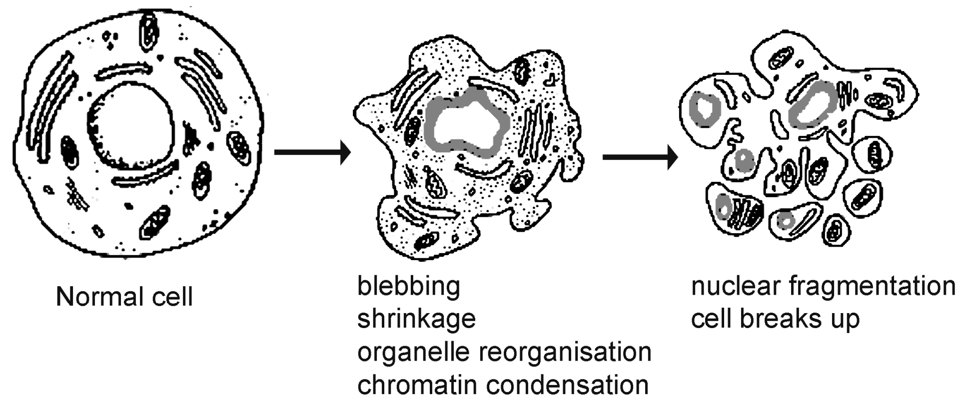

Figure 6: *Morphological changes during apoptosis (modified from Cotran, 1997)*

1.4.1 Regulation of apoptosis

Apoptosis is traditionally known to be initiated either through an extrinsic signal or an intrinsic signal. In the extrinsic pathway, ligands like those of the tumor necrosis factor (TNF) family bind receptors on the cell surface to initiate an intracellular death signal through various death receptors (Ashkenazi and Dixit, 1998, Danial and Korsmeyer, 2004). In the intrinsic pathway, the death signal originates from the intracellular structures such as the nucleus, lysosomes, endoplasmic reticulum and cytosol. This signal may result from stressor factors like DNA damage, cytoskeletal damage and growth factor withdrawal (Danial and Korsmeyer, 2004). There are various points where the extrinsic and intrinsic pathways are interconnected (Fig. 7). After initiation, the process can proceed along different and usually redundant biochemical pathways with varying morphological features. The apoptotic process itself can be divided into three phases; initiation, integration/decision and execution.

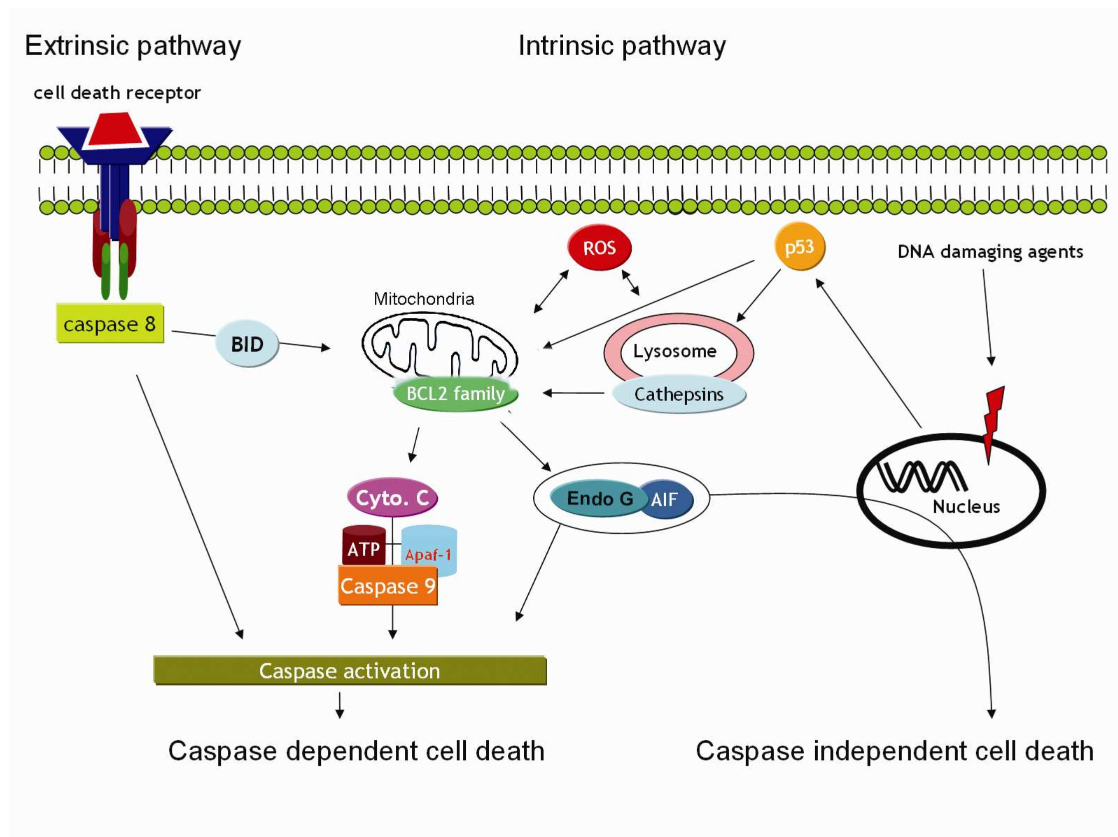

Figure 7: *Simplified schematic presentation of major factors and organelles involved in the regulation of apoptosis*

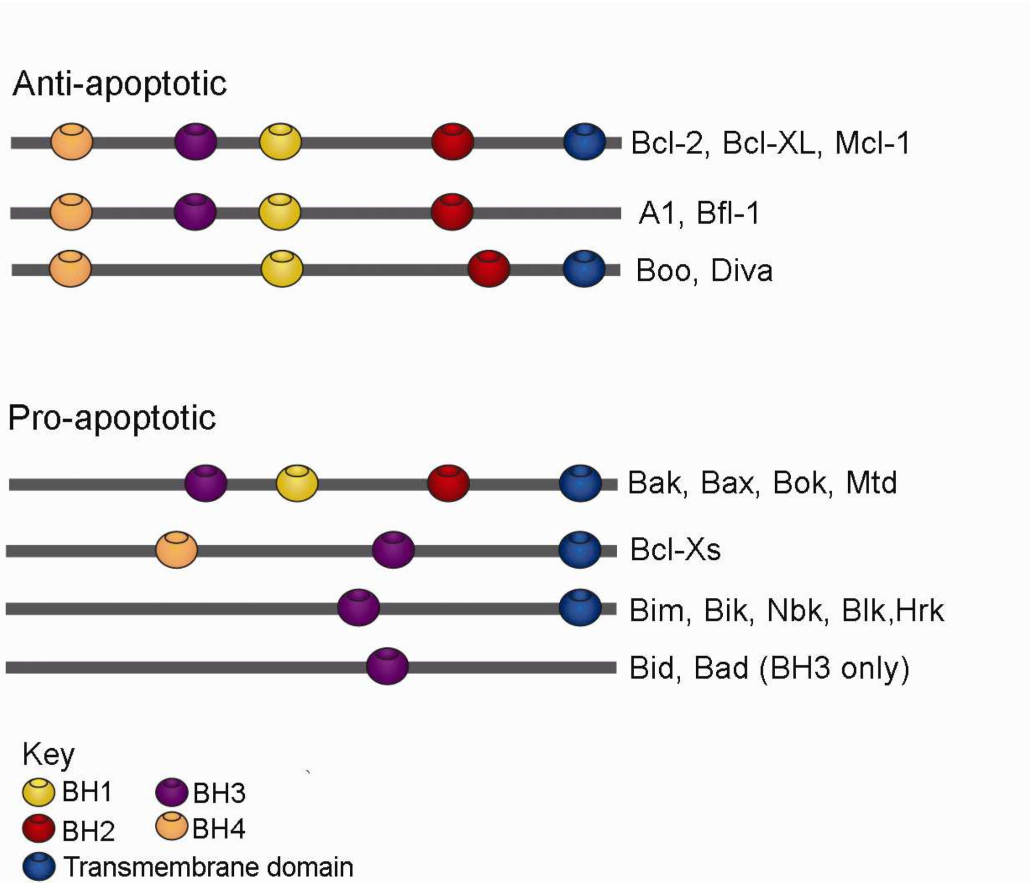

Figure 8: *Pro- and anti-apoptotic members of the Bcl-2 family (modified from Gross, McDonnell et al. 1999)*

The integration of these signals involves a complex interplay between various pro- and anti-apoptotic factors within the cell. The Bcl-2 family of proteins plays a major role in this integration due their dual function as both pro-apoptotic (Bax, BAD and Bak) and anti-apoptotic (Bcl-2 and Bcl-XL) (Adams and Cory, 1998, Reed et al., 1998, Youle and Strasser, 2008) (Fig. 8). These proteins are closely associated with mitochondrial functions and control permeabilization of the mitochondrial membranes. Mitochondria therefore play a key role in this integration as well as initiating the execution phase. Once the point of no return is reached the cell will enter a common execution phase that is largely independent of the initiator signal, but can vary depending on local factors such as availability of energy (ATP). Caspases (cysteine proteases) are a group of enzymes that are known to play essential roles in apoptosis and other cellular functions (Degterev et al., 2003, Lavrik et al., 2005) (Table 1). These proteases are normally synthesized as zymogens and activated during apoptosis by proteolytic cleavage. Mammalian caspases involved in apoptosis initiation include caspases 2, 8, 9, 10; and those involved in apoptosis execution include caspase 3, 6, and 7. During the execution phase of apoptosis, nucleases are activated which cleave DNA into 180-200 base pair increments, and these can be visualized after DNA gel electrophoresis as DNA laddering.

1.4.2 New challenges in the understanding of apoptosis

Early descriptions of apoptosis were aimed at distinguishing it as a regulated, energy-dependent cell death in contrast to the unregulated and passive form of cell death termed necrosis (Kerr et al., 1972). However, this simple dichotomous classification of cell death became problematic with the discovery of cell death patterns that did not quite fit in either group especially in terms of regulatory mechanisms (Kroemer et al., 2005). Unlike classical apoptosis, the biochemical features in these alternative forms of programmed cell death are still poorly understood.

Table 1*: Some of the known mammalian caspases (Degterev et al, 2003)*

Initiator caspases	Effector caspases	Other functions
Caspase-2	Caspase-3	Caspase-1
Caspase-8	Caspase-6	Caspase-4
Caspase-9	Caspase-7	Caspase-5
Caspase-10		Caspase-11
Caspase-12		Caspase-13
		Caspase-14

There is also lack of clear consensus on which biochemical and at times morphological changes should qualify as apoptosis especially under experimental conditions (Kroemer et al., 2005, Ying and Hacker, 2007). With time, the overwhelming redundancy, interconnectivity and plasticity in new cell death pathways gradually led to an upsurge of confusing terminologies most of which described varying degrees of regulation of cell death as well as variations in the involvement of proteases such as caspases (Sperandio et al., 2004, Fink and Cookson, 2005, Broker et al., 2005, Degterev et al., 2005). The Nomenclature Committee on Cell Death has intervened by recommending a limit on the use of new terminologies to describe different types of cell deaths (Kroemer et al., 2005). A simpler classification of cell death that allows for the emerging variations in cell death regulation, but retains the key words 'apoptosis' and 'necrosis' has been suggested by Leist (Leist and Jaattela, 2001). In the present study, the terms 'apoptosis' and 'apoptosis-like programmed cell death (PCD)' (as described by Leist) are used to define khat induced cell death.

1.4.3 Mitochondria and apoptosis

Mitochondria have long been known as the powerhouses of every cell, and their role in apoptosis was, until about 15 years ago, considered contrary to common sense

(Kroemer et al., 2007). It is now known that mitochondria do play a central role in various forms of cell death and serve to integrate cell death signals from cell surface receptors, cytosol as well as other organelles like nucleus and lysosomes (Kroemer et al., 2007) (Fig. 9). The Bcl-2 family of proteins is central to this integration due to their role as both pro- and anti-apoptotic mediators (Youle and Strasser, 2008). The time point when mitochondria are involved can vary especially between the extrinsic and intrinsic pathways. In some situations, cell death can proceed with little mitochondrial involvement whereas mitochondrial mediated apoptosis can proceed with little of the other features normally associated with classical apoptosis (Ying and Hacker, 2007). However, in all cases of programmed cell death, a point of no return in the signaling occurs when the outer mitochondrial membrane is permeabilized enough to allow the release of various apoptogenic proteins that normally reside within the mitochondrial intermembranous space. These factors may be caspase activators such as cytochrome c (Garrido et al., 2006), Smac/DIABLO (Du et al., 2000) and Omi/HtrA2 (Martins et al., 2002) or factors that act independently of caspases such as apoptosis inducing factor (AIF) (Susin et al., 2000, Lorenzo and Susin, 2007) and endonuclease G (Li et al., 2001)

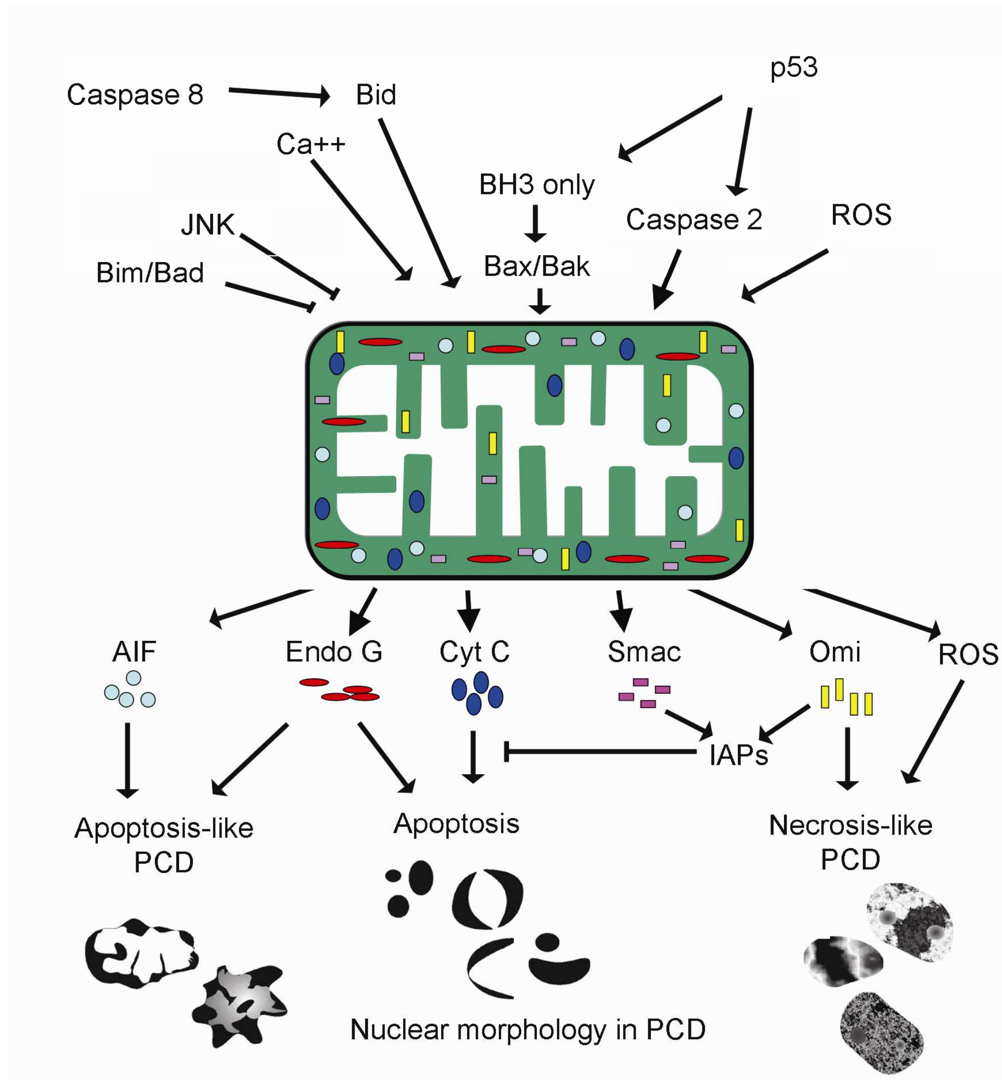

Figure 9: *Role of mitochondria in integration and execution of various forms of cell programmed cell death (PCD) (modified from Jaatella, 2004)*

Mitochondrial membrane permeabilization

Mitochondrial membrane permeabilization (MMP) is a complex multi-stage process that results in the release of apoptogenic proteins from the mitochondrial intermembranous space and has often been proposed as a 'point of no return' during apoptosis (Kroemer et al., 2007). Even though an increased permeability of the outer mitochondrial membrane (OM) alone is enough to cause MMP, the inner mitochondrial membrane (IM) also contributes towards MMP (Galluzzi et al., 2007). OM is normally permeable to small solutes of up to 5 kDa. This permeability increases in response to pro-apoptotic signals working via the Bcl-2 family proteins. These proteins could insert into OM and form large channels or favor the formation of large channels by other proteins (Zamzami and Kroemer, 2001, Galluzzi et al., 2007). Bcl-2 family proteins could also interact with members of the mitochondrial multiprotein channels to cause MMP. Unlike OM, IM is normally impermeable to ions and movement across it is facilitated by selective channels and transporters. Permeabilization of the IM is achieved through changes in mitochondrial permeability transition pore complex (mPTPC) (Galluzzi et al., 2007).

The permeability transition pore in cell death

The so called 'mitochondrial permeability transition' (mPT) is a sudden sustained increase in the permeability of IM (Zamzami and Kroemer, 2001, Grimm and Brdiczka, 2007). The multiprotein channel responsible for this permeability is called the mitochondrial permeability transition pore complex (mPTPC) (Rasola and Bernardi, 2007). One protein that helps form this complex is the voltage-dependent anion channel (VDAC), which is an OM trans-membrane protein that functions as a nonspecific pore to allow diffusion of solutes of up to 5 kDa. Other proteins that help form mPTPC include adenine nucleotide traslocator (ANT), cyclophilin D (Cyp-D), and hexokinase II (HK II) (Zamzami and Kroemer, 2001). The composition of mPTPC also includes the peripheral benzodiazepine receptor (PBR), mitochondrial creatine kinase (mtCK) and anti-apoptotic members of the Bcl-2 family (Fig. 10). Apart from these anti-apoptotic members of the Bcl-2 family, other agents such as cyclosporine A (CsA) and bongkrekic acid (BA) can modulate the channel to

stabilize the membranes. To inhibit mPT, CsA acts on cyclophilin D, whereas BA is a ligand of ANT. Periodic opening and closing (flickering) of the mPTPC is a normal physiological process essential for calcium homeostasis, but it is the sustained opening of this channel that constitutes mPT (Rasola and Bernardi, 2007). The respiratory chain generates a proton gradient and an electrochemical potential of about 120 to 180 mV across IM which is then used to drive oxidative phosphorylation (ATP synthesis) (Grimm and Brdiczka, 2007). During mPT, this proton gradient is equilibrated, a situation that is catastrophic because it leads to a loss of the electrochemical potential ($\Delta\Psi_m$) across IM thereby affecting the ability to synthesize ATP. It also causes an accumulation of intracellular ROS and depletion of glutathione. Moreover, the ensuing osmotic gradient results in an influx of water into the mitochondrial matrix, mitochondrial swelling, unfolding of the cristae and bursting of OM. This allows the release of various apoptogenic factors to the cytosol to cause apoptosis (Zamzami and Kroemer, 2001, Joza et al., 2002, Galluzzi et al., 2007). A detailed review on the function of mPTPC in normal physiology and in cell death can be found in references (Brenner and Grimm, 2006, Grimm and Brdiczka, 2007).

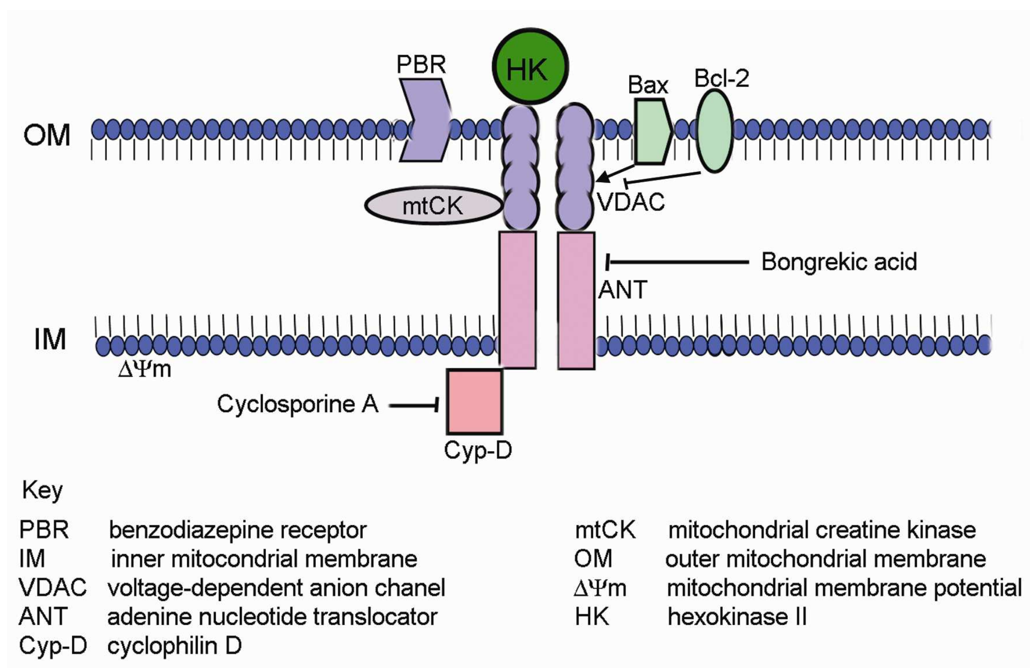

Figure 10: *Permeability transition pore complex and its regulatory proteins. Bongrekic acid and cyclosporine A block the channel by acting on ANT and Cyp-D as shown. Also note the opposite effects of Bcl-2 and Bax on the channel. (Modified from Zamzami and Kroemer, 2001)*

Oxidative stress and cell death

Oxidative stress refers to an abnormal increase in reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals within cells to a point where they interfere with normal cellular functions. At physiological levels, these molecules could be involved in various normal cellular processes like gene expression (Fialkow et al., 2007), proliferation and differentiation (Dumont et al., 2000). Exogenous and endogenous stresses may generate excessive amounts of ROS, which if not detoxified, could lead to damage of DNA, proteins, and lipids. This oxidative damage is often associated with pathologies such as cancer, inflammation and aging. In extreme cases, individual cells that are unable to detoxify ROS or repair the ensuing oxidative damage of the various macromolecules, will eventually undergo cell death. To counter this, cells have antioxidant systems that include enzymes (superoxide dismutase, catalase) and peptides (glutathione) which normally neutralize ROS and prevent disturbances in ROS homeostasis (Valko et al., 2007). Glutathione (GSH) is an essential tripeptide found in mammalian cells where it maintains the intracellular thiol redox status, and detoxifies exogenous and endogenous free radicals and ROS (e.g. hydroxyl radical and hydrogen peroxide). In these reactions, reduced glutathione (GSH) forms oxidized glutathione (GSSG), which is again reduced to GSH to replenish supplies. Depletion of intracellular GSH predisposes cells to pro-apoptotic stimuli and can also activate apoptosis in the absence of such stimuli (Valko et al., 2007). Cellular antioxidant defense systems including superoxide dismutase, catalase and GSH may thus prevent disturbances in ROS homeostasis, or reduce the effect of oxidative stress in cells (Valko et al., 2007).

Role of mPT in ROS generation

There are many ROS production and ROS removal sites within the mitochondria (Andreyev et al., 2005). It has been proposed that normal mitochondria act as net removers rather than net producers of ROS (Andreyev et al., 2005). Notably, factors such as high $\Delta\Psi_m$ and increased respiration enhance ROS production, whereas loss of $\Delta\Psi_m$ reduces ROS production. In the presence of structurally and functionally

compromised mitochondria, extra-mitochondrial accumulation of ROS is possible because of impaired detoxification rather than by increased production. As shown in a recent study (Hansson et al., 2008), mitochondrial emission of ROS can therefore remain intact or even increase mainly due to impaired ROS detoxification following a loss of $\Delta\Psi_m$.

2. Problem statement and aims of the study

Most of the previous studies on khat have focused on the general and medical effects of khat especially on the central nervous, circulatory and reproductive systems. A few studies have also addressed the effects of khat on oral tissues. Khat use is associated with various oral conditions as explained earlier, including the suggestion that khat use could be a potential risk factor for oral cancer. Studies on the toxicological potential of khat remain scarce. The few studies on khat toxicity carried out earlier revealed impaired macromolecule biosynthesis, genotoxicity and induction of apoptosis. However, there is still inadequate information regarding the specific mechanism(s) involved in khat-induced cytotoxicity. It is not even clear whether the effects of khat on the oral mucosa are a result of khat toxicity or frictional injury due to mastication or if both mechanisms play a role. Determination of mechanisms involved in khat toxicity could assist in understanding the potential risk for khat to induce various oral lesions and in the development of appropriate policy responses towards any such risk.

2.1 General objective

To investigate the toxic effects induced by an extract of khat on primary normal human oral keratinocytes and fibroblasts in monolayer and in organotypic models.

2.2 Specific objectives of the study

1) To investigate the effects of an extract of khat on growth of primary normal human oral keratinocytes and fibroblasts *in vitro.*

2) To investigate the effects of an extract of khat on growth and differentiation of an organotypic model reconstituted from primary normal human keratinocytes and fibroblasts.

3) To investigate the effects of an extract of khat on viability and survival of primary normal human keratinocytes and fibroblasts.

4) To investigate the possible mechanisms of cell death induced by khat in primary normal human keratinocytes and fibroblasts.

3. Methodological considerations

The materials and methods used in this study are outlined in the original papers included in this thesis and also in the references provided. This section will summarize main aspects of the methods used in various experiments, and focus more on the relevance of the selected methods to the study.

3.1 Khat extraction and handling

In this study, we endeavored to generate an extract that contained appreciable levels of cathinone because 1) it is the most widely studied and main active ingredient in khat, 2) it is the most unstable compound and its loss would suggest a degenerated extract, 3) it has been implicated to play a role in khat induced cytotoxicity in various experimental systems. Special care was taken in order to have khat extracted as soon as possible after harvest to prevent loss of important bioactive constituents (Table 2). The methanolic extraction has been proven to maintain cathinone within the extract. The extraction protocol used in this study was described by Dimba (Dimba et al., 2004). One criticism of the methanolic method of khat extraction is that it is different from the extraction that occurs in saliva within the oral cavity during khat chewing. In paper II, we attempted to compare this organic extract with an aqueous extract based on an alternative extraction protocol in which fresh khat was air-dried and extracted in water at 37ºC (Al-Hebshi et al., 2005).

In our hands, the organic extract of khat remained stable, and its potency in inducing cellular effects was not affected even when stored for long periods (up to 2 yr) at -80 ºC. Prior to each experiment, a new batch of khat stock solution was thawed at room temperature, diluted in cell culture media to a concentration of 10 mg/ml and centrifuged at 3000 g_{av} (average gravity) for 10 min at room temperature. These conditions were established through a series of pilot studies carried out to compare the levels of cytotoxicity induced by different pellet and supernatant fractions. The

supernatant fraction was collected after centrifugation and adjusted to the appropriate concentrations to be tested in each experiment (range 1000 – 1 µg/ml khat) by performing logarithmic serial dilution. In all experiments (monolayer and organotypic), khat exposure was performed by diluting the khat extract within the culture media. In toxicity studies where organic solvents are used, it is recommended that solvent-treated control cultures be used, and that solvents should not exceed 0.5% concentration (Freshney 1994). In this study, control cultures were routinely supplemented with DMSO equal to the amount in the highest khat concentration tested, and this never exceeded 0.3%.

Table 2: Alkaloid content (in mg/ml) in some of the samples used in the study

	Cathinone	Cathine	Norephedrine
Asili 2004	0 ± 0	2.3 ± 0.1	0.3 ± 0.0
Asili 17-03-05	10.5 ± 0.4	13.5 ± 0.5	1.2 ± 0.1
Asili 22-03-05	27.6 ± 0.8	16.5 ± 0.7	2.0 ± 0.1
Kangeta 12-04-05	26.0 ± 1.2	22.3 ± 0.2	3.4 ± 0.0

3.2 Cells and organotypic tissues

All cells used in this study were isolated from tissue samples taken from normal buccal mucosa of clinically healthy adult volunteers undergoing surgical removal of wisdom teeth. All patients included were informed of the purpose of the study and were requested to sign consent forms. The study was approved by the Regional Committee for Medical Ethics in Research.

3.2.1 Choice of primary oral cells

Many cytotoxicity studies use cells that are well characterized, available commercially and easier to handle such as leukemia or carcinoma derived cell lines.

However, these cells lines are usually transformed and genetically unstable. Their response to test agents such as khat could therefore differ from that of normal cells. In order to investigate the potential toxic effects of khat on normal oral mucosa, we chose to work on primary oral keratinocytes and fibroblasts isolated from normal human oral tissue samples. There were a few challenges such as the difficulty in obtaining a constant supply of cells necessary to run toxicity experiments. There were also variations between different samples in terms of growth rates. Some samples yielded keratinocytes that differentiated rapidly in culture thus becoming less responsive to khat. In general, despite our use of low calcium media, there was a tendency for keratinocytes to differentiate early. A vigorous selection process was therefore done to ensure use of strains that had comparable growth rates and morphological appearance for all experiments. Keratinocytes were used while in their 1st to 4th passage whereas fibroblasts were used while in the 1st to 6th passage.

3.2.2 Choice of organotypic cultures and handling

Although they are easier to work with, oral keratinocytes growing in two dimensions (monolayer) lack the functional structure and cell-cell interactions that occur *in vivo*. Besides, additional interaction of these cells with mesenchymal cells and the tissue matrix may also influence their response to khat. Thus, we decided to test the potential toxicity of khat using an *in vitro* three dimensional model of normal buccal mucosa in which cells resemble the structure and also interact with each other as they do in the native tissue. This model had previously been established by Costea *et al*. (Costea et al., 2005) in our laboratory and has now been standardized to allow its use for various experimental purposes including toxicity studies. However, one limitation of this model is its length of growth in culture which is currently not possible beyond 12 days. Pilot studies that assessed the maturity of the model in terms of epithelial thickness and differentiation indicated that optimal maturity occurs on day 7 of culture. Organotypic cultures were therefore allowed to grow for 7 days before treatment with khat. It has been reported that khat chewing sessions normally last between 4 and 8 h and some chewers may continue chewing for up to 10 h (Al-

Motarreb et al., 2002b, Sawair et al., 2007). We attempted to mimic this exposure status *in vivo* by treating the tissues with khat for only 6 h per day over the last five days prior to harvesting (Fig. 11). In this study, khat was diluted within the culture media as it enabled better control on test concentrations of khat and also better maintenance of the liquid-air interface necessary for normal epithelial growth.

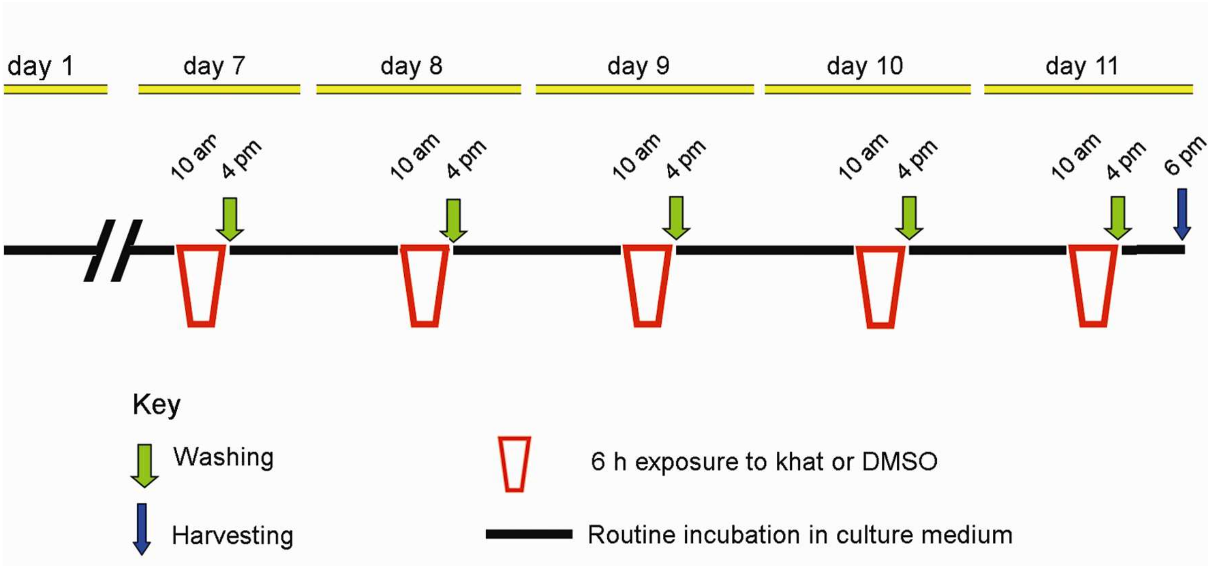

Figure 11: *Schematic presentation of time and handling procedures of organotypic cultures during their exposure to khat*

3.3 Assessment of cell proliferation and differentiation

3.3.1 Cell proliferation in monolayer

The first goal of our experiments was to assess the effect of sublethal concentrations of khat on oral cells. Alteration of proliferation rate was considered as an indicator of toxicity induced by khat. In this study, cell proliferation was determined by cell counts performed over time in oral cells exposed to various concentrations of khat. In our hands, two methods provided reproducible results, i.e 1) cell counts done on attached cells while still in culture and 2) cell counts done using a hemocytometer after enzymatic detachment. The former method enabled us to use fewer cells. After observing an apparent reduction in cell proliferation in cell cultures treated with khat, we then wished to know if there was a distinguishable cell cycle pattern associated with exposure to khat. Cell cycle analysis by flow cytometry is normally preferred for this kind of study. In our case, the cells needed to be detached first, and the best results were achieved when both keratinocytes and fibroblasts were fixed and permeabilized in 70% ethanol (in PBS) and stained for their DNA content using propidium iodide. Later, western blotting was used to assess expression levels of various cell cycle related proteins.

3.3.2 Cell proliferation and differentiation in organotypic models

The proliferation and morphological changes we observed in khat exposed cells in monolayer as well as the expression profiles of specific proteins suggested a potential induction of differentiation in these cells. We explored this parameters further using organotypic cultures. The tissues were harvested on day 11 of culture and fixed in 4% buffered formalin (pH 7.2), paraffin-embedded and later sectioned (5 µm thick). The samples were stained with hematoxylin-eosin (H&E) and examined under light microscopy for structural changes, differentiation pattern and degree of keratinization.

Histomorphometry

For histomorphometric analysis, tissue sections prepared as described above were analyzed by computer based digital image analysis software (analySIS-Pro 11.0, Soft Imaging System GmbH, Munster, Germany). Analysis was done at 200-fold magnification under a Leica DMLM microscope (Leica Microsystems GmbH, Wetzlar, Germany). The epithelium was divided into three components (basal cell layer, spinous cell layer and superficial cell layer) as described previously (Costea et al., 2003). The epithelial thickness was determined by drawing an arbitrary line running vertically from the basement membrane to the surface and reading off its length as was indicated by the software. The three layers were also assessed for their thickness and compared with each other and with the total thickness of the epithelium.

Immunohistochemistry

Formalin fixed, paraffin-embedded sections were dewaxed in xylene and graded ethanol. Antigen retrieval was done by microwave treatment (900W for 8 min, then 450W for 15 min) in Tris-ETDA (pH 9). The indirect method was used in all cases for its better sensitivity, and the peroxidase block, secondary antibodies and 3,3'-Diaminobenzidine (DAB) were part of the ready-to-use, peroxidase-based EnVision™ kits (DAKO) which was used in all cases. The protocols were however routinely optimized for each antibody. For instance, some antibodies, like those against TGase-K and p21, gave better results with a similar microwave treatment in 10 mM citrate buffer (pH 6) when compared with heat treatment in Tris-ETDA.

Other methods

Alternative methods were needed in order to confirm our findings from routine H&E stained slides and immunohistochemistry, and also to allow for easy quantification of the proteins whose expression was assessed in this study. We chose western blotting for most of the proteins, but a colorimetric assessment of enzyme activity was preferred for TGase-K protein. In both of these methods, the epithelial component was gently peeled off the connective tissue component of the organotypic cultures

during harvesting and washed three times in PBS. The epithelium was then minced using a scalpel on a plastic cell culture dish into tiny pieces, collected into an aliquot, mixed with lysis buffer (10mM Tris (pH 7.5), 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP-40 and 5 mM NaF) supplemented with Complete Protease inhibitor (Roche Molecular Biochemicals) and ground (100 passes) using Dounce tissue grinder (Cole-Parmer, Vernon Hills, IL, USA) while on ice. Equal protein loading was ensured by assaying for protein content using the Bradford protein assay method (Bio-Rad Laboratories, Richmond, CA).

3.4 Morphological and biochemical determination of cell death

3.4.1 Determination of cell viability and apoptosis

General viability assays

Morphological assessment of cells under light microscopy was routinely used in this study to monitor cell growth and viability. Since growth and viability of oral cells is dependent on attachment onto a substrate, exposure to lethal agents often results in rounding up of cells and their detachment to float in the media. In addition, cells were assessed for specific morphological changes associated with apoptotic cell death such as plasma membrane blebbing. In the early stages of the study, the proportion of morphologically altered cells was used to give as a crude assessment of khat induced cytotoxicity. Additional methods were also used, such as dye exclusion assays (trypan blue and propidium iodide) to assess plasma membrane integrity. Fluorescence microscopy (Hoechst 33342 staining) was used to study and quantify nuclear changes such as chromatin condensation and nuclear fragmentation. Additional assays were often used to confirm the results we had with any one method. In some instances, variability in the results from two separate methods provided us with information about the mechanisms involved. For instance, in the early phase of cell death, cells still excluded trypan blue even though they were

morphologically affected, indicating an intact plasma membrane as would be expected during the early phase of apoptosis. Electron microscopy was used in this study only to assess late ultrastructural changes in khat exposed cells. In later studies, the reagent 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium otherwise known as WST-1 was used to assess cell viability. Enzymes in viable (metabolically active) cells cleave the tetrazolium salt into colored soluble formazan. This method was chosen mainly due to its advantage of testing different study conditions while using only a small number of cells.

Assays for apoptosis

Annexin V is a protein that binds to phosphatidylserine which is normally situated on the inner surface of the plasma membrane. During apoptosis, phosphatidylserine is translocated to the outer surface, and can be detected by annexin V staining. In our hands, annexin V assay provided a good assessment of apoptosis in both keratinocytes and fibroblasts. To confirm these results, we attempted the TUNEL method in both cell types, but this did not work well at that time in keratinocytes probably because of inadequate permeabilization. Therefore, in keratinocytes, an alternative method using YO-PRO-1 dye was used to confirm the annexin V results. Assays based on the determination of caspase zymogen processing to form active enzymes were done using western immunoblotting. We also used potent caspase inhibitors to test if cell death induced by khat was dependent on caspase activation, and the DNA ladder assay to assess the presence of intranucleosomal DNA cleavage.

3.4.2 Determination of early changes in khat induced cell death

The methods outlined above are commonly used in the assessment of cell death and particularly apoptosis. However, these methods measure events that occur late during the cell death process. In the course of this study, we became particularly interested in studying events that occur early in the cell death process prior to features such as changes in cellular and organelle morphology, DNA fragmentation and loss of plasma membrane integrity. Apart from providing information regarding the

mechanism of cell death, studying these early changes was supposed to help us understand our initial observations which seemed to differ from work published earlier, including the apparent absence of caspase activation and DNA laddering.

Cell attachment assay

At times, cells exposed to lethal agents may not die in short periods, but accumulate irreversible changes that are not compatible with long term survival. The cell attachment assay used in this study was chosen in an attempt to address this issue. In addition, the assay helped to answer two key questions 1) whether cells can survive extremely brief (0.5 h) exposures to khat i.e. whether the early effects of khat in these cells are reversible upon removal of khat, and 2) the time point beyond which the effects of khat become irreversible. The later question was to determine which biochemical change in these cells was a possible determinant of the 'point of no return'. Cell survival (retention of the regenerative potential of cells) is best measured by plating efficiency (colony forming or clonogenic assay). It was difficult to do this test in our case since the fibroblasts did not assume appreciable colonies after trypsination, while the few keratinocytes that remained viable could grow after plating probably due to sensitivity to seeding density. We therefore used the total number of cells that were able to plate (seeding efficiency or cell attachment assay) as a measure of cell survival following khat exposure. In this method care must be taken to count the cells when maximum attachment has occurred but before mitosis begins.

Determination of mitochondrial membrane potential

Normal inner mitochondrial membrane potential ($\Delta\Psi_m$) ranges from 120 to 180 mV and it is negative on the inside (Galluzzi et al., 2007). Positively charged (cationic) fluorochromes will therefore accumulate in the mitochondrial matrix. Loss of $\Delta\Psi_m$ will occur at some stage in all types of cell death, but the time point when it occurs depends on the role of mitochondria in the cell death process (e.g. intrinsic versus extrinsic pathway). To understand the mechanism of khat induced cell death, determination of the temporal relationship of key changes in cells exposed to khat

was necessary. Initial tests showed that mitochondria were affected quite early in the cell death process. Time lapse confocal microscopy enabled us to determine the time point when loss of $\Delta\Psi_m$ began. The choice of dyes to be used was crucial here. There are several commercially available flourochromes used in assessment of changes in $\Delta\Psi_m$ such as 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), chloromethyl-X-rosamine (CMXRos), tetramethyl rhodamine methyl ester (TMRM), and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$). In this study, we chose to use $DiOC_6(3)$ since it is known to have a rapid mitochondrial equilibration. $DiOC_6(3)$ also has limited quenching effects (Galluzzi et al., 2007), an important factor when performing long exposures like the overnight time lapse confocal microscopy. Chemicals known to inhibit mitochondrial membrane depolarization such as CsA and BA were also employed in this study to test the role of mitochondrial membrane depolarization in the cell death process.

Cell fractionation and immunofluorescence

Loss in $\Delta\Psi_m$ alone is not considered adequate to show mitochondrial involvement in cell death. To confirm the degree and time frame of mitochondrial involvement in khat induced cell death, it was necessary to assess for changes in the permeability of the outer mitochondrial membrane. Identification of subcellular redistribution of intermembranous space proteins within the cytosol or even in the nucleus is one of the methods used to ascertain that the OM has been permeabilized. Cell fractionation was used to study translocation of Cyt C and AIF to the cytosol. In subcellular fractionation, over 30 million cells per sample were required in order to isolate sufficient quantities of mitochondria for analysis. It was not possible to perform this procedure in keratinocytes due to the difficulty in obtaining such quantities of cells at an early passage. We used immunofluorescence to confirm the release of AIF to the cytosol and further into the nucleus. By performing immunofluorescence on cells grown on cover-slips, we were able to use confocal microcopy allowing for quantification of nuclear AIF. This also enabled us to visualize translocation of AIF at early time points (2-4 h). For fixation of these cells, a mixture of acetone and

methanol (1:1) provided better results when compared with 4% formalin. We also had difficulties achieving adequate permeabilization of the cells when using Triton X-100 and saponin alone, especially for keratinocytes. After several trials, a combination of saponin (0.1%) and hydrochloric acid (1%) was found to give the best results. In our hands, several anti-AIF antibodies available commercially did not yield good results. The antibody used was chosen in consultation with Dr. Santos Susin (Institut Pasteur, Paris, France) who has spent many years studying the role of AIF in cell death (Lorenzo and Susin, 2007).

Determination of oxidative stress

Different ROS species can be detected selectively in fluorescence-based cell assays, with little interference from other ROS species. The superoxide sensitive dye dihydroethidium (DHE) and the hydrogen peroxide sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) were chosen mainly because they are freely permeable to cells and are easily metabolized within cells to give off fluorescent moieties (Carter et al., 1994). To study GSH levels within cells exposed to khat, the dye 5-chromomethylfluorescein diacetate (CMF-DA) was also chosen because it is freely permeable to cells and easy to handle. In our hands, this dye provided good results, even though under ordinary fluorescence microscopy, there appeared to be a lot of background fluorescence. Other researchers have experienced similar background fluorescence, and have attributed it to fluorescence emanating from the unbound dye (Sebastia et al., 2003). It has also been claimed that CMF-DA measures the total level of free intracellular thiol rather than GSH specifically (Sebastia et al., 2003). For future studies, an alternative dye like 7-amino-4-chromomethylcoumarin (CMAC) may therefore be a better option. Antioxidants Tiron and NAC were used to evaluate the potential role of ROS in khat induced cell death. Tiron gave the best results by countering the rise in ROS and the fall in GSH and also by protecting against cell death. In our hands, NAC was effective in countering the rise in ROS and depletion of GSH induced by khat. However, the

protective effects against cell death were not significant. NAC was also toxic to the cells on its own at higher concentrations.

3.5 Issues of clinical relevance in this study

The methanolic method of khat extraction used in this study makes use of an organic solvent, and could be different from the extraction that occurs in saliva within the oral cavity during khat chewing. In paper II, we compared this organic extract with an aqueous extract based on an alternative extraction protocol. The two extracts showed comparable cytotoxicity in oral fibroblasts.

2) Khat is chewed for its euphoric effects on the central nervous system. These effects are mainly caused by the khat-specific chemical cathinone. In an effort to mimic the clinical extract, we adopted the methanolic extraction protocol that preserves cathinone and the other alkaloids within the extract.

3) We are not aware of any study where the actual concentrations of khat-specific contents in saliva have been assessed. We estimated the possible range of concentrations of cathinone in saliva of khat chewers based on known values of ingested cathinone and stimulated saliva production. Khat concentrations used in this study were within this range.

4) Khat is not chewed continuously, and khat chewing sessions are known to vary between 4 and 10 h (Sawair et al., 2007). In our experiments in paper II, we used intermittent exposure to khat each lasting 6 h in order to mimic this in vivo exposure.

4. Results and discussion

4.1 Effects of low concentrations of khat on oral cells and tissues

4.1.1 Growth inhibition induced by khat (paper I and II)

In the first part of the study, we used sublethal concentrations (3.16 to 31.6 µg/ml) of khat to study the potential of khat to modulate cellular functions such as growth and differentiation in normal oral cells. In monolayer cultures, these low concentrations of khat induced a concentration dependent inhibition of cell growth, and the cells accumulated in the G1 phase of the cell cycle. The cells also showed an increased expression of cell cycle inhibitor proteins like p53, p21 and p16. The increased expression of p16 occurred earlier and was higher in fibroblasts whereas the highest increase in p21 expression was observed in keratinocytes. The growth inhibition occurred earlier in fibroblasts when compared to keratinocytes. Unlike keratinocytes, fibroblasts also showed recovery of their proliferative potential on prolonged exposure. The reduced proliferation shown here is in agreement with what was observed to occur in animal studies and plant cells following exposure to khat (de Hondt et al., 1984, Qureshi et al., 1988) or khat-specific alkaloids (Al-Meshal, 1987). However, these previous studies did not specifically address the potential mechanisms involved in the reduced proliferation.

The inhibitory effect of khat on cell proliferation was also detected in organotypic models of human buccal mucosa. A concentration dependent reduction in number of cycling (Ki67 positive) cells and a reduction in total epithelial thickness was observed. Moreover, there was an increased expression of cell cycle inhibitor protein p21 in khat treated epithelia when compared with controls. With regard to p16 expression, no difference was observed between khat exposed and control organotypic models, in contrast to what was found in monolayer cultures.

4.1.2 Premature differentiation and keratinization induced by khat (paper I and II)

The proteins p53, p21 and p16 are known to play a role in cell differentiation and senescence. In monolayer cultures, keratinocytes exposed to sublethal concentrations of khat acquired a phenotype suggestive of increased differentiation. They became enlarged, more flattened and had a tendency to remain as individual cells rather than grow into larger aggregates or colonies. A few cells acquired an elongated or bipolar shape. Fibroblasts lost their spindle (bipolar) shape to a multipolar shape. These features are commonly associated with increased differentiation especially as a consequence of stress induced by toxic agents (Lu, Chang et al. 2006). Interestingly, there appeared to be a differential expression of p16 and p21 in keratinocytes when compared to that in fibroblasts. Keratinocytes showed a much higher increase in the p21 than p16 and the opposite effect was seen in fibroblasts. The protein p21 is thought to be associated with induction of an irreversible cell cycle arrest and terminal differentiation (Weinberg and Denning, 2002). Since the inhibitory effect in fibroblasts appeared to be reversible, it is possible that fibroblasts treated with khat undergo a temporary cell cycle arrest whereas keratinocytes undergo a permanent arrest as part of differentiation and senescence. Together, these findings demonstrated the potential of an extract of khat to modulate the cell cycle and differentiation pattern in primary normal oral cells

The issue of differentiation was also studied in organotypic cultures of primary normal oral cells. Besides the obvious decrease in epithelial thickness, khat exposed tissues also showed a decrease in the thickness of the spinous cell layer, and an increase in the basal and the superficial cell layers relative to the total epithelial thickness. This gave an impression of a rapid change from the basal layer to the superficial layer, as would be expected in premature differentiation.

The expression of p21 protein in khat exposed tissues was found to be increased when compared to control cultures. In an immunohistochemical test, control cultures showed weak nuclear staining for p21, and generally fewer cells were affected,

whereas khat exposed tissues had stronger staining affecting more nuclei and extending more into the basal layer. When stained for TGase-K, khat exposed tissues generally showed a stronger staining located along the cell membranes, whereas control cells showed weak staining. Basal cells were negative for TGase-K in both control and treated groups. One notable difference was the pattern where khat exposed samples showed intensely stained parabasal and lower spinous layer cells, whereas control cultures showed weaker staining in this region and intense staining in superficial regions. In an alternative colorimetric assay, an increase in TGase-K activity was observed in khat treated tissues when compared with control samples suggesting increased expression of TGase-K. Involucrin, another differentiation marker, also showed increased expression in khat exposed cells. Together, these results suggested a potential for khat to exert some pro-differentiation signaling in organotypic tissues of normal buccal mucosa, with a possible consequence being induction of premature differentiation.

Organotypic tissues not treated with khat showed nonkeratinizing superficial layer, whereas khat exposed tissues showed increased keratin formation towards the surface. Most samples showed parakeratinized epithelium while a few had regions of loss of nuclei within the superficial layer resembling orthokeratinization. In addition, khat exposed tissues showed an increased presence of keratohyaline granules within the cytoplasm of individual cells mainly in the spinous and superficial layers, but without forming distinct granular cell layers. Tissues exposed to khat showed increased immunoreactivity for filaggrin, in a manner that was dependent on concentration of khat used. The positive regions were located mainly in keratohyaline granules within the spinous and superficial layers, but some cells in the superficial layer also showed strong positive staining for filaggrin within their cytoplasm. Khat exposed epithelia showed an increase in p38 as well as phosphorylated p38, and inhibition of p38 (using 2 μM each of SB202190 and SB203580) reversed the effects induced by khat suggesting a possible involvement of p38 MAP kinase in khat induced signaling.

Together, the observations described above suggested a possible switch from nonkeratinizing to keratinizing epithelium in khat treated tissues. Increased keratin formation (hyperkeratosis) has been reported to occur in the oral mucosa of people chewing khat (Ali et al., 2004, Ali et al., 2006). Some researchers have suggested that the hyperkeratosis found in khat chewers could be due to frictional injury associated with the habit (Ali et al., 2004, Hill and Gibson, 1987). In this study, it is shown that the chemical components in khat have the potential to induce increased differentiation and keratinization in otherwise nonkeratinizing epithelial models where such mechanical forces are absent. Moreover, unlike the oral cavity where effects attributed to khat could be confounded by other factors like concomitant tobacco use and khat additives, the *in vitro* environment in this study was clearly defined. However, whether these findings could explain the hyperkeratosis seen in khat chewers will become clearer with further studies. Oral biopsies taken from khat chewers tend to show normal thickness and enlarged rete ridges which could indicate increased proliferation (Ali et al., 2006, Ali, 2007). In contrast, results in organotypic tissues in this study showed a reduced proliferation and reduced epithelial thickness. The reduced proliferation and decrease in epithelial thickness could be an *in vitro* phenomenon probably caused by a shortage of epithelial stem cells that would normally be present in higher numbers *in vivo*. Also, a similar toxicity *in vivo* has the potential to induce compensatory hyperproliferation.

4.2 Effects of high concentrations of khat or oral cells

4.2.1 Features of khat induced cell death in oral cells (paper III)

In the second part of the study, we used lethal concentrations (100 to 316 µg/ml) of khat in order to investigate the mechanisms of khat induced cell death in normal oral cells. The morphological features of dying cells were monitored over time. Cells exposed to khat showed rounding up, loss of pseudopodia and blebbing of the plasma membrane (Fig. 12). Blebs were more obvious in keratinocytes than fibroblasts.

These cellular changes appeared after 3 h of exposure to khat, and were followed by nuclear chromatin condensation. Generally, the affected nuclei appeared smaller, and some had peripherally condensed or clumped chromatin whereas others showed fragmented nuclear chromatin. These changes were also observed under transmission electron microscopy (TEM). In addition, TEM showed a consistent loss of microvilli and vacuolization within the cytoplasm. Nuclear changes were a late stage morphological effect of khat induced cell death. The morphological features were therefore consistent with cell death by apoptosis.

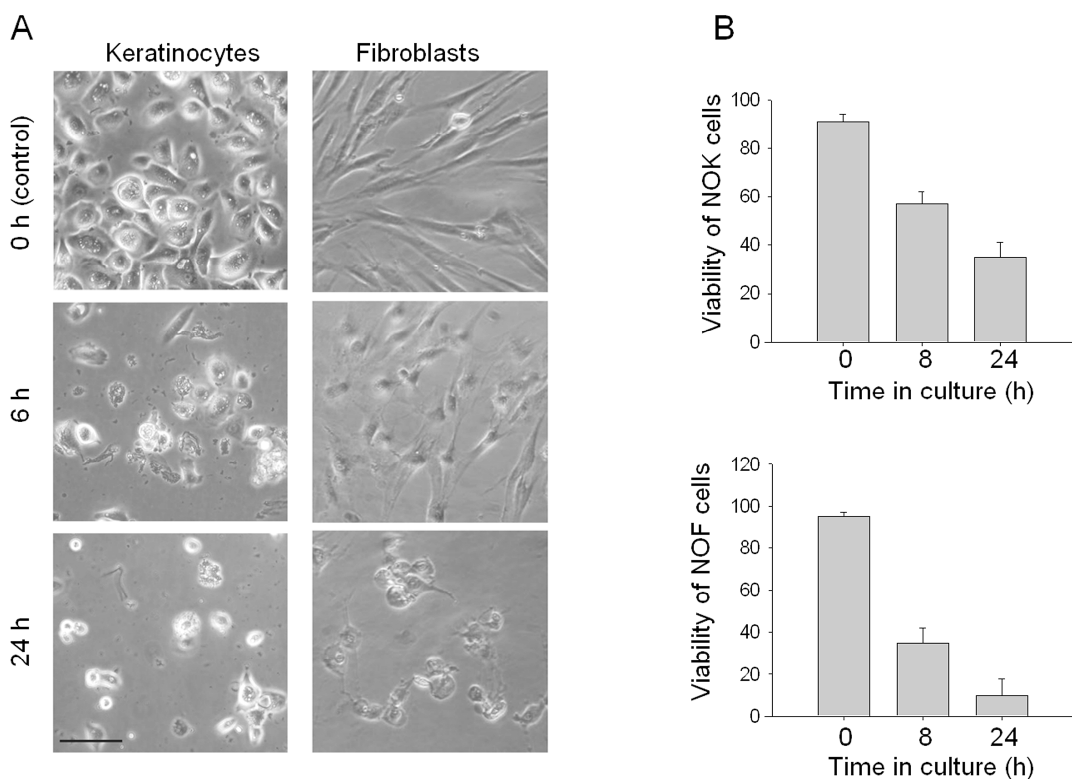

Figure 12. *Morphological features (A) and loss of viability (B) in oral cells following exposure to 316 μg/ml khat.*

The proportion of cells that were permeable to trypan blue dye was lower when compared to the proportion of morphologically altered cells. This was observed in both cell types, but in general, fibroblasts were more sensitive to khat than were keratinocytes in both assessments. Furthermore, the morphologically altered keratinocytes were impermeable to propidium iodide, but were permeable to the fluorescent dye YO-PRO-1, in contrast to cells not exposed to khat which remained

impermeable to both dyes. The exclusion of trypan blue and propidium iodide suggested maintenance of an intact plasma membrane even as the cell underwent cell death, as reported to occur in the early phases of apoptosis. Other biochemical features of cell death seen in these cells were externalization of the plasma membrane phosphatidylserine (annexin V assay) and fragmentation of nuclear DNA (TUNEL assay). All these features of cytotoxicity were dependent on duration of exposure and concentration of khat used.

4.2.2 A critical exposure time required for induction of cell death by khat (paper III and IV)

Primary oral cells exposed to lethal concentrations of khat for only 0.5 h did not show signs of cell death, and were able to reattach to a growth matrix and grow normally. Exposure to khat for longer time periods (0.5 – 2 h) led to cell death in some cells, while the rest seemed to remain unaffected and even to proliferate further. Thus, the initiation of specific irreversible cell death signals and actual commitment to cell death occurred after a critical exposure time period of 0.5 h, but prior to onset of any obvious morphological and biochemical signs of cytotoxicity.

4.2.3 Absence of caspase involvement in khat induced cell death in oral cells (Paper III and IV)

Induction of apoptotic cell death by khat was first reported by Dimba et al in leukemic cell lines (Dimba et al, 2003). However, in contrast to what was reported to occur in leukemic cell lines, potent caspase inhibitors (z-VAD-fmk, a pan-caspase inhibitor, and z-DEVD-fmk, a caspase-3 inhibitor) did not protect against khat induced cell death in primary oral cells in the present study. Moreover, analysis by western immunoblotting did not show cleavage of pro-caspase-3 in khat-exposed fibroblasts, suggesting lack of activation of this protein. Also, at variance with what was reported in leukemic cell lines, khat induced cell death in oral cells was not inhibitable by a protein synthesis inhibitor. Khat specific alkaloids like cathinone and cathine have been suggested as contributing to the cytotoxicity of khat, but these did

not induce cell death in oral cells. A decreased expression of the anti-apoptotic protein Bcl-2 and an increased expression of pro-apoptotic protein p53 were observed in khat treated fibroblasts, partly in agreement with what has been described in other cell types (Fig. 13). It is possible that some of the differences described in these studies may have been caused by differences in cell type and culture conditions (Boujrad, Gubkina et al. 2007), and also differences in response between transformed cells and normal cells.

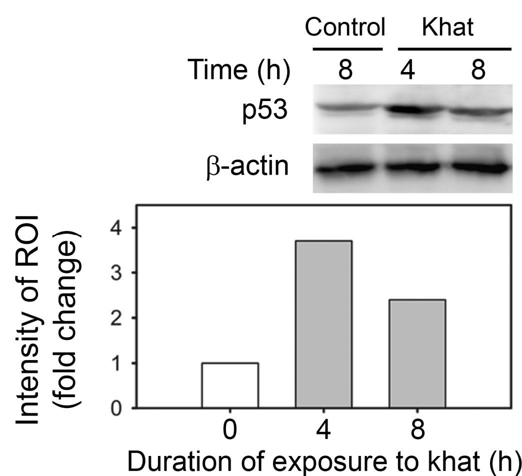

Figure 13. *Increased levels of p53 in khat treated cells*

4.2.4 Induction of cell death by an aqueous extract of khat (paper III)

An aqueous extract, closely mimicking the extraction of khat in saliva during khat chewing, was also evaluated for its cytotoxicity. The aqueous extract induced cell death in fibroblasts in a manner that was comparable to the organic extract, although with a little less potency at low concentrations. Our results may therefore indicate that during khat chewing, the active ingredients in khat responsible for its cytotoxicity could still be adequately extracted into saliva, posing a risk to oral tissues.

4.2.5 Key role of mitochondria in khat-induced cell death (paper IV)

The potential role of mitochondria in the cell death process was closely studied. Exposure of oral cells to lethal concentrations of khat led to a sustained decrease in mitochondrial membrane potential ($\Delta\Psi_m$) that first occurred 0.75 h after exposure to khat, making it the earliest effect induced by khat in oral cells. According to some studies, loss of $\Delta\Psi_m$ as a consequence of mPT may not be part of the central apoptotic machinery and may not always be required during apoptosis (Finucane et al., 1999). The loss in $\Delta\Psi_m$ may therefore occur much later in the process. In such circumstances, mPT can be preceded by activation of various caspases which act on the mPTPC to cause loss of $\Delta\Psi_m$ (Bossy-Wetzel et al., 1998, Grimm and Brdiczka, 2007). However, even when the mPT occurs downstream of caspase activation, it still constitutes the point of no return (Deshmukh et al., 2000, Grimm and Brdiczka, 2007), and the cell death process can therefore be inhibited by caspase antagonists. On the other hand, several other studies have shown that loss of $\Delta\Psi_m$ can be an early and central event in the coordination of the apoptotic process (Zamzami et al., 1995a, Zamzami et al., 1995b, Petit et al., 1995) and that inhibiting mitochondrial depolarization can actually enhance survival of the cells (Zamzami et al., 1995a, Chen et al., 1998).

In the present study, loss of $\Delta\Psi_m$ in oral cells treated with khat occurred very early and preceded all other parameters of cell death. This effect by khat was inhibited by CsA and BA. CsA and BA also delayed onset of cellular rounding up, condensation of nuclear chromatin and enhanced cell viability after exposure to khat. The loss of $\Delta\Psi_m$ also had a very close temporal association with cell survival, indicating that the early mPT and loss of $\Delta\Psi_m$ was central to the apoptotic process and possibly constituted the point of no return in the cell death. Apoptosis inducing factor (AIF) and Cytochrome c (Cyt C) were to be released from the mitochondria into the cytosol within 2 to 4 h of khat exposure. AIF was found to translocate further into the nuclei in khat treated cells. The translocation of Cyt C to the cytosol appeared to occur faster than that of AIF. Despite the translocation of Cyt C, there was no further signaling

leading to caspase involvement since 1) there was no detectable cleavage of pro-caspase-3 protein, 2) co-treatment with potent caspase inhibitors failed to block the cell death process and 3) cells exposed to khat lacked oligonucleosomal DNA fragmentation normally associated with caspase signaling. Therefore, of the mitochondrial apoptogenic proteins, AIF appeared to be more involved in the cell death process than Cyt C which normally leads to caspase signaling. Taken together, these observations point to an early effect on mPTPC and loss of $\Delta\Psi_m$ as the central event in khat induced cell death in primary normal oral cells.

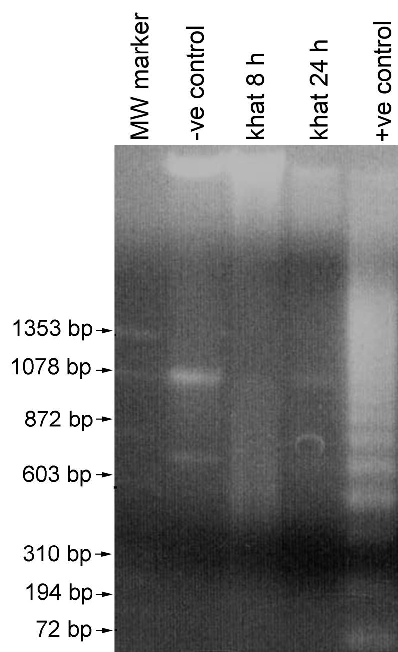

Figure 14. *Absence of DNA laddering in fibroblasts treated with khat*

4.2.6 Oxidative stress induced by khat (Paper III)

Oral cells exposed to khat showed a concentration dependent increase in intracellular superoxide and hydrogen peroxide accompanied by a decrease in intracellular GSH. These effects of khat were inhibited by antioxidants (Tiron and NAC). Tiron was more effective than NAC in protecting against accumulation of ROS and depletion of GSH. In addition, Tiron protected against cell death in both keratinocytes and

fibroblasts exposed to khat, but the cyto-protective effects of Tiron decreased upon long term exposure and were not observed after 24 h. ROS are known to induce increased expression of p21 leading to cell differentiation and senescence (Dumont et al., 2000). It is therefore possible that the ROS production induced by khat could have contributed to the premature differentiation observed in this study, but this was not specifically tested.

With regard to lethal concentrations of khat, the observed sustained loss of $\Delta\Psi_m$ could induce accumulation of ROS in the cells and may also affect energy generation by its effect on the respiratory chain function(s) (Andreyev et al., 2005). An increase in intracellular ROS can induce cell death and depletion of intracellular GSH predisposes cell to apoptosis by other pro-apoptotic signals (Chiba et al., 1996, Hammond et al., 2001). To counter oxidative stress, mitochondria are equipped with multiple ROS removal sites. But mitochondria have multiple ROS production sites as well. Functionally and structurally intact mitochondria could serve as net removers rather than net producers of ROS, and it has been shown that dysfunctional ROS removal as a consequence of a loss in $\Delta\Psi_m$ could be the most important factor leading to the net ROS production in cell death (Andreyev et al., 2005, Hansson et al., 2008). Net ROS production in mitochondria could exacerbate the cell death process by activating further ROS production sites and inducing further loss of $\Delta\Psi_m$ in more mitochondria (Schild and Reiser, 2005, Andreyev et al., 2005). This viscous cycle could be countered by ROS scavengers and thus delay the cell death process.

An interesting phenomenon observed in this study was the differential protective effect of Tiron and NAC, where Tiron was much more effective even though both are potent antioxidants. A recent study has shown that the mechanisms of action of these two antioxidants are quite different. In addition to its antioxidant activity, Tiron was shown to increase NF-kB and Bcl-2 protein thereby protecting mitochondria against depolarization (Yang et al., 2007). On the other hand, NAC was found to have opposite effects on mitochondria. This suggests that the pro-survival effects of Tiron may be partly independent of its ROS scavenging effects. This could explain why

Tiron was significantly more protective against khat induced cell death in the present study whereas NAC was not. Moreover, the observation may explain why NAC was toxic to oral cells whereas Tiron was not. More importantly, it may further support our view that the key event in khat induced cell death in oral cells was the effect on mPTPC and loss of $\Delta\Psi_m$ rather than the increased ROS and depleted GSH. A similar finding was suggested in another study (Zamzami et al., 1995a) in which analysis of the sequence of events during apoptosis clearly showed that an increase in intracellular ROS occurs secondary to loss of $\Delta\Psi_m$.

5. Concluding remarks

An extract of khat was found to be toxic to primary normal human oral cells. Low concentrations of khat induced (a) cell cycle arrest at the G1 phase through increased expression of p53, p21 and p16 in primary normal oral cells and (b) premature differentiation and keratinization in oral keratinocytes grown in co-cultures with normal fibroblasts, possibly through p38 MAP kinase signaling. Higher concentrations of khat induced (a) oxidative stress and (b) caspase-independent cell death initiated through an early and sustained effect on the mitochondrial permeability transition pore complex leading to release of mitochondrial apoptogenic proteins to the cytoplasm.

From the literature, it is still not clear whether khat could be a risk factor in the development of oral cancer. This debate will probably continue until more laboratory and epidemiological studies are carried out in this field. In the present study, we can only contribute towards this debate by highlighting potential clinical implications of our findings. It is shown in our study that the chemical components found in khat have the potential to modulate key cellular functions like proliferation, differentiation and cell death through specific signaling pathways. Modulation of these processes could be responsible for the hyperkeratosis that has widely been reported among khat chewers. The present study also found that oxidative stress can occur in oral cells exposed to khat. Should this occur *in vivo*, it could lead to a host of cellular and tissue responses such as increased or decreased proliferation, increased and keratotic differentiation and damage to macromolecules like DNA. Lastly, the findings described in this study were observed at concentrations of khat comparable to those found in saliva among people chewing khat. The results show that khat has toxic effects on human oral cells and tissues and raises concerns about khat use and the development of various oral lesions.

6. Future study areas

What constituent of khat is responsible for its toxicity?

As explained in this study, the specific constituents of khat responsible for its toxicity on oral cells and tissues were not determined. There is need for fractionation of the khat extract and testing of various fractions and khat specific pure constituents in order to determine the actual factors in khat responsible for khat induced cytotoxicity in oral and other cells. In addition, the concentrations at which these constituents induce cytotoxicity need to be determined and thereafter compared with the concentration found in the saliva during khat chewing in order to assess their potential toxicity *in vivo*.

Are these findings simply an in vitro phenomenon?

There are always difficulties using findings from *in vitro* studies to draw conclusions about the situation in vivo. Therefore, there is need to compare the findings in this and other *in vitro* studies with those derived from histopathological and molecular tests on biopsies taken from the oral mucosa of khat chewers. Another important study would be to use xenografts where organotypic tissues like those used in this study are grafted in mice and exposed to khat and analyzed against controls.

What is the pre-mitochondrial signaling in khat induced cell death?

There are still many unknowns in the signaling pathways in khat induced cell death and even differentiation. In relation to this study, it is necessary to determine the pre-mitochondrial signaling in cell death induced by khat and also the pathways that lead to cell cycle arrest and differentiation. Knowing these pathways could help us understand the potential for khat to induce abnormal development (neoplasia) and to also explain some of the oral lesions associated with khat chewing.

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8. Original papers